DEFINING THE MAMMARY GLAND AS A MUCOSAL ORGAN AND POTENTIAL ROLES OF THE EXTRACELLULAR MATRIX

By

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CCL-28	C-C motif ligand-28
CCR-7, 10	C-C Chemokine receptor – 7, 10
CD-	Cluster of differentiation –
COX	Cyclooxygenase enzyme
CXCL12	C-X-C motif chemokine ligand 12
DC	Dendritic cell
DDR	Discoidin domain receptor
ECM	Extracellular matrix
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
Flt3	Fms Related Tyrosine Kinase 3
FN	Fibronectin
FoxP3	Forkhead box protein P3
Gata3	Gata binding protein 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HER2	Human epidermal growth factor receptor 2
IFN	Interferon
IFNγ	Interferon gamma
IRF	Interferon response factor
IL-	Interleukin –
LPS	lipopolysaccharide
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteinases
MHC	Major Histocompatibility factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDs	Nonsteroidal anti-inflammatory drugs
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
pDC	plasmacytoid dendritic cell
PGE2	Prostaglandin E2
PPBC	Postpartum breast cancer
PR	Progesterone receptor

qRT-PCR	quantitative real-time polymerase chain reaction
RORγT	RAR-related orphan receptor gamma transcription factor
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TNC	Tenascin-C
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
PI3-K	phosphoinositide 3-kinase activation
PIP3	phosphatidylinositol (3, 4, 5)-triphosphate
AKT	protein kinase B
GSK3	glycogen synthase kinase 3
mTOR	mammalian target of rapamycin
CREB	cAMP response element binding
Bcl-2	B-cell lymphoma 2
BAD	Bcl-2 associated death promoter
Bcl-XL	B-cell lymphoma-extra large
FOXO	the forkhead box (FOX) O family of transcription factors
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IDO	idoleamine 2,3- dioxygenase

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ABSTRACT

Mammary gland development, which primarily occurs postnatally, requires coordination between epithelium, stroma, and immune cells. The main goal of this thesis was to extend our understanding of immune contributions to normal adult mammary gland development, specifically during pregancy, lactation, and weaning. We chose to interrogate the adult mammary gland for mucosal immune attributes, as this assessment has not been previously reported even though the mammary gland has external exposure that increases with lactation. For these studies we focused on mucosal immune attributes associated with mucosal barrrier function, including barrier supportive T cells, tolerogenic dendritic cells, and evidence for functional immune tolerance. Understanding mucosal immune attributes of the adult mammary gland are expected to shed light on why reproductive history influences breast cancer progression as well as the understudied problem of lactation failure.

We found that the adult mouse mammary gland exhibits mucosal hallmarks at all reproductive stages evaluated, including Th17 $CD4^+$ skewed T cells and immature dendritic cell phenotypes. However, we also show that mucosal attributes are highly regulated by reproductive state, a paradigm shifting result. Specifically, we find that the mucosal attributes of Th17 skewed $CD4^+$ T cells and immune tolerance are enhanced during lactation. These attributes extend into the period of time post-weaning when the gland undergoes involution with the addition of Th2, Treg, and memory Th17/Treg $CD4^+$ T cell recruitment. After completion of weaning-induced involution, when the gland is considered regressed, we find mucosal attributes similar to the nulliparous gland indicating a return to a steady-state mucosal program. Our results strengthen the classification of the mammary gland as a mucosal organ. Further, our studies imply that

dendritic cell-driven immune tolerance during lactation and involution may be necessary for tissue function, for example successful lactation, however at the potential expense of tumor surveillance.

CHAPTER 1: IMMUNE CONTRIBUTIONS TO POSTNATAL MAMMARY GLAND DEVELOPMENT

The mammary gland is a skin appendage that develops both embryonically and postnatally. Embryonic development involves the invagination of thickened epithelial layers (placode) into the underlying stroma. This process results in a nascent ductal tree, which is present at birth in both females and males [1]. However, since subsequent gland development is dependent on ovarian hormones, postnatal gland development occurs in females. Further, since the mammary gland is one of the only organs that predominately develops postnatally, it has been extensively studied.

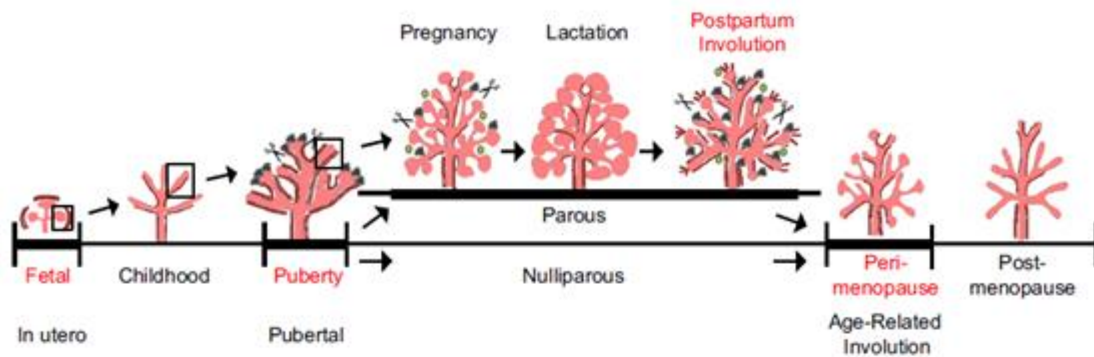

Figure 1-1: Stages of mammary gland development. Adapted from [2]. Major stages of postnatal mammary gland development are during puberty and a pregnancy cycle. It is also important to note that age-related involution also occurs, however our studies will not address this developmental stage.

What has become evident is that all mammary gland developmental stages require the coordinated activity of epithelium and immune cells [3]. Hence, the mammary gland is a superior model to understand the coordinated activity of epithelium and immune cells for the generation of specific tissue programs. In addition, understanding these interactions informs studies of breast pathologies, such as cancer. Below I review mammary gland development followed by a

review of the literature demonstrating the requirement of specific immune cells for each major stage of postnatal mammary gland development.

Major stages of mammary gland development and hormonal regulation

There are several well defined phases of postnatal development in the mammary gland: ductal elongation during puberty, alveolar development during pregnancy, production and secretion of milk during lactation, and ductal regression during involution [4, 5]. At puberty, which occurs around 3 weeks in mice, ovarian estrogen is *de novo* generated and results in the development of terminal end buds (TEBs) which are composed of layered epithelium undergoing high rates of proliferation. These TEBs invade the fat pad and branch in order to generate a highly ordered ductal tree which fill or "invade" the entire fat pad. A primary mechanism of this invasion is through the formation of TEBs, club like structures that form at the tips of developing ducts (epithelium). During ductal outgrowth, high levels of proliferation in a single layer of cap cells in the TEB drive epithelial expansion. This process is controlled by growth hormone, insulin-like growth factor 1, and estrogen [5]. However, high proliferation alone is not enough for ductal elongation to occur. Digestion and invasion of the stroma must also occur for the TEB to successfully elongate [6]. Ductal branching and fat pad filling is complete around 8 weeks of age in the mouse. Further, with each estrus cycle in the mouse, there are small bursts of ductal expansion and regression that occur respectively with cyclic estrogen and progesterone exposure.

With pregnancy, there is a significant increase in the levels of progesterone and prolactin which stimulate the epithelium to undergo a secondary phase of branching morphogenesis and lobuloalveolar expansion. By the end of pregnancy, the gland is dominated by epithelium and is poised to produce milk. Upon parturition there is a drop in progesterone, although prolactin

persists, and this generates the lactational switch needed for milk secretion. In rodent models, where weaning can be synchronized as a result of pup removal, the process of involution is initiated in a manner that permits detailed description of the involution process. Involution is a multi-step process including massive epithelial cell death, immune cell influx, and tissue remodeling. Together, these functions return the gland to a pre-pregnant-like quiescent state that is ready for a subsequent pregnancy [1, 6].

Innate immune cell contributions to ductal elongation (mast cells, eosinophils, macrophages)

Mast cells increase in abundance in mouse mammary tissue around the time of puberty. A function for mast cells in ductal elongation at puberty has been investigating through the use of a mast cell deficient mouse model ($Kit^{W\text{-}sh}$). Specifically, there was a loss of TEB formation and ductal elongation in mast cell deficient mice [7]. Further analysis revealed that mast cell degranulation and release of dipeptidyl peptidase I (DPPI), an enzyme involved in matrix digestion, was likely the mechanism by which mast cells support TEB elongation into the surrounding stroma during ductal elongation [7].

Macrophages and eosinophils are also necessary for ductal elongation during puberty. At the onset of puberty, both macrophages and eosinophils are recruited to the mammary gland. In a mouse model where macrophages (CSF1 cytokine null, $Csfm^{op}/Csfm^{op}$) were severely depleted there was a dramatic reduction in ductal elongation [8-10]. Further experimentation revealed that macrophages provide structural support and guidance to the developing ductal tree, as evidenced by macrophages being localized around the duct under the TEB intermingled with collagen I fibrils [8, 11]. Macrophage deficiency did not reduce the overall abundance of collagen I, but

reduced the linear organization around the neck of the TEB, as detected by second harmonic generation imaging. The necessity of macrophages for ductal expansion has been corroborated by experiments of mammary epithelium transplantation. In these experimental models, bi-potent mammary stem cells, defined as $Lin^{-}CD29^{hi}CD24^{+}$ mammary epithelial cells, can recapitulate the entire mammary gland when placed into a mammary fat pad that has previously been cleared of all epithelium. However, mammary gland outgrowth from mammary stem cell is dramatically reduced when performed in a mouse that has been depleted of macrophages ($Csfm^{op}/Csfm^{op}$ or Clodronate liposomes) [12]. This data indicate that macrophages are necessary for mammary gland development.

Similar to a necessity for macrophages in ductal elongation, eosinophils are also necessary. Studies of eosinophils in ductal elongation utilized a transgenic mouse that is homozygous null for a mutation in the eotaxin gene. Eotaxin is a chemokine involved in the recruitment of eosinophils into tissues, hence the mouse model utilized lacks eosinophils in the mammary tissue. When eosinophils were depleted, there was dramatically reduced numbers of TEBs and branching [9]. This indicates that eosinophils are an important component in regulating branching decisions, specifically [9]. All together this evidence strongly supports the conclusion that the innate immune system, namely mast cells, macrophages, and eosinophils are absolutely essential for ductal elongation during puberty.

Antigen presenting cells and $CD4^{+}$ T cells regulate ductal elongation

A role for antigen presenting cells and the adaptive immune system in supporting ductal elongation during puberty remained elusive until a recent paradigm-shifting report. This seminal

report is important because roles of APCs and adaptive immune cells in mammary gland development have largely been missing. Further, elucidating the role of APCs and the adaptive immune system in mammary gland development will expand our understanding of how the system may be perturbed in the case of breast pathologies, such as cancer [6]. Plaks and colleagues reported that $CD11c^+$ cells negatively regulated ductal elongation during puberty; because removing $CD11c^+$ cells resulted in precocious epithelial branching [13]. In their rodent model, $CD11c^+$ cells in the mammary gland likely represent a mixture of myeloid cells, including dendritic cells and macrophages [13, 14] which both have antigen presentation capacity, i.e. the ability to activate T cells. The authors then questioned whether $CD11c^+$ antigen presentation to T cells was involved in ductal elongation.

These authors tested the hypothesis that $CD11c^+$ cells communicate with T cells to negatively regulate ductal elongation. Using an *ex vivo* model it was found that mammary $CD11c^+$ cells activated naïve $CD4^+$ T cells in an antigen-dependent manner. Furthermore, the newly activated $CD4^+$ T cells produced IFNγ (Th1 skewed) which acted as a negative regulator of branching morphogenesis and luminal differentiation [13]. This APC and $CD4^+$ T cell dependent mechanism of negative regulation of branching morphogenesis likely acts in concert with positive regulators (mast cells, eosinophils, macrophages) to bring about proper organ development. Furthermore, this study is one of the only descriptions of APC function in the mammary gland during normal development, as opposed to many papers describing APCs in the context of cancer.

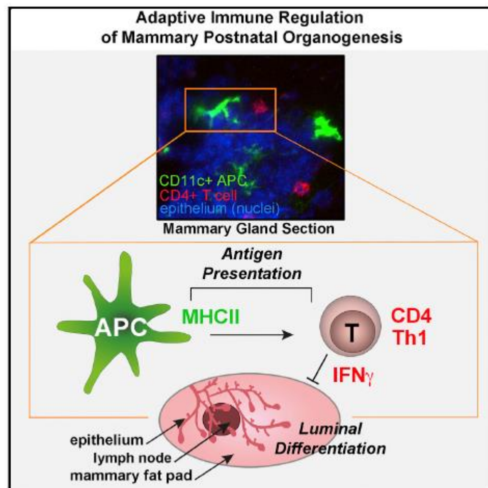

Figure 1-2: Antigen presenting cell activation of Th1 $CD4^+$ T cells is negative regulator of ductal elongation during puberty [13]. Th1 $CD4^+$ T cells produce IFNγ which negatively regulates ductal elongation and luminal differentiation during pubertal mammary gland development.

In sum, studies have revealed that immune cells are absolutely essential in ductal elongation during puberty. Following pubertal development, the gland undergoes some minor development with hormonal cycling (menstrual cycle in humans). It is at pregnancy that the mammary gland undergoes the next major phase of development.

Gland expansion during pregnancy requires macrophages

During pregnancy, the mammary gland expands dramatically, eventually becoming 7-10 times heavier than the virgin gland [15], with progesterone and prolactin supporting secondary branching morphogenesis and lobuloalveolar expansion. During pregnancy mast cells and macrophages increase in abundance and surround the epithelium, in part to prepare the gland for milk production. This is evidenced by the fact that macrophage depletion during pregnancy results in reduced alveolar expansion and premature differentiation. This means that macrophages are a positive regulator of branching morphogenesis in the mammary gland during pregnancy. However, functional studies assessing mast cells, eosinophils, APCs, or adaptive immune cells in gland expansion during pregnancy have not been reported. The next major step in mammary gland development occurs upon the switch from pregnancy to lactation.

Lactation has been well studied as it relates to infant health

The process of lactation has been most well described as related to infant health. The transfer of immunity from mother to infant through breast milk provides the infant with passive immunity. In developing countries, breast feeding reduces infant mortality associated with intestinal parasites and diarrheal illness by almost 20-fold [16, 17]. Mature human milk contains macromolecular nutrients as well as macrophages, neutrophils, B cells [18], and many bioactive molecules such as antimicrobials [19, 20] including mucins and immunoglobulin A (IgA). By ingesting these bioactive agents, the infant gains systemic immunity without having to generate it themselves. The specific protection of the neonate gut by breast milk has been well described and termed the mammary-entero link [21, 22]. Specifically, the transfer of IgA from mother to infant is essential for the infant's first line defense to intestinal and lung pathogens [16].

Since milk IgA has a dominant role in infant health, the mechanism by which IgA is deposited in milk has been described. The source of milk IgA is from IgA antibody secreting cells (ASCs) that have are specifically recruited to the mammary gland during lactation through the bloodstream [20]. Specifically, the lactating mammary gland has enhanced abundance of the chemokine CCL28 which binds to the receptor CCR10 located on IgA ASCs. This mechanism promotes chemotaxis of ASCs into the mammary gland and accumulation of IgA in milk [23]. Also, MAdCAM-1 and VCAM-1 expression were found to be enhanced in blood vessels during lactation and putatively are involved in recruiting systemic IgA producing cells to the mammary tissue during lactation [20, 24]. Further, it was found that gut-primed IgA producing cells were

recruited to the lactating mammary gland. In contrast, IgA producing cells that were derived from the periphery did not accumulate in the lactating mammary gland [25, 26].

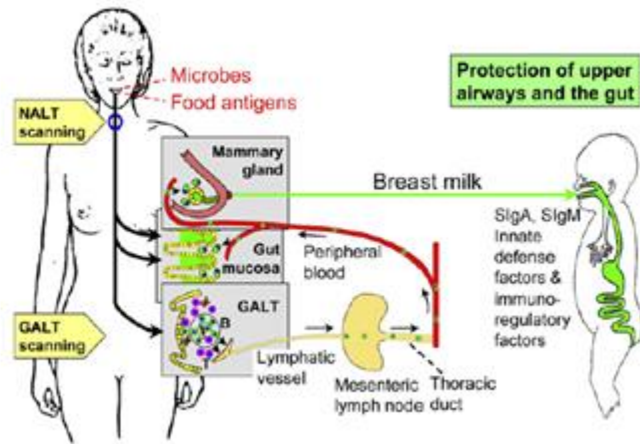

Figure 1-3: The entero-mammary link provides protective immunity to infant [22]. Gut associated lymphoid tissue (GALT) produces immunity in a female that can be transferred to infant via breast milk. Of note, IgA producing cells antibodies are recruited from the gut to the mammary gland during lactation, then IgA is deposited in milk. The passive transfer of IgA confers essential immunity in the breastfed infant.

All together these studies support the conclusion that milk IgA assures infant health. However, understanding how IgA contributes to gland health as it relates to lactation success is much less well understood.

Immune tolerance may be required for lactation, but definitive evidence is lacking

A direct role for immune cells in supporting successful lactation has not been reported, however immune modulation is evident. In particular, Th1 cytokines are known to limit milk production in *in vitro* models. Conversely Th2 cytokines enhance milk production [3, 27, 28]. Consistent with Th1 immunity limiting lactation, bacteria activation of Toll-like receptor (TLR) 4 during lactation results in reduced milk output in mice [29]. Also, NFκB activκation during lactation induces mammary epithelial cell apoptosis and milk decline [30]. These studies identify that a Th1 immune milieu suppresses lactation.

Immune suppression has been noted as likely being necessary for successful lactation, however definitive evidence of this statement has not been shown [31, 32]. However, important

supporting evidence for this argument was recently found in a mouse model of induced autoimmunity during lactation.

To investigate the possibility of autoimmune interruption of lactation, Kesaraju and colleagues immunized mature SWR/J females with alpha lactalbumin protein prior to mating [33]. Alpha lactalbumin is a protein that is expressed only during lactation for the purpose of lactose production. Following immunization, they observed a Th1 immune milieu (IFNγ) and T cell infiltrate only in females who were lactating. They did not observe any inflammation in non-immunized or non-lactating female mice. Further, mice exhibiting Th1 autoimmunity in the lactating gland failed to sustain their pups, leading to pup alopecia, runting, and liver toxicity [33]. The results of this study supports several important conclusions:

1. Th1 T cell inflammation in the mammary gland results in lactation failure. This means that Th1 inflammation may be an underpinning of lactation failure that has not been appreciated.
2. Autoimmunity to a lactation specific antigen is possible. This means that not all lactation specific proteins may be expressed in the thymus, and thus not all milk-reactive T cells removed by the process of central tolerance.
3. Peripheral tolerance is essential to the success of lactation. This means that immunological mechanisms that drive immune tolerance may be essential to lactation success.

The finding that autoimmunity can interrupt lactation may underlie the poorly understood condition of idiopathic granulomatous mastitis [34]. Idiopathic granulomatous mastitis is a local inflammatory reaction that limits lactation and does not respond to antibiotics but does respond

to steroids [35]. This has led to the acceptance of idiopathic granulomatous mastitis as an autoimmune disease of the breast [36, 37]. These results imply that immune tolerance in the form of dampened T cell activation is necessary for lactation, however the mechanisms are unknown.

In summary, lactation has been well studied for how it ensures infant health. However, the biological mechanisms underlying successful lactation programs in the mammary tissue are less described, but Th2 cytokines and immune tolerance are suggested.

Mammary gland involution requires Transforming growth factor-β and macrophages

Upon the cessation of lactation, the gland undergoes yet another developmentally regulated process, called involution. Involution is a tissue remodeling process that restores the gland back to a pre-pregnant like state in order to prepare for subsequent pregnancies. The process of involution has been well described in rodent models. Importantly, a similar program of involution has been observed in the human postpartum breast [38]. In rodents, involution is triggered upon synchronized pup removal which results in an abrupt cessation of lactation. For a period of 24-48 hours following pup removal there is milk stasis and reversible involution, where if pups are re-applied lactation can resume. Once 48 hours have passed since last nursing, an irreversible program of involution ensues. Irreversible involution is characterized by massive epithelial cell death, immune cell influx programs similar to classic wound healing, and a final stage of tissue remodeling and resolution of inflammation.

There are well known epithelial cell intrinsic mechanisms to initiate involution. For example, lipid toxicity resulting from prolonged milk stasis is implicated in inducing epithelial cell death

[39]. However, aside from or in addition to epithelial cell intrinsic triggers of cell death, the contribution of immune cells has also been described. Notably, macrophages have been found to be required for the onset of involution. An example of this dependence can be seen when macrophages are specifically and transiently depleted at the time of weaning. This results in a prolonged delay of epithelial cell death and gland remodeling [40]. Epithelial cell death during involution also requires transforming growth factor-β (TGF-β). Macrophages can be a major producer of TGF-β, indicating an immunological pathway may be essential in triggering involution. In addition to epithelial cell death, involution is also a wound healing-like program of tissue remodeling with input from various immune cells.

Immune changes of involution are similar to cutaneous wound healing

There are many similarities between cutaneous wound healing and mammary gland involution with respect to immune cell function. An example is an initial influx of neutrophils, which occurs during early involution [41]. Following neutrophils, macrophages and T cells influx the involution mammary gland [42].

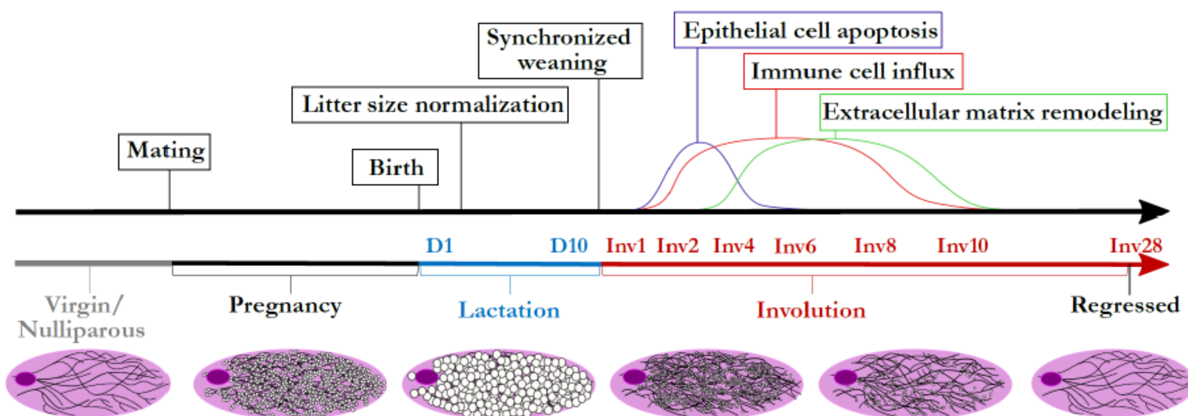

Figure 1-4: Rodent model used to study mammary gland involution reveals distinct stages of epithelial cell apoptosis, immune cell infiltration, and extracellular matrix remodeling following

weaning. This process of events share similarities to cutaneous wound healing. Model published in [43].

Also consistent with wound healing, the macrophages and T cells that influx the involution gland have immune suppressive qualities. For example, macrophages during involution have been found to express arginase I and Interleukin-10 (IL-10), molecules which suppress the function of effector T cells and dendritic cells [42, 44]. In addition, various immune modulators associated with Th2 or immunosuppressive functions are specifically up-regulated during involution, including Prostaglandin E2 (PGE2), TGF-β, Interleukin-4 (IL-4), and Interleukin-13 (IL-13) [44-46]. Further, T regulatory cells are also specifically enhanced during involution [42]. These results indicate the presence of immune suppressive programs during involution. Immune suppression is a hallmark of wound healing and is needed to resolve inflammation, limit immune reactivity to dying cells, and support tissue remodeling.

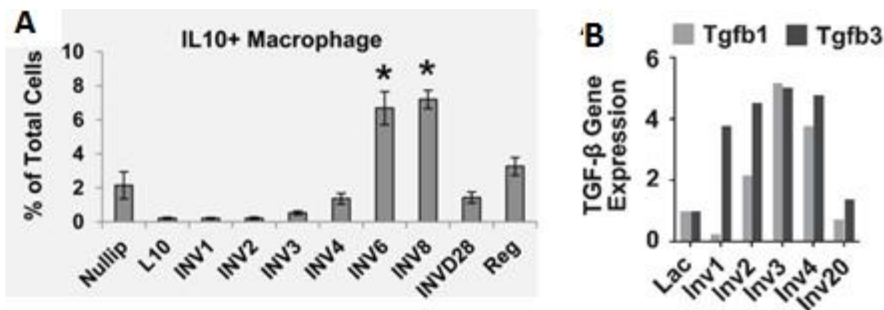

Figure 1-5: Immunosuppressive mediators are specifically up-regulated during mammary gland involution. A) The incidence of IL-10$^+$ macrophages increases during involution as determined by flow cytometry and previously published [42]. B) Gene expression of TGF-β1 and TGF-β3 are increased in the whole mammary gland as determined by qPCR and previously published [46].

The role of immune suppression in the mammary gland during involution is expected to support tissue health and remodeling. However, this necessary immune suppression may come at the expense of immune surveillance. In fact, the immune microenvironment during involution has been found to contribute to a unique type of deadly breast cancer, postpartum breast cancer. In

the next section I will review how reproductive state alters both breast tumor risk and progression with a specific focus on immune contributions to postpartum breast cancer.

CHAPTER II: RELATIONSHIPS BETWEEN REPRODUCTIVE STATE AND RISK AND PROGRESSION OF BREAST CANCER

Breast cancer is the most common cancer in women and is a heterogeneous disease that broadly falls into several molecular subtypes, based largely on the expression of estrogen (ER) and progesterone receptors (PR) and amplification of the human epidermal growth factor receptor 2 (HER2) gene. Triple negative breast cancers, which do not express ER, PR, or HER2 amplification, have especially poor prognosis [47]. There is a complicated relationship between risk and prognosis of breast cancer based on reproductive state.

A relationship between a woman' reproductive history and risk for breast cancer was observed as early as the 1700s, when it was noted that breast cancer was more prevalent in childless nuns [48, 49]. These initial observations of nuns provided the foundation for a paradigm that pregnancy protected against breast cancer. The protective effect of pregnancy was long considered the only effect of pregnancy on breast cancer risk, and even now this relationship is the most widely known. However, paradigm shifting research performed in recent years, in part by my thesis mentor Dr. Pepper Schedin, has lead to the appreciation that the relationship between reproductive state and breast cancer risk is complicated.

In recent years, it has been discovered that a recent pregnancy confers a transient increased risk of breast cancer [50-52]. Breast cancer that arises within 5-10 years following a childbirth has been termed postpartum breast cancer (PR). The length of increased risk is variable based on the age of the woman at her first birth (Figure 1) [53]. For example, populations of women over 30 at their first birth have an increased risk of breast cancer that lasts for about 30 years, at which time risk falls below that of a nulliparous woman (Figure 1). With the shift towards delayed

childbearing the incidence of postpartum breast cancer is expected to increase leading to the confinment of any protective effect of pregnancy to the latter decades of a woman's life [54].

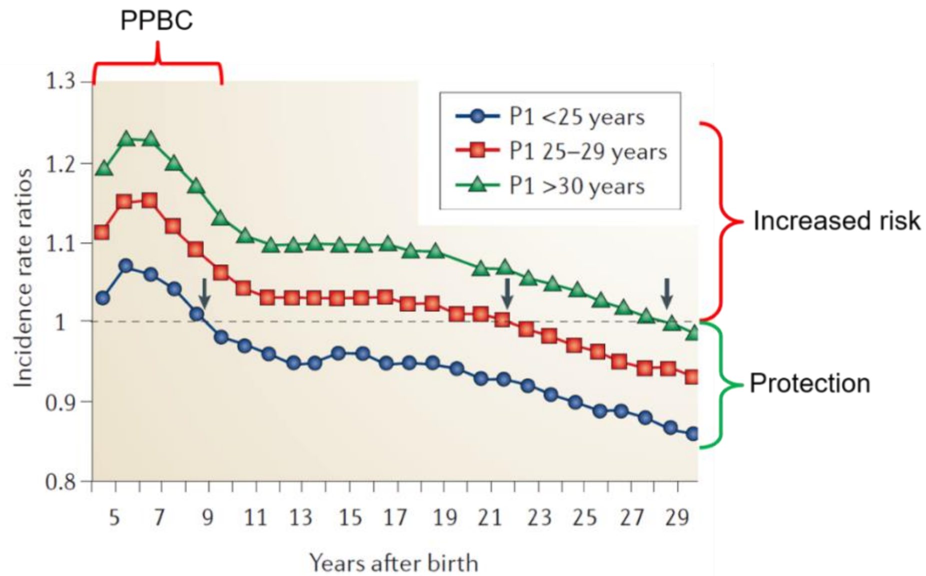

Figure 2-1: Relative rate of breast cancer in women based on parity status. Risk of breats cancer in nulliparous women, or women who had never had a child, is set to 1 (dotted line). There is an increase in the relative risk of breast cancer following childbirth, regardless of age of the mother at first birth (green, blue, and red lines). Breast cancer diagnosed within 10 years of a recent pregnancy is termed postpartum breast cancer (PPBC). Following a period of increased risk of breast cancer, that lasts between 10-30 years, pregnancy can result in a relative protection from risk of breast cancer. Figure has been adapted from the original published version [53].

In sum, we understand that pregnancy has a complicated relationship with cancer; pregnancy provides protection in the long term but first there is an extended window of time where risk of breast cancer is increased following a pregnancy. This complex relationship of pregnancy and breast cancer risk has been termed the dual effect of pregnancy.

The relationship between a recent pregnancy and breast cancer is even further complicated by studies showing that lactation may alter breast cancer risk. Some studies conclude that lactation is associated with a slight protective effect against breast cancer [55-58]. Although, any protective effect of lactation may be maximal against triple negative disease in African American

women [59-61], as reported by recent epidemiological findings. The biological mechanism is currently unknown. One biological mechanism that may account for the protective effect of lactation could be terminal differentiation of the epithelium, which occurs with lactation [55].

Reproductive state affects breast cancer prognosis

Above I discussed how reproductive state impacts the risk of developing breast cancer. A separate but interrelated topic is the effect of reproductive state on the outcome of established breast cancer, i.e. prognosis. Similar to breast cancer risk, reproductive state has a complicated relationship with breast cancer prognosis.

Overall ~2-4% of breast cancer is concurrent with pregnancy, but in women under 40 this increases to ~7-14% [62]. These numbers indicate that breast cancer diagnosis during pregnancy is a significant health problem for younger women. As a result, many efforts have been made to understand how pregnancy affects breast cancer outcome. Some initial reports concluded that breast cancer diagnosed during pregnancy had worse prognosis than breast cancer that was diagnosed in non-pregnant young women [63, 64]. However, it has since been understood that pregnancy often leads to delayed breast cancer diagnosis or treatment [62, 63, 65-67], two factors which alone contribute to worse prognosis. After adjusting for known prognostic factors, such as stage and tumor size, it has since been concluded that a breast cancer diagnosis during pregnancy does not confer worse prognosis compared to breast cancer in non-pregnant women [68].

Instead of pregnancy driving poor prognosis, what has been found is that patients diagnosed with postpartum breast cancer have a significantly worse prognosis compared to those diagnosed

while pregnant or in young women who have never been pregnant. Initially, the postpartum period was defined by close proximity to pregnancy, at ≤ 6 months following a childbirth [50, 51, 57, 64]. However, in recent studies performed by the Schedin lab in collaboration with Dr. Virginia Borges and others, the postpartum effect of poor prognosis has been observed for up to 5 years following a recent pregnancy [51, 52, 69-71]. In one of the first PPBC cohorts, it was found that there is a nearly three-fold increase in distant recurrence and death in women with PPBC compared to breast cancer in nulliparous women. Further, there is a significant decrease in 5 year overall survival probability in women with PPBC, around 65%, compared to rates of survival as high as 98% in nulliparous women with breast cancer [52]. More recent data from our laboratory finds that the poor prognosis of postpartum breast cancer may extend to PPBC diagnosed within 10 years of a recent childbirth, in essence at least doubling the influence of a recent pregnancy on poor breast cancer outcome.

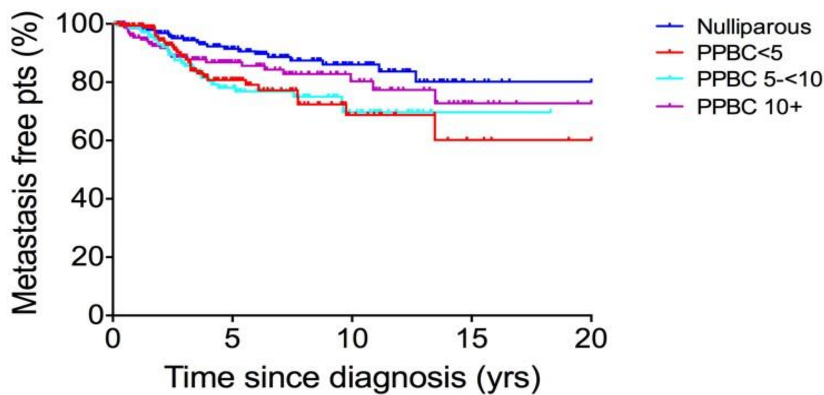

Figure 2-2: Risk of metastasis as a function of reproductive state. Nulliparous women, defined as women who had never had a child, have the lowest incidence of distant metastasis. In comparison, women who have had a child within 5 years (red), or within 5-10 years (teal) within a breast cancer diagnosis have significantly increased risks of metastasis. Schedin and Borges labs unpublished.

It has been estimated that postpartum breast cancer could account for up to 50% of all breast cancers diagnosed in young women (under 45 years of age), with breast cancer in young women

accounting for about 11% of all breast cancers [52]. This equates to about 12,000 cases of postpartum breast cancer diagnosed each year, a non-negligible number of women with young families who are at a substantially increased risk of dying from breast cancer.

Postpartum cancers have been reported to have enhanced grade, elevated mitosis, positive nodes, and progesterone receptor (PR) negative and p53 positive profiles [66]. However, even when influencing factors such as biologic subtype, stage, and year of diagnosis are accounted for, postpartum breast cancers still have a poor prognosis, including about a 3-fold increased risk of distant recurrence and death, when compared to breast cancers in young women who did not have a recent pregnancy [52]. These data indicate that postpartum host biology is a contributing factor to the poor prognosis of postpartum breast cancer. This led to the involution hypothesis, proposed by Dr. Schedin and corroborated in rodent studies, which proposes that the postpartum event of mammary gland involution, which follows the cessation of lactation, is tumor promotional [42, 53, 72, 73].

Lactation, like pregnancy, is often associated with delayed breast cancer diagnosis or treatment. This is because many breast changes, including increased breast lumps, are expected during lactation which might mask potential pathologies. Further, breast imaging is complicated by increased breast density associated with pregnancy and lactation [62]. However, even when cohorts are adjusted for tumor size, stage, and age at diagnosis, a correlation between a breast cancer diagnosis during lactation and worse overall survival is evident [50, 74, 75]. These studies identify that lactation, although always confounded by proximity to a pregnancy and involution, is an independent factor contributing to breast cancer progression, however there is no

mechanistic understanding of how this occurs. Also, a potential contribution of involution to the poor prognosis of cancer diagnosed during lactation is likely, since weaning is highly encouraged prior to breast cancer treatment.

Contributions of involution immune milieu to tumor progression in rodent models

The Schedin lab has been at the forefront in investigating multiple immune centric pathways which contribute to tumor promotion in the involution host. Firstly, it is important to note that orthotopic injection of tumor cells into involution hosts results in increased tumor growth [42, 72]. Involution supported tumor growth advantage has been reported in multiple mouse strains and cell lines, including MCF10DCIS.com human tumor cells orthotopically injected into immunocompromised SCID mice and D2.A1 and 66cl4 mouse tumor cells orthotopically injected into immunocompetent Balbc hosts. In addition, tumor cell escape into vasculature and lymphatics [45, 72], increased lung micro metastases [45, 72], and increased liver metastasis have also been reported across various mouse models [76]. These results are in comparison to genetically identical tumor cells placed into nulliparous or regressed hosts. These results indicate that the involution host contributes to tumor progression.

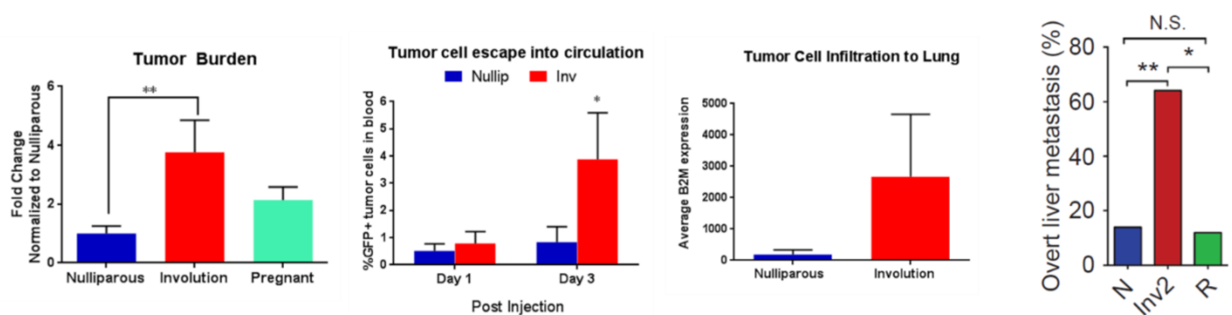

Figure 2-3: Involution is tumor promotional in rodent models. Increased tumor burden, tumor cell escape into circulation, tumor cell arrival into lung tissue, and liver metastasis are enhanced during involution compared to nulliparous hosts. Data have been adapted from [72, 76].

As previously mentioned the involution mammary gland displays wound healing characteristics. Of note, tissue remodeling programs are evidenced by fibroblast activation, de novo extracellular matrix deposition, and activation of matrix metalloproteinases (MMPs) [44, 72, 73, 77, 78]. Further, immune alterations are evidenced by leukocyte infiltration and enhanced PGE2, TGF-β, IL-4, IL-13, and IL-10, among others [42, 44-46]. Importantly, targeting some of these inflammatory mediators has proved to be effective in reducing involution-tumor promotion.

Martinson et al reported that IL-10 macrophages are specifically enhanced in the mammary gland during involution [42]. IL-10 is a cytokine that can limit dendritic cell function and the production of Th1 anti-tumor responses [79, 80]. In fact, targeting IL-10 during involution with a cytokine neutralizing antibody reversed involution-tumor promotion [42]. This result shows that IL-10 immune suppression contributes to tumor promotion during involution, although a role for dendritic cells, T cells, or other immune cells is unexplored in this model.

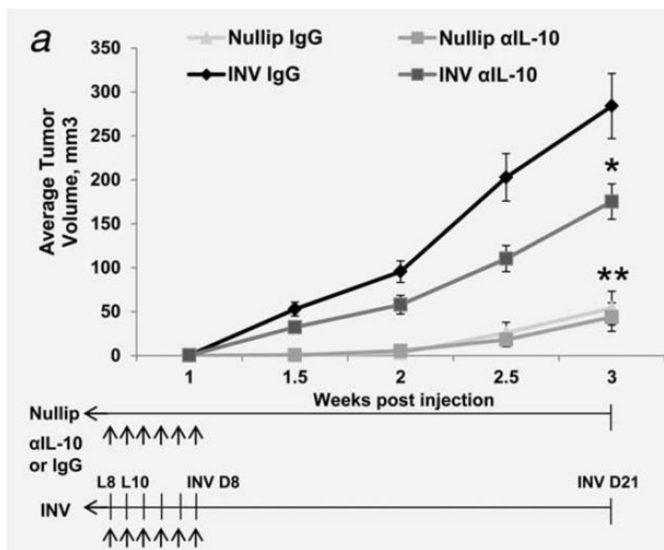

Figure 2-4: Involution promotes tumor growth which can be blocked with anti-IL-10 antibody administration which depletes IL-10 in the environment. Involution promotes tumor growth, but systemic administration of anti-IL-10 antibody significantly reduces the tumor growth advantage of involution. Anti-IL-10 antibody treatment did not affect tumor growth in nulliparous hosts. Adapted from [42].

The Schedin lab has also found that nonsteroidal anti-inflammatory drug (NSAID) treatment during involution results in decreased tumor growth and metastasis. This effect of NSAIDs is specifically effective during involution, as NSAID treatment in nulliparous hosts with tumors does not result in reduced tumor growth.

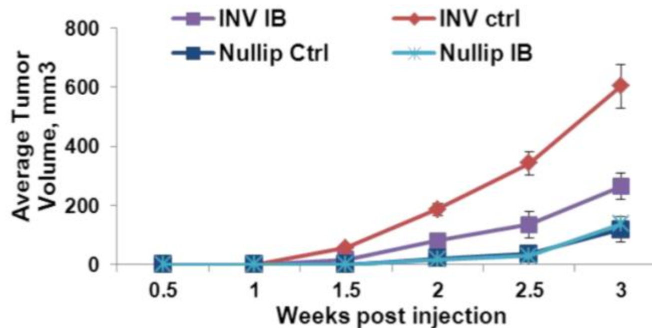

Figure 2-5: Tumor growth advantage of involution is abrogated by Ibuprofen (IB) administration. Ibuprofen does not alter tumor growth in nulliparous hosts. Adapted from [2].

This observation indicates that Cyclooxygenase (COX) enzymes lead to the production of pro-tumor mediators during involution. Of note, the COX-2 enzyme in particular is implicated as the major driver within the pro-tumor involution environment [72]. Further, it has been found that the specific COX-2 product PGE2 is enhanced specifically during involution [45]. However, given the pleotropic effects of COX products on tumor cells, immune cells, and stromal cells, the precise mechanisms underlying COX promotion of tumors in involution could be multiple. Our lab has demonstrated that targeting COX-2 enzymes during involution reduces *de novo* collagen and tenascin deposition [72, 78, 81]. Thus, a contribution of COX-2 enzymes to tumor-promotional extracellular matrix alterations is likely. Further, a recent study identified a COX-2-fibroblast-ECM-immune mechanism underlying tumor promotion in the involution host.

Guo et al found that fibroblasts are physiologically activated during involution by TGF-β, resulting in de novo collagen deposition and tumor promotion via CXCL12 recruitment of

monocytes and dampening of $CD8^+$ T cell cytotoxicity [78]. Further, NSAID treatment of involution fibroblasts resulted in reversal of involution-tumor promotion [78], indicating that COX enzymes drive tumor promoting inflammation during involution in part through fibroblasts.

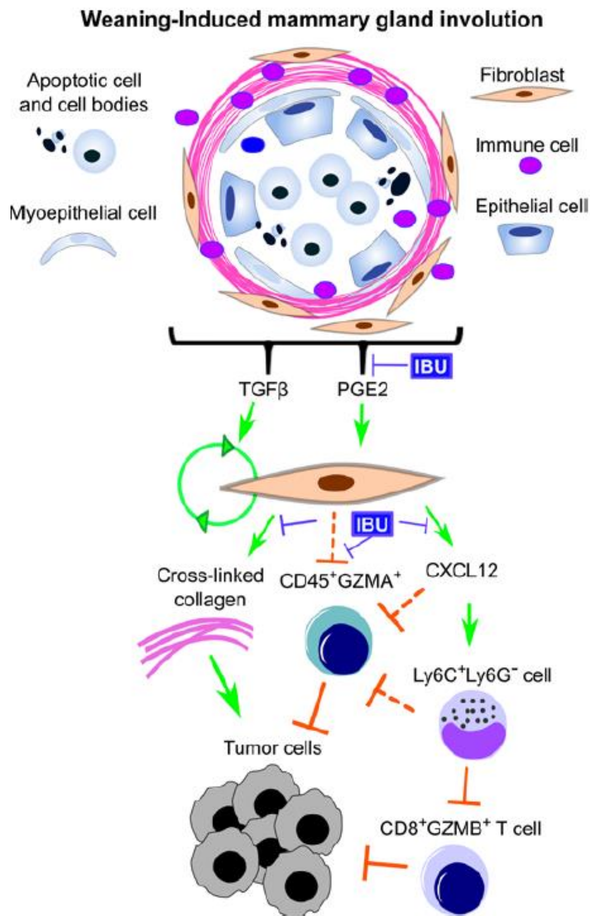

Figure 2-6: Involution drives mammary tumor growth via TGF-β and PGE2 activation of fibroblasts, collagen deposition, monocyte recruitment, and inhibition of $CD8^+$ T cell cytotoxic activities. Ibuprofen (IBU) results in relief of fibroblast pro tumor pathways. Published in [78].

Altogether involution has been shown to promote tumor progression via immune suppression, namely through COX metabolites and IL-10. Unknown is how dendritic cells are altered by involution and how this may contribute to tumor promotion. Given that dendritic cells are central to regulating immune activation versus tolerance, and because COX metabolites and IL-10 can influence dendritic cells to promote tolerance, we chose to focus on dendritic cell alterations in

our current study. Specifically, we hypothesize that reduced dendritic cell - naïve T cell activation may favor immune tolerance during involution to result in tumor promotion.

Mucosal immune programs may contribution to mammary gland development

Overall there are significant contributions of immune cells to multiple stages of postnatal mammary gland development. However, there is less appreciation for large overarching immune programs as they relate to mammary gland development. For example, the mammary gland is sometimes considered a mucosal organ, but a consideration of mucosal immune programs as related to mammary gland development is largely missing. This rationale formed the basis for our current studies. Further, we postulate that our study will shed light on mechanisms of immune regulation as they relate to lactation success and reproductive state dependent differences in tumor risk and progression. I will next provide a literature review of mucosal organs and their associated immune hallmarks, with a specific focus on information that has been reported in the mammary gland when available.

CHAPTER III: UTILIZING A MUCOSAL IMMUNOLOGY FRAMEWORK TO STUDY THE MAMMARY GLAND

Rationale for interrogating the mammary gland for mucosal immunological attributes

A rudimentary definition of a mucosal tissue is one with exposure to the external environment. Because the mammary gland has external exposure it is sometimes considered a mucosal organ. However, the body of literature investigating the mammary gland in the context of mucosal biology is relatively small. Evidence for mammary gland external exposure comes from an understanding of mammary gland development. The mammary gland develops a nascent ductal tree from an epithelial invagination into the underlying tissue during embryogenesis. The nascent ductal tree, and therefore the mature gland, are exposed to the external environment via the nipple aperture. Further evidence of this external exposure is the incidence of mammary infection by exogenous bacteria.

None the less, the mammary gland has not been systematically studied for mucosal immunological programs. This inquiry is important because it provides a novel framework, namely mucosal immunology, with which to study normal mammary gland development. Further, the mucosal immune state of the mammary gland may be variable with reproductive state. Of note, the risk of infection by exogenous bacteria is highest during lactation and involution, indicating increased external exposure at these reproductive stages.

Investigating the mammary gland for mucosal immune programs is expected to enrich our understanding of normal mammary biology and shed light on breast pathologies, such as

lactation failure and cancer. I will now provide a review of mucosal organs and their associated immune hallmarks.

Immunological hallmarks of mucosal organs and the mucosal organ system

Mucosal organs can be broadly defined as having an interface with the external environment, but this rudimentary definition is debated. For example, skin is included in this general definition of a mucosal organ. While the skin can be considered a mucosal organ based on external exposure, there are clear functional differences between internal mucosal organs such as the lung and gut and the skin. The primary difference is the nature of the physical barrier. In internal organs an aqueous mucus layer is the first line of defense from the external environment. In comparison a highly organized keratinized layer of skin forms the barrier to external pathogens. This distinction is important as it relates to how we may classify the mammary gland as a mucosal organ.

Since the mammary gland is a skin derivative (ectoderm), one may presume to find similar barrier functions to what has been described in the skin. However, the lack of a keratinized barrier at the mammary epithelium distinguishes it from skin. In fact, a mucus barrier at the mammary epithelium likens the mammary gland to the lung and gut. For the purposes of this thesis I will utilize the gut for instruction as to how to interrogate the mammary gland for mucosal biological attributes. This rationale is dual. First, the lactating mammary gland produces milk which transfers passive immunity directly into the neonate gut. Second, the source of IgA in milk during lactation is from the gut. These lines of evidence support a functional link between the mammary gland and the gut.

Our inquiry of the mammary gland as a reproductive state-dependent mucosal tissue commenced by understanding the unique needs of mucosal tissues. I will review these attributes and provide information that is known about the mammary gland when available. This will help us to understand the frequent gaps in knowledge as it pertains to mucosal immune hallmarks in the mammary gland. There are two broad immunological hallmarks that I will describe.

Physical barrier

The mucosal epithelium forms an impenetrable physical barrier which is a mechanism of protection to limit interaction with viruses or bacteria. There is also secretion of antimicrobial products and antibodies at the mucosal-external surface which bind to foreign materials to prevent their penetration into the underlying tissue. All together these mechanisms are called epithelial barrier function. Barrier function is supported by physical epithelial junctions, the production of mucus and antimicrobials, and IgA immunity.

Epithelial cellular junctions overview

Epithelial cells exposed to the external environment form a protective barrier from potential environment insults. One of the main components of the barrier is the formation of a severely impenetrable epithelial cell sheet. This occurs when epithelial cells form cell to cell junctions. These junctions separate apical and basal epithelial surfaces and do not allow for transfer of materials between surfaces. Epithelial junctions come in several varieties, including tight junctions, adherens junctions, and desmosomes. Tight junctions are the most apical of all the junctions and include occludin and claudin proteins which connect to the cellular actin cytoskeleton. Adherens junctions are positioned under tight junctions and are attached to the

cellular actin cytoskeleton. Adherens junction proteins include B-catenin, plakoglobin, p120, and cadherins. Desmosomes are located most basally and are composed of desmogleins, desmocollins, and desmoplakin.

It is generally thought that epithelial barriers at mucosal surfaces are static structures, and that perturbation of these barriers can increase the risk of infection. Mammary epithelial junctions are unlike other mucosal barriers. Namely, mammary epithelial junctions are highly dynamic based on reproductive state.

Mammary epithelial junctions are dynamic with reproductive state

Most of the studies on the effects of pregnancy and lactation on mammary epithelial junctions comes from the dairy industry. During pregnancy it has been reported that there is increased leakiness of tight junctions [82-84]. Consistent with this finding there is enhanced extravasation of large molecules from pregnant ducts [82, 85]. Supportive evidence of leakiness was found in that pre-partum milk has higher concentrations of the interstitial ions sodium and chloride, as well as proteins. Using another measure of epithelial junction barrier function, electrical resistance, it was noted that there was a loss of trans-epithelial resistance in pregnant goats and mice [82, 86]. Finally, morphological alterations in tight junctions, including fewer numbers of strands and less branching complexity, in mammary epithelium derived from pregnant sheep were reported [87]. In sum these data indicate increased permeability of epithelial junctions during pregnancy. As the gland transitions from pregnancy to lactation, termed the lactogenic switch, epithelial junctions tighten in order to successfully contain milk within the mammary ducts.

As the lactogenic switch is primarily driven by changes in hormones at the end of pregnancy, it is no surprise that hormones can regulate epithelial cell junctions. Specifically, mammary epithelial tight junctions are altered by glucocorticoids [83, 88-90]. The fall of progesterone in the later part of pregnancy allows for enhanced tight junction closure to provide a leak-proof duct needed for lactation [85]. Further, it has been shown that tight junction organization and connectedness to the actin cytoskeleton are enhanced at the lactogenic switch [91].
In addition to hormonal regulation of epithelial junction integrity during lactation, other influences are also well described. Prolonged milk stasis, up to 21 hours in goat models, increases epithelial junctional leakiness and reduces milk output. The mechanism involves a build-up of luminal pressure with milk stasis, leading to junctional loosening and the backflow of milk products into the mammary gland [88, 89, 92, 93]. Therefore, relative milk storage and emptying can regulate tight junction integrity during lactation.

It is also important to mention that milk stasis is common at the early stages of involution. This likely leads to the increased permeability of mammary epithelium that is reported during involution [46, 85]. However, the physical loss of epithelium because of epithelial cell apoptosis is also expected to compromise the epithelial barrier during involution, although this hypothesis has not been tested [85]. Using an *in vitro* model system it has been shown that TGF-β3, which is specifically up-regulated in the mammary gland upon weaning, coordinates epithelial cell junctional reorganization. Specifically, TGF-β3 treatment of mammary epithelial cell monolayers resulted in decreased electrical resistance, disorganized tight junctions, and down-regulation of genes that encode proteins of adherens junctions. Importantly, TGF-β3 treatment of mammary epithelium also drove phagocytic phenotypes via the release of B-catenin downstream

of γ-secretase cleavage of p120 of adherens junctions *in vitro* and *in vivo* [46]. Altogether these findings provide a TGF-β3-dependent mechanism by which epithelial junctions may lose integrity during involution.

However, it is clear that there are pleiotropic effects of TGF-β on epithelial junctions. In contrast to the mammary gland, TGF-β is well known to promote barrier function in classical mucosal organs such as the gut. In fact, in mouse models where the cytokine TGF-β1 is absent (*Tgfb1*$^{-/-}$) junctional integrity is reduced in the intestine and there is enhanced trans-epithelial migration of luminal bacteria [94, 95]. This elicits a Th1 pro inflammatory response leading to ROS generation, epithelial cell damage, and tumor initiation and progression [43, 94-97].

Other epithelial cell-extrinsic regulators of epithelial barriers are Th17 skewed CD4^{+} T cells, which are common in mucosal tissues. Lee and colleagues found that the Th17 derived cytokine, Interleukin-17 (IL-17), regulates the localization of the tight junction protein occludin during intestinal injury. This IL-17 dependent process limited epithelial permeability, enhanced barrier activity, and limited inflammation following injury [98]. Similar findings have corroborated the role of IL-17 cytokines in epithelial tight junction integrity [99, 100]. The question as to whether Th17 skewed CD4^{+} T cells are present in the mammary gland and may regulate epithelial junctional integrity is unknown.

Mucus and antimicrobial protection of epithelium

A protective barrier composed of mucus and antimicrobials is common in mucosal tissues. In the gut an outer mucus layer is responsible for physically trapping bacteria and severely reducing bacteria-epithelial interactions. A second mucus layer is generally devoid of bacteria and instead has high concentrations of antimicrobial peptides [101]. The mucus layers are primarily

composed of mucins, a large family of diverse proteins. In general, mucins are highly repetitive proteins containing mostly Serine, Threonine, and Proline amino acid residues. Mucins are highly glycosylated, supporting the formation of aqueous gel-like structures [102]. The outer layer of mucus in the gut is primarily composed of the classic gel-forming mucins MUC2, 5AC, 5B, and 6. The inner layer of mucus, closest to the epithelium, is composed of transmembrane mucins, MUC1, 3, 4, 12, 13, 16, 17. These mucins have a small intracellular domain and large extracellular domain with masses up to more than ten million daltons [102].

Mammary gland has a modified mucus barrier

Of note, bona fide mucus layers have not been described in the mammary gland, however the presence of mucin proteins at the epithelial apical surface has been reported [103, 104]. Muc1 expression is increased dramatically during mid-pregnancy and lactation and is localized to the apical surface of the epithelium. In addition, using a cell culture model, mammary epithelial cell expression of Muc1 was dramatically increased by culturing on matrigel in the presence of insulin, hydrocortisone, and prolactin [104]. These data indicate hormonal regulation of Muc1 in the mammary gland, and may indicate that mucosal function in the mammary gland is regulated by reproductive state. However, the function of Muc1 in the mammary gland has not been described.

Understanding mucin biology in the normal mammary gland is important because mucins are often up-regulated in breast cancer and correlated with poor prognosis [105]. Our results that the genes encoding mucin 1, 3, and 10 are regulated by reproductive state, indicate that a mucus border may also be regulated by reproductive state, although this has not been tested. Further, the unique presence of Muc10 in the mammary gland, and not the gut, indicates potential divergence

from the gut in the composition or function of the mucosal protective barrier. This is expected, especially during lactation, when the passage of milk from epithelium to baby may be limited by an especially impassible mucous layer. In this way one may expect a more permeable mucosal barrier during lactation, but this remains to be tested.

Antimicrobials at mucosal surfaces

As previously mentioned, mucus layers usually contain high concentrations of antimicrobials. There are many classes of antimicrobial peptides, each with distinct methods of action [106]. For example, alpha and beta defensins kill both gram positive and negative bacteria via membrane permeabilization. The Reg proteins kill only gram positive bacteria via direct binding and lysis. Antimicrobials are present in the mammary gland of the lactating cow, for example beta defensin is bioactive in suppressing E. coli proliferation in milk. In addition, the antimicrobials S1007A, cathelicidin, and lactoferrin are also detected in the lactating cow mammary gland, as well as milk [107]. Further, the expression of antimicrobials by the lactating mammary epithelium may be hormone regulated [107].

Th17 $CD4^+$ T cells support barriers via the up-regulation of epithelial cell production of antimicrobials. Th17 $CD4^+$ cells can produce the cytokines Interleukin-17 (IL-17) and Interleukin-22 (IL-22). These cytokines stimulate receptors on mucosal epithelial cells to bring about enhanced antimicrobial secretion [108, 109]. Specifically, IL-17A binds the IL-17 receptor on epithelial cells and induces up-regulation of the gene encoding B-defensin 2 [110]. IL-22 acts on intestinal epithelial cells to induce the production of the Reg family of antimicrobial proteins,

including RegIIIy [111]. The antimicrobial RegIIIy protects intestinal epithelial cells from colonization by the pathogenic bacteria *C. rodentium* [111].

IgA protection of mucosal epithelium

IgA is the most abundant antibody species found at mucosal surfaces. IgA protects the mucosal epithelium by binding pathogens and thereby limiting pathogen-epithelial interactions. IgA is produced by plasma cells in the tissue underlying the epithelium [19]. IgA must be transported to the apical side of the epithelium in order to be effective in barrier protection. This is achieved by epithelial cell uptake of basal IgA, transport, and secretion of apical IgA. This process relies on the polymeric IgA receptor (pIgR) on the basal surface of the epithelium. In the gut, Th17 T cells support IgA production and translocation into the intestinal lumen via the up-regulation of epithelial cell expression of the polymeric IgA receptor (pIgR) [110].

Of note, IgA is increased in the mammary gland during lactation via enhanced recruitment of IgA^+ producing cells. However, this biology (explained above) has been mostly studied from the context of IgA transfer to the neonate for gut health. The question as to the function of IgA in protecting the mammary epithelium from infection during lactation has not been addressed.

Immune tolerance at mucosal sites during homeostasis

Underlying the mucosal epithelium there is connective tissue which contains immune cells. There are conserved immune cell phenotypes found in mucosal tissues, including Th17, Th2, Treg, IgA producing producing cells, and tolerogenic dendritic cells. This immune milieu preferentially instructs immune tolerance during homeostasis. However, the mucosal immune system is poised to respond to unfamiliar foreign antigens.

Tolerogenic dendritic cells are primary executioners of T cell immune tolerance during homeostasis. In order to study dendritic cells in the mammary gland, I will review dendritic cell differentiation, maturation, and T cell activation to understand how these multiple layers of DC biology are altered in mucosal tissues to predominately result in tolerogenic dendritic cell functions. Immune tolerance and tolerogenic dendritic cells have not been described in the mammary gland at any reproductive stage. Further, immune tolerance variability based on reproductive state is also unknown. This gap in knowledge formed the basis of our current studies.

Dendritic cell ontogeny and differentiation

Dendritic cells were initially described by Ralph Steinman and Zanvil Cohn in the late 1970s and their unique functions are still being uncovered [112]. The dominant role of dendritic cells is to function as an antigen presenting cell (APC). APCs induce T cell activation or tolerance, and as such instruct the adaptive immune system how to respond to insults. Dendritic cells are the most superior APC, in comparison to other APCs, for several reasons: anatomical localizations, superior antigen processing and presentation ability, superior migration from peripheral tissues to T cell zones of LNs, and superior ability to prime naïve T cell responses.

To date there are at least five distinct subsets of dendritic cells in the mouse. The five most described dendritic cell subsets in mouse are: plasmacytoid (pDC), $CD11b^+$, $CD103^+$, $CD8a^+$, and $CD8a^-$. $CD8a^+$ and $CD8a^-$ dendritic cells are confined to primary and secondary lymphoid organs, which include the bone marrow, thymus, lymph nodes, and spleen. Since the mammary

gland is a non-lymphoid organ, sometimes also referred to as a peripheral tissue, this discussion will focus on dendritic cell subsets found in these organs, which include plasmacytoid, CD11b$^+$, and CD103$^+$ dendritic cells. We have chosen to focus our studies on dendritic cells localized in mammary tissue. This is because these mammary localized dendritic cells are thought to be conditioned by the local tissue microenvironment. However, we do know that tissue localized immune cells can traffic to tissue draining lymph nodes. For example mammary residing dendritic cells may traffic to the tissue draining lymph node where dendritic cells can interact with naïve T cells to result in naïve T cell priming or tolerance. However naïve T cell priming in lymph nodes is also influenced by lymph node resident immune cell populations, such as resident dendritic cells and subcapsular macrophages. These immune cell populations are not expected to be altered by the reproductive states of lactation and involution, however we have not tested this in our model. Hence, we have focused our efforts on understanding reproductive state dependent alterations in dendritic cell phenotypes and functions on those dendritic cells localized to the mammary tissue.

Plasmacytoid dendritic cells (pDCs) represent a small subset of dendritic cells specialized for anti-viral immunity [112]. Since I did not reliably detect pDCs in mammary tissue at any reproductive state (data not shown), I will not discuss pDCs further in this thesis, however they do deserve investigation of a role in mammary gland development.

CD11b$^+$ and CD103$^+$ dendritic cell subsets are termed conventional dendritic cells and are found in both lymphoid and non-lymphoid tissues. Conventional dendritic cells are largely derived from the myeloid lineage of hematopoiesis. A common myeloid derived progenitor gives rise to

monocytes and a common dendritic cell precursor (CDP) in the bone marrow. CDPs are thought to complete differentiation into mature dendritic cells in peripheral tissues [113-116], under the influence of the local tissue immune milieu. The CDP becomes a pre-dendritic cell which is a precursor that is shared by all classical dendritic cells. Pre-DCs become fully differentiated via binding Flt3 ligands, M-CSF, or GM-CSF. Flt3 ligands primarily drive $CD103^+$ dendritic cell differentiation whereas M-CSF and GM-CSF primarily drive $CD11b^+$ dendritic cell maturation [114, 117-119].

Although some markers are shared amongst all differentiated dendritic cells, each dendritic cell subset also has unique transcriptomes, phenotypes, and functions. Upon transition from pre-dendritic cells to fully differentiated dendritic cells there is up-regulation of cell surface CD11c and major histocompatibility complex II (MHCII). However dendritic cell and macrophages cross-express many cell surface markers, including CD11c, F4/80, and CD11b, making cellular distinction tricky especially during inflammation. However, the use of transcriptional analysis has revealed distinct markers of dendritic cells. For example, dendritic cells, but not macrophages, express Flt3, c-kit, CCR7, and Zbtb46 [112, 120].

Dendritic cell maturation

Once dendritic cells are differentiated in tissues they are still immature. Immature dendritic cells primarily induce immune tolerance. Immature differentiated dendritic cells primarily act as sentinels of the immune system. Dendritic cells mostly perform sentinel observation by constant high levels of phagocytosis, macropinocytosis, and endocytosis of environmental contents. Tissue dendritic cells also express a vast array of cell surface receptors which can sense insults.

Environmental variants are often sensed by pattern recognition receptors of which the toll-like receptors (TLRs) are the largest most well described family. Each TLR recognizes distinct stimuli [121]. Three major signaling pathways contribute to dendritic cell maturation downstream of TLR ligation: mitogen-activated protein kinase (MAPK), nuclear factor-kB (NF-κB), and interferon regulatory factors (IRFs) [122]. However, the nature of the specific TLR stimulus shapes dendritic cell activation via specific cell signaling pathways in order to induce a highly tuned immune response.

In general, upon dendritic cell encounter of an activating stimulus, immature dendritic cells become mature/activated and can elicit T cell activation. Immature dendritic cells express small amounts of MHCII and T cell co-stimulation molecules CD80 and CD86 compared to mature dendritic cells [116, 123-128]. Broadly, dendritic cell maturation results in enhanced dendritic cell antigen processing and presentation, expression of MHCII and T-cell co-stimulatory molecules CD80 and CD86, and migration to tissue draining lymph nodes via up-regulation of CCR7 [112, 126, 129-131]. Altogether the process of dendritic cell maturation results in dendritic cell-mediated naïve T cell activation in the lymph node. This is largely because dendritic cell maturation in tissues results in the upregulation of the lymph node homing molecule CCR7 on dendritic cells which responds to lymphatic endothelial expression of the chemokine CCL21.

Three signal hypothesis of dendritic cell activation of naïve T cells

Overall the activation of T cells requires 3 signals from the dendritic cell, and this results in a proto-typical T cell activation program. And describe the timing of this- brief- and generation of memory.

Signal 1 is the handshake

You can imagine signal 1 as a handshake between two individuals occurring in a room full of people. Two entities have come together and acknowledged each other with a physical interactions.

Specifically, signal 1 occurs when there is a specific interaction between a dendritic cell presenting a peptide antigen in the context of either major histocompatibility complex I (MHCI) or II with a $CD8^+$ or $CD4^+$ T cell, respectively, that specifically recognizes this combination of peptide and major histocompatibility complex (MHC). This is also called an interaction of T cell with a dendritic cell that carries its cognate antigen. MHC with antigen is recognized by the T cell receptor, which is composed of alpha and beta chains. The interaction between the T cell and dendritic cell is stabilized by either the $CD4^+$ or $CD8^+$ co-receptor, depending on the class of T cell [132]. Also, the T cell receptor complex is composed of CD3 and the T cell receptor, which together form the antigen recognizing region of a T cell. The T cell and dendritic cell often remodel their actin cytoskeleton in order to cluster the TCR, CD8/4, CD3 and MHC with antigen into what is called the immunological synapse [133, 134].

Downstream signaling emanating from the T cell receptor complex is responsible for T cell activation by signal 1. Engagement of a T cell with antigen-presenting cell sourced MHC bound antigen results in T cell phosphorylation of ITAMS in the T cell receptor complex by The Src

family kinases Lck and Fyn [135]. CD3 phosphorylation leads to the recruitment and phosphorylation of the adaptor protein Zap70. This leads to a cascade of downstream signaling events that in conjunction with signal 2 (discussed below) coordinate T cell activation. Signal 1 is not sufficient to lead to T cell activation. In fact, stimulating a naïve T cell only with signal 1 (TCR-antigen-MHC) in the absence of signal 2 results in a T cell that is unresponsive, broadly termed anergy [136, 137].

Signal 2 is the conversation

An introduction usually follows a handshake with a stranger at a party. The nature of this conversation shapes your opinion of the person you have just met. More specifically, this conversation may either be positive or negative, shaping all your subsequent interactions with the stranger you have just met. This information is similar to signal 2 between dendritic cells and T cells.

Positive regulators of Signal 2

Signal 2 is called T cell co-stimulation and this is essential for T cell activation. T cell receptor ligation in the absence of co-stimulation renders T cell anergic [138]. The molecules responsible for T cell co-stimulation were discovered by the use of a monoclonal antibody screen. It was found that monoclonal antibodies that disrupted CD28 binding to its ligands CD80 and CD86 blocked T cell activation and resulted in T cell anergy, similar to what is observed when only signal 1 is delivered [139]. Specifically, CD28 is expressed by the T cell and interacts with CD80 and CD86 on the dendritic cell surface.

CD28 ligation in T cells results in the activation of downstream signaling pathways that coordinate T cell activation by enhancing T cell survival and proliferation. In short CD28 ligation leads to phosphoinositide 3-kinase (PI3-K) activation, generation of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) lipids, recruitment of protein kinase B (Akt) and activation of glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), cAMP response element binding (CREB), B-cell lymphoma 2 (Bcl-2), Bcl-2 associated death promoter (BAD), B-cell lymphoma-extra large (Bcl-XL), the forkhead box (FOX) O family of transcription factors, and Nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) [135]. Therefore, PI3K signaling downstream of CD28 ligation promotes cell cycle progression, cell survival, alterations in cellular metabolism, and Interleukin-2 (IL-2) transcription. The primary consequences of these events are enhanced T cell survival and proliferation.

Negative regulators of Signal 2

Altogether this simple paradigm described above assumes that dendritic cell sourced CD80 or CD86 can similarly deliver signal 2 during naïve T cell activation. However, it has become clear that dendritic cell CD80 and CD86 have different propensities to support T cell CD28 stimulation (signal 2). This is primarily because these ligands also bind T cell CD152 (CTLA-4), which is a T cell negative regulator. This biology deserves a review since I have found that mammary dendritic cell expression of CD80 and CD86 are distinctly regulated by reproductive state, supporting potentially divergent functions in regulating T cell responses based on reproductive state.

CD80 and CD86 are dendritic cell receptors that can bind T cell CD28 to lead to T cell activation (explained above) but can also bind T cell CTLA-4 to lead to T cell inhibition [140, 141]. The divergent functions of CD80 and CD86 lie in their differential abilities to bind T cell CD28 (activating) and CTLA-4 (inhibiting). Both CD80 and CD86 have enhanced affinity for CTLA-4 in comparison to CD28, as a means to shut off co-stimulation upon sufficient T cell activation. However, CD80 has higher affinity and avidity for CTLA-4 compared to CD86, indicating a more T cell suppressive role of CD80 compared to CD86 [141]. In addition to CD80/CD86-CTLA-4 there are other negative regulatory interactions to contradict signal 2.

Aside from CD80 and CD86 also having the capability to transmit negative signals to T cells, other negative dendritic cell -T cell interactions exist. Dendritic cells can express PD-L1 which can bind to T cell PD-1 and this interaction limits co-stimulation by dampening T cell activating signals [142, 143]. In sum, Signal 2 is the integration of negative and positive influences which together determine the strength of T cell co-stimulation which translates into a relative amount of T cell survival and proliferation [144].

Signal 3 is an influence of the room full of people

Signal 3 is an input from the environment on the feelings you have about meeting a stranger at a party. For example, if the people in the room were all happy versus angry this would influence your final perceptions and future actions concerning the stranger you just met.

Although Signal 2 has been shown to control T cell survival and proliferation it is clear that the generation of specific T cell response, such as Th1 or Th2, require additional signals. Signal 3

provides information to regulate T cell polarization and acquisition of unique programs tuned for the specific inflammatory insult, for example the generation of Th1 and cytotoxic responses in viral infection. Signal 3 is extremely diverse and is mostly required for T cell polarization, also known as differentiation. $CD4^+$ T cells, also called helper T cells, can be polarization to several distinct differentiation states: Th1, Th2, Th9, Th17, Tfh, and Treg, although more subsets are being discovered all the time. These $CD4^+$ T cells are specialized for unique roles in providing immunity against viruses and intracellular bacteria (Th1), parasites (Th2 and Th9), and extracellular bacteria and fungi (Th17). Treg skewed $CD4^+$ T cells suppress T cell proliferation and cytokine production and are critical in the maintenance of central and peripheral tolerance. For the most part the cytokines which drive the polarization of these distinct $CD4^+$ T cell subsets are well defined: Type I IFN and Interleukin-12 (IL-12) drive Th1; Interleukin-4 (IL-4) and TGF-β drive Th2 and Th9; Interleukin-6 (IL-6), TGF-β, Interleukin-21 (IL-21), and Interleukin-23 (IL-23) drive Th17; Interleukin-21 (IL-21) and Interleukin-27 (IL-27) drive Tfh; TGF-β and retinoic acid drive Tregs [135, 145, 146]. These cytokines are provided by the dendritic cell or the tissue environment in which T cell activation is occurring. $CD4^+$ T cell polarization is highly regulated by master transcription factors. For example, the transcription factor Tbet is essential for Th1 $CD4^+$ T cell development; Gata3 for Th2 and Th9; RORγT for Th17; Bcl-6 for Tfh; and FoxP3 for Treg [135].

Signal 3 also drives the functional specialization of $CD8^+$ T cells, termed cytotoxic T cells. $CD8^+$ T cell polarization occurs in response to cytokines and is supported by the transcription factors Tbet and eomesodermin [135]. Tbet supports cytotoxic effector functions and eomesodermin

supports long-lived memory. Cytokines affect the balance of these transcription factors, and thus the amplitude of cytotoxic response.

Tolerogenic dendritic cells are common at mucosal surfaces during homeostasis

Mucosal organs at homeostasis largely promote immune tolerance, and this is majorly controlled by the activity of tolerogenic dendritic cells. It is clear that there are many mechanisms which support tolerogenic dendritic cells. Further, tolerogenic dendritic cells can promote immune tolerance through various mechanisms, leading to a high level of complexity. A summary will follow of the dominant theories of tolerogenic dendritic cells and their promotion of immune tolerance.

A dedicated tolerogenic dendritic cell subset has not been described despite attempts [147, 148]. Tolerogenic dendritic cells are most accurately described in terms of their function, which is promoting T cell tolerance. Despite lack of definitive markers of tolerogenic dendritic cells, much effort has been exerted in trying to understand tolerogenic dendritic cell characteristics. The simplest description of tolerogenic dendritic cells comes from Steinman and colleagues, who posed that tolerogenic dendritic cells are immature. Consistent with this argument, dendritic cells under steady state conditions exhibit mostly immature phenotypes [149]. And further, homeostatic dendritic cell maturation preferentially induces tolerance [126, 127, 150-153].

However, a potential conundrum of this simple hypothesis is that dogma state that dendritic cells need to be mature “enough” to be able to travel to lymph nodes, via CCR7, to interact with naïve T cells. In fact, there is experimental evidence that tolerogenic dendritic cells are mature

“enough” to travel to lymph nodes and deliver TCR stimulation (signal 1) in the absence of adequate co-stimulation (signal 2). A prime example of this paradigm occurs when dendritic cells ingest apoptotic cells. Immature dendritic cells can uptake apoptotic cells via αVβ3 and αVβ5 integrins [154] and other cell surface molecules [155]. In this scenario dendritic cells are not fully matured because there is suppression of NF-κB activation in the absence of TLR and cytokine receptor ligation. The resulting partially mature dendritic cell results in T cell tolerance to apoptotic cell antigens [156-160].

In sum, the discussion to this point supports the hypothesis that tolerogenic dendritic cells are either immature or partially mature. However, this hypothesis has proved to be too limited, as fully mature dendritic cells can also have tolerogenic functions. Albert and colleagues showed that fully matured dendritic cells can also induce tolerance [161]. They showed that dendritic cells matured by *in vitro* treatment with tumor necrosis factor and prostaglandin E2 expressed high levels of maturation markers, including CD86, but still resulted in the deletion of responding T cells, i.e. tolerance [161].

In support of tolerogenic dendritic cells being alternatively matured, it has been shown that dendritic cell maturation in the presence of a certain immune milieu induces mature but tolerogenic dendritic cells. For example, dendritic cell maturation in the presence of corticosteroids, IL-10, and TGF-β preferentially results in tolerogenic functions [162]. In support of the inhibitory effects of IL-10, dendritic cells are known to down-regulate the Interleukin-10 receptor (IL-10R) upon TLR activation in order to evade the effects of this immunosuppressive cytokine [124, 163]. This hypothesis may represent the primary mechanism by which mucosal

tissues at homeostasis promote tolerogenic dendritic cells and immune tolerance. Dendritic cells located in the lung or gut, bona fide mucosal organs, are under the constant influence of IL-10 and TGF-β, an immune milieu that promotes tolerogenic dendritic cells in order to avoid pathologic inflammation. Thus dendritic cells in these environments preferentially promote tolerance rather than immunity [164, 165]. Another way to say this is that the threshold for immune activation is higher in mucosal tissues. Importantly, immune tolerance has not been definitely described in the mammary gland. However, IL-10 and TGF-β are greatly up-regulated during lactation and involution, supporting the prediction that immune tolerance may be greatest during these reproductive stages.

Dendritic cell subsets as they relate to immune activation and tolerance with a specific focus on mucosal organs

Another layer of complexity in studying tolerogenic dendritic cells is that the individual dendritic cell $CD11b^+$ and $CD103^+$ subtypes may have variable propensities for supporting immune activation or tolerance. $CD11b^+$ and $CD103^+$ dendritic cells differ in their routes of differentiation, antigen presentation, maturation, and function. However, it is important to keep in mind that distinct $CD11b^+$ and $CD103^+$ functions appear extremely context dependent.

Overall $CD11b^+$ dendritic cells primarily differentiate from monocytes through the action of GM-CSF and the IRF4 transcription factor [166-168]. $CD11b^+$ cells are inferior to $CD103^+$ dendritic cell subsets in antigen cross presentation on MHCI (to activate $CD8^+$ T cells). For this reason, it has been postulated that $CD11b^+$ dendritic cells primarily regulate $CD4^+$ T cell responses through antigen presentation on MHCII [169]. $CD11b^+$ dendritic cells express TLRs 1, 2, 4, 5, and 6 which recognize bacteria and flagella primarily and can lead to dendritic cell

maturation [126]. Altogether this supports CD11b$^+$ dendritic cells to be specialized for CD4$^+$ T cell mediated bacterial and parasite defense. Although of course exceptions to this simple conclusion are abundant.

CD103$^+$ dendritic cells are primarily generated from pre-dendritic cells through the action of Flt3L and activation of IRF8 and Batf3 transcription factors [166]. However, Batf3 has been termed the “master regulator” of CD103$^+$ dendritic cell differentiation [170]. In general, the CD103$^+$ dendritic cell subset is superior to CD11b$^+$ in antigen cross presentation onto MHCI which allows for CD8$^+$ T cell activation. CD103$^+$ dendritic cells uniquely express TLR11 and 3, and only lowly express TLR4, 7, or 8. Viral double stranded RNA binds TLR3 and causes CD103$^+$ dendritic cell maturation, MHCI antigen presentation, CD8$^+$ T cell activation, and potent anti-viral immunity [126, 170]

The generalizations above must again be softened, because the specific propensity for CD11b$^+$ and CD103$^+$ dendritic cell subsets is extremely context dependent. Further, the distinction between CD11b$^+$ and CD103$^+$ dendritic cells has been near impossible until the recent generation of specific research tools. A good example of this complexity is the role of CD103$^+$ dendritic cells in anti-tumor immunity via the generation of potent cytotoxic CD8$^+$ T cells activity [14, 130, 170-172] versus the central role of gut CD103$^+$ in promoting immune tolerance in the gut [173, 174].

In sum, while dendritic cell subsets may have a propensity for eliciting immunity towards specific pathogens, exceptions to these “rules” occur almost as frequently as the “rules”. Therefore, it is imperative to delineate the functions of dendritic cell subsets in each model

system. This can be achieved through the use of mouse models that specifically lack $CD11b^+$ (IRF4 KO) or $CD103^+$ dendritic cells (IRF8, Batf3, or Xcr1 KO).

Treg vs Th17 balance regulates the homeostatic gut

Although all $CD4^+$ T cell subsets can be found in mucosal tissues, T regulatory (Tregs) and T-Helper 17 (Th17) cells specifically predominate during homeostasis [146, 175]. In general, Tregs are favored during homeostasis and exert immune tolerance. When insults occur, Th17 $CD4^+$ T cell differentiation is favored in order to up-regulate barrier function. This represents a balance in the gut between tolerance and being poised to activate an immune response.

The balance between $CD4^+$ Tregs and Th17 can be explained by the fact that TGF-β can polarize both Tregs and Th17 $CD4^+$ T cells. TGF-β is very abundant in the intestine and as a single agent supports Treg development [176]. However, TGF-β in combination with the pro-inflammatory cytokines Interleukin-6 (IL-6), Interleukin-1B (IL-1B), or Interleukin-21 (IL-21) favors Th17 $CD4^+$ T cell differentiation [177]. It is also clear that specific dendritic cell subsets in the intestine may favor Treg or Th17 generation. $CD103^+$ dendritic cells are specifically involved in the generation of Tregs in the gut [173] whereas $CD11b^+$ dendritic cells specifically induce Th17 $CD4^+$ T cells [178-180].

Tregs constitute 30-40% of all $CD4^+$ T cells in the gut and are essential to the maintenance of homeostasis. The functional consequences of Treg polarization are overall immune suppression. Specifically, Tregs suppress T cell activation, production of Th1 and Th2 cytokines, proliferation, and survival. Many of these effects are mediated by Treg interruption of IL-2

production during T cell activation [181]. Loss of Treg-derived cytokines IL-10 [182] and TGF-β [183] result in spontaneous intestinal inflammation in mice. Further, in mouse models where intestinal inflammation is induced, transfer of Tregs results in disease amelioration [184, 185].

The simple paradigm of Tregs suppressing immune activation has become much more complex as of late. It is now clear that Tregs exhibit plasticity and can give rise to either homeostasis or inflammation. FoxP3 expressing cells in the gut can also express STAT3, an essential regulator of Th17 differentiation, Tbet, the Th1 polarization transcription factor, and Gata3, the Th2 polarization transcription factor [186-189]. This observation supports the existence of Treg functional plasticity in response to alterations in tissue immune milieu.

Similar to Tregs, Th17 $CD4^+$ T cells exhibit profound plasticity and are capable of directing either homeostatic or inflammatory programs. Some of the first lines of evidence showed that Th17 support pathogenic inflammation, leading to the generation of dogma that Th17 T cells were primarily pathogenic. Th17 $CD4^+$ T cells and their associated cytokines, Interleukins 17A, 17F, 21, and 22, are implicated as drivers in various autoimmune diseases included psoriasis, inflammatory bowel disease, rheumatoid arthritis, type I diabetes, and multiple sclerosis [190]. Th17 promotes autoimmunity in similar mechanisms to what has been described for Th1, with a primary role for IFNγ in tissue damage [191]. However, it has since become appreciated that Th17 cells also have important roles in maintaining homeostasis, especially in mucosal organs [98, 146, 175, 192].

Th17 CD4^{+} T cells are required for gut homeostasis because of their role in supporting barrier functions in mucosal organs. In mouse models lacking the Th17 effector cytokines, Interleukins 17A and 17F, there was a dramatic increased propensity for gut infection by *Staphylococcus aureus* and *Citrobacter rodentium* [193]. In a similar mouse model, Th17 and Th17 receptor-deficient animals have increased colonization by the fungus *candida albicans* (yeast). These results indicate that Th17 cells support tissue responses that limit infection by commensals. Th17 are through to limit infection by supporting barrier function in mucosal epithelium (reviewed in chapter above). In sum, Th17 dependent mechanisms largely promote homeostasis to commensal bacterium. However, in a game of chicken-and-egg commensal bacterium may also regulate Th17 CD4^{+} T cells.

Understanding how bacteria regulates Th17 CD4^{+} T cell generation in the gut could inform our studies of the mammary gland since bacteria is expected to be highly dependent on reproductive state, with lactation representing a peak in bacterial load. Ivanov and colleagues show that Th17 appears in animals as they are colonized with commensal microbiota, and this can be inhibited by antibiotic treatment [194]. Further, segmented filamentous bacteria (SFB) were shown to bind to intestinal epithelial cells and lead to the up-regulation of serum amyloid A protein (SAA) [195]. SAA in the presence of reactive oxygen species programmed dendritic cells to produce IL-6 and IL-23 which supported the generation of Th17 CD4^{+} T cells [196]. Altogether these studies suggest that commensal bacteria can induce dendritic cell generation of Th17 CD4^{+} T cell differentiation and this can contribute to increased mucosal barrier function and homeostasis.

Acute Th1 at mucosal surfaces for the elimination of bacteria and viruses versus chronic Th1 in autoimmunity

Th1 $CD4^+$ T cells are polarized by IFNγ and Interleukin-12 (IL-12) via activation of Stat1 and Stat4 signaling pathways and the master transcription factor Tbet. Th1 $CD4^+$ T cells specifically produce IFNγ, an effector cytokine with direct effects on bacteria, viruses, and cells of the host immune system to result in pathogen clearance (REF). Upon successful pathogen clearance, acute Th1 programs undergo contraction and are quieted via Th2 and Treg immunity.

However, aberrant Th1 activity drives tissue destruction common in autoimmune disease. Neutralization of the Th1 effector cytokine, IFNγ, can prevent the onset of chronic IBD in a mouse model, implicating Th1 in the onset of this pathology [184, 197]. This finding is confirmed by studies that transferred naïve T cells deficient in Th1 polarization failing to develop disease in a mouse model of colitis [198, 199]. Relevance to human disease is found in the accumulation of Th1 cells in crohn's disease patients. In sum, Th1 responses in a mucosal tissue, the gut, support eradication of infectious agents and this pathway is chronic in autoimmune disease.

Th2 immunity in the gut mostly regulates defense to parasites

Th2 polarized $CD4^+$ T cells are programmed by TGF-β and IL-4 and produce IL-4. These specialized T cells are absent from parasite-free mice [200]. This observation indicates that parasites specifically lead to the development of Th2 in the gut. Th2 $CD4^+$ T cells coordinate the activities of eosinophils, mast cells, basophils, production of IgE, M2 macrophage polarization, and activated fibroblasts. Together these effects elicit anti parasite immunity by inducting parasite stress and mechanisms that result in parasite physical removal [201].

However, as with all specific axes of immunity, if left unregulated aberrant Th2 responses can result in immunopathology. Tregs are a natural negative regulator of Th2 responses. In fact, Treg deficiency can result in aberrant Th2 inflammation in the gut and lung [202]. Overall Th2 immunity occurs in the gut in the specific context of responding to and clearing parasitic infections and can lead to pathology if not properly regulated.

The integrated mucosal organ system and shared immunity

While it is clear that mucosal organs independently execute programs to support barriers and immune tolerance, there is also a crosstalk between mucosal organs which deserves consideration. Most recently this has been termed the mucosal immune system which is an integrated view where mucosal organs share information in order to better protect the organism [203]. One such example is the finding that antibiotic use in neonates is associated with an increased risk of developing asthma [204]. These data indicate that gut microbiome and mucosal biology may impact the lungs, although the mechanisms are unclear. Another example is the finding that intranasal immunization results in vaginal protection from infection by herpes simplex virus type 2 [205]. These examples show the interconnectedness of mucosal organs, and this is supported by the finding of shared homing molecules. Evidence for the mammary gland as a component of the integrated immune system is the finding that IgA^{+} antibody producing cells influx the mammary gland from the gut during lactation [20]. Future investigation of the mammary gland as a member of the mucosal organ system is sure to result in paradigm shifting research work in the fields of development, immunology, and cancer.

Applying mucosal immunology hallmarks to the mammary gland in our current study

The mammary gland is a hormonally regulated organ that relies on immune cells for postnatal development. However, overarching dominant immune programs have not been described in the mammary gland. We postulate that the mammary gland is a mucosal organ because of external exposure and evidence of some mucosal hallmarks, such as epithelial cell junctions, mucins, and antimicrobial molecules. In our current study we have interrogated the mammary gland as a mucosal organ. Specifically, we have focused on studying immune tolerance during homeostasis, as this is a central theme in mucosal organs. Further, we inquire if mammary gland mucosal function may be dynamic with mammary gland developmental state. The results of our studies are expected to lead to significant gains in knowledge in the fields of mammary gland development, mucosal immunology, mucosal organ systems, and breast cancer.

Chapter IV: MANUSCRIPT ENTITLED "DEFINING THE MAMMARY GLAND AS A MUCOSAL ORGAN"

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Title: Defining the mammary gland as a mucosal organ

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Abstract

The mammary gland is not classically considered a mucosal organ even though it exhibits some features common to mucosal tissues. Notably, the mammary epithelium is contiguous with the external environment, is exposed to bacteria during lactation, and displays antimicrobial features. Nonetheless, immunological hallmarks predictive of mucosal function have not been demonstrated in the mammary gland, including immune tolerance to foreign antigens under homeostasis. This inquiry is important, as mucosal immunity in the mammary gland likely assures infant and woman's health during lactation. Further, such mucosal immune programs may protect mammary function at the expense of breast cancer promotion via decreased immune surveillance. Here, using murine models, we evaluated for mammary specific mucosal attributes focusing on two reproductive states at increased risk for foreign and self-antigen exposure: lactation and weaning-induced involution. We find a baseline mucosal program of Th17 $CD4^{+}$ T cells that is elevated within lactating and involuting mammary glands and extended to include tolerogenic dendritic cell phenotypes, barrier-supportive antimicrobials and immunosuppressive Treg $CD4^{+}$T cells. Further, we demonstrate suppression of antigen-dependent $CD4^{+}$ T cell activation, data consistent with immune tolerance. We also find antigen-independent, accumulation of memory Th17- Treg $CD4^{+}$ T cells specifically within the involution mammary gland, consistent with inflammation. Overall, these studies support the conclusion of strong mucosal immune programs within lactating and involuting mammary glands. Our findings support the classification of the mammary gland as a mucosal organ and open new avenues for exploration into breast pathologies including compromised lactation and breast cancer.

Impact statement

Elucidation of developmentally regulated mucosal immune programs within the lactating and involuting mammary glands reveals T cell tolerance which may limit autoimmunity at the potential expense of breast cancer progression.

Introduction

Under conditions of homeostasis, classical mucosal organs such as the skin, lung, and gut harbor unique immunological properties in which epithelial and immune cells function as a unit to protect the organ from external insult [203]. Specifically, Th17 $CD4^{+}$ T cells and various antimicrobial products support epithelial barrier function and limit infection [206]. Another key attribute of mucosal immunity is the presence of tolerogenic dendritic cells and T regulatory $CD4^{+}$ T cells, which promote immune tolerance and dampen response to frequently encountered antigens [207, 208]. While not classically considered mucosal, the mammary gland is a skin appendage [1] and exposure to the external environment is evident by increased risk of infection during nursing. Further anecdotal evidence for mucosal classification is the dependence of the mammary epithelium on immune cells during development. Specifically, dendritic cells and $CD4^{+}$ T cells coordinate pubertal branching [13], and macrophages are essential for pregnancy dependent alveolar expansion [8] and weaning-induced epithelial cell death [40]. Because of these potential mucosal attributes, we elected to systematically study the murine mammary gland using a mucosal immunology framework. We focused on two developmental states that impact infant and mother health: lactation and weaning-induced mammary gland involution. This work may lead to new avenues of investigation into lactation failure and postpartum breast cancer, two critical and understudied public health concerns [52, 53, 209].

To date, studies supportive of mucosal biology in the mammary gland have focused on lactation, as an increased risk of mastitis in dairy cows is a significant health as well as economic problem. One proposed mechanism of increased infection in lactating cows is active immune suppression, a biology that would be consistent with mucosal function. However active immune suppression has not been explicitly demonstrated [32, 210, 211]. Further, immune suppression is not the only

possible explanation for increased infection rate in lactating cows. Notably, heightened pathogen exposure due to teat damage from mechanical milking [212] could contribute to increased infection rates independent of immune suppression. Overall, studies from the bovine field describe the mammary gland's exposure to the external pathogens, however the role of other mucosal attributes including immune tolerance have not been demonstrated.

Supporting evidence for mucosal function in the lactating mammary gland has also been reported in the context of human neonatal health and corroborated in murine studies. Specifically, expression of antimicrobial molecules found at mucosal epithelial borders, including IgAs and mucins, are present in milk [23, 104, 213]. In mice, milk IgA is the product of developmentally regulated antibody producing cell influx into the mammary gland via the chemokine CCL28 [23]. Importantly, milk IgA has been demonstrated to play a critical role in maintenance of infant gut health by providing maternal-derived antimicrobial function [21, 22]. However, it is unknown if IgA also plays a protective, antimicrobial role in the lactating mammary epithelium, a role consistent with mammary mucosal function. Indeed, mammary epithelium may require additional barrier function and immune tolerance because of the bioactive components of milk, including lactoferrin, bacteria and leukocytes [18]. Thus, while there is strong rationale for proposing the presence of mucosal immunologic programs in the lactating gland, definitive demonstration is lacking, especially for active induction of immune tolerance.

In contrast to lactation, the reproductive state of weaning induced involution has not been studied in the context of mucosal immunology. Weaning is a developmentally regulated process characterized by the death of ~80-90% of secretory mammary epithelial cells followed by wound healing-like tissue repair and immune cell influx [42, 44, 77, 214]. We predict that weaning induced mammary gland involution will be characterized by mucosal immune features similar to

but likely distinct from lactation. One rationale for this is that the risk of self-antigen exposure as a consequence of epithelial cell death likely necessitates the mucosal hallmark of immune tolerance. Further, tissue restoration and maintenance of barrier function are expected to be particularly prominent during involution. Study of the involuting mammary gland with a mucosal framework will improve understanding of baseline immune programs during involution, and may inform the development or progression of postpartum breast cancer, a particularly life-threatening form of young women's breast cancer [52].

In this study, we find evidence for baseline mucosal features within the mammary gland at all reproductive stages evaluated. This is evidenced by immature dendritic cell populations and phenotypes as well as Th17 skewed T cells within the nulliparous mammary gland. Further, during lactation we find evidence for enhanced mucosal features, chiefly the presence of dendritic cells with tolerogenic phenotypes and decreased antigen-dependent T cell expansion. This immunologic program is anticipated to assure successful lactation by providing enhanced protection against bacterial penetration and suppressing autoimmunity. With weaning we find an extension of immune tolerance with the addition of immune regulatory programs, including the inflammation-based recruitment of memory Th17/Treg $CD4^+$ T cells. During involution these mucosal hallmarks are anticipated to provide immunosuppression and tissue repair. Unknown is whether these mucosal attributes might also inhibit anti-tumor immune surveillance.

In sum, the data presented in this study support the classification of the mammary gland as an immune-tolerant mucosal organ. Further investigation of the mammary gland within a mucosal framework may lead to new understanding of lactation failure and provide insight into known links between lactation, involution and breast cancer risk.

Materials and Methods

Human breast tissue collection

Formalin fixed tissues and paraffin embedded breast tissue specimens from premenopausal women who underwent clinically indicated biopsies were obtained with OHSU IRB approval. All cases were de-identified to the research team.

Preclinical mouse models and tissue collection

Oregon Health & Science University IACUC approved all mouse procedures in compliance with current NIH guidelines. In order to obtain mice of various reproductive stages, female Balb/c were outbred with male C57BL/6 mice (Jackson laboratories) in ventilated cages with 12 hr. light/dark cycles. For *ex-vivo* antigen cross presentation studies (described below) C57BL/6 females were mated to C57BL/6 males. Pup number was normalized two-three days' post parturition to 6-7 pups/dam in order to ensure equal lactational load across groups. Synchronized weaning was initiated at lactation day 9-14 (denoted involution day 0) to generate groups of animals for various postpartum (involution) time points. Age-matched nulliparous animals were either never bred or bred without detectable pregnancy. Mice were sacrificed across reproductive groups, with CO_2 and cervical dislocation, and tissues collected. Left and right #s 4 and 5 mammary glands, inguinal lymph nodes, and spleens were harvested and fixed in formalin for IHC applications, or placed in Hank's balanced salt solution (HBSS) on ice to be processed for *ex vivo* assays and flow cytometry.

H+E and Immunohistochemistry

Formalin fixed tissues were processed and paraffin embedded, sectioned to 4 um and subjected to hematoxylin and eosin staining or immunohistochemistry for CD45 (Clones 2B11^{+}PD7/26

Dako for human, Clone 30-F11 BD for mouse), CK18 (polyclonal, Abcam), or E-cadherin (24E10, Cell Signaling) and visualized with a DAB chromogen (Dako). H+E and IHC stained slides were scanned on an AperioScanScope AT and images captured using Aperio ImageScope Software (Leica Biosystems). CD45 IHC images were pseudo colored in Aperio using a color deconvolution algorithm where blue represents epithelium negative for DAB signal, and yellow, orange, and red represent increasing intensity of DAB staining (in $CD45^+$ immune cells).

Analysis of publicly available microarray data set

A publicly available data set of microarray gene expression in wild type Balb/c mice at various reproductive stages [213] was analyzed for various genes. Each data point represents the average of three replicates each composed of mammary glands from three Balb/c female mice.

Tissue digestion and staining for flow cytometry

Fresh tissues were collected as above and finely minced, then digested with collagenases II and IV (2.5 mg ml^{-1} Worthington) and DNase I (0.5-2.5 mg ml^{-1} Worthington) in HBSS with calcium and magnesium for 30 minutes with agitation at 37C. Digests were filtered (100 µm) and red blood cells lysed (eBioscience) per manufacturer's instructions. Samples were counted and blocked with CD16/32 (eBioscience 1:100) and stained with Live Dead (aqua, Invitrogen 1:500) for 30 minutes, then washed 1x with PBS. Cells were stained with antibodies for extracellular proteins (CD45, 30-F11; MHCII, M5/114.15.2; B220, RA3-6B2; CD11b, M1/70; CD11c, N418; F480, BM8; Ly6C, HK1.4; Ly6G, 1A8; CD103, 2E7; CD80, 16-10A1; CD86, GL-1; CD4, RM4-5; PD-1, 29F.1A12; CD3e, 145-2C11) diluted in buffer (1% BSA in 1x PBS) for 30 minutes at RT. Cells were fixed (BD cytofix) and permeabilized (eBioscience) per

manufacturer's instructions and stained with antibodies for intracellular proteins (Gata3, 16E10A23; RORγT, AFKJS-9; FoxP3, FJK-16s), diluted in permeabilization buffer (eBioscience) for 2 hours at 37C or overnight at 4C. Cells were fixed again and run on an 18 color flow cytometer (Fortessa BD) in the OHSU flow cytometry shared resource. Data were analyzed using FlowJo software.

Ex vivo antigen uptake and processing assays

Mammary digests from reproductively distinct mice were incubated for 4 hours at 37C with Alexa-Fluor 488 labeled ovalbumin (100 µg ml^{-1} ThermoFisher), or DQ-ovalbumin (100 µg ml^{-1} ThermoFisher), to test antigen binding/uptake and antigen processing, respectively. Cells were stained with extracellular antibodies (CD11c, N418; F480, BM8; Ly6C, HK1.4; MHCII, M5/114.15.2; CD45, 30-F11) and flow cytometry performed.

Ex vivo antigen cross presentation assay

C57BL/6 females were used for this assay in order to utilize a strain specific antibody (eBioscience clone eBio25-D1.16) that can specifically detect ovalbumin antigen peptide (SIINFEKL) loaded into MHCI (Kb). Mammary digests were incubated with unlabeled whole ovalbumin protein (100 µg ml^{-1}) for 4 hours at 37C, then stained with extracellular antibodies (same as above with the addition of eBio25-D1.16) and flow cytometry performed.

Adoptive transfer *in vivo* T cell experiments

$CD4^+$ splenocytes were isolated from DO11.10 transgenic female mice (Jackson Laboratories Stock #003303 - Stock Name C.CG-Tg(DO11.10)10Dlo/J) and enriched (to >95%) using a $CD4^+$

negative selection kit (MACS miltenyi). $CD4^+$ T cells were either unstimulated (putative naïve T cells), or stimulated for 10 days *in vitro* with plate bound CD3 (2 μg ml^{-1} eBioscience) and CD28 (5 μg ml^{-1} eBioscience) in IMDM base media containing TGF-β (1 ng ml^{-1}), IL-1B (10 ng ml^{-1}), and IL-6 (50 ng ml^{-1}) [215]. The method of activation generates resting effector memory T cells [216], referred to as memory T cells herein. The resulting T cell pool was >95% $ROR\gamma T^+$ $FoxP3^+$ (Th17/Treg) (Supplementary Figure 4E). Naïve and memory T cells were adoptively transferred (150,000 T cells) via tail vein into syngeneic involution Day 0 or 4, lactation day 10, regressed 6 weeks, or nulliparous age-matched hosts. Two days later ovalbumin protein antigen (10 μg in 10 μl, Worthington) was injected into either the left or right #4 mammary fat pad, and the opposite mammary fat pad received equal volume PBS. Five days later tissues were harvested, digested and stained with extracellular antibodies (CD45, 30-F11; CD4, RM4-5; D011.10 TCR, KJ1-26) and intracellular antibodies (Gata3, 16E10A23; RORγT, AFKJS-9; FoxP3, FJK-16s) as described above. A known quantity of absolute counting beads were added to samples (C36950 Invitrogen) and flow cytometry performed. Naïve or memory T cell counts in inguinal LNs, mammary glands, and spleens were calculated by normalizing to the known abundance of counting beads. Data are either represented as transgenic T cell number, or by ratio of T cell number (with antigen: without).

Statistics

If not indicated otherwise data are represented as mean ± s.e.m. Significance was determined by performing one-way ANOVA, two-way ANOVA, or two-tailed unpaired or paired T tests in GraphPad Prism. Statistical test employed and key for significance values are described in each Figure legend.

Results

$CD45^+$ immune cells and antimicrobial barrier function in the normal mammary gland

Close physical interactions between epithelium and immune cells are found at mucosal surfaces, hence we predict this physical proximity in the normal mammary gland. Here we find, even in quiescent nulliparous glands, that $CD45^+$ leukocytes are closely associated with mammary epithelium ($CK18^+$ or E-cadherin$^+$) in human (Figure 1A) and murine (Figure 1B) mammary glands. These observations support mucosal biology and are consistent with previous reports [38, 44, 217]. We next assessed if $CD45^+$ abundance within the mammary gland changes with reproductive state, as the murine mammary gland structure and function are dynamic across the reproductive cycle. The nulliparous murine mammary gland is composed of sparse epithelium, an adipose-rich stroma, and a collagen-rich extracellular matrix [44, 218]. During pregnancy, the epithelium proliferates, differentiates, and replaces the fat pad. With lactation, glandular epithelium dominates and milk is secreted into the ductal lumen. Upon weaning, up to 90% of the secretory epithelium undergoes programmed cell death and the fat pad re-emerges in a tissue-remodeling process referred to as involution. By histologic criteria, involution is essentially complete by 8-10 days post weaning, with 6 weeks post-weaning considered a full return to a pre-pregnant like state [213, 219-221]. Of note, similar hormone dependent structural and functional changes occur in the human breast [38].

As determined by two distinct methods, IHC staining (Figure 1C) and flow cytometry (Figure 1D-E), we observe a decrease in abundance of total immune cells ($CD45^+$ as % of live cells) during lactation and an increase during involution (Figure 1E and Supplementary Figure 1A), confirming regulation of mammary immune cell abundance by reproductive state.

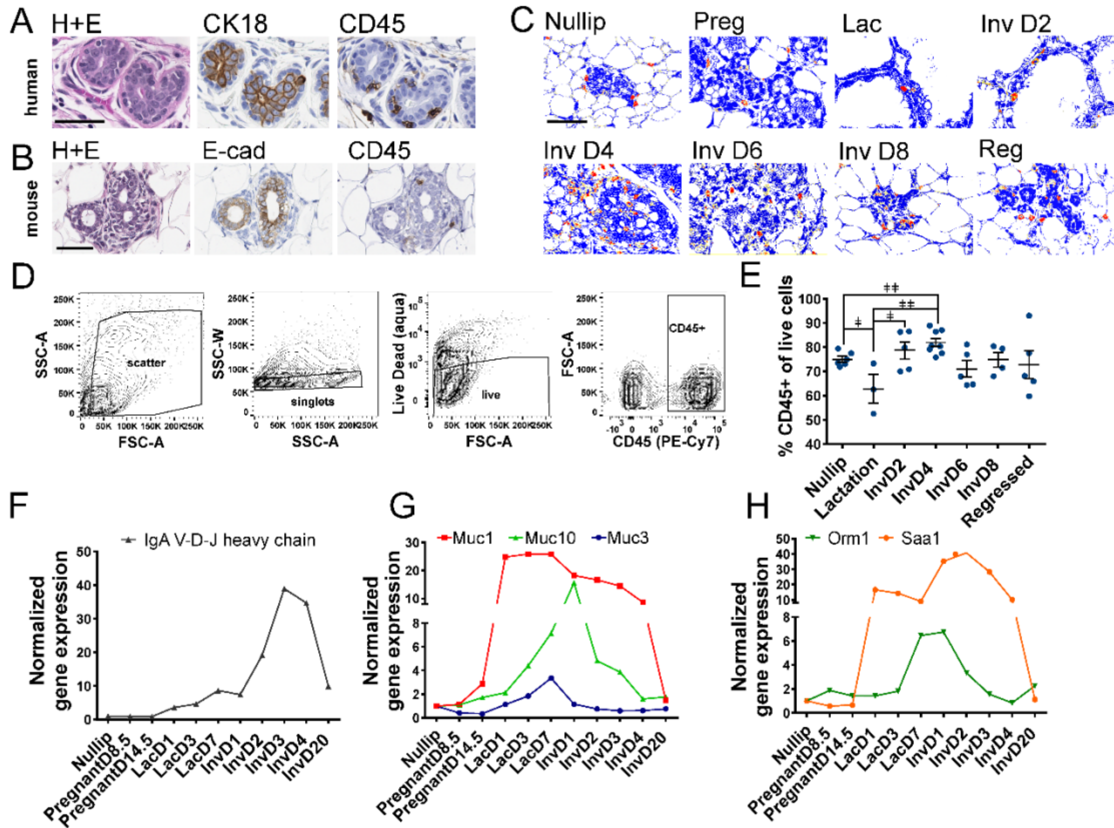

Figure 1: Evidence for coordinated mammary epithelial and immune cell regulation across a reproductive cycle. (A) H+E and IHC for CK18 and CD45 in human nulliparous breast tissue (40x images, scale bar equals 50 μm). (B) H+E and IHC for E-cadherin and CD45 in nulliparous mouse mammary tissue (40x images, scale bar equals 50 μm). (C) Immunohistochemistry for CD45 in mouse mammary tissue from Balb/c nulliparous (Nullip), pregnant Day 16 (Preg), lactation Day 10 (Lac), involution days 2, 4, 6, 8 (Inv D2, 4, 6, 8) and 6 weeks regressed (Reg) animals (40x images, scale bar equals 50 μm). Color deconvolution using an algorithm on Aperio ScanScope was used where blue indicates negative epithelium and yellow, orange, and red increasing intensities of DAB signal (CD45). (D) Identification of live CD45+ cells in mammary digests from a nulliparous host by flow cytometry. (E) Quantitation of total %CD45+ of all live cells in mammary digests from distinct reproductive hosts. N=3-7 per group, ‡ indicates $p < 0.05$ and ‡‡ $p < 0.01$ by two-tailed unpaired student's T test. (F-H) Expression of mucosal mRNAs during lactation and involution, (F) IgA heavy chain, (G) mucins 3, 10, 1 (Muc), and (H) orosomucoid 1 (Orm1) and serum amyloid A1 (Saa1). Data gathered from publicly available microarray data set, and normalized to expression in nulliparous tissue.

To further investigate the mucosal state of the mammary gland, we assessed for expression of various mRNA transcripts associated with mucosal function using publically available data [213]. We find that IgA heavy chain mRNA is increased in the murine mammary gland during lactation (Figure 1F), as expected based upon previous characterizations of milk [18, 23]. Somewhat unexpectedly, we find even further increases in IgA heavy chain mRNA expression

post-weaning, suggesting extension and expansion of antimicrobial function with gland involution (Figure 1F). In addition, mRNA for mucins, which aid in barrier function, are dynamically but distinctly upregulated during lactation and involution (Figure 1G). Further, orosomucoid 1 (Orm1) and serum amyloid A1 (Saa1) mRNAs, acute early phase inflammatory mediators induced at liver and gut barrier surfaces [222, 223], are more abundant in lactating and involuting glands (Figure 1H). In total, these gene expression features are consistent with enhanced, but distinct barrier function in the mammary gland during lactation and involution.

Dynamic regulation of dendritic cell abundance and activation with reproductive state

Mucosal mediated immune suppression is dependent on active suppression of T cells by antigen presenting cells, of which tolerogenic dendritic cells are a major contributor. Thus, increased dendritic cell influx is anticipated under conditions of increased need for immune tolerance such as lactation and weaning. To assess for mammary dendritic cell abundance as a function of reproductive state, we utilized flow cytometry; mammary dendritic cells were identified as $CD45^{+}$, $CD11c^{+}$, and $MHCII^{+}$ and Ly6C-, Ly6G-, B220-, and F480- (Figure 2A, Supplementary Figure 1B). In nulliparous and lactating mice, the relative abundance of mammary dendritic cells were comparable, at ~2.5% of the total immune cell ($CD45^{+}$)

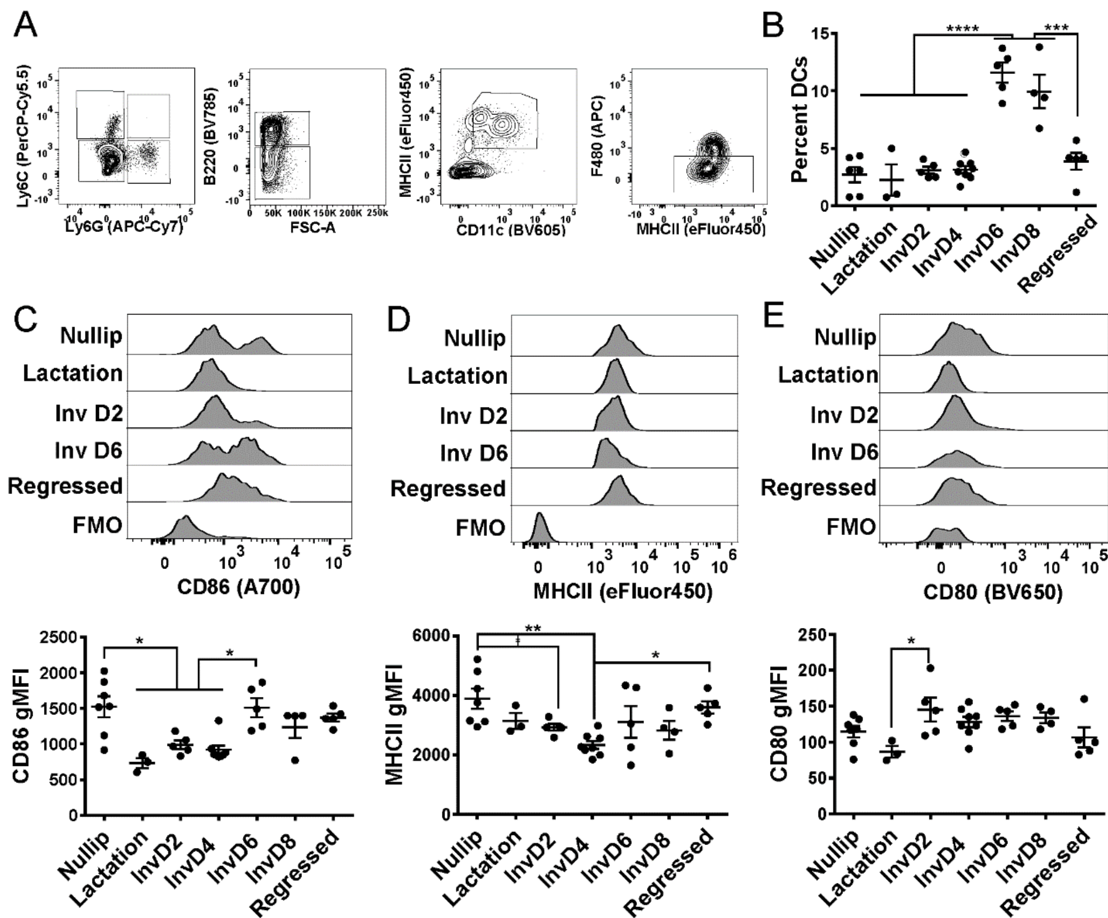

Figure 2: Mammary gland dendritic cell abundance and activation state are altered by reproductive state. (A) Representative flow gating schema to identify mammary dendritic cells in nulliparous mouse mammary digest, inguinal LN removed. Gates downstream of CD45+ cells (Figure 1E) are shown. (B) Quantitation of total DCs (CD45+ Ly6C- Ly6G- B220- F480- CD11c+ MHCII+) as percent of total CD45+ cells. (C-E) Flow histograms and quantitation of DC activation markers CD86, MHCII, and CD80 as average fluoresence (gMFI) at various reproductive stages. N=3-7 per group, * indicates p <0.05, ** p <0.01, *** p <0.001 and **** p <0.0001 by one way ANOVA with Tukey's multiple comparison test and ‡ indicates p <0.05 by two-tailed unpaired student's T test. Nullip= nulliparous, and Inv D2, D4, D6, D8= Involution days 2, 4, 6, and 8 days post weaning.

population (Figure 2B). After weaning, dendritic cell abundance remained unchanged for 2-4 days, and then abundance increased by ~5 fold at 6-8 days post weaning, accounting for ~10% of the total immune cell population (Figure 2B and Supplementary Figure 1C). This increase in dendritic cell abundance was transient; at 6 weeks post-weaning (i.e., regressed state), dendritic

cell numbers returned to baseline, nulliparous levels (Figure 2B and Supplementary Figure 1C). The abundance of other myeloid immune cells including monocytes and macrophages were also altered by reproductive state (Supplementary Figure 2A-B), as previously reported [42, 44]. These results provide evidence, for the first time, that there are increased numbers of dendritic cells in the mammary gland during lactation and involution. We next assessed whether these dendritic cells are tolerogenic, as anticipated for a mucosal organ.

A hallmark of tolerogenic dendritic cells is decreased activation state as measured by reduced cell surface expression of MHCII and co-stimulatory molecules CD80 and CD86 [224-226]. Thus, we analyzed for the expression of these molecules by flow cytometry. During lactation, dendritic cell expression of CD86 and CD80 was greatly reduced. In the early post-weaning (Inv D2/D4) mammary glands CD86 remained reduced on dendritic cells and only returned to baseline nulliparous levels by InvD6 (Figure 2C, Supplementary Figure 1D). Dendritic cell expression of CD80 returned to nulliparous levels earlier, on InvD2. Unlike CD86 and CD80, MHCII expression was not diminished during lactation but was reduced during early involution (D2-4), before returning to nulliparous levels by InvD6 (Figure 2D). Cumulatively, these data indicate dendritic cells with tolerogenic phenotypes in the mammary gland during lactation and early stages of involution. These data also indicate distinct mechanisms of dendritic cell tolerance in the lactating, compared to the involuting mammary gland.

To further delineate the regulation of mammary dendritic cell populations by reproductive state the total mammary dendritic cell pool was evaluated for CD11b and CD103, markers that delineate dendritic cell subsets in non-lymphoid tissues [112]. Determining subset abundance is

important because CD11b and CD103 dendritic cells have differing propensities for T cell activation and tolerance [121, 173, 224, 227]. To our surprise, we found that ~50% of the

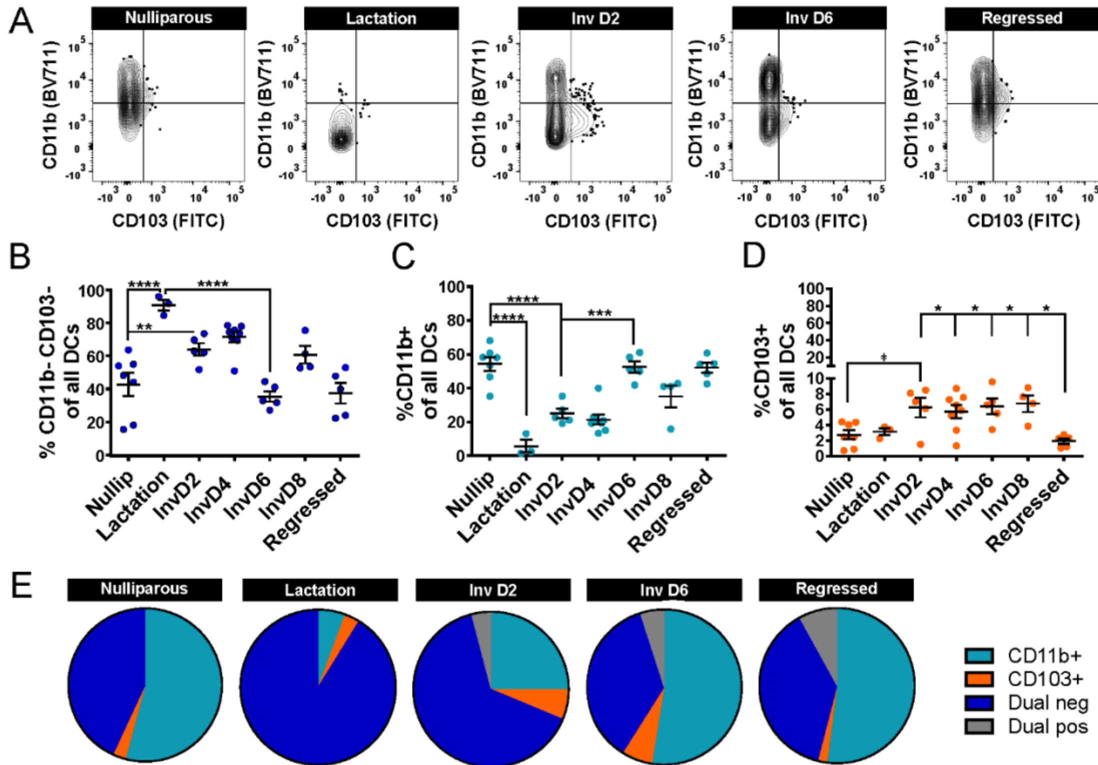

Figure 3: Dendritic cell subtypes are dynamically regulated during lactation and involution. (A) Representative flow plots from Nulliparous, Lactation, Inv D2, Inv D6, and Regressed hosts showing CD11b and CD103 expression in the total DC population (Figure 2B). (B) Quantitation of CD11b-/CD103- DCs (CD45+ Ly6C- Ly6G- B220- F480- CD11c+ MHCII+CD11b-CD103-), (C) CD11b+ DCs (CD45+ Ly6C- Ly6G- B220- F480- CD11c+ MHCII+CD11b+CD103-), and (D) CD103+ DCs (CD45+ Ly6C- Ly6G- B220- F480- CD11c+ MHCII+CD11b-CD103+) as percent of total DCs (Figure 2B). (E) Pie charts depicting average abundance of DC populations at various reproductive stages. N=3-7 per group, * indicates p <0.05, ** p< 0.01, *** p <0.001 and **** p <0.0001 by one way ANOVA with Tukey's multiple comparison test, and ‡ indicates p <0.05 by two-tailed unpaired student's T test. Nullip= nulliparous, and Inv D2, D4, D6, D8= Involution days 2, 4, 6, and 8 days post weaning.

dendritic cells in the nulliparous mammary gland did not express CD11b or CD103 (Figure 3A lower left quadrant). We confirmed that these dual negative dendritic cells (CD11b- and CD103-) are not Ly6C^{+} monocytes or F480^{+} macrophages (Supplementary Figure 2A-B). Further, these dual negative dendritic cells are likely not contaminated with plasmacytoid dendritic cells, which are reported to express PDCA-1 and low levels of CD11c (Supplementary

Figure 2C-D). During lactation and early involution, these dual negative dendritic cells constituted almost the entire mammary dendritic cell pool before returning to nulliparous, baseline levels by day 4 post-weaning (Figure 3B and E).

For the singly positive CD11b and CD103 dendritic cell populations, we also find dynamic regulation of abundance with varying reproductive state. Notably, $CD11b^+$ dendritic cells dominated over $CD103^+$ dendritic cells at all reproductive stages (Figure 3C-E), similar to subset ratios reported in the homeostatic gut [178]. Also, the abundance of mammary $CD11b^+$ dendritic cell during lactation was remarkably low and modestly increased during early involution, before returning to baseline levels by InvD6 (Figure 3A, C, E). In contrast, the $CD103^+$ dendritic cells, while a smaller subset of the overall mammary dendritic cell population, was increased during involution (Figure 3A, D, E). To better understand the potential functional status of these distinct mammary dendritic cells, we next assessed each dendritic cell subtype for expression of MHCII and CD80 and CD86 co-stimulatory markers.

We first assessed activation state in the dual negative (CD11b- and CD103-) dendritic cells. We observed relatively low levels of CD80 and CD86 expression in these cells at all reproductive stages, consistent with an immature state (Figure 4B-C left panels, Supplementary Figure 3A). However, during involution, we observed an even further depression of MHCII and CD86 expression in the dual negative dendritic cells (Figure 4A-B left panels). We next assessed the activation state in the $CD11b^+$ and $CD103^+$ dendritic cell subsets. Similarly, these dendritic cell subsets showed decreased activation states during lactation and early involution as measured by lower MHCII and CD86 expression (Figure 4A-B middle and right panels). Interestingly, during

lactation and involution, CD80 was enhanced in both CD11b^{+} and CD103^{+} dendritic cell subsets (Figure 4C middle and right panels). CD80 and CD86 are both ligands for CD28 and CTLA-4 (CD152) on T cells, with binding to CD28 leading to T cell activation and binding to

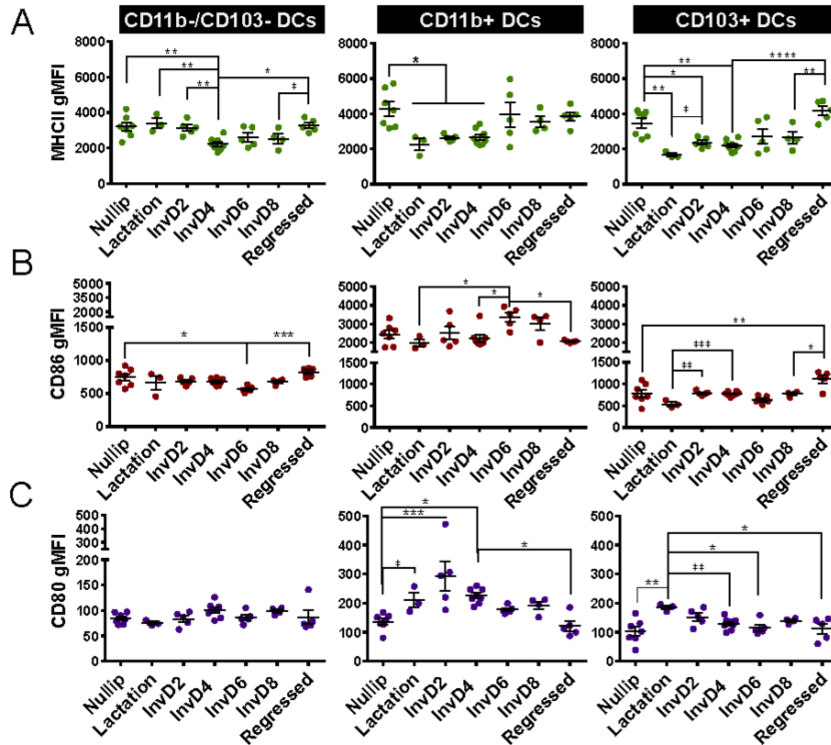

Figure 4: Reduced dendritic cell activation during lactation and involution. Quantitation of expression as measured by geometric mean fluorescence intensity (gMFI) of activation markers (A) MHCII, (B) CD86, and (C) CD80 in CD11b-/CD103- (left column), CD11b+ (middle column), and CD103+ (right column) DC populations. N=3-7 per group, * indicates $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ and **** $p < 0.0001$ by one way ANOVA with Tukey's multiple comparison test and ‡ indicates $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ by two-tailed unpaired student's T test. Nullip= nulliparous, and Inv D2, D4, D6, D8= Involution days 2, 4, 6, and 8 days post weaning.

CTLA4 leading to inhibition. Of note, compared to CD86, CD80 has increased affinity for CTLA-4 [141]. In this context CD80, which is elevated during involution, may be anticipated to lead to increased CTLA-4 binding and T cell inhibition. Combined, our data indicate a diverse population of tolerogenic dendritic cells in the mammary gland, with increased tolerogenic phenotypes during lactation and weaning-induced involution.

Dendritic cell antigen binding, uptake, and presentation are not blocked during lactation and involution

One mechanism by which dendritic cells can exert tolerogenic programs is through active T cell suppression, which is dependent on dendritic cell antigen uptake, processing and presentation. Thus, we assessed these functional capabilities in mammary dendritic cells from lactating and involuting glands in comparison to nulliparous glands. Lymph node free mammary gland digests prepared from nulliparous, lactating and involuting hosts were incubated with fluorescently labeled ovalbumin proteins. Dendritic cells were then assessed for antigen binding and uptake by fluorescent ovalbumin (AF488) signal, and for antigen processing by DQ-ovalbumin signal, which is produced in the lysosome because of reduced pH. Antigen uptake and binding (AF488-ova) were evident at all reproductive stages, however lactation and involution (D2) dendritic cells had ~2 fold reduction in binding and uptake compared to nulliparous mammary dendritic cells (Supplementary Figure 3B). With respect to antigen processing, we found that dendritic cells had an equivalent ability to process antigen at all reproductive stages tested, consistent with intact dendritic cell functionality (Supplementary Figure 3C).

To assess the ability of mammary dendritic cells to cross present antigen, we utilized an *ex vivo* antigen presentation assay that detects the final step in the process: the presence of OVA peptide-MHCI complex (MHCI-SIINFEKL) at the cell surface (Figure 5A cartoon). Representative flow plots of MHCI-SIINFEKL expression from lactation and Inv D6 stages are shown (Figure 5B). We found that mammary dendritic cells from all reproductive stages tested had the ability to cross present antigen (Figure 5C, all values above zero), with dendritic cells from Inv D6 having the highest level of antigen presentation (Figure 5C). In sum, mammary dendritic cells retain the ability to present antigen at all reproductive stages and may be superior at this ability during mid-

involution. We next tested for the ability of antigen presenting cells to support antigen-dependent naïve T cell activation. Based on our evidence that mammary dendritic cells have reduced

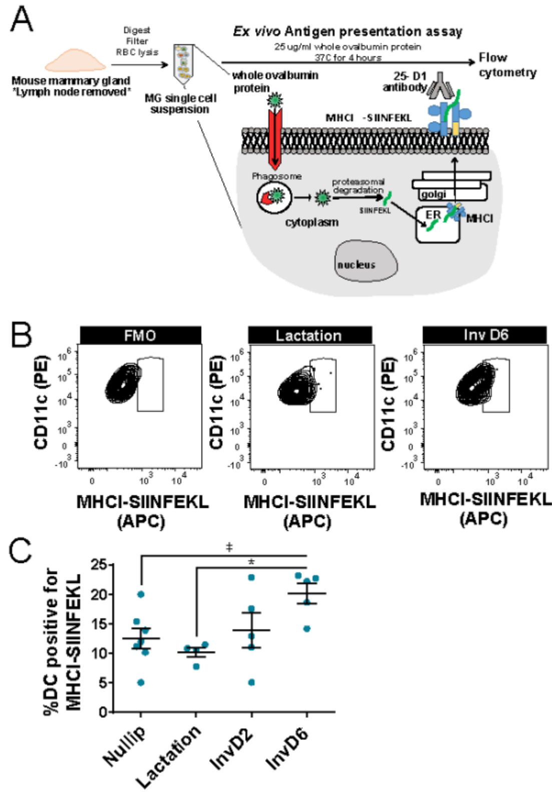

Figure 5: Dendritic cell antigen cross presentation is enhanced during involution. (A) Schematic of *ex vivo* antigen cross presentation assay. (B) Representative flow charts showing MHCI-SIINFEKL as detected by the 25-D1 antibody in lactation (middle) and Inv D6 (right) as well as a sample without 25-D1 antibody staining (FMO control, left). (C) Quantitation of % total DCs that are positive for 25-D1 staining is shown. N=4-7 per group, * indicates p <0.05 by one way ANOVA with Tukey's multiple comparison test, and ‡ indicates p <0.05 by unpaired two-tailed student's T test. Nullip= nulliparous, and InvD2 and InvD6= 2 and 6 days post weaning.

expression of T cell activation markers (MHCII, CD80, and CD86, Figure 2C-E and Figure 4), and mucosal organs are characterized by tolerogenic dendritic cells, we predicted that lactating and involuting hosts are skewed toward inducing T cell tolerance.

Mammary antigen presenting cells lack ability to activate naïve T cells during involution

To address dendritic cell functionality, we employed an *in vivo* T cell activation assay. This assay evaluates the ability of mammary antigen presenting cells (APCs), with dendritic cells presumably being the most potent, to present antigen to adoptively transferred $CD4^+$ naïve T cells within the mammary draining lymph node (Figure 6A, and Supplementary Figure 4A). This assay utilized naïve DO11.10 TCR Transgenic $CD4^+$ T cells, which specifically recognize ovalbumin antigen. Transgenic $CD4^+$T cells were purified (Supplementary Figure 4B) and transferred by tail vein injection into syngeneic, wildtype nulliparous, lactation, involution, or regressed hosts. Two days later ovalbumin antigen was injected unilaterally into the #4 mammary fat pad with PBS injected into the contralateral gland to serve as the internal negative control. Finally, $CD4^+$ transgenic T cells in the mammary draining lymph nodes (LN) were then detected via specific staining for DO11.10 TCR by flow cytometry (Figure 6B and Supplementary Figure 4C). To determine absolute transgenic T cell count, we utilized counting beads (Figure 6B top left panel). In nulliparous and regressed hosts we observed an antigen-dependent increase in DO11.10 T cell abundance in the draining lymph nodes, whereas increases in T cell abundance were not observed in lactation or involution hosts (Figure 6C). To assess the magnitude of T cell accumulation we calculated the ratio of T cell abundance in ova to PBS conditions. We observed an antigen-dependent 15-20 fold increase in T cells in the draining LNs of nulliparous and regressed hosts and only a 2-5 fold increase in antigen-dependent T cell accumulation in lactation and involution hosts (Figure 6D). In contrast to the data observed in mammary draining lymph nodes, the abundance of DO11.10 $CD4^+$ T cells in the spleens of animals did not differ based on reproductive state or antigen treatment (Supplementary Figure 4D). In sum, these data indicate local, mammary specific suppression of antigen-dependent T

cell activation during lactation and involution, consistent with mucosal immune programs.

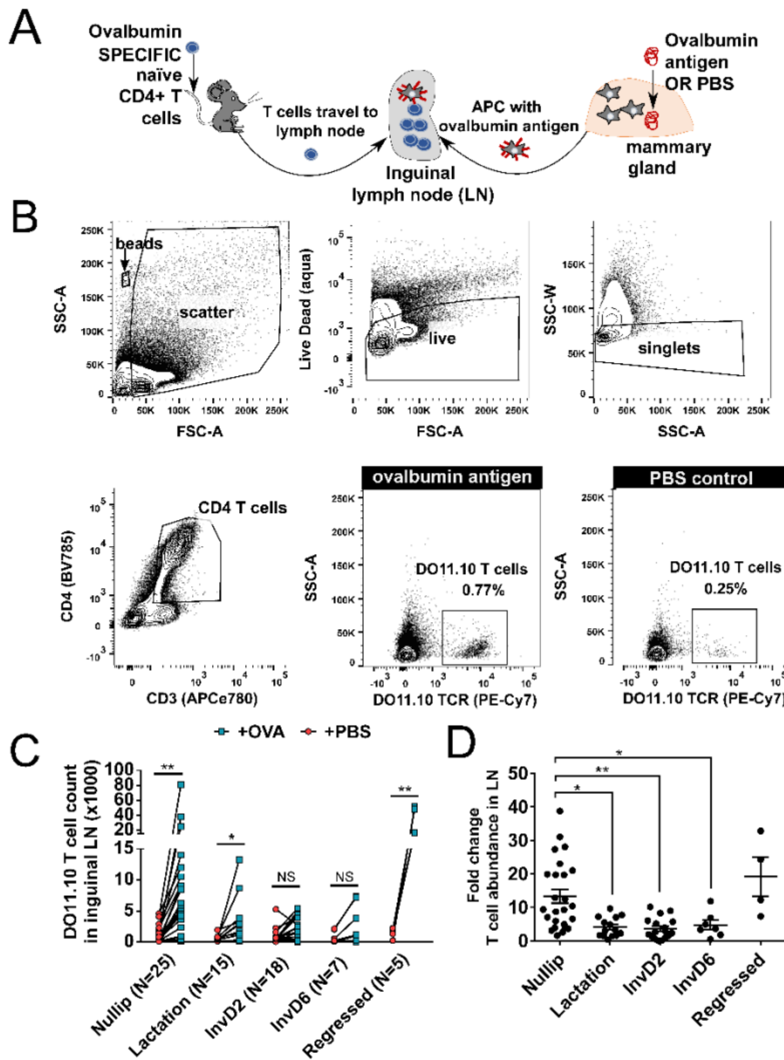

Figure 6: Lactation and involution hosts have reduced ability to activate naive CD4+ T cells to mammary antigen. (A) Schematic of experiment where CD4+ T cells specific for ovalbumin antigen (DO11.10) were adoptively transferred into Balb/c hosts, then ovalbumin antigen OR PBS was introduced locally into either the left or right #4 mammary gland. (B) DO11.10 CD4+ T cells were detected in the inguinal (mammary tissue draining) lymph node (LN) and absolute cell counts calculated by the addition of counting beads (top left panel). Representative flow data from inguinal lymph nodes draining mammary glands that received ovalbumin antigen or PBS are shown. (C) DO11.10 T cell count in inguinal LNs draining mammary tissue that received PBS (red) or ovalbumin (blue) in various reproductive hosts. Each mouse received PBS in one MG and ovalbumin in the other, and are joined by a line. Two-tailed paired T tests were performed, where * indicates $p < 0.05$ and ** indicates $p < 0.01$. Ns are denoted next to the group along the X-axis. (D) The ratio of T cell counts in the inguinal lymph node (LN) that drain mammary glands that received ova antigen to those that received PBS. One way ANOVA with Tukey's multiple comparison test was performed and * indicates $p < 0.05$ and ** indicates $p < 0.01$. Combined results of three independent experiments are shown, statistically significant outliers have been removed. N=25 for nulliparous, N=14 for lactation, N=17 for Inv D2, N=7 for Inv D6, N=4 for regressed hosts. Nullip= nulliparous, and InvD2 and InvD6= 2 and 6 days post weaning.

Phenotyping of mammary tissue CD4$^+$ T cells

Since we observe a decrease in CD4$^+$ T cell expansion in the mammary draining lymph nodes of lactation and involution hosts, we next sought to determine if the endogenous CD4$^+$ T cell compartment in the mammary gland was similarly reduced. Compared to nulliparous mammary glands, lactating glands had decreased abundance of total CD4$^+$ T cells (CD45$^+$ CD4$^+$ CD11b-) (Figure 7A-B). In contrast, we observed an increase in CD4$^+$ T cells during involution (Figure 7B). We next assessed for programming of CD4$^+$ T cells in the mammary gland at various reproductive states for those commonly found at mucosal barriers, including Th17, Th2, and Treg.

We found the existence of a baseline Th17 mucosal state in the nulliparous mammary gland with evidence for an immune regulatory milieu during lactation, as measured by increased Th17 and PD-1$^+$ CD4$^+$ T cell populations (Figures 7C-D, 7H-I, and7J, PD-1$^+$ T cell populations are outlined in red). With the onset of weaning (InvD2) we found significant expansion of the Th17 population (Figure 7D), consistent with the need for increased barrier protection. By InvD6, the gland is characterized by the presence of Th17, Th2, and Treg populations with high PD-1 positivity (Figures 7D, E, G, I, J). Of note, many of the Gata3$^+$ CD4$^+$ T cells were also positive for RORγT, indicating a potential Th17 to Th2 transition specific to late involution. These involution associated T cell populations likely reflect active immune suppression coincident with the programed cell death of involution and tissue remodeling necessary to return the gland to a non-lactating state. Since our previous result described reduced naïve T cell activation during involution (Figure 6), and given that we observe T cell abundance increases two days following cessation of weaning, not long enough for a naïve response to be mounted, we hypothesized that

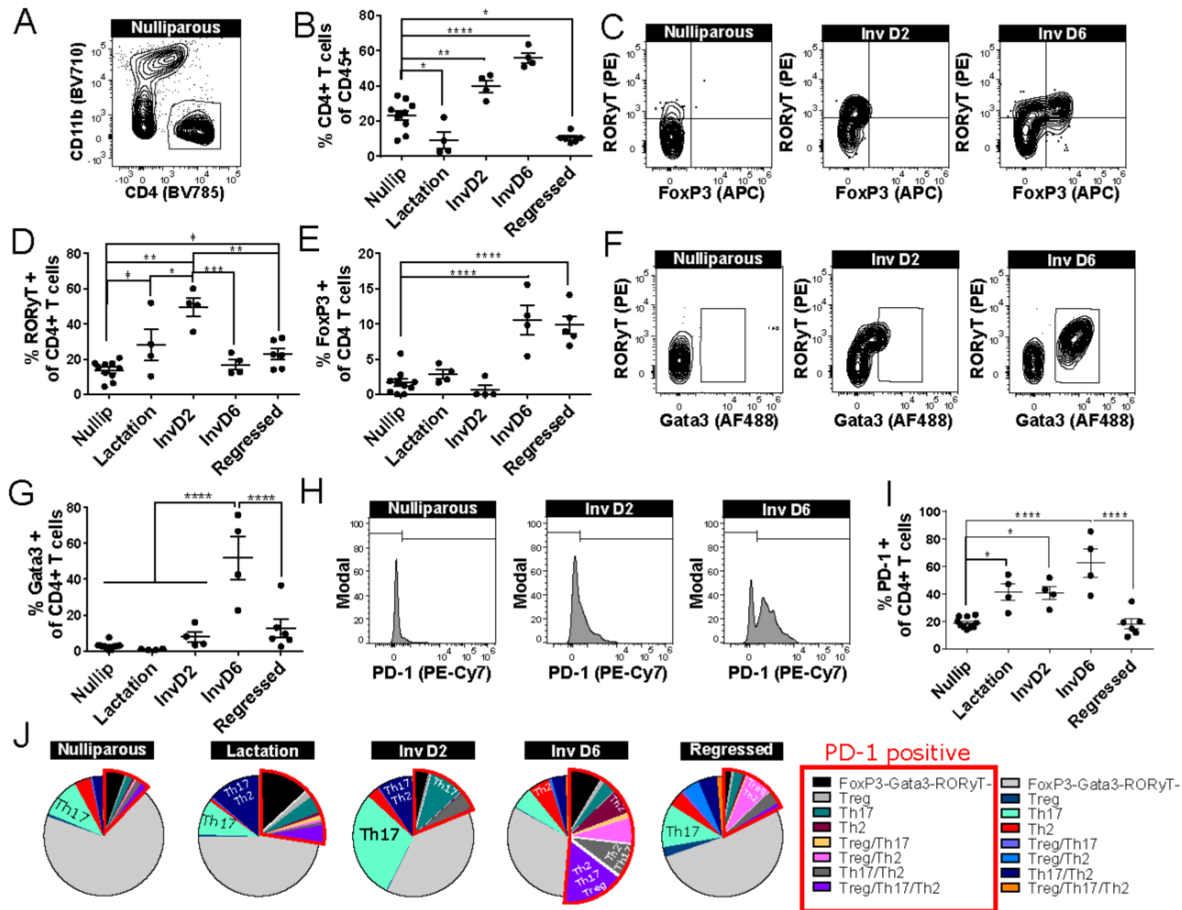

Figure 7: Phenotyping of mammary gland CD4+ T cells. (A) Identification of CD4+ T cells in nulliparous mammary gland digest (Live, CD45+ CD4+ CD11b-) where LN was excised. (B) CD4+ T cell abundance as % of total CD45+ cells. (C) Representative flow plots of RORγT and FoxP3. (D) Abundance of RORγT+ (FoxP3-) and (E) FoxP3+ (RORγT-) CD4+ T cells at various reproductive stages. (F) Representative flow plots of Gata3. (G) Incidence of Gata3+ CD4+ T cells in the mammary gland. (H) Representative flow plots of PD-1. (I) Incidence of PD-1+ CD4+ T cells in the mammary gland at various reproductive states. For panels B, D, E, G, and I there are N=4-10 mice per group, * indicates p <0.05, ** p <0.01, *** p <0.001 and **** p <0.0001 by one way ANOVA with Tukey's multiple comparison test and ǂ indicates p <0.05 by unpaired two-tailed student's T test. (J) Average incidence of 16 variants of CD4+ T cells are shown for each reproductive stage. Pie slices that are exploding and outlined in red indicate PD-1 positivity. Each pie slice represents the average of 4-10 animals in that reproductive group. Nullip= nulliparous, and InvD2 and InvD6= 2 and 6 days post weaning.

a putative source of $CD4^+$ T cells in the involution mammary gland may be recruitment from a systemic memory pool.

Th17 and Treg memory $CD4^+$ T cells are recruited to involution mammary tissue

In order to test the hypothesis that systemic memory T cells accumulate specifically in the involuting mammary gland, we devised an *in vivo*, adoptive T cell transfer experiment where memory T cells could be enumerated. Also, we tested antigen dependence of memory T cell accumulation, as antigen independent accumulation is consistent with an inflammatory environment. Ovalbumin specific (DO11.10 TCR transgenic) memory $CD4^+$ T cells skewed to Th17-Treg polarization were generated as described (Methods section and Supplementary Figure 4E) and transferred into involution (D0) or nulliparous age-matched hosts. Two days later, ovalbumin protein (i.e., antigen) was injected into the mammary fat pad with PBS injected into the contraleral gland (Figure 8A), and abundance of the adoptively transferred memory $CD4^+$ T cells assessed five days later using flow cytometry.

We found significantly more memory $CD4^+$ T cells in the involution mammary glands compared to the nulliparous glands (Figure 8B). Further, this memory $CD4^+$ T cell recruitment was independent of the presence of ovalbumin antigen (Figure 8C). In sum, these data indicate an antigen-independent, inflammation based accumulation of memory Th17- Treg $CD4^+$ T cells specifically to the involution mammary gland. Additionally we did not observe antigen-independent accumulation of memory T cells into the lymph node or spleen of the involution hosts (Figure 8E-F). These data are inconsistent with systemic inflammation during involution, and combined with mammary specific T cell accumulation support the existence of tightly regulated and localized inflammatory immune milieu during involution. Also we did not observe antigen dependent memory T cell accumulation in the mammary draining LN during involution, data consistent with tolerogenic dendritic cell functions and our observed lack of naïve T cell activation during involution (Figure 6). Altogether these results show that the involuting

mammary gland is capable of accumulating Th17 and Treg skewed memory T cells via an antigen-independent mechanism, and further support heightened mucosal barrier function and immune tolerance during involution.

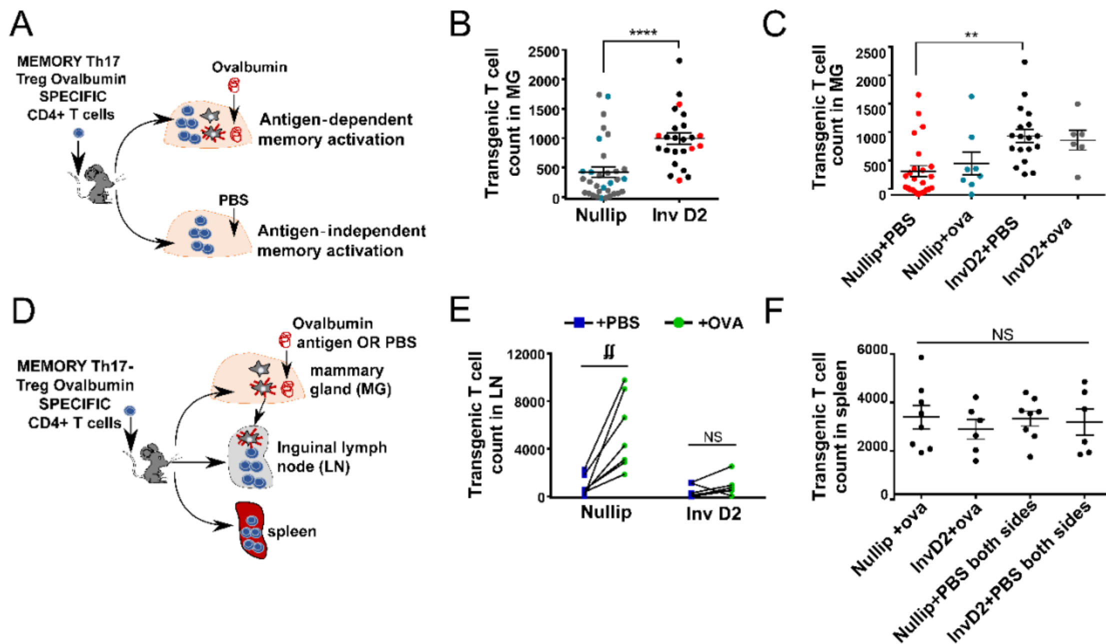

Figure 8: Involution hosts support antigen-independent Th17/Treg memory CD4+ T cell accumulation in mammary tissue and fail to accumulate antigen-dependent memory T cells in lymph nodes. (A) Experimental schema where memory Th17 /Treg CD4+ T cells specific for ovalbumin antigen (DO11.10) were generated in vitro and then adoptively transferred into Balb/c nulliparous and involution hosts, then ovalbumin antigen or PBS was injected into the mammary gland. The absolute abundance of transferred T cells in various tissues was calculating by the addition of counting beads in flow cytometry. (B) Number of DO11.10 transgenic memory T cells in mammary glands of nulliparous and involution hosts. Two-tailed unpaired student's T test was performed, where **** indicates p <0.0001. (C) Data from panel B separated by delivery of ovalbumin or PBS to mammary tissue. Two-way ANOVA with Tukey's multiple comparison test where ** indicates p <0.01 (D) Schema to identify memory T cell abundance in lymph node and spleen in the presence or absence of mammary delivered antigen or PBS. (E) Number of DO11.10 transgenic memory CD4+ T cells in LNs of nulliparous and Inv D2 hosts with or without mammary antigen introduction. Each mouse received PBS in one MG and ovalbumin in the other, and are joined by a line. Two-tailed paired T tests were performed, where ∫∫ indicates p <0.01. (F) Number of DO11.10 transgenic CD4+ T cells in spleens. NS= not significant by two-way ANOVA. Combined results of two independent experiments, N=8-24 for nulliparous and N= 6-18 for involution (InvD2) hosts with ova one MG and PBS one MG, or PBS in both MGs (both sides), respectively. Nullip= nulliparous, and InvD2 =2 days post weaning.

Discussion

Our study is the first to describe a baseline mucosal immune profile in the murine mammary gland and provide evidence that immune tolerance to foreign antigens is reproductive state dependent. Specifically, we find the nulliparous mammary gland is dominated by immature dendritic cells and Th17 skewed $CD4^+$ T cells. During lactation we find increases in antimicrobial IgA and mucin mRNAs, dendritic cells with tolerogenic features, Th17 $CD4^+$ T cells, and functional evidence for antigen dependent immune tolerance. During involution these mucosal features are enhanced to include increased antimicrobial gene expression, and extended to include antigen-independent accumulation of memory Th17/Treg, and elevated levels of Th2, Treg, and $PD\text{-}1^+$ $CD4^+$ T cells. These studies support the mammary gland as a mucosal organ, and further identify reproductive control over these mucosal attributes.

Our data are consistent with mammary barrier function and immune tolerance being essential for maintaining women's health during lactation, an argument pioneered by others [32, 90]. One reason that enhanced barrier function and immune tolerance may be needed is because milk represents a foreign antigen risk. Even though milk is a natural endogenous product of the mammary gland, milk proteins may represent neoantigens and represent challenges to self-tolerance [104, 228-230]. Further, risk of foreign antigen exposure during lactation is due to the presence of a wide variety of viable bacteria in milk [18, 231], likening the lactating mammary gland to the gut. These bacteria are supported by milk sugars and are thought to play important roles in infant gut health. Thus, throughout lactation, it can be argued that it is important to prevent an immune reaction towards milk as well as minimize reactivity to milk-associated bacteria. This argument is congruent with the increases in IgA and mucin mRNAs, tolerogenic

dendritic cells and Th17 CD4$^+$ T cells that we describe, since these are key factors in maintaining immune tolerance and healthy epithelial barriers in mucosal organs [203, 206-208]. This study is also the first of its kind to identify mucosal barrier and immune tolerance programs that extend past lactation and into weaning-induced involution. Weaning-induced mucosal programs likely support the remodeling of the lactation competent mammary gland to its non-secretory, pre-pregnant like state. During involution, we propose that the stimulus for tolerance mechanisms is the massive alveolar cell death that occurs post weaning [232]. This is because the ingestion of dying cells induces tolerogenic dendritic cell functions, and is thought to be a primary mechanism to avoid the generation of self-reactive immunity [156, 158, 233]. Specifically, dendritic cell phagocytosis of apoptotic cells inhibits dendritic cell maturation, as measured by reduced CD86, which altogether leads to a reduced capacity to activate naïve T cells [156, 158, 233].

The primary mechanism by which antigen presenting cells, with dendritic cells being most potent, suppress naïve T cell activation is through antigen presentation in the absence of co-stimulatory signals. The results of this dendritic cell-T cell interaction are deletion of T cells via apoptosis, T cell anergy (or paralyzation), or T cell polarization to a regulatory phenotype (Treg) [224]. While we show antigen-dependent naïve T cell accumulation is suppressed during lactation and involution and demonstrate T cell polarization to regulatory phenotypes, the particular role of the dendritic cell in executing these T cell responses is unknown, as other immune cells are also capable of these functions. However, decreased dendritic cell expression of MHCII and CD86 during lactation and involution is consistent with reduced antigen presentation to T cells and reduced co-stimulation. Furthermore, while the cytokine milieu of the

lactating murine gland is poorly understood, the cytokine milieu within the involution mammary gland has high levels of TGF-β, COX2, IL-10, IL-4, and IL-13 [42, 44, 46, 72, 234]. This cytokine milieu is consistent with the promotion of tolerogenic dendritic cell functions and/or direct suppression of T cells.

Surprisingly, in spite of observing antigen-dependent T cell suppression during lactation and involution, we also observed antigen-independent accumulation of memory T cells to the mammary gland specifically during involution. The function of these memory T cells is likely to skew the environment towards immune suppression (Treg, PD-1^{+}) and maintain epithelial barrier integrity (Th17) during gland collapse. These programs are also expected to limit Th1 damaging inflammation that could be elicited during the massive epithelial cell loss and gland remodeling that occurs post weaning. The mechanism of how memory CD4^{+} T cell are recruited during involution is unknown, although activated vasculature may be predicted [235]. Indeed mammary vasculature is highly dynamic across a reproductive cycle [45, 236], and could play a role in differential recruitment and drainage of immune cells. Further, the source of these memory CD4^{+} T cells is also unknown. Recruitment from other mucosal sites is possible, as integration across mucosal tissues is reported for lung and gut and evidenced by alteration of systemic immune responses by the gut microbiome [237, 238]. One interesting developmental window that we did not assess for mucosal attributes is the mammary gland during pregnancy. However, it is known that systemic immune modulation/suppression occurs to permit tolerance to fetal non-self-antigens [239]. How systemic immune suppression during pregnancy impacts the mammary immune milieu remains to be determined.

This research may have far reaching impacts on understanding pathologies that can arise during lactation and involution. For example, these two reproductive windows are characterized by increased self and foreign antigen exposure and autoimmunity may occur if immune tolerance is not executed properly. While a role for autoimmunity in lactation failure has not been explored, tissue destruction is a common result of autoimmune disease, and insufficient glandular tissue correlates with lactation failure in women [240, 241]. The potential implications warrant further investigation, as lactation failure occurs in 5-15% of all parous women, leading to serious infant malnutrition if formula supplementation is not possible [240]. Our study also may provide new avenues to investigate breast cancer risk and promotion, since immune tolerance is the antithesis to immune surveillance. Of potential relevance, the risk of developing breast cancer is increased in the postpartum window and cancers that develop postpartum have poorer prognoses than breast cancers matched for stage and biologic subtype in age-matched nulliparous women [53]. Further, in rodent models, the immune milieu of the involuting gland has been implicated in progression of postpartum tumors [42, 72, 76]. Our study finds that immune tolerance, which may be executed by tolerogenic dendritic cells, is enhanced during involution, implicating immune tolerance as one potential mechanism of mammary tumor promotion during weaning-induced involution.

Altogether this study describes, for the first time, the immune milieu of the normal murine mammary gland to be mucosal in nature at all reproductive stages assessed, as indicated by baseline immature dendritic cell phenotypes and Th17 $CD4^+$ T cells. These mucosal immune programs are enhanced and extended to include immunosuppressive and immune tolerant programs during lactation and involution. This study provides rationale for including the

mammary gland as a mucosal organ, which may lead to new investigative avenues not only within the mammary gland but within the greater mucosal organ system.

Acknowledgements

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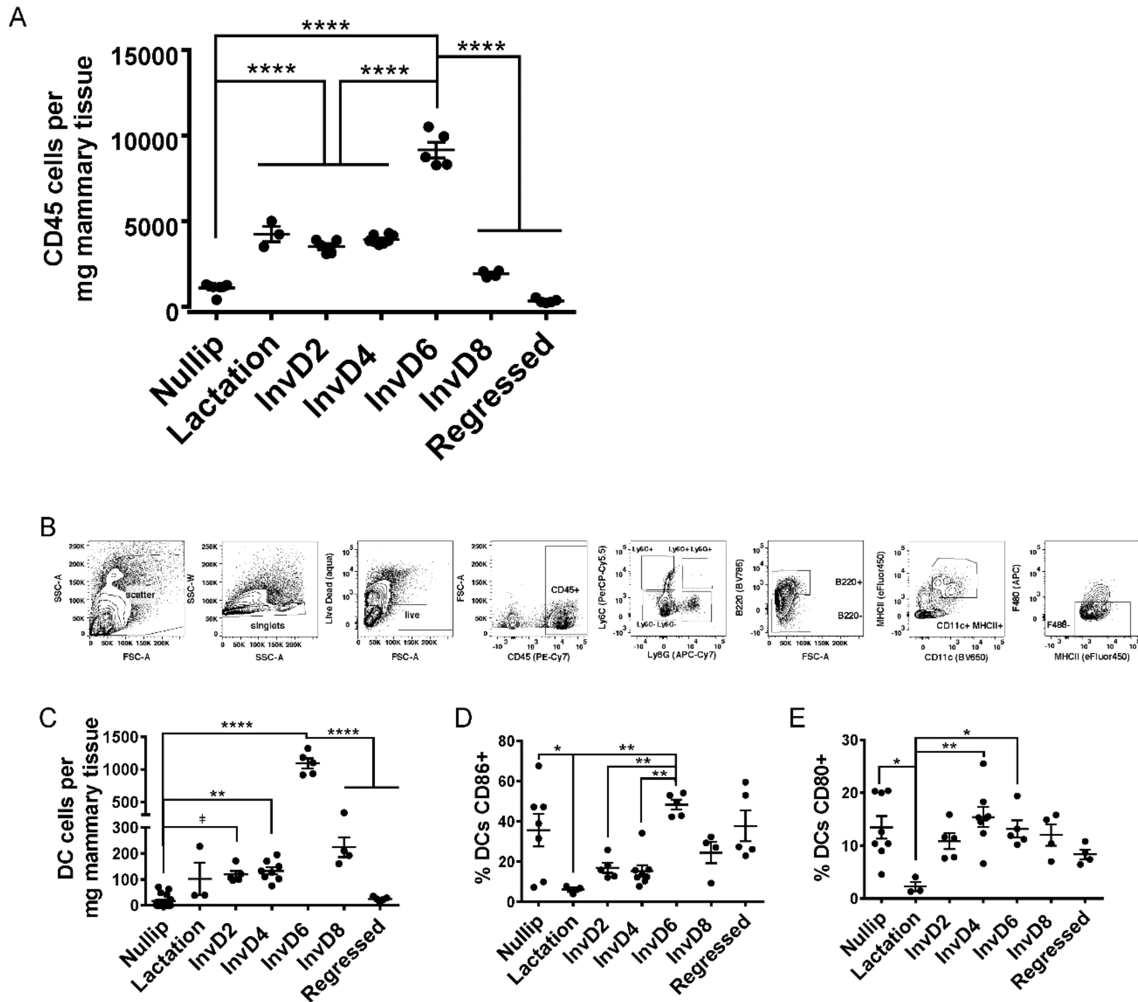
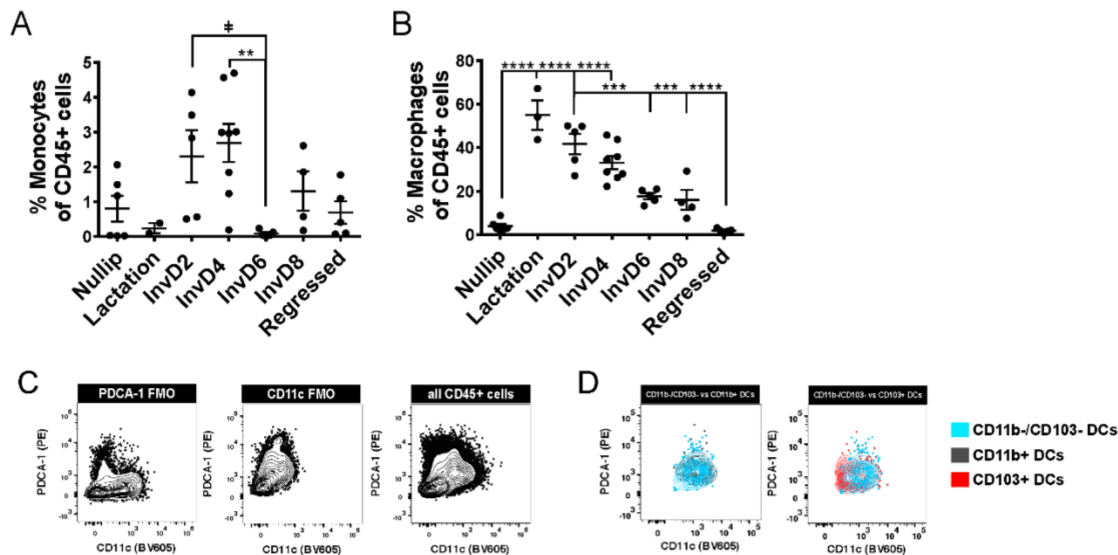
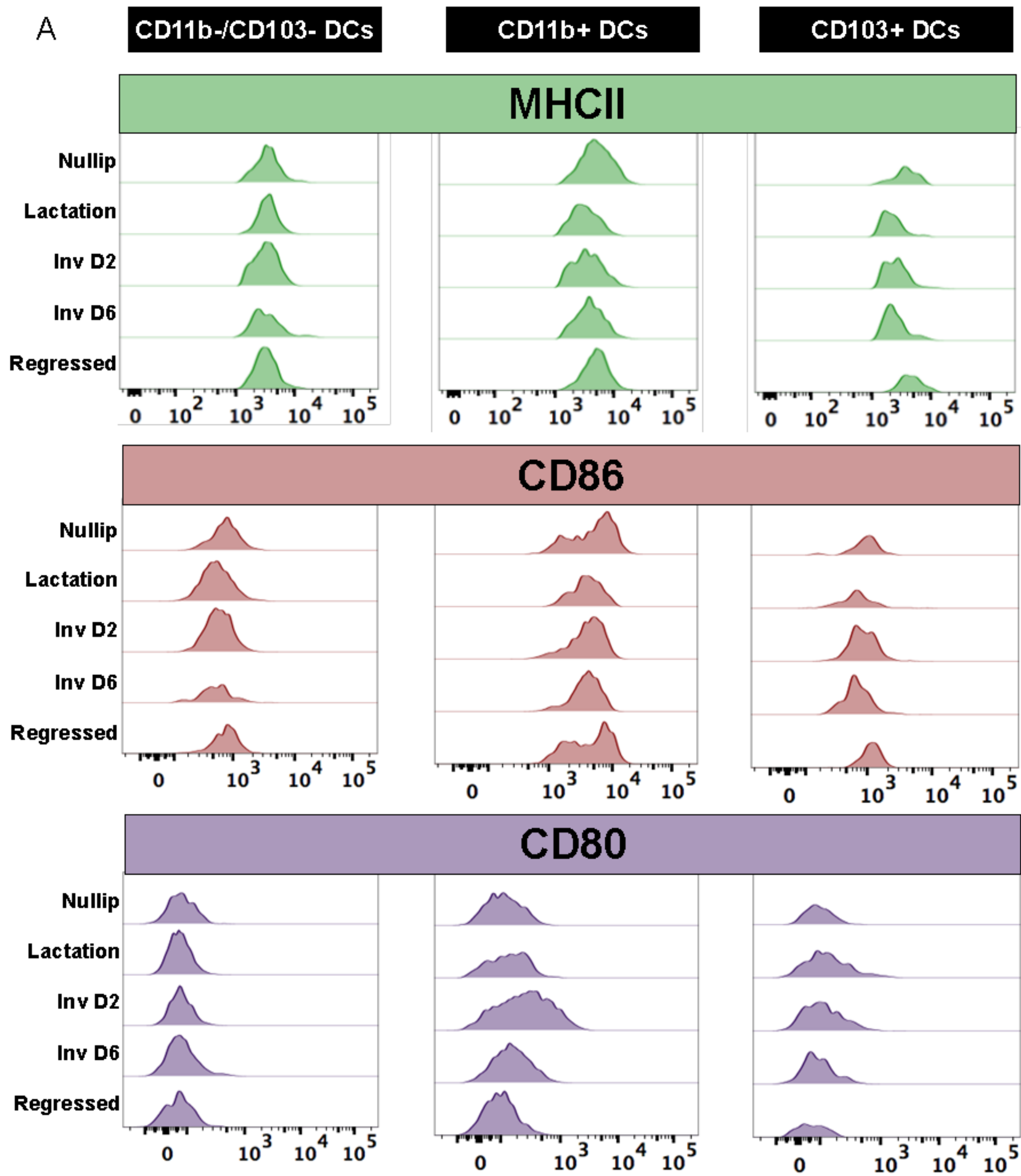


Supplementary Figure 1: Extended analysis of mammary immune cells and DC abundance and activation at various reproductive stages. (A) Total CD45+ cells were calculated by multiplying the % of CD45+ cells of all live cells detected by flow cytometry by the total number of live cells in the tissue digest. The total number of CD45+ cells was divided by tissue wet weight to determine CD45+ density within the mammary tissue. (B) Flow gating schema to identify mammary dendritic cells in a Balb/c wild type InvD6 mouse is shown as an example to compare with nulliparous tissue (Figure 2A), inguinal LN was removed. (C) Calculation of DC density in mammary tissue at various reproductive stages. Total number of DCs were calculated by multiplying the %DCs of total live cells detected by flow cytometry by the total number of live cells in the tissue digest. The total number of DCs was divided by tissue wet weight to determine density of DCs. (D) Quantitation of percent DCs positive for CD86. Results are similar to when gMFI for CD86 is used for quantification (Figure 2C). (E) Quantitation of percent DCs positive for CD80. N=3-7 per group, * indicates p <0.05, ** p <0.01, and **** p <0.0001 by one way ANOVA with Tukey's multiple comparison test and ‡ indicates p <0.05 by unpaired two-tailed student's T test. Nullip= nulliparous, and Inv D2, D4, D6, D8= Involution days 2, 4, 6, and 8 days post weaning.



Supplementary Figure 2: Dynamic regulation of monocyte and macrophage abundance based on reproductive state in Balb/c wild type hosts and confirmation of mammary dendritic cell identification in nulliparous hosts. (A) Monocytes (CD45+ Ly6C+ Ly6G-) increase as a percent of all CD45+ cells 2-4 days post weaning compared to Inv D6. (B) Macrophages (CD45+ Ly6C- Ly6G- F480+) increase significantly during lactation and early involution compared to nulliparous and later involution (D6-8) and regressed. N=3-7 per group, ** indicates $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by one way ANOVA with Tukey's multiple comparison test and ǂ indicates $p < 0.05$ by two-tailed unpaired student's T test. Nullip= nulliparous, and Inv D2, D4, D6, D8= Involution days 2, 4, 6, and 8 days post weaning. (C) Flow cytometry plots downstream of Live CD45+ cells for PDCA-1 and CD11c. Staining FMOs and a stained Inv D6 sample are shown. CD11c and PDCA-1 positive populations are visible. (D) Overlay plots showing CD11b-/CD103- DCs (blue), CD11b+ DCs (gray), and CD103+ DCs (red) are negative for PDCA-1 and express relatively similar levels of CD11c. Representative overlay dot plots from an Inv D6 sample are shown.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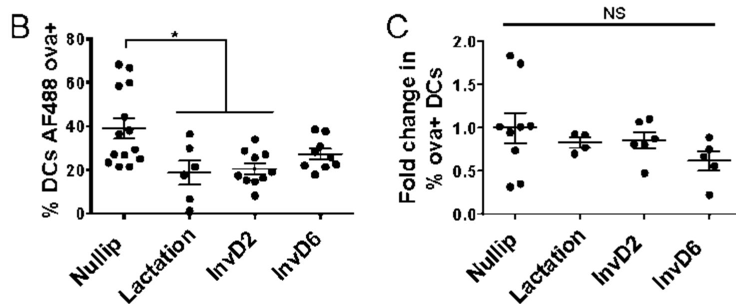

Supplementary Figure 3: Dendritic cell activation, antigen uptake, and antigen processing by reproductive state. (A) Histograms of MHCII, CD86, and CD80 in CD11b-/CD103-, CD11b+, and CD103+ DC populations that were detected in the mammary gland at various reproductive stages. CD11b-/CD103- DCs (left column) have relatively low levels of CD86 and CD80 compared to the other DC populations. Since most activation markers show a single population shifting in fluorescence intensity with reproductive state, rather than clear positive and negative populations, quantitation of geometric mean fluorescence intensity (gMFI) of the entire population was used for quantitation in Figure 4. (B) Antigen binding and/or uptake was reduced by ~half in DCs from either the lactation or Inv D2 mammary gland as compared to nulliparous. (C) Antigen processing by DCs was unaltered by reproductive state. N=4-14 per group, * indicates $p < 0.05$, by one way ANOVA with Tukey's multiple comparison test, and NS= not significant.

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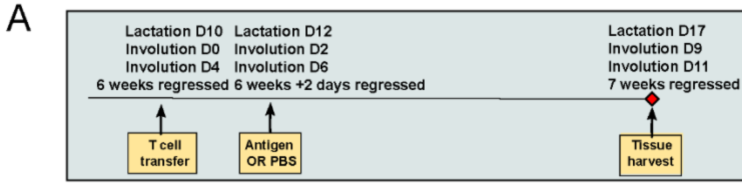

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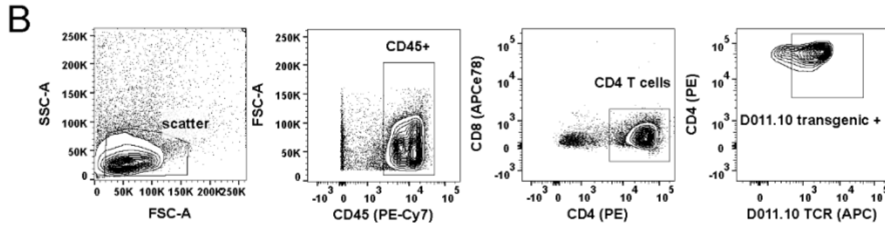

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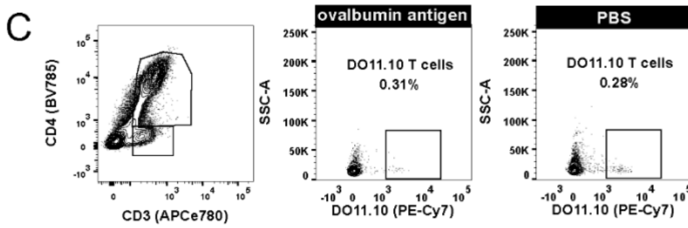

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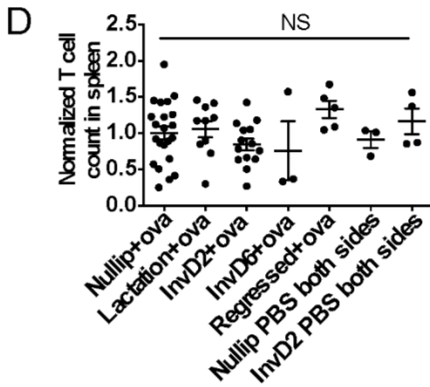
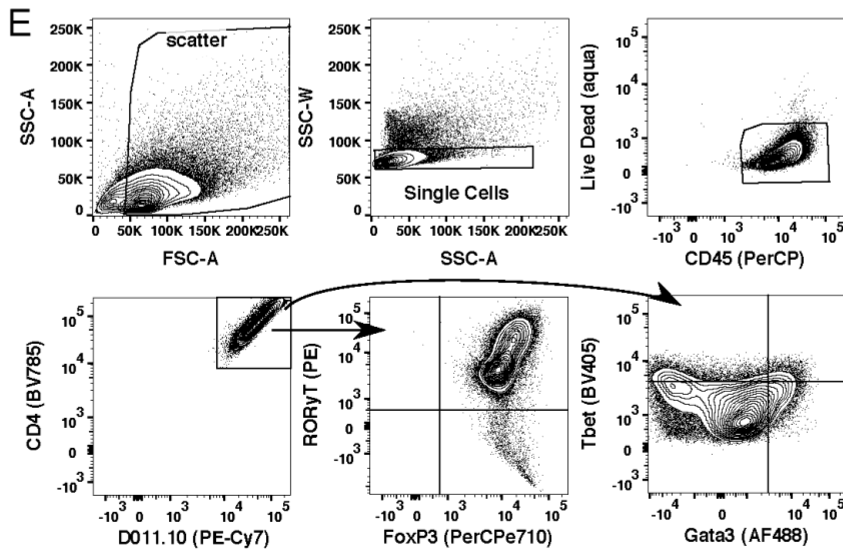




Supplementary Figure 4: Details of naïve and memory T cell activation in vivo assays. (A) T cell transfer into Balb/c hosts occurred at various reproductive states two days before antigen or PBS was administered. Five days following antigen administration in the mammary gland tissues were harvested, a time point where CD4 + naïve T cell activation should be at its peak. (B) CD4+ T cells specific for ovalbumin were enriched for adoptive transfer from the spleens of female Balb/c DO110.10 transgenic mice to greater than 95% pure (middle right panel) using magnetic bead negative selection (macs miltenyi). The CD4+ T cell pool was largely positive for the DO11.10 transgene (right panel). (C) DO11.10 staining in CD3+ CD4- T cells in LNs draining mammary glands that received antigen or PBS. DO11.10 staining is at background levels in CD3+ CD4- T cells detected in the lymph node, indicating specificity in using this method of detection for CD4+ transgenic T cells only. (D) Counts of DO11.10 transgenic T cells in the spleen were comparable across all groups and treatments, and similar to animals that did not receive antigen (PBS both sides), consistent with a lack of naïve T cell activation in the spleen in this model. N=3-15 per group, NS= not significant by two way ANOVA. (E) Characterization of ovalbumin transgenic memory CD4+ T cells for in vivo memory T cell experiments. CD4+ T cells were isolated from the spleens of female DO110.10 transgenic mice, and purified to greater than 95% using magnetic bead negative selection (macs miltenyi, as displayed in Supplementary Figure 7B), plated and incubated for 10 days with TGFB (1 ng/ml), IL-1B (10 ng/ml), and IL-6 (50 ng/ml). Figure shows flow cytometry analysis of T cells at the end of culture, and at the time of adoptive T cell transfer. T cells were greater than 95% pure CD4+ T cells (top right panel), nearly 100% positive for the DO11.10 transgenic T cell receptor (bottom left panel) and were mostly RORγT+ FoxP3+ (bottom middle panel) indicating a dominant Th17/Treg programming. As such, these cells are largely negative for Th1 (Tbet) and Th2 (Gata3) programming factors (bottom right panel).

Chapter V: Extended Discussion

A summary of major findings

Our study is the first of its kind to systemically assess the mammary gland for mucosal immunological hallmarks. We found that the adult mammary gland exhibits numerous mucosal hallmarks, including Th17 CD4$^+$ skewed T cells and immature dendritic cell phenotypes, at all reproductive stages evaluated. Additionally, we report mucosal attributes are highly regulated by reproductive state. Specifically, we found that Th17 CD4$^+$ T cells, tolerogenic dendritic cell phenotypes, and immune tolerance are enhanced during lactation in comparison to the nulliparous gland. We also find evidence that the mucosal state extends into involution with an increased abundance of Th17 CD4$^+$ skewed T cells and the appearance of Th2 and Treg CD4$^+$ T cells. Immune tolerance is also evident during involution in the form of dampened naïve T cell activation. Further there is systemic recruitment of memory CD4$^+$ T cells that are Th17/Treg skewed to the involution gland (Figure 4-1).

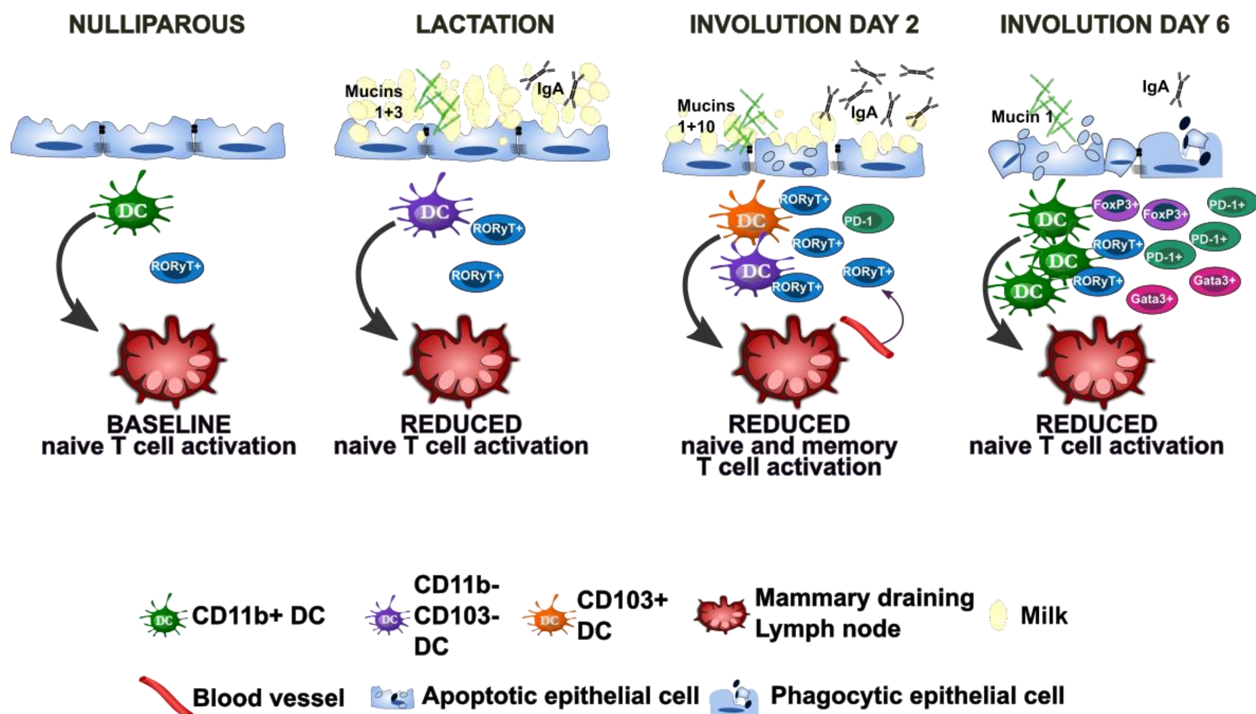


Figure 4-1: Model figure of major findings related to tolerogenic dendritic cells, T cell attributes, and T cell activation in mammary draining lymph nodes in nulliparous, lactation, involution day 2, and involution day 6 hosts.

The regressed gland exhibits mucosal attributes similar to the nulliparous gland indicating a return to a steady-state mucosal program. Our results not only strengthen the classification of the mammary gland as a mucosal organ, but have implications for altered risk of infection and breast cancer based on reproductive state. Further, our results may shed light on the understudied problem of lactation insufficiency.

Reproductive state changes in epithelial barrier function and immune tolerance may alter risk of infection

Previous studies by others have identified components of barrier function in the mammary gland to include epithelial junctions, mucins, and IgA. Our study has extended the understanding of the mammary epithelial border by discovering border-supportive T cells (Th17) and immune tolerance. Previous work by our lab and others have found that barrier integrity is highly variable based on reproductive state. While barrier integrity is known to influence the risk of infection, our contributions to the understanding of the mammary barrier will expand understanding of this relationship. Namely, reproductive state dependent alterations in Th17 $CD4^+$ T cells and immune tolerance may also underlie observed differences in risk of infection.

We have reported an increased abundance of Th17 $CD4^+$ T cells in the mammary tissue during lactation and involution in comparison to nulliparous and regressed stages. We postulate that this is a mechanism by which the body is attempting to strengthen barrier function in order to limit infection. However, since there is enhanced risk of infection during both lactation and involution, it may be that Th17 $CD4^+$ T cells are not always successful in limiting infection. Instead the

increased incidence of Th17 $CD4^+$ T cells during lactation and involution may be an indicator of enhanced bacterial exposure. This paradigm would fit with what is known in the gut, where particular bacteria species are known to increase the abundance of Th17 $CD4^+$ T cells in an effort to enhance barrier defenses [195]. The functional role of Th17 $CD4^+$ T cells in the mammary gland during lactation and involution is currently unknown.

Variable epithelial barrier function with reproductive state may alter tumor initiation and progression

Epithelial barrier function may also influence the reproductive state dependent differences in tumor initiation. For example, increased epithelial cell permeability in the gut allows for translocation of bacteria into the tissue, Th1 immune responses and generation of ROS. ROS generation can cause genetic mutations which can initiate cancer [95-97]. The existence of a breast microbiome is tantalizing and only beginning to be understood [242]. And further, a link between bacteria and breast tumor initiation and progression has been suggested, but not robustly demonstrated [243].

It might be argued that a role for bacteria in tumor risk and progression is enhanced during lactation and involution. Specifically, enhanced Th17 $CD4^+$ T cells during lactation and involution may be a symptom of enhanced bacterial exposure that might enhance tumor risk. A first line of inquiry for future studies may be to assess whether enhanced bacterial exposure to the mammary tissue is enhanced during lactation and involution compared to other reproductive states, as well as assess for evidence of ROS-induced DNA damage. Further, a connection between bacteria and tumor risk in lactation and involution may be assessed by the use of

antibiotics. These findings may support the use of antibiotics in lactating and involuting women at high risk for developing breast cancer.

Tumor progression may be enhanced during lactation and involution because of mucosal immunological attributes

Our finding of enhanced immune tolerance during lactation and involution may contribute also to the tumor promotion, distinct from tumor risk that has been reported at these distinct reproductive states. In particular, we found that $CD4^+$ naïve T cell activation was significantly reduced in lactation and involution hosts compared to nulliparous or regressed. Of note $CD4^+$ T cells are important in supporting anti-tumor immunity in the form of cytokine production resulting in tumor cell killing [244, 245]. One may postulate that the blunted ability to activate a new T cell response to tumor antigen during lactation and involution may contribute to the tumor-promotional attributes of these developmental stages. And although lactation and involution are relatively short developmental windows that occur many years before a tumor may be discovered (in a woman), tumor promotional aspects of these microenvironments are thought to shape the development of a tumor for the extended future. Therefore, therapeutics aimed at boosting naïve T cell priming may prove efficacious for the treatment of tumors developing in lactation and involution hosts for longer-term tumor control and slowed tumor growth. These efforts may be expected to reduce the poor prognoses of postpartum breast cancers, by potentially allowing more latency or resulting in a poised immunogenic tumor microenvironment.

Immunomodulation of breast cancers in the lactation and involution hosts may be achieved in several ways. For one, converting tolerogenic dendritic cell phenotypes to activating dendritic

cells is expected to boost naïve T cell activation. This may be achieved by inhibiting factors known to support tolerogenic dendritic cell functions, such as TGF-β and PGE2 [121, 162, 224, 246, 247]. Or, one may be able to block tolerogenic dendritic cell signals that transmit negative signals to T cells, such as PD-L1, in order to reduce the need for strong co-stimulation. Conversely, administration of an agent that promotes dendritic cell maturation and reversal of tolerogenic phenotypes may be advantageous, such as anti-CD40. Dendritic cells express CD40 that bind to CD40L on activated T cells. Ligation of CD40 on the surface of dendritic cells results in dendritic cell up-regulation of co-stimulatory molecules and T cell activation [248]. These methods of boosting dendritic cell -T cell immunity with the goal of generating tumor reactive T cells may be especially useful for the treatment and even prevention of postpartum breast cancer.

Another method to boost anti-tumor T cell responses in lactation and involution hosts would be to bypass the need for dendritic cells in naïve T cell priming. This could be achieved by administering fully activated effector T cells to the patient, otherwise known as adoptive T cell therapy [249]. However, the microenvironments of lactation and involution may serve to limit the efficacy of engineered anti-tumor T cells. For example, TGF-β and IL-10 are dominant during involution and can dampen T cell function [250, 251]. Further, Tregs are increased in abundance during involution and can dampen both $CD4^+$ and $CD8^+$ T cell proliferation. Therefore, it is likely that it will be necessary to block the immune suppressive nature of the lactating and involution glands in conjunction with adoptive T cell therapy for maximal therapeutic efficacy. In summary, in the context of breast cancer, alleviation of immune tolerance during lactation and involution is anticipated to provide therapeutic benefit. The rationale for this

argument is that although lactation and involution are brief, the immunosuppressive nature of these developmental windows may help to shape the tumor to have worse prognosis. Therefore, it is postulated that short term interventions may slow tumor growth and progression perhaps through activation of tumor immune surveillance. However, in the context of breast feeding, suppression of immune tolerance may be pathologic.

Immune tolerance may be necessary for lactation success

Lactation insufficiency or failure is an understudied prevalent global health problem. Further, the biological underpinnings of lactation problems are poorly defined. While the dairy cow industry has proposed a role for immune tolerance in lactation success, a definitive demonstration of immune tolerance during lactation has not been displayed until our current study. Consistent with a necessity for immune tolerance for lactation success, a seminal rodent study showed that autoimmunity to the milk protein alpha lactoalbumin can result in failed lactation [33]. This finding relates to immune tolerance. While central tolerance results in T cell tolerance to self-antigens that are expressed by a random and stochastic process in the thymus during T cell development (Aire and Fezf2 in medullary thymic epithelial cells), this does not create fail-proof self-tolerance. Therefore, peripheral tolerance to tissue antigens is a safe guard mechanism to avoid autoimmunity. Further, some self-antigens are not expressed robustly until specific times and places, for example alpha lactalbumin during lactation. Hence, immune tolerant mechanisms in the periphery are expected to be necessary to deal with the expression of this protein, and potentially others, during lactation. This rationale supports the idea that it is at least possible for autoimmunity to interfere with lactation, however whether this occurs in humans has not been documented.

We have revealed that immune tolerance, in the form of dampened naïve T cell activation, is enhanced during lactation in the rodent. This likely represents a mechanism by which the body limits reactions to neoantigens in milk that have not been presented in the thymus during the process of central tolerance so as to ensure successful lactation. Otherwise stated, peripheral tolerance to milk neoantigens through the functions of tolerogenic dendritic cells may be essential for lactation. This finding may impact future studies of lactation insufficiencies in the hopes of understanding how to treat humans to ensure lactation success. This is especially important in areas of the world where breastmilk is essential for neonatal survival.

Speculating on mechanisms by which reproductive state regulates dendritic cell differentiation

A major finding of our work is that mammary localized dendritic cell differentiation and maturation are variable based on reproductive state. Specifically, lactation was enriched for CD11b- CD103- dual negative dendritic cells, which had relatively immature phenotypes compared to fully differentiated $CD11b^{+}$ or $CD103^{+}$ dendritic cell subsets. In contrast, involution had a higher abundance of fully differentiated $CD11b^{+}$ or $CD103^{+}$ dendritic cells. However, consistent with lactation, dendritic cells in involuting mammary tissues also appeared largely immature. These dendritic cell phenotypes were in contrast to nulliparous or regressed hosts, where dendritic cells were relatively more differentiated and mature than lactation or involution hosts. These data indicate that dendritic cell differentiation and maturation status are relatively dampened in involution and lactation hosts, although the mechanisms behind this observation are currently unknown.

Dendritic cells differentiate in peripheral tissues from Pre-DCs by the action of Flt3 ligands, M-CSF, and GM-CSF [114, 117-119, 252]. Lactation and early involution are dominated by CD11b- CD103- dual negative dendritic cells, which may be considered a pre-DCs. The relative paucity of differentiated dendritic cells during lactation and early involution may indicate a relative lull in the abundance of Flt3 ligands, M-CSF, or GM-CSF, although this has not been tested. An initial assessment of Flt3 ligands, M-CSF, or GM-CSF levels across a pregnancy cycle would begin to address this possibility.

It is also possible that the immune milieu of lactation and involution actively limit dendritic cell differentiation. Of note, involution has been shown to have enhanced levels of IL-10 and TGF-β [42, 46] which are known to actively limit dendritic cell differentiation. IL-10 and TGF-β interference of dendritic cell differentiation is described in the gut as a tolerance mechanism [164, 165]. A similar mechanism of TGF-β mediated reduction of dendritic cell differentiation may occur in mammary tissue. However, modulating TGF-β levels during involution induces a myriad of phenotypes [43], making a direct assessment of this hypothesis difficult.

Speculating on mechanisms by which reproductive state regulates dendritic cell maturation

A summary of our results concerning dendritic cell maturation at various reproductive states is visually represented in Figure 4-2 below, with red indicating gain of function and green depicting loss of function of various dendritic cell functions.

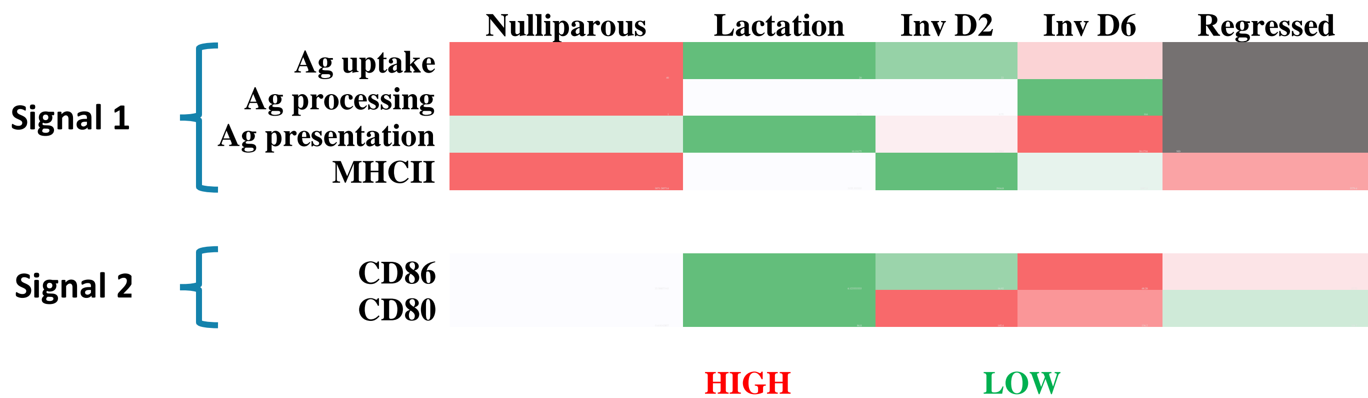


Figure 4-2: Heat map of reproductive state dependent differences in dendritic cell maturation markers. Each parameter (row) was scaled individually, and red indicates high values and green indicates low values. Gray means data is not available. Parameters involved in dendritic cell signal 1 and 2 are denoted as they related to T cell activation.

As discussed above, we observed relatively low levels of dendritic cell maturation during lactation and involution compared to nulliparous or regressed hosts. Broadly, immature dendritic cells express small amounts of MHCII and T cell co-stimulation molecules CD80, and CD86. In addition, immature dendritic cells prioritize antigen uptake but do not process or present antigen robustly. Lastly, immature dendritic cells express low levels of the lymph node (LN) homing molecule CCR7, which we have not tested in our current study. Dendritic cell maturation results in enhanced dendritic cell expression of MHCII, CD80, CD86, antigen processing and presentation, and migration to tissue draining lymph node.

Dendritic cell maturation often occurs in response to environmental variants by pattern recognition receptors, of which the toll-like receptors (TLRs) are the most well described family. Each TLR recognizes distinct stimuli for example TLR4 recognizes lipopolysaccharide on bacteria, TLR3 recognizes dsRNA common to viral infection, and TLR9 recognized un-methylated CpG oligonucleotide fragments common in infection. Three major signaling pathways contribute to dendritic cell maturation downstream of TLR ligation: mitogen-activated

protein kinase (MAPK), nuclear factor-κB (NF-κB), and interferon regulatory factors (IRFs) [122].

A possible explanation for low dendritic cell maturation in lactation and involution hosts is a relative lack of TLR stimuli. However, this is very unlikely given the facts that bacteria are commonly found in "healthy" breast milk, and that bacterial induced mastitis is most common during lactation and involution. As a reminder, bacteria are TLR ligands. Further, the existence of bacteria in the mammary gland, or the mammary microbiome, is an emerging field of research [242]. Another possibility to explain our experimental observation of decreased dendritic cell maturation during lactation and involution may be the interruption of dendritic cell maturation by immune micro-environmental factors.

It has become appreciated that numerous local tissue factors can block dendritic cell maturation. Of potential relevance, TGF-β, IL-10, IL-4, and PGE2 have all been reported to contribute to dampened dendritic cell maturation [121, 162, 246, 247, 253]. These tissue influences are all enhanced during involution with the addition of apoptotic cells, which are also reported to dampen dendritic cell maturation and lead to immune tolerance [156, 158-160, 233]. Although the precise mechanisms by which these factors dampen dendritic cell maturation are somewhat elusive, interference of NF-κB signaling has been reported [246]. Further, these molecules may actively enhance dendritic cell expression of cell surface markers that translate negative signals to dendritic cells, such as idoleamine 2,3- dioxygenase (IDO) [246, 247]. All together these microenvironmental influences produce a dendritic cell that has reduced maturation state and is

poised to induce T cell tolerance rather than activation. These factors are good candidates to investigate in our studies for a role in dampening dendritic cell maturation during involution.

Speculating on mechanisms of T cell immune tolerance during lactation and involution

We found evidence of tolerogenic dendritic cell phenotypes and lack of naïve T cell activation in lactation and involution hosts relative to nulliparous and regressed hosts. However, we are unsure of the mechanisms by which tolerogenic dendritic cells may preferentially dampen naïve T cell activation in lactation and involution hosts.

Further, we observed that lactation and involution dendritic cell phenotypes are not exactly the same, most notably dendritic cell expression of CD80 is higher during involution than lactation. One may expect this to result in more naïve T cell activation during involution compared to lactation, however we did not observe this. One interpretation of this result is that CD80 expression during involution may not be sufficient to overcome the relative weakness of other co-stimulatory signals. Specifically, CD80 expression in the absence of CD86 may not be sufficient to drive T cell activation during involution, although we have not tested this in our model.

Altogether in our studies, we found that $CD4^+$ T cell accumulation in response to mammary localized antigen was reduced in lactation and involution hosts compared to nulliparous and regressed. $CD4^+$ T cell accumulation is a sign of naïve T cell activation, as T cell proliferation and survival are directly downstream of TCR and co-stimulation, otherwise known as signals 1 and 2. We attribute the reduced $CD4^+$ T cell accumulation in lactation and involution to tolerogenic dendritic cell phenotypes and relative lack of signal 2 during T cell activation,

however the exact mechanisms of this immune tolerance are unknown. Lack of signal 2 can lead to immune tolerance in the form of T cell anergy, broadly defined as a state of T cell hypo responsiveness [254]. However, T cell anergy is usually accompanied with some amount of T cell proliferation/expansion [254]. Therefore, our observed lack of T cell expansion in lactation and involution hosts may be more consistent with T cell death by neglect (deletion), another mechanism of peripheral tolerance [157, 255].

Limitations of our studies and alternative approaches

Implications of our study in addressing risk of infection have a notable limitation. We have not tested if Th17-skewed CD4$^+$ T cells influence epithelial cell junctions in the mammary gland, although this biology is well described in the gut. Th17 skewed CD4$^+$ T cells are known to produce IL-17 which enhances epithelial cell junctions and barrier function in the gut [98-100]. While we postulate a similar mechanism may exist in the mammary gland we have not tested this relationship. First we would test if Th17 skewed CD4$^+$ T cells in the mammary gland produce IL-17 cytokines. If they do, we could test the role of Th17 cells in protecting the mammary tissue from bacterial infection.

An additional limitation of our study is the inability to assign our observed result of dampened naïve T cell activation in lactation and involution hosts to the specific activity of mammary tolerogenic dendritic cells. Stated another way, other cell types within the host may dampen T cell activation during lactation and involution, namely macrophages and B cells. Both macrophages and B cells are antigen presenting cells with the ability of presenting exogenous antigen to CD4$^+$ T cells to result in tolerance. Similar to dendritic cells, we know that

macrophages are enhanced in abundance in the mammary gland during involution. In order to test a specific role of mammary experienced dendritic cells in mediating immune tolerance we performed *ex vivo* T cell activation experiments using dendritic cells FACS isolated from reproductively distinct mice. The rationale for studying mammary localized dendritic cells is that these cells have experienced the microenvironments of lactation and involution and can travel to the tissue draining lymph node to influence naïve T cell priming. However, lymph node resident immune cells, including dendritic cells, may also have a role in priming naïve T cells during lactation and/or involution. However, we do not expect lymph node resident immune cells to be altered by the microenvironments of lactation and involution (although we have not specifically tested this) and therefore have elected not to focus on lymph node resident immune cells at this point. Numerous attempts all resulted in a lack of T cell activation even in positive control conditions. Thus, we could not address a specific role for dendritic cells in naïve T cell activation using *ex vivo* models.

Another way to assess a specific role for dendritic cells promoting immune tolerance would be to perform experiments in mice lacking dendritic cells, which we have not done. One such model is the CD11c-diptheria toxin receptor (DTR) transgenic mouse. This mouse expresses DTR on the surface of $CD11c^{+}$ cells, and the administration of diphtheria toxin causes apoptosis of $CD11c^{+}$ cells [256]. This model could be utilized to assess the necessity of dendritic cells in promoting T cell tolerance during lactation and involution. However, CD11c is also expressed on other cell types including macrophages, so this experiment would be complicated by this fact. A better model may be the precise removal of a specific subset of dendritic cell, either the $CD11b^{+}$ or $CD103^{+}$ dendritic cells which can be achieved very effectively with mice lacking transcription

factors necessary for the differentiation of these dendritic cell subsets [170, 256, 257]. In particular, $CD103^{+}$ dendritic cells are specifically depleted in mice lacking Batf3 or XCR1 transcription factors [170, 257].

Another possibility is that our *in vivo* T cell activation assays may not reflect the function of a mammary localized APC. Specifically, ovalbumin antigen that is injected into the mammary tissue may travel through lymphatic vasculature into the lymph node. Once soluble antigen arrives at the lymph node it would be ingested by lymph node resident APCs, such as dendritic cells or subcapsular macrophages which could dampen naïve T cell activation in lactation and involution hosts. This possibility could be addressed by labeling antigen with a dye and looking for accumulation in the lymph node, or assessing antigen uptake *in vivo* by cells in the mammary gland and lymph node.

I proposed several follow-up experiments above that could decipher the role of dendritic cells in promoting immune tolerance during lactation and involution. If other cell types besides dendritic cells are implicated, this would indicate even broader immune suppressive microenvironments during lactation and involution where multiple APCs and tissues may be able to preferentially support T cell tolerance. While this would be very insightful to delineate, the overall endpoint of T cell tolerance may be most important as it relates to risk of infection and tumor outcome.

Future Directions

My studies have focused on dendritic cell regulation of $CD4^{+}$ T cell priming. $CD4^{+}$ T cells are involved in supporting anti-tumor immunity by supporting cytotoxic T cell functions or

participating in direct tumor cell killing [244, 245]. We would like to assess dendritic cell regulation of $CD8^+$ T cell priming as a function of reproductive state to extend our current studies. We hypothesize that dendritic cells will support $CD8^+$ T cell tolerance most during lactation and involution, consistent with the tolerogenic dendritic cell phenotypes we have described. In order to test this we could utilize OT-I transgenic animals where the $CD8^+$ T cells recognize ovalbumin antigen and perform similar experiments as we have for $CD4^+$ T cell naïve priming. However, OT-I transgenic animals are on a C57BL/6 background, and most all our studies concerning dendritic cell phenotype and naïve T cell priming have been performed in Balb/c mice until this point. While this may not represent a problem, C57BL/6 animals are known to be more Th1 skewed versus Balb/c animals which are more Th2 skewed [258]. Thus, we may need to perform dendritic cell and T cell phenotyping studies in C57BL/6 hosts in the future in order to ascertain strain versus reproductive-state differences. Testing for enhanced $CD8^+$ T cell tolerance during lactation and involution will expand our understanding of immune contributions to tumor promotion at these reproductive states.

Another future direction would be to determine what microenvironmental factors may support enhanced tolerogenic dendritic cell functions during lactation and involution. One such factor is the COX metabolite PGE2 which is enhanced during involution. PGE2 has been reported to limit dendritic cell differentiation and maturation, and support dendritic cell induction of immune tolerance rather than activation [247, 259]. To reduce PGE2 in the involution microenvironment, NSAIDs are an effective strategy. Our hypothesis is that NSAID treatment during involution will restore naïve T cell priming to levels observed in nulliparous hosts through alleviation of tolerogenic dendritic cell functions.

Finally, we could test for a contribution of dendritic cells to the observed effect of NSAIDs in reducing tumor growth in involution hosts [2, 72]. In this model we could treat tumor bearing involution hosts with NSAIDs, which usually slows tumor growth, in the absence of dendritic cells using methods described above. If dendritic cells are a mechanism by which NSAIDs are effective in slowing tumor growth in involution hosts we would see less NSAID effect when dendritic cells are absent. It is important to note that in a recently published study from our lab NSAIDs resulted in enhanced peri-tumoral granzyme B^+ $CD8^+$ T cells, consistent with a potential role for dendritic cells in mediating NSAID effects of reducing tumor growth during involution.

Another possible mechanism by which dendritic cell function is altered by reproductive state is through changes in the extracellular matrix (ECM) composition. The ECM composition of the mammary gland is dynamically and dramatically dependent on reproductive stage, such that the mammary epithelial cell and its ECM have been defined as the functional unit of the epithelial cell (X). The role of the ECM in regulating immune cell function is a relatively unexplored area of research. Because lactation and involution states of the mammary gland have coordinated changes in both ECM composition and immune cell infiltrate/function, I explored potential causal relationships in an independent set of experiments. While these studies did not mature to completion, data obtained was sufficiently provocative to warrant inclusion, as follows.

Chapter VI: Extracellular matrix as a regulator of dendritic cell and epithelial cell biology; impact of reproductive state

Introduction

The mammary gland is unique because epithelial content and function are dynamically altered throughout the pregnancy, lactation, and weaning-induced involution cycle. Considering the functional unit of the mammary gland is defined as the epithelial cell plus the surrounding extracellular matrix [260-263], it is not surprising that mammary extracellular matrix, with collagen being the most abundant, undergoes similarly dramatic transformation throughout the reproductive cycle [44, 77, 81, 218, 264]. Numerous observations regarding the extracellular matrix throughout the reproductive cycle provides evidence for reproductive state dependent changes. For example, extracellular matrix remodeling occurs as part of ductal elongation during puberty, during gland expansion of pregnancy, with weaning, and with loss of ovarian hormone function of menopause, demonstrating that extracellular matrix changes accompany epithelial changes [265].

In addition to the epithelium and extracellular matrix, it has been argued that immune cells should be included into the functional unit of the mammary gland. Rationale for this inclusion is supported by evidence that all stages of mammary gland development are supported by, or coincident with, immune changes [6]. Further, interactions between these components—the epithelium, extracellular matrix, and immune cells—have proven to play essential roles during development. Additionally, all three are also involved in mammary tumor development and progression, making study of their interactions of high importance.

In the current study we chose to examine the mammary gland as a functional unit consisting of epithelium, extracellular matrix, and immune cells. Specifically, we investigated a role of

reproductive state dependent alterations in extracellular matrix structure and composition as it relates to epithelial and immune cell changes. In particular, we present data that mammary epithelial cells may respond to changes in collagen organization by altering production of type I IFN, a cytokine which supports Th1 immunity. We postulate that extracellular matrix-epithelial cell-immune cell crosstalk may regulate reproductive state differences in tumor outcome, and established *in vitro* cell culture models to begin to test this hypothesis. Further, we investigated a direct role for reproductive state changes in extracellular matrix on the regulation of immune cell phenotypes and functions. Because dendritic cells and T cells have well described roles in tumor rejection, we chose to focus on dendritic cells and T cells in this study.

The purpose of our inquiry is multifocal. First, this inquiry expands our current knowledge of dendritic cells and T cells in the mammary gland. Second, studying the role of extracellular matrix in regulating dendritic cell and T cell function is understudied. Greater knowledge of dendritic cell and T cell regulation is an important inquiry to understand normal biology and tumor progression in the mammary gland. Lastly, this inquiry allows us to understand how immune surveillance may be regulated in the mammary gland and could shed light on how immune function becomes disrupted when cancer develops.

We focused our experimentation on extracellular matrix proteins that are altered by reproductive state and have known roles in tumor progression—namely collagen, fibronectin, and tenascin-C. We show that these extracellular matrix proteins directly affect epithelium, T cells, and dendritic cells. Further, we predict that the interaction of these parameters will coordinate normal reproductive state development as well as alter the risk and progression of cancer.

Background

Reproductive state dependent changes in global extracellular matrix composition influences tumor progression

In order to study global alterations in mammary extracellular matrix as a function of reproductive state, the Schedin lab developed a method of mammary extracellular matrix extraction for use in various biological assays. This method relies on the unique biological properties of extracellular matrix proteins, specifically their high molecular weight and relative insolubility, which lends themselves to extraction via high concentrations of salt and urea. The resulting fraction is vastly enriched for non-cellular components and largely depleted of cellular contaminants [266]. Use of this semi-purified, tissue specific extracellular matrix has led to a functional understanding of how reproductive state dependent changes in extracellular matrix affects normal and tumor cellular behavior [73, 81, 267]. The potential use of theses extracellular matrices in cell culture models to test for effects on immune cells or stromal cells is exciting but currently not reported.

Using extracellular matrix isolated from mammary glands, our lab has implicated changes in extracellular matrix as a dominant factor in reproductive state dependent differences in tumor outcome. Notably, mammary extracellular matrix isolated from involution hosts supports enhanced tumor growth and metastasis when orthotopically co-injected with tumor cells into nulliparous hosts [73, 81]. Consistently with this observation, whole mammary extracellular matrix isolated from involution hosts supports tumor cell invasive phenotypes in 3D *in vitro* culture models [81]. Conversely, mammary matrix from parous hosts reduces tumor growth and burden when used in orthotropic tumor studies in nulliparous hosts. Further, parous mammary matrix suppressed tumor cell invasion, enhanced tumor cell expression of e-cadherin at cellular junctions, and reduced proliferation in 3D *in vitro* culture models [267]. These findings support a

dominant role for reproductive state dependent changes in extracellular matrix in mediating differences in tumor outcome. So far, a role for extracellular matrix in mediating stromal or immune cell function has not been specifically investigated.

With the paradigm-shifting observation that reproductive state dependent mammary matrix can regulate tumor outcome in rodent models, the next question was to interrogate which specific extracellular proteins may be involved in this profound biology. Mammary extracellular matrix is a complex mixture of proteins which fall into these broad classifications: fibrillar collagen, basement membrane, matricellular, and 'other' structural [264]. Using proteomics methods, and most recently with the advent of targeted quantitative proteomics [268], we have gained a more complete understanding of individual extracellular matrix proteins that change with the reproductive cycle [264]. In particular, previous studies from our lab have discovered that changes in mammary collagen, fibronectin, and tenascin-C are highly regulated by reproductive state. Given the well-established links between these proteins and tumor outcome, we have tried to understand if they contribute to reproductive state differences in tumor outcome. In the sections that follow I will discuss the known links between collagen, fibronectin, tenascin and tumor outcome, tumor cell phenotypes, and immunomodulation. In addition, I will discuss how these proteins are specifically altered by the reproductive cycle. I will first discuss mammary collagen since it is highly correlated with breast cancer risk and progression.

Collagen density as a risk factor for breast cancer

High breast density is a strong risk factor for the development of breast cancer, superseding other menstrual or reproductive factors [269]. It is estimated that 10% of postmenopausal women, and

20% of premenopausal women, have high breast density and that 1/3 of all breast cancers might be complicated by high breast density [270].

Much effort has been exerted to understand what contributes to breast density. Breasts predominated by cellular and stromal content have higher density than those composed mainly of adipose tissue. There is a great amount of variation in the relative abundance of the adipose, epithelial, and stromal compartments within the female breast [271]. These compartments vary in their x-ray attenuation characteristics, producing greatly variable radiological mammograms. Adipose tissue is radiologically lucent and appears dark on a mammogram, versus stromal and epithelial tissues which are radiologically dense and appear light on a mammogram. The appearance of light, or dense, regions can be expressed as a percentage of the whole tissue, resulting in what is called percentage mammographic density (PMD) [269-271]. High PMD, compared to low, is consistently, strongly, and significantly associated with an increased risk of developing breast cancer (odds ratios range from 3-6) [269, 272].

Since both epithelial and stromal components contribute to density, it is important to understand the relative contributions of these components as they relate to the biology of tumor initiation and progression. So far, previous research has yielded circumstantial evidence of a connection between epithelial cells, the extracellular matrix and breast density. Previous research found that epithelial cells are more abundant in areas of tissue that are denser, but that epithelial cell proliferation is not enhanced [273-275]. It has also been found that stromal fibrosis, and more specifically collagen content, is higher in denser breast tissue [273, 275-278]. In addition, other extracellular matrix molecules such as laminin and decorin, which can act as collagen cross linkers, are enhanced in tissue with high density [273]. Also, in mouse models where cells (epithelial or stromal) or matrigel, composed of extracellular matrix proteins laminin, collagen

IV, and enactin, were injected into the mammary gland, there was an enhanced density observed by mammography [279]. Together these data imply that both the epithelial and stromal compartments of the mammary gland can contribute to increased mammographic density. However, collagen has since been found to be a greater contributor to breast density than epithelium [275].

Aside from inherent differences in breast density amongst women [269], it is evident that molecules which contribute to breast density may also be regulated by reproductive state. Chief among these molecules is collagen, which we have shown to contribute to postpartum breast cancer in mouse models [44, 72, 267]. Understanding the intersection of these breast cancer risk factors—collagen density and reproductive state—may reveal novel therapeutic avenues for the treatment and diagnosis of postpartum breast cancer.

Collagen alterations with reproductive state are related to windows of tumor progression and protection

Fibrillar collagens are the most abundant extracellular matrix proteins in the mammary gland at all reproductive states, with collagen I specifically dominating. Because of the established role of collagen in breast cancer risk, our lab has thoroughly investigated dynamic regulation of collagen with respect to reproductive state. We previously reported that baseline fibrillar collagen abundance decreases during pregnancy and lactation, then increases during involution. Further, in the regressed gland 4 weeks post-weaning, there is a sustained enhanced abundance of fibrillar collagen in comparison to the nulliparous gland [44, 264, 267]. This finding was initially surprising because increased collagen density is associated with increased breast cancer risk, tumor progression in animal models [280, 281], and enhanced tumor cell invasion in 3D *in vitro*

culture models [72, 280]. The finding of enhanced collagen abundance in parity seemed to be in conflict with the protective effect of parity on breast cancer. However it has been reported that collagen organization, as an independent parameter from density, also regulates tumor progression [280]. The consideration of collagen organization as a predictor of breast cancer outcome led us to investigate reproductive state dependent alterations in collagen organization.

Fibrillar collagens (I, II, III, V, XI) can be organized into linear bundles with various tensile strengths and orientations with respect to the epithelium. Specifically, during weaning-induced mammary gland involution, a process dominated by epithelial regression, there is an enhanced deposition of collagen I that assumes a linear organization [72] as well as enhanced stiffness compared to other reproductive states (Schedin lab unpublished). Linearly arranged 'involution collagen' resembles the collagen found at the invasive fronts of breast cancers, which independently predicts poor patient outcomes [280-282]. In contrast, after the completion of mammary gland involution, when the mammary gland returns to a pre-pregnant state, collagen I reaches its highest abundance but has a lower tensile strength (soft) and is less linear [267].

In order to address a specific role for collagen organization in regulation of tumor cell invasive phenotypes, we developed a simplistic 3D *in vitro* model system. In order to generate relatively soft collagen, mimicking collagen found in the parous mammary gland, sonication was used to mechanically dissociate collagen. This resulted in collagen that alone was unable to polymerize (i.e. form long chains) and was mechanically softer than collagen that was not sonicated. In our experiments, soft, sonicated collagen mimicked parous collagen and fibrillar collagen mimicked nulliparous or involution collagen.

This model allowed us to test how collagen stiffness affected tumor cell behavior. We found that tumor cells cultured on fibrillar collagen had enhanced invasive phenotypes and expression of the poor prognostic marker COX-2 [72]. In contrast we found that sonicated collagen, which models the dense but softer collagen of the parous mammary gland, induced a quiescent phenotype in tumor cells, reduced invasive phenotypes, and up-regulated an immune activating gene signature [267]. These observations are consistent with the parous mammary gland exhibiting resistance to tumorigenesis [283-288]. The summation of our observations that collagen organization alters tumor cell phenotypes indicates that collagen organization is likely a contributor to reproductive state differences in tumor outcome, a powerful finding for highlighting the role of extracellular matrix in tumor biology. Given the powerful role for collagen in tumor biology, we have tried to understand if reproductive state dependent changes in other extracellular matrix proteins may relate to tumor progression or suppression.

Reproductive state dependent changes in fibronectin may contribute to differences in tumor outcome

Fibronectin has well described roles in tumor progression and is highly regulated by reproductive state. This makes fibronectin an attractive candidate for investigation as a contributing factor to reproductive state dependent differences in tumor outcome. Previous reports have shown that fibronectin is aberrantly expressed in many human cancers including colorectal and breast, where high expression serves as a poor prognostic marker [289-291]. Additionally, fibronectin enhances tumor cell growth, migration, invasion, and reduces therapeutic efficacy [291].

Fibronectin is a ubiquitous extracellular glycoprotein that is incorporated into a fibrillar matrix. Fibronectin self assembles into fibrils, networks, and bundles, but also interacts with glycosaminoglycans, cell surface receptors, and other extracellular matrix proteins including collagen [292]. Fibronectin is deposited by platelets and fibroblasts following cutaneous injury, and is a large component of the provisional wound matrix. The provisional matrix serves as a scaffold for cellular movement and eventual deposition of a mature matrix dominated by collagen which restores tissue strength. The EIIIA containing splice variant of fibronectin also specifically appears during wound healing, with roles in facilitating tissue repair [293].

Wound healing has provided a valuable framework for understanding how fibronectin may be involved in mammary gland involution, a wound-healing like process. In fact, as in wound healing, fibronectin is enhanced in the mammary gland following weaning. Further, the EIIIA containing splice variant of fibronectin, as well as fibronectin cleavage also specifically increase during involution [77, 294-296]. A role for fibronectin fragments in supporting mammary epithelial cell death has been demonstrated [294], however other roles related to tissue remodeling have not been investigated.

The discovery of a smoking gun: Tenascin-C may contribute to reproductive state dependent differences in tumor outcome

Another developmentally regulated extracellular matrix variation occurs in tenascin-C, which also has clear links to cancer. Tenascin-C is an onco-fetal extracellular matrix protein, meaning it is expressed during embryonic development and in the adult mostly during tissue repair or pathological situations, such as the progression of cancer [297-301]. High expression of tenascin C in breast tumors is associated with poor clinical outcome [290, 298]. It is important to note, however, that tenascin-C in tumors has been described to drive tumor promotion primarily

through a tumor cell centric mechanism; although, immune modulatory mechanisms are presumed but not described.

Tenascin-C is a glycoprotein comprised of EGF repeats, fibronectin type III repeats, and a fibrinogen globe domain. Individual monomers assemble into hexameric units via disulfide bonds. Tenascin-C interacts with fibronectin, perlecan, heparin, and cell surface receptors including various integrins and toll-like receptor 4 (TLR4). TLR4 expression by immune, stromal, and epithelial cells indicate that tenascin-C may impact a wide array of cell types and processes [302]. Tenascin-C is mostly deposited by stromal cells in response to cytokines, hypoxia, mechanical stress, or reactive oxygen species, i.e. tenascin-C expression usually accompanies tissue injury [303, 304]. In addition, tenascin-C can harbor alternatively spliced exons or be cleaved by matrix proteases to result in the release of bioactive fragments with specific functions on a wide range of cell types including epithelium, stromal cells, and immune cells [303, 305], with an equally wide range of effects.

Biological effects of tenascin-C include enhancing epithelial cell migration during wound healing and cancer cell migration. Tenascin-C can also lead to stromal cell activation and has immune modulatory functions on myeloid and lymphoid cells [305]. While tenascin-C expression in adult tissues is usually associated with pathology, our lab has reported a specific up-regulation of tenascin C in the mammary gland and liver during involution, a developmentally regulated normal process [81]. However, high levels of tenascin-C have also been observed during lactation, consistent with reports that tenascin-C is a milk protein [306, 307]. A role for tenascin-C in mammary gland involution has not been explicitly demonstrated, although presumed activities are tissue remodeling and immune modulation.

Demonstration that these ‘pro-tumorigenic’ extracellular matrix proteins are up-regulated in the normal mammary gland during the reproductive cycle, implicate changes in extracellular matrix in regulating tumor outcome across the cycle. In addition to the direct effects of reproductive state changes in extracellular matrix on epithelial and tumor cells, there are likely extracellular matrix effects on other cell types within the microenvironment such as immune or stromal cells. The field of extracellular matrix-immune cell regulation is not robustly established, however recent reports shed light on to this important topic.

Extracellular matrices can modulate dendritic and T cell functions

Since dendritic cells and T cells are major regulators of the body’s response to a developing tumor, my research has focused on these cell types when interrogating how extracellular matrix proteins influence immune cell function. However, even for these cell types, reports are limited concerning their extracellular matrix regulation. Thus, further exploration of this line of inquiry is expected to reveal new biological mechanisms underlying extracellular matrix influences on tumor progression, with a particular focus on reproductive state alterations.

A role for extracellular matrix in regulating general inflammation is established. For example, extracellular matrix stores growth factors and cytokines which, when released, control the spatiotemporal activation of a broad range of cell types and biological processes. Specifically, inactivate TGF-β is sequestered by the extracellular matrix, and upon tissue injury, protease activation, or mechanical pulling of the extracellular matrix releases active TGF-β. Active TGF-β influences almost every cell type within the body, because of the broad expression of TGF-β receptors, and brings about specific cellular processes based on cell type and tissue context. For example, TGF-β has been shown to suppress epithelial transformation or promote tumorigenesis depending on epithelial and tissue state [43]. Furthermore, TGF-β influences immune cells,

which then influences the development and progression of cancer. For example, TGF-β inhibits dendritic cell and T cell functions [131, 308, 309]. Specifically, TGF-β can reduce $CD8^+$ T cell upregulation of Tbet, a transcription factor associated with cytotoxic effector function [308]. Thus, TGF-β during lactation and involution may result in the loss of a potent anti-tumor mechanism.

In addition to indirect effects of extracellular matrix on immune cells, such as the release of cytokines, there are reports of direct extracellular matrix modulation of immune cell phenotypes and functions. This may represent an important component of how reproductive state dependent changes in extracellular matrix exert differences in tumor outcome, since immune function and the development and progression of cancer are intimately linked. In particular, we are interested in understanding how dendritic cells and T cells may be affected by reproductive state dependent changes in extracellular matrix. This is because dendritic cells are the most potent activator of naive T cell responses, which can eliminate tumor cells. Conversely, dampening of the dendritic cell -T cell activation axis is expected as tumors develop and progress. Understanding how to alleviate dendritic cell-T cell suppression may be a powerful therapeutic avenue. I will review what is known about how collagen, fibronectin, and tenascin C can regulate dendritic cells and T cells.

Collagen may regulate dendritic cell and T cell biology

As collagen is the most abundant extracellular matrix protein in the body, one may expect extensive studies into how collagen influences dendritic cells and T cells; however, this is not the case. Based on the available evidence, it is currently difficult to discern how collagen alters the dendritic cell-T cell axis of immunity, meaning more experimentation is needed.

Several studies have characterized how dendritic cell and T cells interact with and migrate through collagen, although a consensus on the mechanisms involved has not been reached. Several studies identified that a tyrosine kinase collagen receptor, discoidin domain receptor 1 (DDR1), may be utilized by both T cells and myeloid cells alike to interact with collagen [310, 311]. Consistent with this observation, another study showed that integrin subunits were not required by dendritic cells to navigate through 3D matrices but rather cytoskeletal rearrangements were more dominant [312]. In addition, there are conflicting reports of whether dendritic cells express β1 collagen binding integrins [312-314]. Taken altogether, there is insufficient data to determine how collagen interacting receptors may be utilized by dendritic cells and T cells.

In terms of how collagen may influence dendritic cell or T cell functionality, even fewer studies have been performed. Several studies have shown that collagen up-regulates dendritic cell maturation and T cell activation [315-317], although these studies utilized an allogeneic mixed lymphocyte reaction (MLR), which has been argued to have limited physiological value in assessing dendritic cell function. Allogeneic MLRs, like the ones used in these publications, primarily tests the ability of T cells to respond to a foreign MHC complex on the dendritic cells' surface to result in T cell expansion; this process is thought to happen in graft-versus-host disease. This reaction does not specifically test the ability of the dendritic cell to activate a T cell in the traditional manner of foreign antigen presentation, co-stimulation, and cytokine stimulation.

A single more recent study tested for an effect of collagen on dendritic cell-mediated T cell activation assays in the presence of a known antigen. However, the results were not quantitated, making a conclusion challenging [313, 314]. Furthermore, an effect of collagen structure, i.e.

fibrillar vs sonicated, on dendritic cell function has not been reported. These critical gaps in knowledge are important to address as collagen is the most abundant protein in the body, and is highly abundant in many tumor types.

Established role for fibronectin in activating dendritic cells

The EIIIA splice variant of fibronectin is a specialized molecule associated with tissue damage, and as such has been shown to activate the innate immune system. The splice variant EDA FN that is specifically up-regulated during wound healing and involution has been found to be a TLR4 agonist [318-320]. TLR4 is conserved across vertebrates and invertebrates and is mostly expressed by innate immune cells, including macrophages and dendritic cells, as a sensor for exogenous bacterial and viral PAMPs [302]. However, it has become evident that endogenous ligands for TLR4 also exist, such as heat shock proteins, saturated fatty acids, and EDA FN [302, 320].

In accordance with EDA FN being a ligand for TLR4, EDA-FN was found to activate dendritic cells *in vivo* to the same levels as LPS. In fact, it has been found that EDA FN can be utilized to deliver antigens to dendritic cells for the purposes of vaccination. Specifically, an EDA-FN ovalbumin fusion protein delivered *in vitro* was successful in rejection of ovalbumin expressing tumor cells, i.e. a successful dendritic cell vaccination strategy [320].

Since full length FN or other FN fragments do not bind TLR4, this is consistent with a unique immune modulatory capacity of the damage-associated EDA FN specifically [319]. However, there are several reports that dendritic cells and T cells can bind to full length FN through various receptors [321-323], and the effects of these interactions are variable [313-315] as they

relate to dendritic cell or T cell phenotype or function. In conclusion, an effect of FN is most well described in the context of EDA FN acting as a TLR4 agonist, and studies of full length FN or other FN fragments are very few, meaning more experimentation is needed to address this gap in knowledge.

Tenascin-C: A dichotomous tale concerning effects on dendritic cells and T cells

Like EIIIA fibronectin, Tenascin C has been shown to activate the innate immune system as a TLR4 agonist. However, in seeming opposition to the previous statement, Tenascin C has strong inhibitory roles on T cells. These dichotomous effects of tenascin C warrant further exploration to gain a more complete picture of this interesting biology.

Tenascin C has been most well studied for how it regulates macrophages and T cells, with an effect on dendritic cells notably lacking. Tenascin C, like EIIA FN, has been shown to be a TLR4 agonist which has shown to activate macrophages and fibroblasts in mouse models of rheumatoid arthritis, leading to disease progression via the production of pro-inflammatory cytokines [324]. Further, tenascin C was shown to support macrophage response to LPS via up-regulation of the miRNA155 in animal models of sepsis [325]. Together these studies implicate that tenascin C may enhance myeloid function similar to a response to infection, but a role for T cells has not been described in these models.

Tenascin C has been reported to activate innate immune cells, which may be expected to result in downstream activation of naïve T cell priming and/or memory T cell activation. However, to the contrary, studies testing an effect on tenascin C on T cell functionality have mostly found suppressive effects. Specifically, tenascin C was found to suppress human T cell activation in response to CD3 ligation or activation with phorbol ester, maybe because of blockade of

Interleukin 2 receptor (IL-2R) up-regulation that usually accompanies T cell activation [326]. This finding was corroborated in another study where the fibronectin type III domain of tenascin-C (TnFnIIIA-D) was responsible for inhibiting T cell activation via down-regulation of TCR and CD8 expression by T cells [327].

These findings of tenascin C inhibition of T cell activation are in accordance with observations of de novo expression of tenascin-C in many types of human and mouse cancer [298]. An important study explored the presumed suppressive action of tumor microenvironment tenascin-C on tumor infiltrating T cells. They found that tumor infiltrating T cells from tenascin-C high tumors had decreased proliferative capacity and IFNγ production when isolated and stimulated *ex vivo* [328], consistent with tumor tenascin-C suppressing cytotoxic T cell functions. Further, these studies utilized tenascin-C *in vitro* and found a direct suppressive effect on T cell activation. They performed gene expression analysis of T cells stimulated in the presence of tenascin-C and found suppression of IL2, IL2 receptor, IL4, IL4 receptor, TNF-beta, interferon regulatory factor, ICAM, and VCAM, among others [328]. These molecules are up-regulated with T cell activation via the activation of numerous downstream signaling pathways, indicating that tenascin-C interferes with early events in T cell activation [328], although the precise mechanisms are not known.

In sum, these publications indicate that tenascin-C may enhance innate immune cell function via activation of TLR4 but may directly inhibit T cell activation. Importantly, an integrated approach to understand how the entire APC-T cell axis may be altered by tenascin-C is also lacking. Further, a role for tenascin-C in specifically modulating dendritic cell function has not been described. This inquiry represents a missing link in the field given the known potency of dendritic cells in activating T cells. Better understanding of how innate and T cell immunity are

linked and how this axis may be altered in environments with tenascin C, for example mammary gland involution, would be a valuable addition to the fields of normal and cancer biology alike.

Type I Interferon (IFN) regulates dendritic cell and T cell immunity and responses to cancer

In addition to direct effects of extracellular matrix on dendritic cells, the extracellular matrix may also indirectly influence dendritic cell biology through cell crosstalk. Specifically, we postulate that collagen organization alters mammary tumor and normal epithelial cell type I IFN signaling and production, which may influence dendritic cells and T cells in the environment to regulate tumor outcome. To better understand the potential power of this postulated cell-crosstalk, it is important to understand how type I IFN regulates dendritic cells and T cells.

Dendritic cells both produce and respond to type I IFNs [329]. Specifically, dendritic cell differentiation and maturation is enhanced by type I IFN, resulting in enhanced T cell activation and humoral immunity [329-331]. Specifically, type I IFNs supports enhanced dendritic cell activation as measured by up-regulation of dendritic cell activation markers CD80, CD86, MHCII, and CD40 [329]. Further, numerous studies have identified that dendritic cell antigen processing is supported by type I IFN [332] [333]. Further, type I IFNs enhance dendritic cell ability to activate T cells [330]. Several studies have shown that type I IFNs can operate through dendritic cells to mediate early immune mediated tumor elimination [334-338]. Because of the well described roles of type I IFN in promoting dendritic cell immunity, we chose to investigate indirect effects of extracellular matrix on dendritic cells through type I IFN regulation.

Materials and Methods

Mammary tumor and epithelial cells

D2.OR mammary tumor cells were originally isolated from a spontaneous mammary tumor in Balb/c mice by Dr. Henry Miller. Our laboratory obtained D2.OR cells from Dr. Ann Chambers (University of Western Ontario). D2.OR cells were cultured and used as previously described [267]. MCF10DCIS.com tumor cell lines were generously obtained from Dr. Kornelia Polyak (Dana-Farber Cancer Institute) and used as previously described [72, 267].

Bone marrow dendritic cell cultures

Mouse bone marrow was harvested from the femur and tibia of Balb/c of C57BL/6 female mice between the ages of 8-20 weeks. Material was filtered (100 µm) and red blood cells lysed (eBioscience) per manufacturer's instructions, then live cells were counted as trypan blue negative on a hemocytometer. Cells were plated at 1 million cells per well of a 24 well tissue culture plate in RPMI-1640 media supplemented with 5% heat inactivated FBS, 1x HEPES, 20 ug/ml gentamicin, 50 uM beta -mercaptoethanol, and 10 µg/ml of each mouse purified GM-CSF and IL-4 (Worthington biochemical). Old media was removed and replaced with fresh every other day. On day 4, floating and loosely adherent cells were collected with warm PBS by gently

washing the plate. This fraction is enriched for dendritic cells, whereas the cells stuck on the plate are enriched with macrophages [339]. Cells were then counted and 60,000 bone marrow cells per well of a 96 well plate were plated on either plastic or pre-made extracellular matrix pads (see below) with or without the addition of 10 ug/ml lipopolysaccharide (Sigma) for 24 hours.

Extracellular matrix pad preparation

Purified rat tail Collagen I (Corning) was neutralized and diluted to 4 mg/ml in dendritic cell media without GM-CSF or IL-4. Half the volume of collagen was subjected to sonication using a Branson sonifier 450W model (Danbury, CT) set to duty cycle of 60% and output of 6. A total of 6 minutes of sonication was performed, in 1 minute increments with 5 minute breaks in between each cycle. Sonication was performed in a pre-chilled cooler rack, to prevent collagen warming and polymerization. To confirm sonication, collagen was run on a SDS-PAGE tris-acetate gel and fragments no larger than ~120 kDa were observed. The fibrillar collagen aliquot was placed on the sonicator probe for the same length of time as the sonicated collagen in order to control for this variable; however, the fibrillar collagen was not sonicated. Fibrillar or sonicated collagen were then mixed with 4 mg/ml matrigel at a ratio of 40% to 60% respectively. Mixing matrigel with collagen allowed for polymerization in the sonicated collagen condition, which is not possible with sonicated collagen alone. Addition of matrigel to fibrillar collagen kept polymerization consistent between all conditions. A condition of 100% matrigel was used as a control. Fibronectin or tenascin-C were added to 4 mg/ml matrigel at a final concentration of 10 ug/ml. Thus the total protein concentration in the extracellular matrix pad was around 4 mg/ml but composed of various mixtures of matrigel with collagen, fibronectin, or tenascin-C. These

extracellular matrices were mixed and kept on ice, then utilized in various 3D culture models (below).

Tuft's 3D culture model

For microarray and qPCR analyses, D2.OR, Eph4, MCF10DCIS.com, and bone marrow derived dendritic cells were cultured using a "tuft's" 3D model where cells are embedded in extracellular matrix. First a 50µl layer of pure matrigel was placed atop a 0.4µM filter placed in well of a 24-well plate and allowed to solidify for 90 minutes at 37°C. Then 70,000 cells per well were re-suspended in 200µl of pre-mixed extracellular matrices (as explained above) and placed atop the matrigel layer and allowed to solidify for 90 minutes two hours, media was then placed on top of the culture and replaced every 2-3 days.

Overlay 3D culture model

For assessing dendritic cell phenotype and for dendritic cell -T cell activation assays, or T cell activation assays without dendritic cells, an overlay 3D model was used. For this assay, 50µl of pre-mixed extracellular matrices (as explained above) were added to one well of a 96-well plate and allowed to solidify at 37C for 2 hours- overnight then cells were plated a top this pad and cultured for 1 day in the case of assessing dendritic cell activation and 4 days in the case of assessing T cell proliferation.

Ex vivo T cell activation assays

$CD4^+$ or $CD8^+$ splenocytes were isolated from OT-II or OT-I transgenic female mice, respectively (Obtained from Lisa Coussens' laboratory) and enriched (to >95%) using a $CD4^+$ or

CD8$^+$ T cell negative selection kit (MACS miltenyi). T cells were then stained with CellTrace (Invitrogen) tagged with either CFSE or far red. Dendritic cells were loaded with antigen: either peptide (SIINFEKL) or whole protein antigen. For peptide antigen loading 1 fg/ml of SIINFEKL peptide (Worthington Biochemicals) was added to dendritic cells and incubated for 30 minutes at 37C, then dendritic cells were washed 3-4 times with PBS to remove all unbound peptide. For protein antigen loading 100 μg/ml of ovalbumin protein (Worthington) was incubated with dendritic cells for 8 hours, then dendritic cells were washed 3-4 times. After antigen loading dendritic cells were mixed with T cells at a ratio of 1:6 on plastic or extracellular matrix pads in T cell media which consists of RPMI-1640 with the addition of 10% heat inactivated FBS, 2mM L-glutamine, 50 μM beta-mercaptoethanol, and 1x pen/strep. Three or four days later, for OT-I or OT-II cells, respectively, cells were harvested and flow cytometry staining was performed as described below.

Flow cytometry

Cells were harvested by either collagenase digestion (in the case of extracellular matrix pads) or by using warm versene (EDTA) in the case of culture on plastic. This ensured that all cells were captured for endpoint analyses. Samples were blocked with CD16/32 (eBioscience 1:100) and stained with Live Dead (aqua, Invitrogen 1:500) for 30 minutes, then washed 1x with PBS. Cells were stained with antibodies for extracellular proteins (CD45, 30-F11; MHCII, M5/114.15.2; B220, RA3-6B2; CD11b, M1/70; CD11c, N418; F480, BM8; Ly6C, HK1.4; Ly6G, 1A8; CD103, 2E7; CD80, 16-10A1; CD86, GL-1; CD4, RM4-5; PD-1, 29F.1A12; CD3e, 145-2C11) diluted in buffer (1% BSA in 1x PBS) for 30 minutes at RT. Cells were fixed (BD cytofix) and permeabilized (eBioscience) per manufacturer’s instructions and stained with antibodies for

intracellular proteins (Gata3, 16E10A23; RORγT, AFKJS-9; FoxP3, FJK-16s), diluted in permeabilization buffer (eBioscience) for 2 hours at 37C or overnight at 4C. Cells were fixed again and run on an 18 color flow cytometer (Fortessa BD) in the OHSU flow cytometry shared resource. Data were analyzed using FlowJo software.

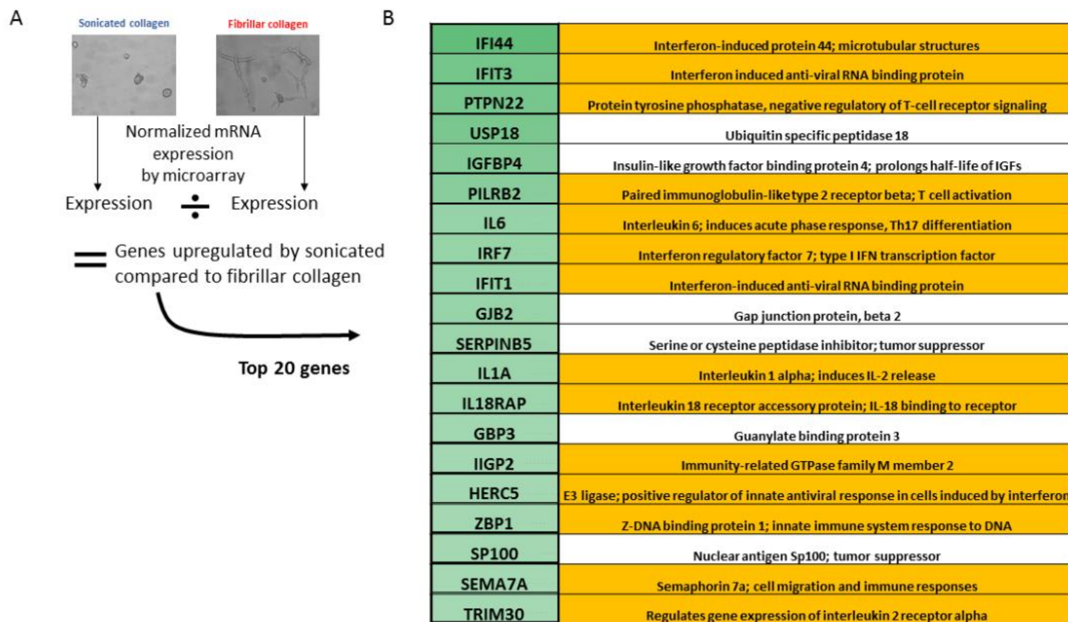
RNA extraction and qPCR

Media was removed from 3D pads gently to not disrupt the cells. 750µl of TriZol LS (Invitrogen) was added to the pad and pipetted up and down in order to break-up the pad. The suspension was then passed through a homogenization column (QiaShredder, Qiagen), and spun at 12,000 x g for 10 minutes. TriZol-LPS chloroform extraction was performed. To generate one sample 3-4 replicates were pooled at this stage. An equal volume of 70% ethanol was added to the resulting upper (aqueous) layer containing RNA. The precipitated RNA was added to an RNeasy micro kit (Qiagen) to purify the RNA and digest contaminating DNA per the manufacturer's protocol. RNA was assessed using the NanoDrop for concentration and contamination with organics and proteins. cDNA was prepared using the BioRad iScript cDNA synthesis kit per the manufacturer's protocol and using random primers. cDNA was used in RT-PCR reactions run on a myiQ Single-Color Real Time PCR detection system from Biorad with custom designed and validated primers from IDT Technologies. Gene expression was calculated using the delta-delta Ct method [340] relative to actin. Fold changes in gene expression relative to fibrillar collagen were calculated.

Results

Type I IFN gene signature enhanced in mouse mammary normal and tumor cells cultured on sonicated collagen

I began these studies by assessing the effects of collagen organization as a modulator of gene expression changes in mouse mammary tumor cells, with the goal of interrogating genes that might influence immune cell function. Notably, we found a type I IFN gene signature that was up-regulated by sonicated collagen, indicating epithelial cell-dendritic cell crosstalk. This study relied on a previously performed microarray analysis of mouse mammary tumor cells (D2.OR) cultured in fibrillar or sonicated collagen [267]. As part of my study, a ratio of normalized expression values of cDNA targets was calculated to determine genes specifically up-regulated in D2.OR mammary tumor cells cultured in sonicated collagen compared to fibrillar collagen (Figure 1A).



Gene	Description
IFI44	Interferon-induced protein 44; microtubular structures
IFIT3	Interferon induced anti-viral RNA binding protein
PTPN22	Protein tyrosine phosphatase, negative regulatory of T-cell receptor signaling
USP18	Ubiquitin specific peptidase 18
IGFBP4	Insulin-like growth factor binding protein 4; prolongs half-life of IGFs
PILRB2	Paired immunoglobulin-like type 2 receptor beta; T cell activation
IL6	Interleukin 6; induces acute phase response, Th17 differentiation
IRF7	Interferon regulatory factor 7; type I IFN transcription factor
IFIT1	Interferon-induced anti-viral RNA binding protein
GJB2	Gap junction protein, beta 2
SERPINB5	Serine or cysteine peptidase inhibitor; tumor suppressor
IL1A	Interleukin 1 alpha; induces IL-2 release
IL18RAP	Interleukin 18 receptor accessory protein; IL-18 binding to receptor
GBP3	Guanylate binding protein 3
IIGP2	Immunity-related GTPase family M member 2
HERC5	E3 ligase; positive regulator of innate antiviral response in cells induced by interferon
ZBP1	Z-DNA binding protein 1; innate immune system response to DNA
SP100	Nuclear antigen Sp100; tumor suppressor
SEMA7A	Semaphorin 7a; cell migration and immune responses
TRIM30	Regulates gene expression of interleukin 2 receptor alpha

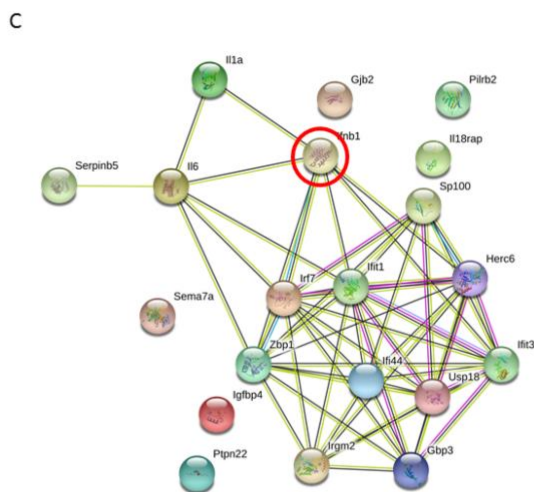


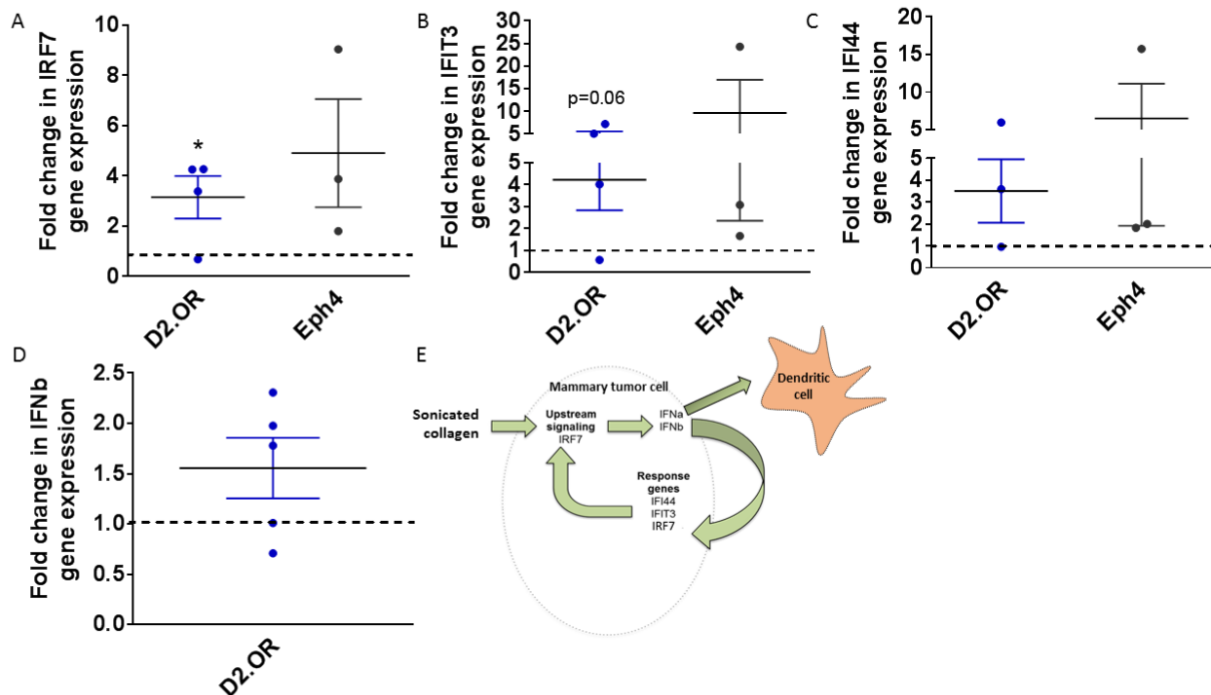
Figure 1: A) Experimental schematic. Mouse mammary tumor cells (D2.OR) were cultured in sonicated or fibrillar collagen for four days, then total RNA was isolated, reverse transcribed and cDNA was applied to an affymetrix microarray chip, as described and published previously. Relative expression of genes was calculated and a ratio was determined, as shown to result in a lists of genes up-regulated or down-regulated in D2.OR cells cultured on sonicated collagen. B) The top twenty most up-regulated genes in D2.OR cells cultured on sonicated compared to fibrillar collagen. Brief gene descriptions are provided. Gene descriptions highlighted yellow indicate a function related to immunomodulation. Further, many genes are involved in either the response or regulation of type I interferons.

The top 20 most up-regulated genes in D2.OR mouse tumor cells cultured on sonicated collagen are listed in Figure 1B. To our surprise, only 6 of these 20 genes have primarily epithelial cell intrinsic effects, whereas 14 of these 20 genes have known roles in immune modulation (shaded

orange, Figure 1B). More specifically, many of these 14 genes are involved in type I interferon (IFN) response or production. Upon STRING gene network analysis, a strong association with IFN-beta emerged (circled in red, Figure 1C).

Several of the type I IFN related, namely IRF7, IFIT3, and IFI44, were validated to be up-regulated by sonicated collagen in D2.OR mouse mammary tumor cells in independent experiments as detected by qRT-PCR (Figure 2A-C). Surprisingly, these gene changes induced by sonicated collagen in tumor cells also may be induced in normal mouse mammary epithelial cells (Eph4) (Figure 2A-C), indicating a potentially conserved biological response.

To confirm a type I IFN response in D2.OR tumor cells on sonicated collagen, we performed qPCR analysis for IFNb. We observed a trend towards increased mRNA expression of IFNb in



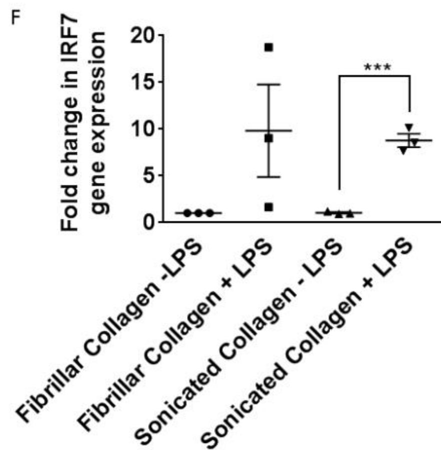


Figure 2: Type I IFN related genes are up-regulated in response to sonicated collagen. Mouse mammary tumor (D2.OR) or normal epithelial (Eph4) cells were cultured on sonicated or fibrillar collagen for four days, then RNA was isolated, reverse transcribed, and subjected to real time qPCR analysis for various genes identified in a microarray database (Figure 1). Relative expression of genes was determine as related to actin, then fold change expression in cells on sonicated collagen calculated as related to expression on fibrillar collagen (set to 1, dotted line). There was up-regulation of IRF7 (A) and a trend towards increased IFIT3 (B), IFI44 (C), and Interferon beta 1 (D) in D2.OR tumors cells on sonicated collagen, and similar trends in normal mammary epithelial cells (A-C). (D) Schematic shows genes as related to upstream signaling in induction of type I IFN (IRF7), and downstream response to type I IFN (IFI44, IFIT3, IRF7), and a feed forward loop of type I IFN up-regulation (IRF7) supports the enhanced production of type I IFN in cells on sonicated collagen.

D2.OR mouse mammary tumor cells on sonicated collagen (Figure 2D). Together these data indicate that sonicated collagen may promote a type I IFN epithelial cell-dendritic cell crosstalk mechanism (Figure 2E).

Type I IFN related gene expression by sonicated collagen lead us to question whether immune cells themselves may respond similarly to changes in collagen structure. We focused on dendritic cells since type I IFNs results in increased maturation, activation, antigen presentation, and dendritic cell mediated T cell activation. We cultured mouse bone marrow derived dendritic cells on fibrillar or sonicated collagen in the absence or presence of lipopolysaccharide (LPS), an established TLR4 agonist known to induce dendritic cell activation. We found that LPS results in up-regulation of IRF7, the master type I IFN regulator, in dendritic cells on both collagens (Figure 2F).

Sonicated collagen enhances dendritic cell maturation

We next questioned whether collagen organization could alter dendritic cells as measured by established parameters, including abundance and up-regulation of co-stimulatory molecules CD80 and CD86. To this end, we cultured bone marrow derived immature dendritic cells on fibrillar or sonicated collagen with or without the addition of lipopolysaccharide (LPS). We found that dendritic cell abundance as measured by flow cytometry was enhanced by sonicated collagen although only significantly in Balb/c bone marrow cultures, with similar trends observed in C57BL/6 cultures (Figure 3A-B). Further analysis revealed that dendritic cell expression of T cell co-stimulatory molecules CD80 and CD86 were enhanced upon culture on sonicated collagen, in the absence of LPS (Figure 3C-D). These data indicate that dendritic cell maturation and activation are enhanced by sonicated collagen. We also assessed for dendritic cell expression of IL-12, a cytokine which supports dendritic cell activation of cytotoxic $CD8^+$ T cells or Th1 skewed $CD4^+$ T cells [150]. We found enhanced dendritic cell expression of IL-12 when cultured on sonicated collagen (Figure 3E). These data indicate that dendritic cells may have an enhanced ability to activate T cells when cultured on sonicated collagen.

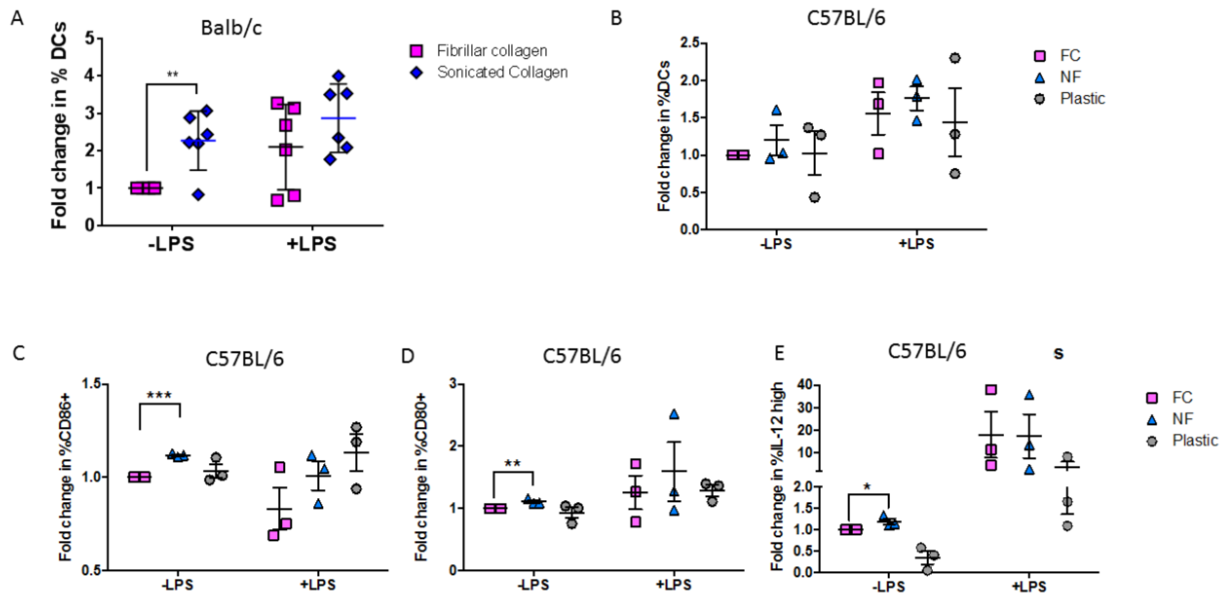


Figure 3: Sonicated collagen is superior at supporting dendritic cell characteristics consistent with T cell activation compared to fibrillar collagen. Bone marrow derived dendritic cells (DCs) were generated from Balb/c (A) or C57BL/6 (B-E) mouse bone marrow using GM-CSF and IL-4. DCs were then exposed to fibrillar or sonicated collagens with or without lipopolysaccharide (LPS) stimulation for 24 hours, then flow cytometry was performed. Fold changes of quantitated parameters were calculated relative to the fibrillar collagen without LPS condition (set to 1). (A) Fold change in %DCs in the final culture was enhanced by sonicated collagen in Balb/c derived cultures, with a similar trend observed in C57BL/6 cultures (B). Dendritic cell activation was enhanced by sonicated collagen as measured by increased expression of CD86 and CD80 (C-D) without LPS stimulation. Similar trends were observed in the presence of LPS. (E) DC expression of IL-12 was enhanced by sonicated collagen in the absence of LPS. N=6 for panel A and N=3 for panels B-E, and graphs represent averages +/- SEM. The symbol * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ by two tailed unpaired student's T Test.

Extracellular matrices modulate dendritic cell activation of T cells

We predicted that direct effects of extracellular matrix on dendritic cells can be measured using T cell activation as a readout of dendritic cell function. In order to test this, we cultured $CD4^+$ or $CD8^+$ T cells that specifically recognize ovalbumin antigens with dendritic cells that had been pre-loaded with ovalbumin antigens on plastic or various extracellular matrix proteins. At the

assay endpoint, flow cytometry was performed and the percent of original T cells that underwent proliferation (% divided) and the average number of divisions in proliferative cells (Division

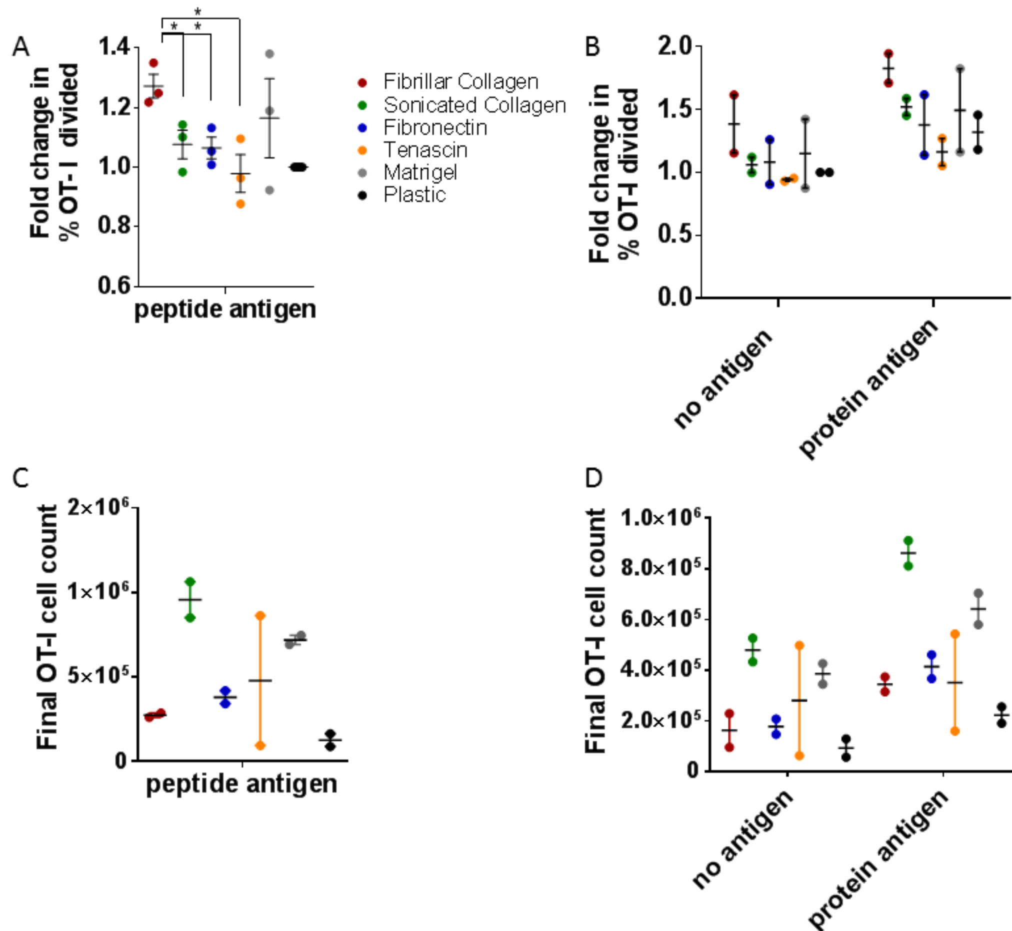


Figure 4: CD8+ T cell proliferation and accumulation is altered by dendritic cell (DC)-T cell culture on variable ECM substrates. (A) Percent CD8+ (OT-I) T cells that divided when DCs were loaded with peptide antigen or (B) protein antigen and mixed with T cells on various ECM substrates. Data are normalized to DC-T cell interactions on plastic. (C) Absolute OT-I T cell count at assay endpoint when mixed with DCs loaded with peptide antigen or (D) protein antigen as determined by the addition of absolute counting beads in flow cytometry. N=3 for panel A and N=2 for panels B-D. Averages +/- SEM are graphed. For panel A the symbol * indicates $p<0.05$ as determined by two tailed unpaired student's T test.

index) was calculated. We found that dendritic cell presentation of peptide antigen resulted in significantly reduced CD8+ T cell proliferation on sonicated collagen, fibronectin, or tenascin-C compared to fibrillar collagen (Figure 4A). This trend was conserved in cultures where protein

antigen was used (Figure 4B). However, we did observe a trend toward increased abundance of T cells from sonicated collagen pads, data supportive of dendritic cell activation on sonicated collagen but inconsistent with the decreased proliferation observed in this condition (Figure 4). We were surprised that fibrillar collagen supported the most T cell proliferation of any extracellular matrix protein tested. A prediction of this result would be enhanced absolute numbers of $CD8^+$ T cells from fibrillar collagen pads compared to the other extracellular matrix proteins, however this was not observed in preliminary experiments (Figures 4C-D). This result shows that fibrillar collagen supports T cell proliferation in the absence of survival. Similar trends were observed when we assessed $CD4^+$ T cell proliferation and accumulation when cultured with dendritic cells loaded with protein antigen on various extracellular matrix pads (Figure 5). Altogether these data indicate the existence of direct extracellular matrix dependent dendritic cell functions that alter T cell activation and survival.

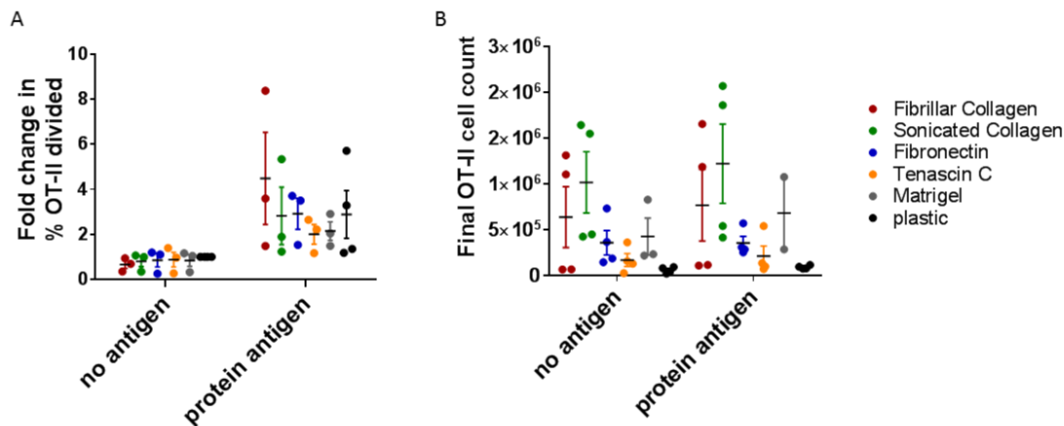


Figure 5: $CD4^+$ T cell proliferation and accumulation is altered by Dendritic cell (DC)-T cell culture on variable ECM substrates. (A) Percent $CD4^+$ (OT-II) T cells that divided when DCs were loaded with protein antigen and mixed with T cells on various ECM substrates. Data are normalized to DC-T cell interactions on plastic. (B) Absolute OT-I T cell count at assay endpoint when mixed with DCs loaded with protein as determined by the addition of absolute counting beads in flow cytometry. N=3 for ECM conditons and N=4 for plastic conditions. Averages +/- SEM are graphed.

Extracellular matrices directly influence T cell activation, independent of a dendritic cell effect

We next wanted to test a direct role for extracellular matrix in altering T cell activation, without the dendritic cells as a conduit. There is a precedence for this question in several scientific reports, and we sought to extend this line of inquiry to determine how established reproductive state dependent mammary extracellular matrix changes may directly influence T cell activation. This question is important since this may relate to reproductive state dependent differences in tumor outcome. We found that tenascin C consistently stunted T cell activation compared to all other extracellular matrix proteins tested.

We cultured $CD4^+$ or $CD8^+$ T cells that specifically recognize ovalbumin antigens on plastic, or various extracellular matrix proteins in the absence or presence of stimulation. We found that fibrillar collagen supported higher levels of proliferation (Figure 6A) and divisions (Figure 6B) compared to all other extracellular matrix proteins. We also found that tenascin C reduced $CD4^+$ and $CD8^+$ T cell proliferation (Figure 6C-D). A role for collagen organization in altering T cell proliferation was not found. Altogether these results show that extracellular matrix composition can directly alter T cell proliferation.

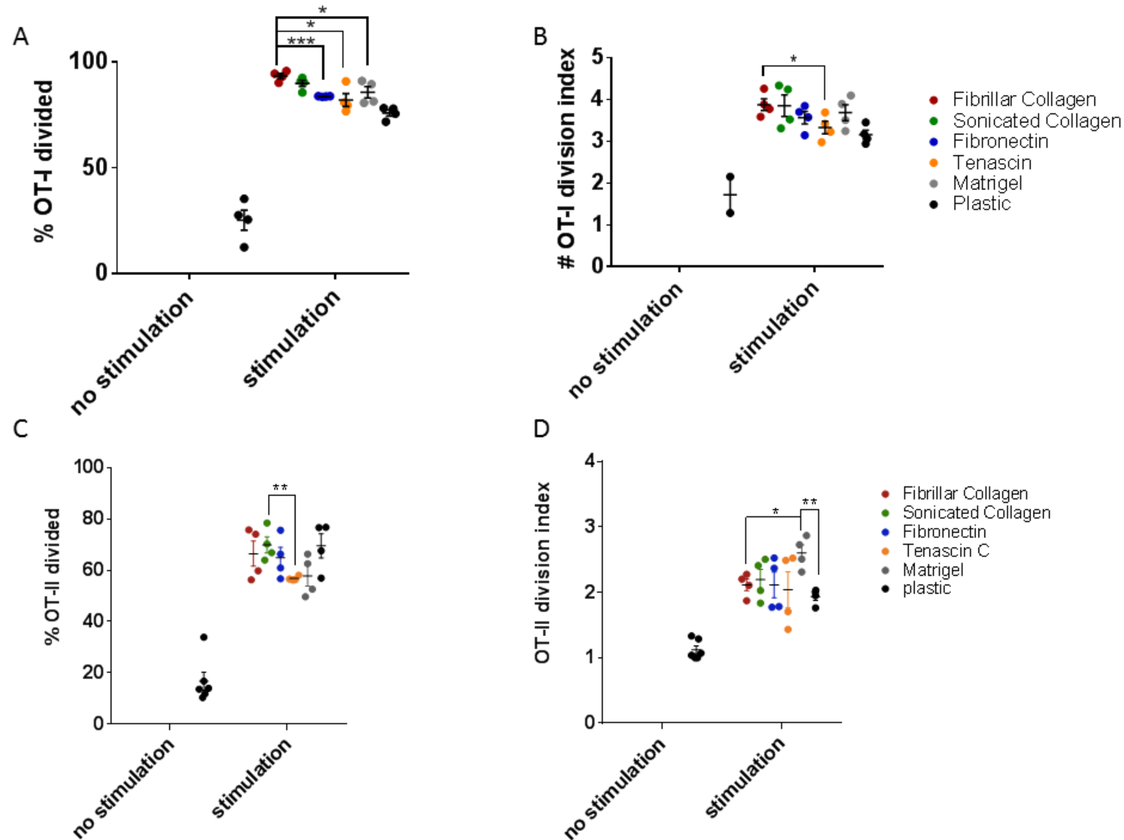


Figure 6: T cell proliferation in response to stimulation is directly altered by extracellular matrix. (A) Percent OT-I T $CD8^+$ T cells divided and (B) division index in response to stimulation (SIINFEKL peptide) when cultured on plastic or various ECM substratum. (C) Percent OT-II $CD4^+$ T cells divided and (D) division index in response to stimulation (anti CD3/28 beads) when cultured on plastic or various ECM substratum. N=4 for all conditions, averages +/- SEM are shown, and * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$ by two tailed unpaired student's T test.

Discussion

We tested a direct effect of extracellular matrix proteins on regulating epithelial, dendritic cell, and T cell phenotypes as well as indirect effects of extracellular matrix proteins in the crosstalk of these cell types (Figure 7). One of our first findings was that sonicated collagen directly enhanced type I IFN signaling in mouse mammary tumor cells. This finding raises the possibility that sonicated collagen may be an endogenous TLR ligand and may be responsible for type I IFN

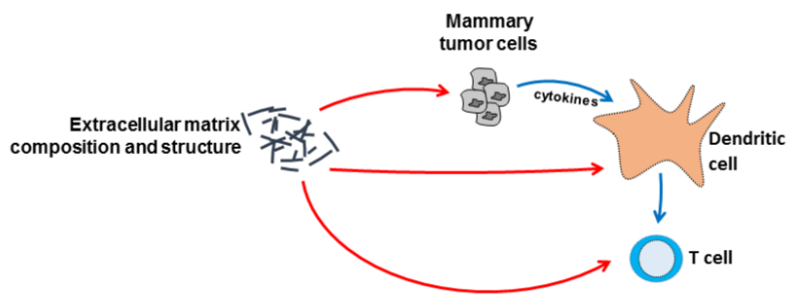


Figure 7: A model of our study of direct and indirect effects of extracellular matrix proteins on epithelial cells, dendritic cells, and T cells. Direct extracellular matrix regulation are shown with red lines. Indirect effects of extracellular that were tested are mediated by cellular crosstalk and shown with blue lines.

induction in epithelial cells, since epithelial cells are known to express TLR receptors. One may postulate that this program may serve to alert the body to the potential of invading pathogens that often occurs with tissue damage and breach of barriers to the external environment. Further, since sonicated collagen is being used to model the parous mammary gland there may be a larger effect of type I IFN induction on reproductive state dependent differences in tumor outcome.

Our result raises the possibility that mammary tumor cells on sonicated collagen may produce more type I IFN than tumor cells plated on pro-tumorigenic fibrillar collagen. We also found a type I IFN gene signature in non-tumor mammary epithelial cells cultured in sonicated collagen.

Since type I IFN affects dendritic cells to become activated, cross presents antigens, and activates dendritic cell mediated anti-tumor $CD8^+$ T cell responses [337, 338] it is possible that an extracellular matrix-epithelial cell- IFN axis plays a previously undescribed role in tumor behavior. Specifically, our data is consistent with enhanced dendritic cell -T cell immune surveillance by sonicated (parous-like) collagen which may eliminate nascent tumor cells and reduce the appearance of tumors. This observation fits with the protective effect of parity against the development of tumors.

There is also the possibility that extracellular matrix structure and composition directly affects dendritic cells. Our results indicate that dendritic cell activation is enhanced when cultured on sonicated collagen. In preliminary studies we tested if β1 integrins mediated this effect, but no change was observed (data not shown). Upon further review we found conflicting reports of beta 1 expression in dendritic cells [312-314]. Further, other collagen receptors are expressed in dendritic cell such as DDR1, DDR2, and OSCAR [311, 341], which we have not tested in our system. In addition, other alterations in dendritic cell activation state or cytokine production are possible with culture on differing substratum and this warrants further investigation. For example, aside from T cell co-stimulation, dendritic cell production of TNF receptor family members enhances T cell activation and survival [342].

Our results of fibrillar collagen reducing dendritic cell activation state but supporting dendritic cell mediated T cell proliferation are in apparent conflict. In fact, fibrillar collagen supported T cell activation to a greater degree than all other extracellular matrix proteins. However, in our studies fibrillar collagen did not result in an increased number of T cells present in the culture at study endpoint, as would be expected with increased proliferation. This phenomenon of proliferation in the absence of survival has been described as a mechanism of inducing T cell

tolerance- i.e. the effective removal of a reactive T cell [254]. Our results are in accordance with the hypothesis that fibrillar collagen enhances immune tolerance via proliferation in the absence of survival. If true, assessment of molecules involved in T cell survival following activation would be expected to be reduced by fibrillar collagen. Of note, the factors that may be involved are IL-2 or TNF receptor family members, which could be tested in future studies.

Our observation of tenascin-C dampening dendritic cell -mediated T cell activation is in accordance with other reports [326, 328]. However, our study is expected to shed light on the mechanisms behind this biology because we performed in depth assessment of proliferation, including percent original divided and number of divisions, as well as overall cell accumulation. We found reduced proliferation and accumulation, consistent with T cell anergy. This result enhances the understanding of how tumor tenascin-C may interrupt dendritic cell mediated T cell activation and further how this axis may be modulated for cancer therapy.

As part of our study we have also tried to address how extracellular matrix proteins may directly alter T cell response to activating signals. Similar to what was observed in the dendritic cell -T cell mixed cultures, as explained above, we saw that fibrillar collagen had the highest ability to support T cell activation compared to any other extracellular matrix protein tested. Fibrillar and sonicated collagen did not differ substantially in these experiments in their ability to alter T cell activation.

Our studies of direct effects of extracellular matrix proteins on dendritic cells and T cells alone are straightforward. However, when ECM-DC-T cell mixtures are analyzed the direct effects of ECM on either DCs or T cells alone is hard to decipher. Comparing mixed cultures to single cultures helps us to understand the relative contributions of ECM-DC and ECM-T cell

relationships to the overall endpoint of T cell activation (Figure 7 red and blue lines both influence T cell endpoint). One such example is the observation that sonicated collagen supports T cell activation in single cultures, but reduces T cell activation in mixed cultures with dendritic cells. This suggests that the negative effects of sonicated collagen on T cell activation are mediated through dendritic cells, rather than through direct regulation of T cells. This paradigm is different than is the case of fibronectin or tenascin-C, which alone both directly limit T cell activation. Thus, in the case where fibronectin or tenascin-C are cultured with dendritic cells and T cell mixtures, there is likely a combinatorial negative effect of extracellular matrix on dendritic cells and T cells to result in the final experimental outcome. One may be able to tease apart the individual effects of ECM-DC and ECM-T cell regulation by interrupting how dendritic cells or T cells interact with extracellular matrices, assuming they do so differently.

Our study of extracellular matrix effects on dendritic mediated T cell activation warrants a review of how it may relate to *in vivo* T cell activation. Dendritic cells reside within tissues where they remain poised to respond to insults or perturbations of homeostasis. When dendritic cells become activated, by a vast array of stimuli, there is an up-regulation of activation markers, including MHCII, CD80, and CD86. Dendritic cell activation also results in a concomitant increase in CCR7, a lymph node homing molecule [130, 135]. Dendritic cells then migrate to tissue draining lymph nodes via CCR7 where they can interact with naïve T cells that recognize the antigen they are presenting to bring about new T cell activation, also called T cell priming [135].

Extracellular matrices may influence this well described process at various points. For instance, dendritic cells in the tissue may be imprinted with a memory of how they were activated,

including potentially an input from the extracellular matrix. However, a role of extracellular matrices in this process has not been described.

Another point at which extracellular matrices may influence dendritic cell - mediated T cell priming is in the lymph node itself, which is supported by a reticular collagen-rich extracellular matrix [343-345]. Furthermore, it is now clear that lymph nodes are dynamic with respect to extracellular matrix composition, including even the presence of tenascin-C [344, 345]. This raises the possibility that altered lymph node extracellular matrix composition or structure may be altered by reproductive state and affect dendritic cell mediated T cell activation. This possibility is currently unexplored.

Another point at which extracellular matrices may influence dendritic cell mediated T cell priming is in tertiary lymphoid structures. There structures are reminiscent of *bona fide* lymph nodes but exist in non-lymphoid peripheral tissues, such as the gut. Although tertiary lymphoid structures have not been described in the mammary gland during normal development, they do appear in mammary tumors [346, 347]. An influence of reproductive state dependent extracellular matrix changes on dendritic cell mediated T cell priming in tertiary lymph nodes structures of the mammary gland remains to be determined.

Finally, another possibility altogether is that naïve T cell activation may occur in the mammary gland, although this is contrary to accepted dogma. This has not been definitely demonstrated but dendritic cells have been shown to interact with T cells in the mammary tissue [13, 14, 130]. In fact about 50% of mammary residing T cells may be naïve [13]. If you are so bold to consider naïve T cell activation in the mammary tissue, then an influence of extracellular matrix is easy to imagine.

Aside from dendritic cells affecting naïve T cell activation, it is also possible that extracellular matrix may influence dendritic cell activation of previously activated T cells, termed memory T cells. Memory T cells reside in peripheral non-lymphoid tissues, such as the mammary gland, and can be re-activated outside of lymph nodes [135]. This inquiry is intriguing giving our results in chapter III where we show that memory T cells are recruited to the mammary tissue specifically during involution. A possibility is that involution specific extracellular matrix changes may recruit and activate memory T cells, a provocative and unexplored hypothesis. Overall, understanding how naïve or memory T cell activation is altered by extracellular matrix and dendritic cells has great implications for our understanding of how baseline immune state is altered with a reproductive cycle. Further, these studies may impact our understanding of reproductive state dependent differences in tumor outcome.

Altogether our study provides rationale for further investigation of how reproductive state dependent changes in collagen, fibronectin, and tenascin C may mediate epithelial cell-immune crosstalk, dendritic cells, T cells, and dendritic cell mediated T cell activation. Further, exploration into how these numerous extracellular matrices controlled processes may contribute to tumor promotion during involution and tumor protection in parity is warranted in the future. This line of inquiry is expected to reveal new inroads in the fields of breast cancer, tumor microenvironment, and immunotherapy.

APPENDIX: AN EXTENDED DISCUSSION OF LACTATION INSUFFICIENCY

Lactation is critical for human health, but poorly defined as a biological process

It is important to understand the biological programs underlying successful lactation because of the impact it has on infant health and survival, particularly in less developed areas of the world. While lactation has been well studied from the aspect of neonatal health, basic biological underpinnings of lactation programs in the mammary gland are largely missing. Our results will expand our understanding of immunological programs that may ensure successful lactation. Breast milk provides nourishment and immunity which is superior to formula supplementation. This is especially true considering that formula supplementation is not possible or safe in many parts of the world. Further, many parts of the world have high incidence of parasites which are 20-fold more likely to result in mortality in non-breast fed infants [240, 348]. Herein lies the importance in understanding biological programs underlying successful lactation.

Measuring successful lactation versus lactation failure is difficult given that the breast is one of the only organs without a diagnostic test to measure function. The lack of information related to lactation physiology as well as a lack of definition of successful lactation is striking. In a 2007 publication under the heading of "Physiology of Lactogenesis" discussion is heavily slanted towards how a mother's mental health, intentions, body image, and spousal support all contribute to the positive breastfeeding experience [240]. While potentially true, this information does not provide the needed biological understanding of lactation success or failure.

Ambiguity pertaining to lactation is especially evident when considering how lactation insufficiency or failure are defined and treated. Some of the clinical indications of lactation

insufficiency or failure are infant weight loss, no audible swallowing from the baby while suckling, reduced infant urine and stool output, infant irritability, minimal breast changes, or painful nipples. Although it is hard to know the exact prevalence of lactation insufficiency or failure, estimates between 5-15% of women have been reported [349, 350]. If lactation insufficiency or failure is suspected, the mother's risk factors are considered in an attempt to find a solution. Risk factors for lactation insufficiencies and failure are primiparity, stress, maternal obesity, diabetes, hypertension, unscheduled cesarean section, low breast feeding frequency, breast surgery, retained placental fragments, cigarette smoking, PCOS, and postpartum hemorrhage [240, 351]. Many of these risk factors indicate hormonal or metabolic underpinnings of lactation insufficiency and failure. And in fact, lactation insufficiency can be treated by hormone or metabolic treatment [240]. However, the biological mechanisms by which lactation insufficiency are propagated or alleviated in the mammary tissue are not defined.

Mastitis is a common condition that limits lactation

Additional evidence suggests that lactation success requires an evasion of breast inflammation, generally termed mastitis. Otherwise stated, mastitis results in decreased lactation. Mastitis is prevalent, occurring in 10-27% of breastfeeding women [348, 352]. Mastitis, technically defined as inflammation (-itis) of the breast (mastos-) is defined as localized breast pain, often coincident with fever, chills, aches, and reduced milk output. Much of our knowledge about mastitis comes from dairy cows, where mastitis is a significant and costly problem. In cows and humans alike, mastitis can be caused by bacterial infection. The most common pathogen associated with mastitis is *Staphylococcus aureus*, although *Streptococcus* or *Escherichia coli* are also noted [353] need cow ref). The use of antibiotics to treat mastitis is standard of care in both humans

and cows. Studies of cow mastitis and treatment with antibiotics have provided a deep understanding of bacterial mastitis that deserves review.

Mastitis is the most common and costly disease in the daily cow industry. The costs of mastitis are estimated to be about 2 billion dollars annually [348]. In addition, it is estimated that mastitis results in about 15% of bovine culling, and about a 911 kg loss of milk production per affected cow per lactation period [354]. The costs of mastitis come in the forms of milk loss, veterinary services, diagnostics, and treatments [355]. Because of the deep losses associated with bovine mastitis, intra-mammary antibiotic treatment is used prophylactically in most cases. As such, massive efforts in understanding the biology underlying bovine mastitis have been put forward with the goals of reducing mastitis incidence and generating improved therapeutics.

Lessons from bacteria induced mastitis in bovine models

The most well described mastitis models in bovine involve the injection of bacteria into the teat canal. This model has been utilized to study the bovine immune response to bacteria-induce mastitis. One of the primary responses to bacterial inoculation into the mammary gland is innate immune cell activation following by accumulation of neutrophils into the mammary gland and milk [210, 356]. Neutrophils act as the first responders to reduce bacteria proliferation and ward off disease. In addition to an innate neutrophil-driven response to intra-mammary bacteria, adaptive and antigen specific immune responses have also been shown to occur.

It has been shown that immunization of cows with specific antigens, in particular ovalbumin or bacteria antigens, leads to a rapid influx of neutrophils into the mammary tissue upon inoculation of the specific antigen into the teat canal. Subsequent studies identified that $CD4^+$ T cells were

required for recruitment of neutrophils to the mammary gland [210]. However, the exact identity of the adaptive immune response to intra-mammary bacteria and mechanism of neutrophil recruitment has remained elusive until recently.

In a series of recent publications, the incidence of IL-17 cytokines (IL-17A and IL-17F) have been discovered in the context of antigen or bacteria specific infection of the mammary gland. These studies are coincident with the observation that IL-17 cytokines influence bovine mammary epithelial cells to up-regulate chemokines and self-defense proteins [357]. Specifically, IL-17 related cytokines in conjunction with IFNγ characterized the early responses of the mammary gland to antigen/bacteria challenge [358]. Further, the authors saw staining of IL-17 in the mammary gland in the absence of infection leading them to postulate that IL-17 cytokines may be part of the mammary gland defense system during homeostasis, as well as infection. These results show that Th-17 related immunity may be involved in the mammary gland response to infection, which is common to mucosal tissues.

Altogether bacteria-causative mastitis that occurs in cows results in a neutrophil and IL-17 related immune response. These responses liken the mammary gland to a mucosal organ because Th17 related defenses are common in mucosal sites. These studies do not address what types of mucosal immunity support the mammary gland during homeostasis, i.e. during lactation in the absence of overt bacterial infection. Further, other mucosal immune programs such as immune suppression are suspected but not well defined in the lactating cow mammary gland [32]. Further, there is low appreciation for how mammary gland mucosal defenses may be altered by reproductive state, even though there are reports of altered immune tolerance and activation

based on bovine reproductive state [359-361].

Mastitis can occur in the absence of overt bacterial infection

The link between mastitis and bacterial infection has been strengthened by extensive bovine studies. This has led to the dominant hypothesis that human mastitis is caused by bacterial infection. However, there are pieces of evidence which suggest that mastitis may occur in the absence of overt bacterial infection. First, there does not seem to be a correlation between mastitis disease severity and bacterial load [362, 363]. Second, similar levels of staphylococcus aureus and streptococci in breast milk of women with and without mastitis has been reported [362]. Third, Staphylococcus aureus and streptococci are common bacterial species in the nasal passages and skin, therefore the mere presence of these species does not always correlate with disease. These evidences suggest that mastitis, and therefore lactation success and milk output, may be a more complex condition than we currently understand.

Mastitis as an inflammatory condition of unknown origin

An alternative theory of the cause of mastitis is inflammation of unknown origin. It is clear that the severity of mastitis is correlated with local and systemic markers of inflammation, including fever, IL-6, IL-1, IL-8, TNFa, and C-reactive protein [364, 365]. One proposal is that bacterial activation of innate immune cells via TLRs jump starts inflammation that can lead to mastitis in the absence of overt infection. In fact, bacteria activation of TLR4 during lactation results in reduced milk output in mice [29]. Also, NF-κB activation during lactation induces mammary epithelial cell apoptosis and milk decline [30]. Consistent with NF-κB as a Th1 inflammatory mediator, Th1 cytokines are known to reduce lactation and Th2 cytokines enhance lactation [27,

28]. These evidences support the relationship between an innate immune system driven Th1 inflammatory milieu and reduced milk production, and may underlie bacterial infection independent mastitis.

An argument that mastitis is autoimmunity in the lactating mammary gland

In particular, a form of mastitis called idiopathic granulomatous mastitis has lead researchers to a deeper understanding of the immune requirements for successful lactation. This form of mastitis is rare and most commonly occurs in women of reproductive age who are lactating or recently lactated [34]. Idiopathic granulomatous mastitis is a local inflammatory reaction that does not respond to antibiotics but does respond to steroids [35]. This has led to the acceptance of idiopathic granulomatous mastitis as an autoimmune disease of the breast [36, 37]. The observation led Kesaraju and colleagues to investigate the possibility of adaptive autoimmune reactions to milk components [33].

To investigate the possibility of an autoimmune disease of the breast, Kesaraju and colleagues immunized mature SWR/J females with alpha lactalbumin protein prior to mating. Alpha lactalbumin is a protein that is expressed only during lactation for the purpose of lactose production. Following immunization they observed a Th1 immune milieu (IFNγ) and T cell infiltrate only in females who were lactating. They did not observe any inflammation in non-immunized or non-lactating female mice. Further, mice exhibiting Th1 autoimmunity in the lactating gland failed to sustain their pups, leading to pup alopecia, runting, and liver toxicity [33]. The results of this study supports several important conclusions:

1. Th1 T cell inflammation in the mammary gland results in lactation failure. This means that Th1 inflammation may be an underpinning of lactation failure and mastitis that has not been appreciated.
2. Autoimmunity to a lactation specific antigen is possible. This means that not all lactation specific proteins may be expressed in the thymus, and thus not all milk-reactive T cells removed by the process of central tolerance.
3. Peripheral tolerance is essential to the success of lactation (if point number 1 above is true). This means that immunological mechanisms that drive immune tolerance may be essential to lactation success.

In sum, the biological understanding of lactation success or failure are ill defined. Lactation insufficiencies or failures may be overcome with hormone treatment, however this does not always work, highlighting the naivety surrounding lactation treatment. Further, mastitis is well described for limiting lactation, however the etiologies underlying mastitis are vast and may include autoimmunity

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