Innate immune cells may be involved in prepubertal bovine mammary development

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ABSTRACT

Pre-pubertal bovine mammary development involves ductal and stromal tissue changes. In mice, this process is impacted by presence of innate immune cells. Whether or not such immune cells are present or involved in bovine mammary development is unknown. We studied the presence, location and changes in numbers of eosinophils, mast cells and macrophages in pre-pubertal bovine mammary tissue. Chemical stains and immunofluorescence were used to identify the cells in formalin fixed, paraffin embedded mammary tissue. The first set (ONT) included samples (n=4/week) from birth to 6 weeks of age. Another set (OVX) determined the influence of ovaries, 19 animals were intact or ovariectomized 30 days before sampling. They were 90, 120 or 150 days old at examination. The third set (EST) allowed examination of the potential influence of exogenous estrogen on innate immune cells in the mammary gland. Samples were from calves given estrogen implants (n=6) or placebo (n=4) at 56 days old, and sampled at 70 days old. We examined 20 images each of NEAR and FAR stroma from every animal. More eosinophils were observed in NEAR versus FAR in the ONT and OVX , more mast cells observed in NEAR versus FAR in ONT. More macrophages were observed in NEAR versus FAR in ONT and EST. We show, for the first time, that innate immune cells are present in prepubertal bovine mammary tissue and that abundance is related to the epithelial structure. We suggest a possible role for these cells in control of bovine mammary development.

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF FIGURES v

LIST OF TABLES vi

CHAPTER 1: REVIEW OF LITERATURE 1

1.1 Overview of Mammary Gland Development 1

1.2 Immune Cells in Mammary Development 5

1.3 Hormonal Control of Early Mammary Development 11

1.4 Conclusions 15

CHAPTER 2: INNATE IMMUNE CELLS MAY BE INVOLVED IN BOVINE MAMMARY DEVELOPMENT 17

2.1 Introduction 17

2.2 Materials and Methods 19

2.3 Results and Discussion 29

2.4 Conclusions 38

CHAPTER 3: EPILOGUE 39

LITERATURE CITED 42

APPENDICES 45

LIST OF FIGURES

FIGURE 2.1: A) Luna stain for eosinophils. Arrow indicates an eosinophil with dark pink/red granules and blue nucleus. Inset is enlargement of the eosinophil. B) May-Grunwald Giemsa stain for mast cells. Arrow indicates a mast cell with distinctive purple granules and blue nucleus, inset is enlargemen of the picture. C) Immunofluorescent stain using anti-macrophage antibody. Green is anti-macrophages, blue is a DAPI counterstain. Arrows indicate macrophages. Scalebars on large images represent 50µm, scalebars on insets represent 10µm. **25**

FIGURE 2.2: Illustration of NEAR (N) stroma, FAR (F) stroma and epithelium (E) in mammary tissue at 4x (A), 10x (B) and 40x (C) magnification. Scale bar represents 50µm. Pictures are of tissue sections stained with May-Grunwald Giemsa, May-Grunwald Giemsa and Luna stains, respectively. **28**

FIGURE 2.3: Significantly more macrophages were observed in the NEAR versus FAR stromal areas in ONT (A) and EST (B) (P=0.0001 and 0.0004, respectively). There was a tendency for a treatment and location effect (greater in NEAR vs FAR) in the OVX set (P=0.081 and 0.083, respectively). No other significant effects were observed. **32**

FIGURE 2.4: eosinophils were observed in the NEAR versus FAR stroma in ONT (A) and OVX (B) sets (P=0.0004 and 0.038, respectively). There was a tendency for an effect of age in the ONT (P=0.08). No significant effects were observed in the EST (C) set. **34**

FIGURE 2.5: More mast cells were observed in the NEAR versus FAR in the ONT set (A, P=0.0001). There was also a trending effect of age in the ONT set (P=0.0594). There were no significant effects in the OVX set (B). There was a tendency for more mast cells in the ACC versus ACCEST in the EST set (C, P=0.076), but no effect of location. **37**

LIST OF TABLES

TABLE 1 Results of antibodies against macrophages tested in bovine tissue. All antibodies were tested in bovine lymph and tonsil tissue prior to use in bovine mammary tissue. - indicates did not work, +, ++, and +++ indicate signal: fair, good and best. NA indicates the antibody was not tested with this antigen retrieval method. Symbol before the slash indicates signal, symbol after the slash indicates background. This is important to note as there were some antibodies which had good signal but which had such high background that computer processing was not practical. **54**

CHAPTER 1: REVIEW OF LITTERATURE

1.1 Overview of mammary gland development

Mammary gland development begins in the fetus prior to birth, but is not completed until the establishment of lactation. The earliest signs are the formation of a mammary bud from the ectoderm and associated primitive mammary spouts (Akers, 2002). Progressive development though fetal life results in mammary fat pad formation by around day 80 of gestation and the canalization of primary and secondary spouts by day 100 (Akers, 2002). At birth, both rodent and bovine species have a mammary fat pad and some ducts (Sheffield, 1988), while bovines also exhibit the formation of a teat, teat cistern and gland cistern (Akers, 2002). Most species display the same rudimentary mammary structure at birth (Akers, 2002). The post-natal period of mammary development is characterized by the formation of a unique mammary fat pad into which epithelial cells later differentiate as well as the presence of a minimal rudimentary ductal system upon which future development will occur (Sheffield, 1988; Akers, 2002).

The developing post-natal bovine mammary gland undergoes a period of isometric growth after birth through 1.5 months of age, which is characterized by rates of increase in mammary DNA content and body mass during this period (Sinha and Tucker, 1968) as well as the appearance of an isolated mass of mammary parenchymal tissue (Capuco and Akers, 2010). This period of isometric growth generally lasts until about 1.5 months of age, when the developing gland enters a period of accelerated growth which is allometric to the rest of the animal (Sinha and Tucker, 1968). This period of allometric growth, which encompasses the peri-pubertal developmental time period, lasts from 1.5-9 months of age or through the first 2-3 estrous cycles (Sinha and

Tucker, 1968). This period of accelerated mammary growth is of interest due to the interrelated developmental events which occur with the potential to affect future production of the animal through direct impacts on regulation of mammary cell proliferation and ductal development as well as alterations to other physiological systems that can impact future mammary function (Tucker, 1981). Specific to the dairy industry, it is of interest to investigate the possible manipulation of mammary development during this period of allometric growth as a tool to maximize future production.

The allometric growth period is characterized by the development of a more complex ductal system in both mice and cattle (Akers, 2002; Macias and Hinck, 2012). Growth of the ductal system during this period occurs in a highly ordered manner (Sheffield, 1988) which provides the framework for the lobulo-alveolar mammary development which occurs in late gestation (Macias and Hinck, 2012). As illustrated in rodents, elongation of the ductal system in mice occurs at a specialized structure on the end of a duct, termed a "Terminal End Bud" (TEB)(Williams and Daniel, 1983). These structures are typically club-shaped and located at the distal end of all elongating mammary ducts (Sheffield, 1988), they serve a dual function of adding new cells and forward movement of the duct through the fat pad from the region of the nipple (Silberstein, 2001). TEBs eventually transition to Terminal End Ducts (TED) as ductal elongation reaches completion, when the cells which form the cap of the TEB differentiate into myoepithelial cells (Williams and Daniel, 1983; Macias and Hinck, 2012). TEBs contain several cell types which allow the elongation of the duct. Mammary stem cells form a cap over the end of the proliferating duct, with a population of

proliferating epithelial cells present underneath which provide the cells needed for elongation of the duct (Sheffield, 1988). These epithelial cells become progressively more differentiated as they become more distal to the cap (Sheffield, 1988). Bovine mammary development differs dramatically from human and rodent development at this point. Extensive ductal branching occurs in bovines in concert with the elongation of ducts (Capuco et al., 2002). Moreover, developing ducts in heifers do not contain TEBs (Rowson et al., 2012), but instead contain clusters of ductules arising from larger ducts which are referred to as Terminal Ductule Lobular Units (TDLU) (Hovey et al., 1999). Bovines also differ in the presence of proliferating cells throughout the branching duct rather than only at the ductal end as in rodents (Capuco et al., 2002). Ductal growth was observed to preferentially expand dorsally away from the teat (Capuco et al., 2002). The advancement of the parenchyma in a rather dense form results in a mature virgin mammary gland consisting primarily of epithelium and associated connective tissue with very few adipocytes in ruminants (Hovey et al., 1999). This is in contrast to the relatively sparse, adipocyte rich mouse mammary gland (Macias and Hinck, 2012).

The surrounding stromal environment is particularly important to the appropriate development of mammary ducts. Not only is there evidence that the mammary fat pad, which forms the stroma surrounding the developing parenchyma, mediates the local response to some hormones, it also houses the vasculature and lymph systems as well as providing a 3-D matrix for the physical development of the ductal system (Hovey et al., 1999). There is an inherent requirement in mouse ductal development for adipose tissue (Hovey et al., 1999). The mammary fat pad, which contains connective tissue and fibroblasts along with a variety of other cell types (Hovey and Aimo, 2010), helps to

define the growth of the parenchyma (Rowson et al., 2012). The mammary fat pad helps to shape the developing ducts through a variety of means, including through inhibitory signals as growing ducts approach either the edge of the fat pad or another duct (Silberstein, 2001). This serves to allow for space for the later growth of side branches. The compact nature of mammary development in the bovine suggests there are fewer inhibitor signals so that branches and buds are more closely aligned even in pre-pubertal development. Regardless, the mammary fat pad provides stromal structure and signaling to developing ducts, playing a significant role in the ultimate 3-D structure of the mammary gland.

Following the period of allometric mammary growth and rapid ductal development pre- and peri-pubertally, the mammary gland returns to an isometric pattern of growth around 9 months of age (Sinha and Tucker, 1968). In rodents, a loose network of mammary ducts with minimal branches fills the entire mammary fat pad as animals approach puberty (Macias and Hinck, 2012). After puberty there is progressive formation of side branches as well as alveolar buds which will give rise to areas of lobulo-alveolar development, especially during gestation. The mammary gland undergoes relatively little change from this point until gestation when, under the influence of pregnancy hormones, lobulo-alveolar development is dramatically stimulated in preparation for lactation (Tucker, 1981; Akers, 2002). The allometric growth period, which occurs peri-pubertally (at 1.5-9 months of age in cattle) is a very important stage in the development of the mammalian mammary gland, and changes to factors such as hormones or the stromal environment during or prior to this period of growth can have lasting effects on the resulting adult mammary gland. The resulting

ductal structure from this period of development provides the framework for the lobuloalveolar development which takes place during pregnancy (Akers, 2002).

1.2 Immune cells in mammary development

Overview

The importance of leukocytes in the mammary cycle is well established in the period of involution following each lactation, as several studies have found different classes of immune cells in the involuting gland, which act to help remodel the stromal environment through digestion of cellular debris (Reed and Schwertfeger, 2010). However, studies in pre-pubertal mice suggest that some classes of immune cells are also involved in at least the initial developmental stages of ductal formation in the mammary gland (Coussens and Pollard, 2011). This is presumably related to their capacity to regulate epithelial cell proliferation, as large numbers of neutrophils and other white blood cells have been shown to aggregate around end buds of growing ducts (Silberstein, 2001). There is substantial evidence of increased numbers of macrophages and eosinophils, though no evidence of altered numbers of B cells, T cells or neutrophils, in the developing mammary stroma (Gouon-Evans et al., 2002). Several studies have evaluated the importance of leukocytes to normal mammary development. One, which supports their general importance, involved the irradiation of mice prior to formation of TEBs to deplete hematopoietic stem cells and the correlated impairment of subsequent mammary development (Gouon-Evans et al., 2000). When leukocytes were depleted to a very low level without killing the mice or directly affecting mammary epithelial or stromal cell proliferation as observed through BrdU incorporation, irradiated

mice exhibited an impaired outgrowth of the epithelial tree and absence of TEBs (Gouon-Evans et al., 2000). However, the mammary development of the treated mice was rescued following bone marrow transplantation, which replaced the leukocyte populations (Gouon-Evans et al., 2000). This confirms the general importance of leukocytes in mammary development. Each specific leukocyte localizes to a different place in the developing mammary gland, and may aid in facilitating specific morphological changes in ductal tree development. Of particular note is the location and proposed roles of macrophages, eosinophils and mast cells.

Macrophages

Macrophages are typically thought of as part of the wound healing process or involution, as their primary role is that of phagocytosis (Reed and Schwertfeger, 2010) However, macrophages are also known to help orchestrate tissue morphogenesis and remodeling in the mammary gland throughout the estrous cycle through secreted factors such as pro-inflammatory cytokines and interleukins (Chua et al., 2010). This closely parallels the proposed function of macrophages in the developing mammary gland, where they could potentially act by supplying trophic factors for epithelial cell growth or matrix remodeling (Gouon-Evans et al., 2002). Macrophages are generally thought to be found at the initiation of mammary development pre-puberty in the stroma surrounding the newly formed TEBs, and to disappear as the TEB transitions into a more mature form (Coussens and Pollard, 2011). In rodents, macrophages are typically recruited during puberty to localize in the neck region of the TEB, and have been shown to be important for ductal elongation, branching, and shape (Reed and Schwertfeger, 2010).

An important molecule in macrophage function is Colony Stimulating Factor (CSF-1). CSF-1 is a major regulator of the mononuclear phagocyte lineage (from which macrophages are derived), as it is required for their survival, differentiation, and proliferation (Lin et al., 2002). Some macrophages are also dependent on CSF-1 for their tissue localization; this subset of cells is associated with the scavenger and trophic functions of macrophages (Lin et al., 2002). Given the importance of CSF-1 to the recruitment and actions of macrophages throughout the body, a CSF-1 null mutant mouse model has been useful in the investigation of macrophage involvement in mammary development. Using that model, macrophages were shown to be an indispensable part of proper mammary ductal tree development and morphogenesis during the pre-pubertal period. The Csf1 gene is the major growth factor required for macrophage proliferation, differentiation and recruitment (Gouon-Evans et al., 2000), therefore Csf1 null ($Csfm^{op}/Csfm^{op}$) mice provide a good model for investigating the necessity of macrophages to mammary development. Csf1 null mutant mice were shown to have a delayed onset of TEB formation as well as changed morphology of major ducts ultimately resulting in fewer ducts overall when compared to the heterozygous control mice (Gouon-Evans et al., 2000). These mice were confirmed to have a reduced number of macrophages based on F4/80 immuno-staining to identify the cells (Gouon-Evans et al., 2000). Csf1 null mutant mice treated with exogenous Csf1 from birth also had partial rescue of both the macrophage number as well as ductal morphogenesis (Gouon-Evans et al., 2000), confirming the involvement of macrophages in the process of mammary development. The use of Csf1 null mutant mice helps to corroborate the necessity of macrophages in proper early mammary

development; however, it does not give a clue as to the mechanisms through which macrophages direct ductal formation.

Macrophages tend to accumulate around the shaft of the developing duct, and have been shown to affect ductal development; it has been proposed that the mechanism of this action is through association with aligned collagen fibers along developing ducts. Ingman et al (2006) used a multi-photon microscope with a Csf1 mouse model to reveal that macrophages tended to be associated with the long collagen fibers which help to direct ductal formation. Through this association with collagen, the macrophages can be seen to help determine the shape of the developing TEBs (Ingman et al., 2006). They showed that the amount of collagen itself is not affected in Csf1 null mice, however, the amount of collagen organized into long fibrils was affected by a macrophage deficiency (Ingman et al., 2006).

Macrophages are known to be involved as phagocytosing cells in the processes of wound healing and involution, but they are also involved in tissue remodeling and help to promote the growth and development of the mammary gland. They do this at least partially through organization of the collagen fibrils which surround developing ducts (Coussens and Pollard, 2011) and have been shown to be key to this process through a Csf1 null mouse model which showed significantly reduced ductal formation and altered morphology (Gouon-Evans et al., 2000). However, macrophages are not the only immune cells involved in the formation of the ductal tree during the pre-pubertal development period.

Eosinophils

Eosinophils are typically involved in the mediation of an inflammatory response during normal wound healing processes through the release of cytokines (Reed and Schwertfeger, 2010). Eosinophils are also involved in the process of mammary development, but the mechanism of their actions is unknown. They locate around the head and cleft of bifurcating TEBs (Coussens and Pollard, 2011) and appear to aid in ductal branching as well as TEB formation (Reed and Schwertfeger, 2010). Eosinophils are recruited to the mammary gland by eotaxin, and are a necessary part of mammary development. Their importance in mammary development has been explored through several experiments in eotaxin deficient mice.

Eotaxin is a powerful chemoattractant molecule that works specifically on eosinophils to both mobilize them from the bone marrow into the blood and recruit them to specific tissues (Gouon-Evans et al., 2002). Eotaxin -/- mice have been shown to have a near complete lack of eosinophils around TEBs, demonstrating that eotaxin is necessary to the recruitment of these leukocytes (Gouon-Evans et al., 2000). Not only did these eotaxin -/- mice have a severe lack of eosinophils, displaying only 2% of the wild type numbers, they also exhibited reduced branching of the developing ductal tree when compared with eotaxin +/+ mice. However, overall ductal length was unaffected (Gouon-Evans et al., 2002). Since eosinophils are known to secrete inflammatory cytokines which are important for cell trafficking and vascular permeability among other things (Reed and Schwertfeger, 2010), it is possible that they help to support ductal tree branching through local secretion of these various cytokines.

Mast Cells

Mast cells are the third major type of leukocyte known to be involved in mammary development. Mast cells are known regulators of the immune response, primarily through the variable contents of the granules by which they can also be identified (Lilla and Werb, 2010). Mast cells have been shown to be present in the microenvironment of the mammary gland at all developmental stages (Reed and Schwertfeger, 2010), but of particular note is their presence at the invasive front of TEBs (Coussens and Pollard, 2011). Mast cells typically appear to be scattered in small numbers throughout the mammary stroma, however they tend to localize in the stroma adjacent to the developing TEB and just ahead of the advancing TEB in rodents (Lilla and Werb, 2010).

Lilla and Werb (2010) demonstrated the necessity for mast cell presence in the developing mammary gland through use of a mast cell deficient mouse model. They showed that mast cells begin to exert effects on the developing mammary ductal system just prior to and around the onset of puberty, as mast cell deficient mice had significantly fewer ductal ends in the invasive front as well as fewer TEBs (Lilla and Werb, 2010). This phenotype persisted until the termination of ductal advancement and was accompanied by a reduced ductal length in mast cell deficient mice (Lilla and Werb, 2010). Through use of an antibody to the phosphorylated serine 10 residue of histone H3, a marker for cells entering mitosis, mast cell deficient developing mammary glands were shown to have reduced levels of proliferation around the TEBs (Lilla and Werb, 2010). This reduced proliferation could account for the reduced ductal length. Finally, the mechanism of action for the mast cells was investigated through use of cromolyn

sodium to stabilize and block the degranulation of mast cells, preventing the release of granule factors. The mice with inhibited degranulation exhibited a similar phenotype to the mast cell deficient mice. That is, they had inhibited TEB formation and reduced duct end number (Lilla and Werb, 2010). To further elucidate the active factors in the mammary mast cells, mice deficient in DPPI(dipeptidyl peptidase I), a protease activator, were also tested. Mast cell granules often contain proteases, many of which are activated by DPPI (Lilla and Werb, 2010), therefore reducing DPPI should effectively inactivate many of the granule proteins. The DPPI deficient mice were also seen to have inhibited TEB and duct end formation (Lilla and Werb, 2010). The mast cell deficient mouse model demonstrates an important role of mast cells in mammary development. Furthermore, the results of prevention of degranulation and DPPI deficient mice suggest a prominent role of granule products, specifically proteases, in the functions of mast cells in mammary development.

1.3 Hormonal control of early mammary development

Peri-pubertal mammary development is a complex, ordered process; one that must be orchestrated on an organismal level with the general growth and development of the animal. This is where hormones contribute to mammary development. Hormones are responsible for the initiation of lactation, as well as the changes that allow the switch from the non-lactating to lactating state. Hormones are also responsible for the period of allometric growth and development of the mammary gland around puberty, preparing the framework for future production. Estrogen is the widely-implicated stimulator of pubertal mammary development, but there is also evidence for the involvement of

pituitary hormones and local factors (Kleinberg, 1997). The hormonal cascade at puberty propels the mammary gland into a period of allometric growth (Li et al., 2006). This period of allometric growth continues through several estrous cycles and is followed by isometric growth until further development of the mammary gland during pregnancy in preparation for lactation (Tucker, 1981).

The use of ovariectomy (removal of all ovarian tissue), with accompanying lack of ovarian hormones, has proven useful in investigating the hormonal influences on the mammary gland. Hodson et al. (2013) used ovariectomized mice to demonstrate the necessity of progesterone for early alveolar development. They first observed a stimulation of development in these mice through treatment with estradiol and progesterone. Subsequent treatment of the stimulated mice with a progesterone antagonist resulted in the regression of alveolar development (Hodson et al., 2013). This study demonstrates the necessity of progesterone to early alveolar development.

Other studies have looked at ovariectomy in bovine mammary development specifically. There was found to be a lower mammary tissue volume, with less epithelial tissue and lumen and more stroma in the ovariectomized heifers (Purup et al., 1993) suggesting a lack of ductal development, the hallmark of allometric mammary growth, in these heifers. They also noted a decrease in mammary parenchyma as measured by a number of factors including weight, volume, DNA and protein content (Purup et al., 1993), indicating that the mammary glands of ovariectomized heifers contain fewer cells, and is not simply more compact. These gross morphological changes in the ovariectomized heifers suggests an important role of ovarian hormones in the events of

mammary development, specifically a failure of ductal elongation and budding of smaller ducts (Purup et al., 1993).

Clearly ovarian hormones, particularly estrogen, have important effects on mammary development, as administration of estradiol can elicit a time-dependent proliferative response in beef heifers, with epithelial cells responding first followed by the adjacent fibroblasts (Woodward et al., 1993). It has also been demonstrated in rats and mice that in animals deprived of pituitary, adrenals and ovaries, the correct combination of estrogen, corticoids and growth hormone will bring about mammary development (Forsyth, 1989), confirming that these hormones are necessary and sufficient to induce mammary development. Li and others (2006) examined the expression of estrogen-responsive genes and found that a majority responded only in the parenchyma or fat pad, indicating that estrogen has the most impact on these areas. They also found a direct link between estrogen and proliferation, noting that estrogen stimulates proliferation in both intact and ovariectomized heifers (Li et al., 2006). The exact mechanism of estrogen's action on the mammary gland is unclear, but studies suggest that estrogen acts through its receptor containing cells to alter gene expression, which could result in paracrine-factor mediated control of surrounding cells (Li et al., 2006).

The literature suggests that Growth Hormone (GH) and estrogen are the most important hormones involved in mammary ductal growth. There also appears to be a time-dependent effect of estrogen, as ovariectomies performed before eight weeks of age in bovine heifers had significant effects on parenchymal abundance and resulted in a rudimentary ductal structure with limited branching while mammary glands of heifers

ovariectomized after eight weeks had significantly more branching, and evidence of growing ducts (Berry et al., 2003). This confirms the role of estrogen for the induction of mammary development, but indicates little further involvement. Studies on mice have shown that estrogen manages local cell growth during puberty, through the estrogen receptor, and can stimulate mammary growth both in ovariectomized and intact individuals (Macias and Hinck, 2012). Though estrogen has been shown to have important effects on mammary growth and development, it is unlikely that estrogen is acting directly to stimulate proliferation, as estrogen receptor positive cells typically do not express proliferation markers or incorporate thymidine analogs such as BrdU (Capuco et al., 2002), this suggests that estrogen activates paracrine factors, and the effects of estrogen are mediated indirectly through these agents.

Growth hormone (GH) has also been shown to have actions in the mammary gland during the developmental period. This includes an increase in epithelial cell proliferation stimulated by GH treatment (Berry et al., 2003). However, growth hormone, similar to estrogen, likely does not act directly on the mammary gland. Instead, the effects of GH are mediated by the paracrine factor IGF-1 (Purup et al., 1993), which has been shown to be important in the mammary development of mice. Mice lacking IGF-1 display a late-developing, sparse ductal tree (Macias and Hinck, 2012). Though these hormones have been shown to be very important to the induction and continuation of pubertal mammary development, the precise mechanism of action is still somewhat unclear, though it seems as though they work through paracrine factors, and by influencing the actions of immune cells.

Immune cells such as B and T lymphocytes have been shown to appear in the mammary tissue in varying numbers which cycle in correspondence to different stages of pregnancy (Ismail et al., 1996). Immune cells are also recruited by and respond to estrogen-related signals, for example, the synthesis of eotaxin has been linked to estrogen levels in mice (Gouon-Evans and Pollard, 2001). The bovine mammary gland is known to secrete cytokines (Atabai et al., 2007), and cytokines are regulated by estrogen in immune cells (Reed and Schwertfeger, 2010). This association provides a direct link between the hormonal regulation of mammary development and remodeling mediated by immune cells. Cyclic changes in circulating concentrations of estrogen and progesterone have also been shown to regulate macrophage distribution in the uterus (Reed and Schwertfeger, 2010), making it entirely plausible that the actions and distributions of immune cells in the mammary are also regulated by hormones.

1.4 Conclusions

Development of the bovine mammary gland is a complex process that occurs in several stages. The pubertal development stage has the potential to influence later production in the individual, and is a period of development which can be manipulated. It is during this period of allometric growth induced by the hormones at the onset of puberty that the basic ductal formation and framework for the adult mammary gland is created. In the mouse mammary gland, the ductal structure is highly influenced not only by hormones, but also by the actions of certain cell types which are present in the developing gland. Various immune cells help to support and direct the development of the mouse mammary system. Macrophages, eosinophils and mast cells have all been

shown to contribute to the proper development of the mammary gland at puberty. These cells can also be influenced by various hormones, most notably estrogen and growth hormone, whether the influence is direct or indirect. Therefore, it is reasonable to extrapolate and investigate the possibility that immune cells under the control of hormones may be involved in proper pubertal mammary development in the bovine, as bovine and mouse mammary growth follow broadly similar patterns.

CHAPTER 2: INNATE IMMUNE CELLS MAY BE INVOLVED IN BOVINE MAMMARY DEVELOPMENT

2.1 Introduction

Bovine mammary development during the peri-pubertal period is important for future milk production (Tucker, 1981). During this period, the bovine mammary gland develops a ductal system which extends into the fat pad and provides a framework for future lobulo-alveolar development (Sheffield, 1988; Akers, 2002). The bovine mammary ductal system which develops during this period arises as terminal ductule lobular units (TDLU), which are clusters of ductules arising from larger ducts (Hovey et al., 1999). In contrast, the mouse mammary gland develops through extension of ducts at terminal end buds (TEB) (Williams and Daniel, 1983; Sheffield, 1988). In the mouse, the TEB is the site of the majority of the proliferation, whereas proliferation in the developing bovine ductal system occurs along the length of the extending duct (Sheffield, 1988; Silberstein, 2001; Capuco et al., 2002).

The stromal area surrounding the developing ducts provides a 3-D matrix for development as well as mediating local response to hormones and housing for vasculature and lymph systems (Hovey et al., 1999). Within this stromal environment, clustered around the mouse TEB, are also innate immune cells which have been shown to be required for normal mouse mammary development (Gouon-Evans et al., 2000). Irradiation studies demonstrate severe impairment of mammary ductal structures in developing mouse mammary glands when innate immune cells are absent. The changes in mouse mammary ductal structure were rescued through bone marrow transplant (Gouon-Evans et al., 2000). Of the innate immune cell types, three have

been identified to play crucial roles in mouse mammary development. Macrophages, which are generally considered professional phagocytes, are thought to be heavily involved in matrix remodeling. They affect the elongation and shape of the duct during development (Reed and Schwertfeger, 2010; Coussens and Pollard, 2011). Removal of the macrophage recruitment factor Colony-Stimulating Factor 1 (CSF 1) during the developmental period reduces macrophage recruitment and causes an overall reduction in duct number (Gouon-Evans et al., 2000). Eosinophils, which mediate immune responses through cytokine release during wound healing, are also important in mouse mammary development (Reed and Schwertfeger, 2010; Coussens and Pollard, 2011). Reduction in eosinophils through reduction in their attractant, eotaxin, results in a ductal tree with reduced branching (Gouon-Evans et al., 2000). Finally, mast cells, which are regulators of the immune response through the contents of their granules, are present in the stroma surrounding the mammary gland at all developmental stages (Reed and Schwertfeger, 2010). Deficiency of mast cells during the peri-pubertal period leads to fewer ductal ends and TEBs (Lilla and Werb, 2010). Each of these three innate immune cell types - macrophages, eosinophils and mast cells - have unique but apparently necessary roles in mouse mammary development. Each immune cell type also has specific localization in relation to the TEB: macrophages align with collagen fibers along the neck region (Ingman et al., 2006), eosinophils locate at branch points (Reed and Schwertfeger, 2010; Coussens and Pollard, 2011), and mast cells are found clustering at the head of advancing TEBs (Lilla and Werb, 2010).

Hormonal changes during the peri-pubertal period can also have profound effects on mammary development. The hormonal cascade at puberty produces a period of

allometric mammary growth (Li et al., 2006), and ovariectomy of animals at an early age leads to lower mammary tissue volume and a reduction in ductal development in heifers (Purup et al., 1993). Estrogen in particular appears to be an important hormonal regulator of mammary development, with the ability to stimulate proliferation (Li et al., 2006). Estrogen-related signals have also been linked to the recruitment of immune cells in the mammary gland, with estrogen possibly regulating cytokine production (Gouon-Evans and Pollard, 2001; Reed and Schwertfeger, 2010). This provides a link between the hormone-mediated changes in the development of the mammary gland and the presence of immune cells.

The present study examines the presence of three innate immune cell types - macrophages, eosinophils and mast cells - in the developing bovine mammary gland as well as examining the relative location of these cells. To our knowledge no one has yet explored the possible presence of innate immune cells in the developing bovine mammary gland.

2.2 Materials and Methods

Tissue samples

Samples for the Ovariectomy (OVX) set were obtained from a previous study (Velayudhan et al., 2011). Briefly, 24 Holstein heifers were purchased after weaning and fed heifer grower with mixed forage pasture and high quality forage. After a 1-week adaptation period, heifers were assigned to a 2x3 factorial treatment design. The first factor was ovariectomy (OVX, n=12) or intact (INT, n=12), with the second factor age at

which surgery was performed (60, 90, or 120 days). Surgery to remove ovaries in OVX calves was performed laproscopically, and INT heifers had their ovaries palpated during a sham operation. Animals were sacrificed humanely thirty days after surgery and mammary tissue was harvested immediately. Tissue for histology was taken from the left front quarter of the udder and fixed in 10% normal formalin. Ovariectomy was confirmed at slaughter, which resulted in the exclusion of two animals that were found to have ovarian fragments with follicles, two other animals had insufficient mammary parenchyma for analysis. Tissues were formalin fixed and paraffin embedded prior to histological analysis. (Velayudhan et al., 2011)

Samples for the ontogeny (ONT) set were obtained from a study conducted at Clemson University. Briefly, neonatal Holstein heifers (n=4/age) were sacrificed and mammary tissue sampled within 12h of birth (d0) and at d7, 14, 21, 28, 35 and 42 of age. Commercial milk replacer and starter ration were fed according to manufacturer's instructions. All procedures were approved and monitored by the Clemson University Institutional Animal Care and Use Committee. Collected tissue samples were fixed, dehydrated, cleared, infiltrated, embedded in paraffin and sectioned following standard procedures.

Samples comprising the estrogen (EST) set were also obtained from a previous study (Geiger, 2015). Briefly, calves were obtained within the first week of life and started immediately on an accelerated milk replacer diet. Animals were administered estrogen and weaned at 8 weeks of age (ACC- animals on an accelerated diet not

receiving estrogen, ACCEST - animals on an accelerated diet receiving estrogen). Estrogen delivery was accomplished through an implant in the ear, with 6 animals receiving implants and 4 animals placebo implants. Animals were humanely slaughtered at 10 weeks of age and the mammary gland was removed immediately. The right front quarter was formalin fixed, tissue samples were collected and paraffin embedded for histological analysis.

Animals which supplied tissues were all obtained from IACUC approved projects at Virginia Polytechnic and State Institute or Clemson University. All tissue samples were processed and embedded in paraffin before being sectioned to 5μm on a microtome (HM 340E, Microm, Walldorf, Germany). The sections were floated in a warm water bath (37-42°C) to remove wrinkles and mounted on a charged slide (superfrost white, VWR, Radnor, PA), with 3-5 sections per slide. Slides were placed on a slide warmer after draining to thoroughly dry. Slides for both staining and immunofluorescence were deparaffinized and rehydrated in the same manner. Deparaffinization was achieved by sending slides through two baths of aliphatic xylene substitute (VWR, Radnor, PA) for five minutes each. Sections were then rehydrated through a graded series of ethanol to distilled water. Slides were taken directly from the distilled water bath for the following procedures.

Immunofluorescence

Rehydrated sections were outlined with a hydrophobic barrier pen (PAP pen©, Ted Pella, Inc, Redding, CA) were subjected to a 0.125% trypsin digest (ultravision trypsin, Thermo Fisher Scientific, Suwanee, GA) at 37°C for 10 minutes for antigen retrieval. Sections were washed 3 x 2 minutes in Dulbecco's Phosphate Buffered Saline, calcium and magnesium free, pH 7.4 (PBS). Sections were outlined with PAP pen, which creates a hydrophobic barrier, and blocked with CAS-Block™ (Invitrogen, Grand Island, NY) by flooding sections and letting them sit 30 minutes at room temperature. Primary Antibody, mouse anti-human macrophage, which recognizes the calprotectin molecule(cat #ab22506, Abcam, Cambridge, MA), was applied at 100µL per positive section. Slides with primary antibody were incubated overnight at 4°C. Primary antibody was aspirated and sections were washed three times in 1x PBS for five minutes. A goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor® 488 (Invitrogen, Grand Island, NY) was used at 1:200 diluted with CAS-Block™. The secondary antibody solution was centrifuged for 10 min at 10,000g to remove aggregates before use, and 100 µl of this solution was pipetted onto all sections. Sections were allowed to incubate for one hour at room temperature. The remaining secondary antibody solution was aspirated and sections were washed three times in 1xPBS for two minutes. After the last wash, the PAP pen markings were removed with xylene substitute and slides were rinsed with 1xPBS before being coverslipped using the SlowFade® Gold with DAPI (4',6-diamidino-2-phenylindole, binds to DNA, Invitrogen, Grand Island, NY) mounting medium. Slides were allowed to sit for 20 minutes before visualization. Macrophages were identified in pictures as positive staining cells with a non-lobulated nucleus (unlike granulocytes which appear multi-nucleated) (figure 2.1).

Modified Luna stain for Eosinophils

Eosinophils can be identified by their distinctive nuclear shape as well as the granules present in their cytoplasm (figure 2.1). Rehydrated sections were stained first in Weigert's Iron Hematoxylin (kit, ENG scientific, Clifton, NJ) for five minutes and blued in 0.5% lithium carbonate (Sigma-Aldrich, St. Louis, MO) until the sections noticeably changed color, and rinsed with tap water. This was followed with a three minute stain in aqueous 1% Biebrich scarlet (Ponceau BS, Sigma-Aldrich, St. Louis, MO). Slides were then differentiated in 1% Hydrochloric acid in 70% ethanol for 5-7 dips. Sections were then rinsed in running tap water until slides turned a noticeable purple-blue color (about 2min). Sections were dehydrated through graded series of ethanol and two changes of histological grade xylene (Thermo Fisher Scientific, Suwanee, GA) before mounting with a resinous medium (Permount,Thermo Fisher Scientific, Suwanee, GA).

May-Grunwald Giemsa staining for Mast cells

The May Grunwald Giemsa staining protocol stains most immune cells with several identifying characteristics. The nuclei stain blue and mast cell granules undergo a metachromatic reaction to turn purple. The colors of these stains are very pH sensitive. To achieve staining which identified mast cells, a pH 6.3 sodium phosphate buffer (0.06 molar) was used. Rehydrated sections were flooded with a working May-Grunwald stain solution and allowed to sit 6 minutes with occasional agitation. The working May Grunwald stain solution was prepared by a 1:1 dilution of stock May-Grunwald stain (Harleco for EMD Millipore, Darmstadt, Germany) in the pH 6.3 sodium phosphate buffer. The excess stain was rinsed off slides with the phosphate buffer and

slides were flooded with working Giemsa stain solution for 13 minutes with agitation. Working Giemsa stain was prepared by a 1:20 stain:buffer dilution of stock Giemsa (Harleco for EMD Millipore, Darmstadt, Germany) in the pH 6.3 phosphate buffer. Stain

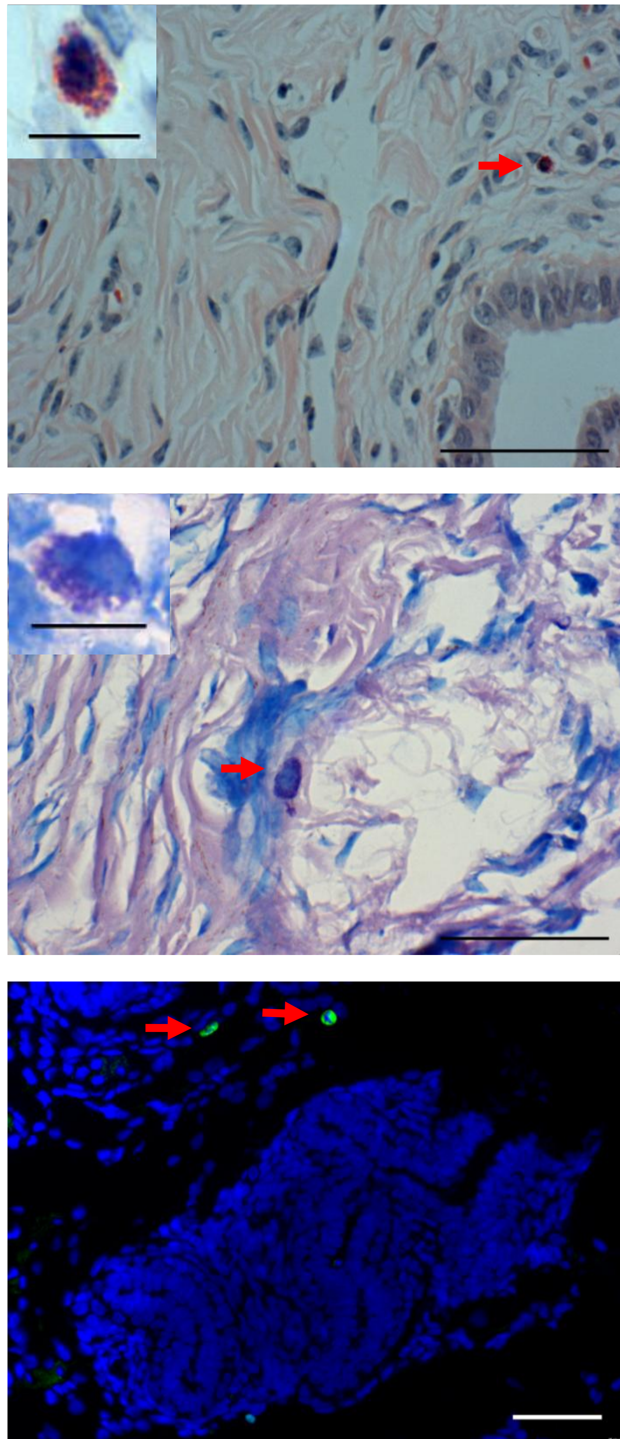


Figure 2.1: A) Luna stain for eosinophils. Arrow indicates an eosinophil with dark pink/red granules and blue nucleus. Inset is enlargement of the eosinophil. B) May-Grunwald Giemsa stain for mast cells. Arrow indicates a mast cell with distinctive purple granules and blue nucleus, inset is enlargemen of the picture. C) Immunofluorescent stain using anti-macrophage antibody. Green is anti-macrophages, blue is a DAPI counterstain. Arrows indicate macrophages. Scalebars on large images represent 50μm, scalebars on insets represent 10μm.

was again rinsed off with buffer. Sections were differentiated by dipping slides in 0.25% acetic acid solution 8-10 times, then rinsed again in buffer. The slides were rinsed very quickly in distilled water prior to air drying. After air drying, slides were cleared in xylene, mounted and coverslipped using Permount (Thermo Fisher Scientific, Suwanee, GA) and allowed to cure before viewing. Mast cells were counted as cells with a blue nucleus and purple granules (figure 2.1).

Determining number of cells per unit area

For each animal, one slide was stained with each method. From these, 40 photos were taken, ideally from a single section and if not from non-adjacent sections to avoid counting a cell twice. All pictures used for counting from non-fluorescent slides were taken on an Olympus microscope with 40x objective lens (Olympus America Inc., Center Valley, PA) using a color camera (Olympus America Inc., Center Valley, PA). Half (20) of the pictures taken for each animal contained epithelium and half (20) were stroma with no epithelium. Pictures were taken in a generalized grid fashion within the confines of stroma or epithelial containing regions to ensure no overlap between pictures and coverage of a representative area. Pictures were then transferred to image-pro plus 7.0 (Media Cybernetics, Rockville, MD) for processing. All pictures were spatially calibrated and relevant areas were measured using the software, with cell counts (cells per outlined tissue area) also being taken at this time.

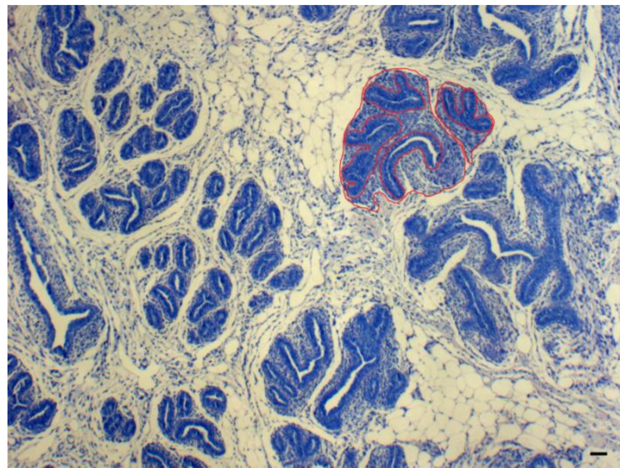
The cell counts are divided into two distinct groups: cells that were determined to be from "far stromal" (FAR) or cells which were determined to be from "near stromal" (NEAR) tissue regions. The distinction between far and near stromal was determined by

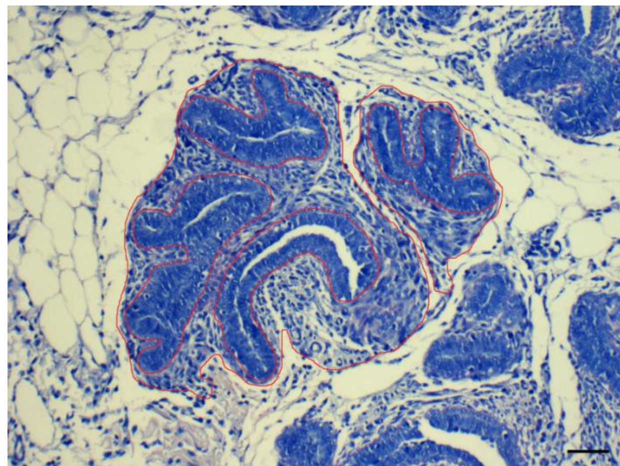
visual examination (figure 2.2). Far stroma was generally more sparsely cellular and contained more fibrous tissue as well as adipose. Near stroma was generally more densely cellular, and typically within 100-150 µm from the nearest epithelium. Areas were measured in image-pro, for far stroma, the area of the entire "stroma" picture was considered, while for near stroma, only the area considered to fall into the near stroma category was considered.

To obtain the values for statistical analysis, the areas of NEAR and FAR stroma, respectively, from 20 pictures for each animal were summed, as were the cells which fell within the respective area. The cell number for NEAR or FAR stroma for each calf was then divided by the area of the respective location (NEAR or FAR) for the calf. The resulting values were expressed as cells per micron squared, which were then multiplied by $1x10^5$ to express values as cells/mm^2 of tissue area.

Statistical Analysis

Data were analyzed in JMP (SAS institute Inc., Cary, NC). All three cell types were evaluated with the same model, however models differed between tissue sets. For the OVX set the model included main effects of treatment, age and location, with calf within treatment, age as a random effect. For the ONT set the model included main effects of age and location along with interaction and a random effect of calf within age. Finally, for the EST set the model included main effects of treatment, location and interactions with calf within treatment as a random effect. Differences were determined to be significant if $P<0.05$, while $P<0.1$ was considered to be a trend.





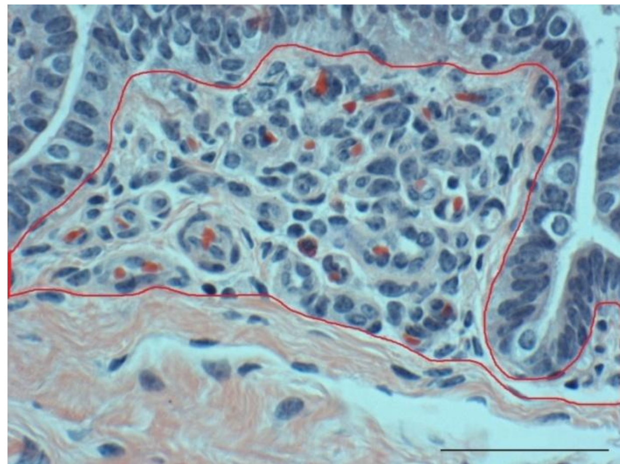


Figure 2.2: Illustration of NEAR (N) stroma, FAR (F) stroma and epithelium (E) in mammary tissue at 4x (A), 10x (B) and 40x (C) magnification. Scale bar represents 50µm. Pictures are of tissue sections stained with May-Grunwald Giemsa, May-Grunwald Giemsa and Luna stains, respectively.

2.3 Results and Discussion

Macrophages

We observed macrophages in animals from all three tissue sets. We observed more macrophages in the NEAR versus FAR locations with more than five times as many in NEAR versus FAR in the ontogeny tissue set and a difference of over four-fold in the estrogen tissue set (P=0.0001 and P=0.0004, respectively). There was also a tendency for more macrophages in NEAR versus FAR (p=0.083) stromal areas in ovariectomized calves (figure 2.3). We found no significant effects of treatment or age on any set related to macrophages. Our finding of more cells in the NEAR versus the FAR stroma suggests that macrophages do indeed cluster near developing ductal structures. This is similar to work demonstrating macrophages preferentially clustering around developing terminal end buds (TEBs) in mice (Gouon-Evans et al., 2000). The larger numbers of macrophages we noted in the NEAR stromal tissue areas indicate a preferential location for the macrophages within the developing bovine mammary gland.

The stromal microenvironment, which contains connective tissue and blood vessels as well as various cell types including immune cells, is important to ductal development in the mammary gland (Cunha and Hom, 1996). Macrophages in the mouse mammary gland are involved in organization of the collagen fibers and aid in the maintenance of the structure of the TEB (Ingman et al., 2006) and are generally found at the neck region of the TEB (Gouon-Evans et al., 2000). The mouse TEB provides a convenient orientation point, and is also the location of the proliferating cells which drive ductal growth forward (Macias and Hinck, 2012). Since developing bovine ducts have proliferating cells along their length (Capuco et al., 2012), it is reasonable to expect a

less defined location for clustering of macrophages in the bovine. Therefore, the NEAR stromal location indicates clustering of cells close to the developing structures containing proliferating cells, similar to the clustering near the proliferating TEB in the mouse model.

It has been suggested that the purpose of macrophage clustering near the neck of the developing TEB is to aid in collagen organization (Ingman et al., 2006). Mice lacking in macrophages through a homozygous recessive mutation typically have delayed TEB formation as well as organizational changes, with some TEBs migrating in a lateral direction or away from the tip of the fat pad (Gouon-Evans et al., 2000). A deficiency in macrophages has also been shown to change the shape of the TEB, causing them to be shorter and rounder (Ingman et al., 2006). Overall the literature demonstrates a need for macrophages and evident role for them in the organization of the developing ductal structure in the mouse mammary gland.

The presence and location of the bovine macrophages which we observed suggests that they may also be involved in bovine mammary development. Considering the differences in the pattern of development between bovine and mouse mammary glands, our findings support the notion that bovine mammary macrophages cluster in a similar location to that seen in mice. This clustering suggests that they too are involved in development, possibly in a similar manner to that in mice.

We did not see any significant effect of age (ontogeny sample set) on macrophages. This could be due to the age ranges examined not adequately covering the developmental period, or the changes in macrophages over time in the developing bovine mammary gland being negligible or too subtle for detection with small animal

numbers. In the mouse gland, they did observe macrophages around ductal structures prior to the appearance of TEBs, however they were at a lower density to those found later in postnatal mammary development (Gouon-Evans et al., 2000). We also did not observe a significant effect of treatment, either exogenous estrogen or ovariectomy, on macrophage number or location. This was unexpected as estrogen can stimulate bovine and murine mammary ductal development (Li et al., 2006; Macias and Hinck, 2012). Immune cells, including those within the mammary gland, have also been shown to be modulated by ovarian hormones (Reed and Schwertfeger, 2010), and the number of macrophages fluctuate over the estrous cycle as they orchestrate mammary remodeling (Chua et al., 2010). It is possible that our small sample sizes as well as the timing of estrogen supplementation did not allow us to see any possible effects of exogenous estrogen.

Although mouse mammary development and the involvement of immune cells has been well characterized, the presence and involvement of immune cells has yet to be described in the bovine, and there may be an important timing component that we were unable to resolve in our experiment. Overall, our results demonstrated the presence of macrophages in the developing bovine mammary gland. We also showed that there are more macrophages/mm^2 in the NEAR versus FAR epithelium, suggesting that they are clustering close to the developing ductal structures and may well be involved in development in a similar manner to those in mice.

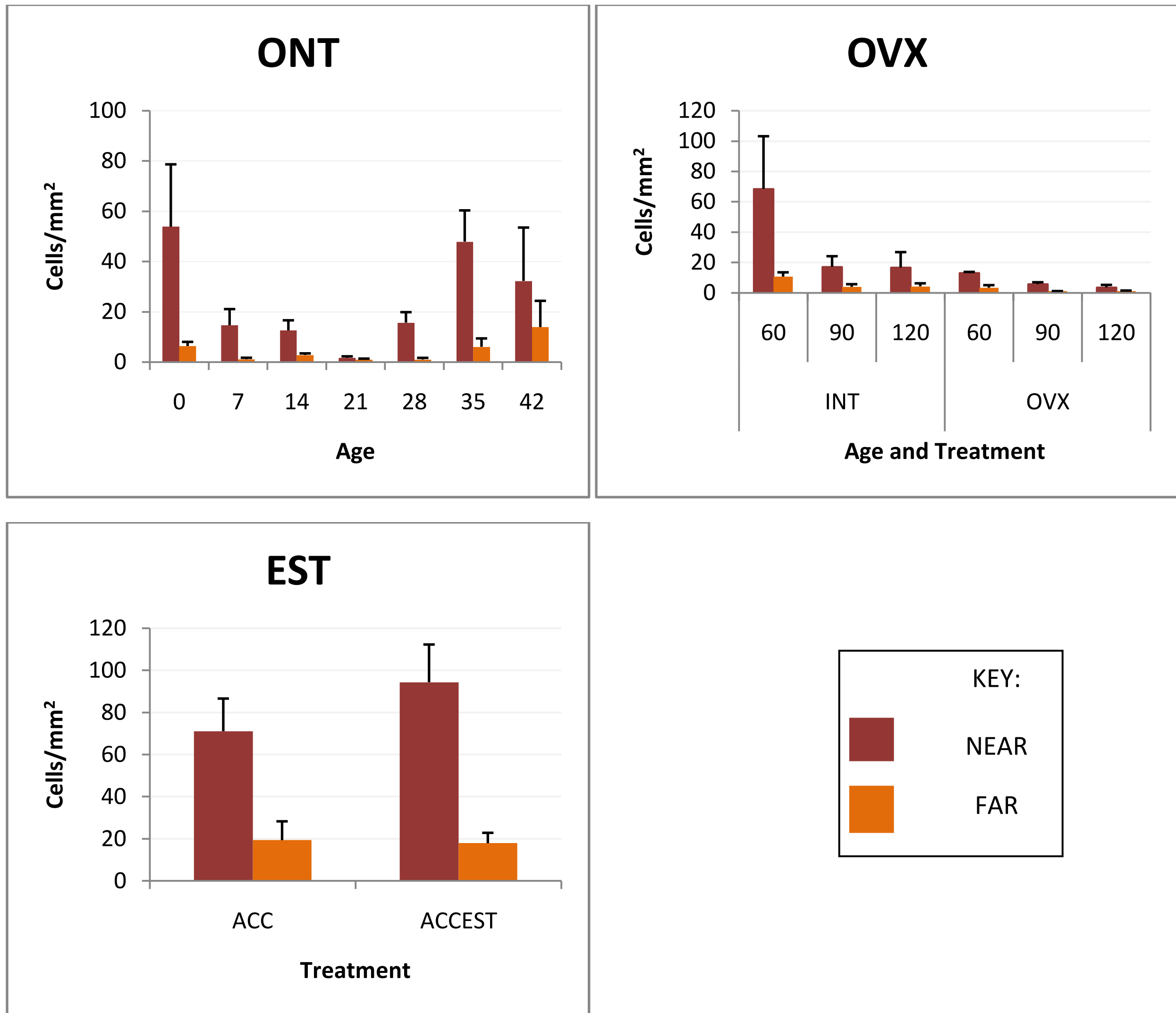


Figure 2.3: Significantly more macrophages were observed in the NEAR versus FAR stromal areas in ONT (A) and EST (B) (P=0.0001 and 0.0004, respectively). There was a tendency for a treatment and location effect (greater in NEAR vs FAR) in the OVX set (P=0.081 and 0.083, respectively). No other significant effects were observed.

Eosinophils

We observed eosinophils in all three sets of animals. We measured more eosinophils in the NEAR versus FAR tissue locations in both the ontogeny (P=0.0004), with an overall mean of 28.1 cells/mm^2 in the NEAR and 0.07 cells/mm^2 in the FAR, and in the ovariectomy tissue set (P=0.038) with 13.5 cells/mm^2 in NEAR compared with 3.5 cells/mm^2 in the FAR stroma (figure 2.4). However, there was no significant effect of location in the estrogen treated calves (figure 2.4). The observation of many more eosinophils within the denser area of stroma close to the developing epithelium and near absence of eosinophils in FAR stroma in ontogeny animals, this strongly suggests very distinct clustering of eosinophils close to the developing ductal structures in these animals. Abundant eosinophils have been observed in specific areas around developing ductal structures in mice (Gouon-Evans et al., 2000). They have also demonstrated that removing locally produced eotaxin results in a lack of recruitment of esoinophils to the mouse TEB as well as defective branching morphogenesis (Gouon-Evans et al., 2000). This local production of eotaxin could explain the specific locations of eosinophils in mice and, if true in calves, could explain the differences we noted between NEAR and FAR stromal areas. The abundance of eosinophils in the NEAR stroma compared to the FAR stroma which we observed suggests that eosinophils have a similar role in bovine mammary development as they do in mice.

We observed no other significant effects of treatment or age on eosinophil number, however we did note a trend for the effect of age (P=0.08) in the ontogeny set. The greatest number of eosinophils was observed at day 14. In mice, the transcript levels of eotaxin, a specific and powerful eosinophil attractant, increase as TEBs form

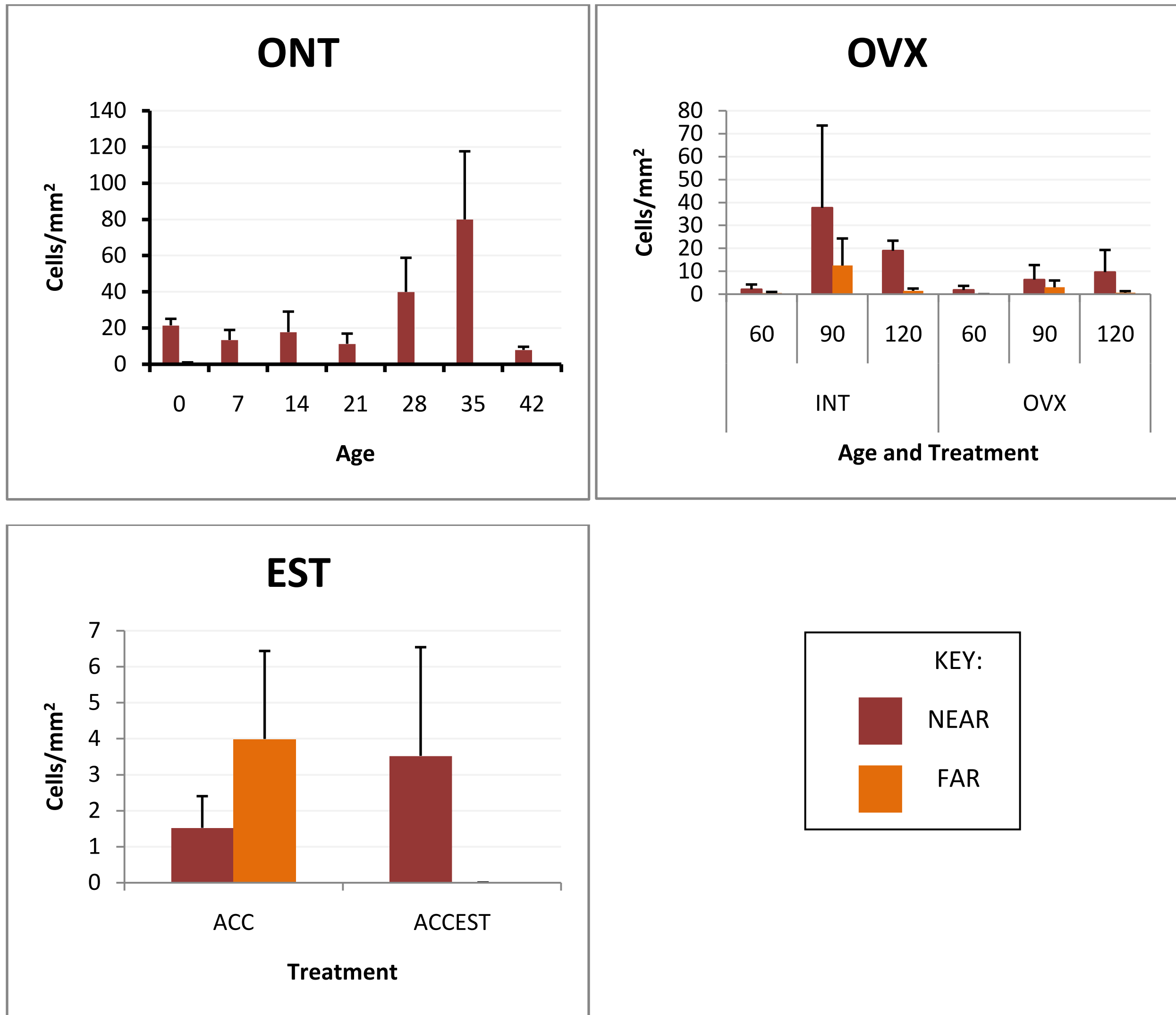


Figure 2.4: More eosinophils were observed in the NEAR versus FAR stroma in ONT (A) and OVX (B) sets (P=0.0004 and 0.038, respectively). There was a tendency for an effect of age in the ONT (P=0.08). No significant effects were observed in the EST (C) set.

(Gouon-Evans et al., 2000). Unfortunately, they do not report eosinophil numbers, but the increase in transcribed eotaxin should lead to an increase in eosinophil number as TEBs develop. Though we did not observe a significant age effect, presence of this trend warrants further exploration, as does the pattern of change in immune cell number with age.

Mast Cells

We observed mast cells in animals from all three experimental sets. We measured a significant effect of location in the ontogeny set (P<0.0001), with nearly four times more mast cells observed in the NEAR versus FAR stromal designations (Figure 2.5). In mice, mast cells have been observed during postnatal development in the mammary stroma (Lilla and Werb, 2010). In the prepubertal gland, mast cells were observed scattered throughout, while mast cells were more frequently observed near TEBs once the gland began to grow and proliferate (Lilla and Werb, 2010). The similarity in mouse and bovine mast cell localization, with more nearer to the developing ductal structures, suggest that bovine mast cells could also be involved in mammary development in a similar manner to mouse mast cells. A mast-cell deficient mouse line suggests a possible role for mast cells in development. Mice lacking stromal mast cells have fewer ducts ends at the invasive front, fewer TEBs, and fewer proliferating cells (Lilla and Werb, 2010). This suggests that mast cells play a role in the invasion of ducts into the mammary fat pad.

We also measured a trend for the effect of age in the ontogeny set (P=0.0824) as well as an effect of estrogen treatment (p=0.0755). These measurements suggest

possible changes in the number of mast cells as the gland develops, which could be related to the relative rate of epithelial cell proliferation occurring in the developing ducts. The trend for an effect of estrogen treatment suggests that estrogen could have a role in mast cell regulation. Estrogen is known to increase bovine mammary ductal development (Woodward et al., 1993). Further work should be done to determine if estrogen indeed does have an effect on immune cell levels in the developing gland.

Generally speaking, overall abundance (Figure 2.5) of mast cells was markedly greater in the youngest calves evaluated (ONT set). Averaged across ages and treatments for NEAR stroma there were 78.9±11.7; 5.4±1.2; and 7.6±2.2 mast cells per mm^2 for calves in the ONT, OVX and EST tissue sets, respectively. When values for ONT and OVX samples were compared by a simple two-sided T-test the difference was highly significant (P=0.0001). This suggests a possible broad effect of age on the numbers of mast cells present.

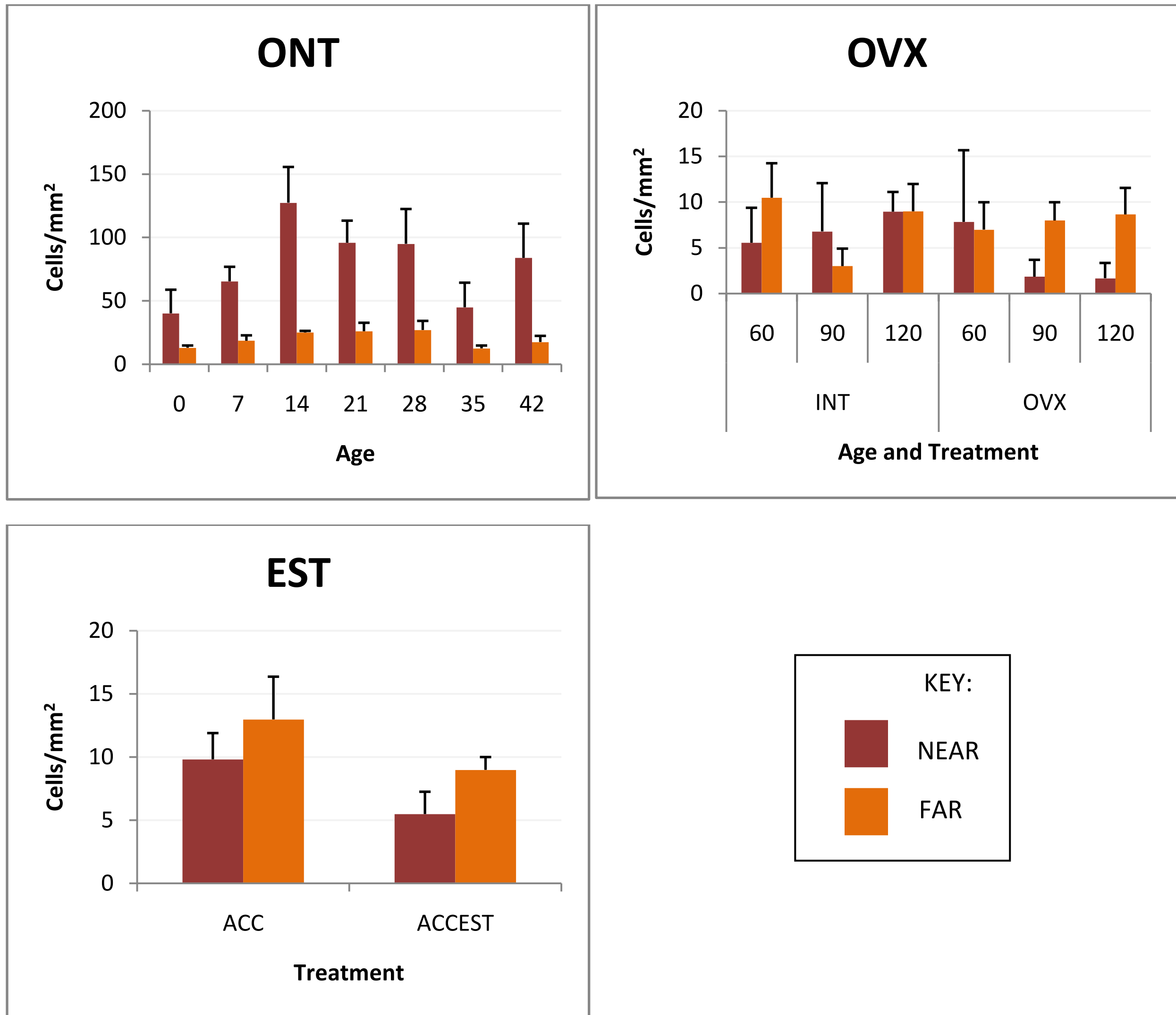


Figure 2.5: More mast cells were observed in the NEAR versus FAR in the ONT set (A, P=0.0001). There was also a trending effect of age in the ONT set (P=0.0594). There were no significant effects in the OVX set (B). There was a tendency for more mast cells in the ACC versus ACCEST in the EST set (C, P=0.076), but no effect of location.

2.4 Conclusions

Overall our findings suggest that all three immune cell types cluster closely with developing ductal structures in the growing bovine mammary gland. When compared with the data from the mouse models, which also show an increased number of all three cell types near TEBs during development, this suggests that bovine eosinophils, mast cells and macrophages could all be involved in mammary development. Especially striking was the 12.1 fold higher number of mast cells in mammary tissue from the youngest calves studied. All three cell types have been shown to be essential to mouse mammary development. Our data support the idea that these immune cells are likely also involved in bovine mammary development.

CHAPTER 3: EPILOGUE

Our objective was to determine the presence and relative locations of macrophages, eosinophils and mast cells in bovine mammary tissue and to relate that to the knowledge of their actions in murine mammary development. We were successful in finding all three cell types in mammary tissues at all ages, indicating that, at the very least, these cells are present while the mammary gland is undergoing dramatic changes in growth and development. We also measured a clustering of these cells close to the epithelial ducts in several sets in what we termed the "NEAR" stroma. The clustering of the cells near the developing ductal structures is broadly similar to what is seen in mouse mammary glands. However, because ductal proliferation in the murine mammary gland is focused in the region of the TEB compared with more widely distributed areas of epithelial cell proliferation in the bovine mammary tree, we found immune cells localized in stroma adjacent to the epithelium throughout the parenchymal tissue. Regardless, this suggests these cells likely play similar roles as in rodents. In mice, macrophages are thought to be involved in shaping the TEB and aiding in the organization of collagen along the developing duct. Mast cells are thought to aid in the advancement of the TEB into the fat pad. Finally, eosinophils are thought to aid in branching of ducts, which occurs at the TEB. All three cell types were observed to cluster in at least one set of tissue. The disparity between tissue sets is possibly due to the fact that the tissue sets are from animals of different ages. For example, the overall 12.1 fold greater number of mast cells in the youngest calves studied (ONT) suggests that mast cells may be especially important in the very early post-natal mammary development, however there is no direct comparison available in mice.

Further work is necessary to define the role of these immune cells in bovine tissues specifically. While our work confirms the presence of these immune cells, we have not truly defined their roles in bovines. Further work should be done to look at the changes over time in cell numbers, especially in relation to the stage of mammary development. There has been an observed change in eosinophils and macrophages in the murine mammary gland during development (Gouon-Evans et al., 2000). Unfortunately the tissue sets we had available were somewhat limited in the ages which they covered, which possibly obscured any age effect.

While mammary whole mounts or knock-out models are impractical in the bovine model, it would be interesting to look at the presence and locations of the chemical attractants shown to be important in the mouse mammary gland. This could be accomplished through gene expression analysis or in situ hybridization. One would specifically look for CSF1, an attractant for macrophages, and eotaxin, an attractant for eosinophils. Although the murine research doesn't have a specific chemical attractant identified for mast cells, these cells are nonetheless implicated in proliferation at the TEB. Therefore, it could be informative to look at a potential correlation between clustering of mast cells and proliferation of epithelial cells in the tissue, perhaps over time. This could be accomplished through dual staining for mast cells and a proliferation marker at different ages of animals.

Although we did not observe any effects of exogenous estrogen on the numbers of any of the three immune cell types in the tissue, we were limited in only looking at estrogen supplementation during a set time period, and it is possible that any influence of estrogen on immune cells specifically occurs at a different time than the one studied.

Therefore it would also be interesting to look at effects of estrogen supplementation at different time points during development.

This research focuses on understanding the processes behind mammary development, though it seems unlikely that immune cells in development would be a viable avenue for modifying mammary development in a practical dairy sense. However, if this research continues, it could be interesting to look at the potential implications of abnormal immune response and early life infection in calves on mammary development, possibly mediated through these immune cells.

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APPENDICES

APPENDIX A:

STAIN PROTOCOLS

A1 May-Grunwald Giemsa stain for Mast cells

The May-Grunwald Giemsa stain is typically used to identify cell types in blood smears, however it can be adapted for use in fixed tissues by changing the pH of the buffer used. We based our protocol for this stain using information from a kit protocol from Diagnostic Biosystems (Diagnostic Biosystems Giemsa Stain Kit instructions for use), as well as several papers which discuss the alterations which can be made to use Giemsa and similar stains in formalin fixed, paraffin embedded tissues (Difford, 2002). This stain gives all cells a blue nucleus. Mast cells can be identified by their purple granules, whose color is formed through a metachromatic reaction with the dye. Ideally one can also identify eosinophils, whose characteristic granules stain a dark pink, and neutrophils, whose cytoplasm stains a lighter pink, but these colors are very sensitive to pH changes and any alterations. To achieve a color which allowed the easiest identification of mast cells, we used a sodium phosphate buffer at pH 6.3 (0.06 molar). Toluidine blue (Toluidine Blue-O, Sigma-Aldrich, St. Louis, MO) is another stain which can be used to successfully identify mast cells in fixed tissue and is very easy to use, however I found it easier to distinguish mast cells in the May-Grunwald Giemsa stain than the toluidine blue stain.

We began by deparaffinization of the tissue sections in a xylene substitute (VWR, Radnor, PA). The tissue sections were then rehydrated in a graded series of ethanol: 1

minute in two changes of 100%, 2 minutes in 95% and 2 minutes in 70% before being placed in distilled water for 5 minutes. The rehydrated tissue sections were then stained by flooding the slides with a 1:1 dilution of stock May-Grunewald (Harleco for EMD Millipore, Darmstadt, Germany) stain in pH 6.3 phosphate buffer. The slides were agitated occasionally and the stain allowed to sit for 6 minutes. Stain was then rinsed off with buffer. The second staining step involved flooding the slides with a 1:20 dilution of Giemsa stain (Harleco for EMD Millipore, Darmstadt, Germany) in sodium phosphate buffer and allowing it to sit with periodic agitation for 13 minutes. This stain was then rinsed from the slides with buffer. The colors were differentiated by dipping in 0.25% acetic acid in distilled water, I found 8-10 dips worked best for the mammary tissue. Slides were then rinsed in phosphate buffer. Following the differentiation and rinse, slides were rinsed very quickly in distilled water. I found that longer rinsing increased the blueness of the colors on the slide, and as I was trying to see some of the red colors I rinsed the slide very briefly. The slides are then allowed to air dry. It is important to note that the dye colors turn bluer when the sections dry, so it is necessary to not over-blue in the previous rinse step. The final step is coverslipping; the slides were first cleared in xylene (Thermo Fisher Scientific, Suwanee, GA), then cover-slipped using Permount (Thermo Fisher Scientific, Suwanee, GA) and allowed to dry.

A2 Luna stain for eosinophils

Eosinophils are easily identified by their granules and unique nuclear shape, which often appears segmented. We based our Luna stain on a protocol found on the

IHC world website (http://www.ihcworld.com/smf/index.php?topic=81.0), however we did alter their protocol in several ways. In the Luna stain, a two-part stain which uses Biebrich scarlet and Weigert's iron hematoxylin, eosinophilic granules stain a deep red-pink while nuclei stain a purple-blue. This stain combination can also identify neutrophils, the cytoplasm of which stain a light pink color.

We began with deparaffinizatin of the tissue in a xylene substitute (VWR, Radnor, PA). The tissue was then rehydrated in a graded series of ethanol: 1 minute in two changes of 100%, 2 minutes in 95% and 2 minutes in 70% before slides were placed in distilled water for 5 minutes. The rehydrated tissue sections were then stained in Weigert's Iron Hematoxylin (kit, ENG scientific, Clifton, NJ) working solution. Staining was achieved by flooding the slides with the prepared solution and allowing it to sit for 6 minutes. This time was specific for the mammary tissue, as I found lymph and tonsil required less time (5 minutes) to achieve adequate coloration. The slides were very quickly rinsed in distilled water to remove excess stain then blued in 0.5% lithium carbonate in distilled water. The sections turned noticeably more blue during this step, I found that 5-7 dips in the solution was adequate for mammary tissue. Slides were then rinsed in tap water, and stained in a 1% aqueous Biebrich scarlet solution made by dissolving 0.25g Biebrich scarlet dye (Ponceau BS, Sigma-Aldrich, St. Louis, MO) in 25 mL distilled water. This was achieved by flooding the slides with the stain solution and allowing them to sit. I found that mammary tissue required 5 minutes of staining while lymph and tonsil required only 3 minutes. After the Biebrich scarlet was rinsed off quickly in distilled water, sections were differentiated in an acidified alcohol solution (1% HCl in 70% ethanol). The slides appear to redden slightly during this step, and I found

that 2 dips was adequate for mammary tissue while 5-7 dips was required for lymph and tonsil. The final staining step is a bluing step. I found the originally suggested bluing step to be too much for my sections, and therefore I blued these sections by rinsing with running tap water until the sections turned noticeably purple-blue in color. The mammary tissue generally required 1.5-2 minutes while the lymph tissue required 2.5 minutes of rinsing. From here the sections were dehydrated through a graded series of ethanol - 2 min in one change of 70%, 2 minutes in 2 changes each of 95% and 100% ethanol. The sections were then cleared in xylene (Thermo Fisher Scientific, Suwanee, GA) and cover-slipped using Permount (Thermo Fisher Scientific, Suwanee, GA).

A3 Immunohistochemistry for macrophages

Macrophages have been identified through the use of antibodies to several different macrophage associated proteins in mice. However, the difficulty lies in finding an antibody which cross-reacts with appropriate bovine antigens. This proved to be a challenge for us, as we tested several before settling on what seemed to be the best and most reliable, though perhaps not the most specific, antibody. Perhaps the most commonly used antibody to identify macrophages is CD68. This protein is in the lysosomal/endosomal associated membrane protein family and scavenger receptor family, which aids in and is associated with the phagocytic actions and activation of macrophages. CD68 is most commonly found in the cytoplasm of macrophages (NCBI, 2014). Unfortunately, we were unable to find a CD68 antibody which cross-reacted well with the bovine protein (see Table 1 in appendix B for antibody testing data). The final

antibody which we settled on was a monoclonal mouse anti-human macrophage antibody found to react with calprotectin (Abcam, cat# ab22506). Calprotectin is a cytoplasmic protein found in granulocytes (eosinophils, neutrophils) and cells of the monocyte lineage (macrophages). This worked for us since we were interested only in counting the number of macrophages and not identifying specific subclasses of macrophages. Helpfully, the eosinophils and neutrophils have a nucleus which appears segmented, while macrophages tend to have a large, round nucleus, meaning that we could exclude all calprotectin positive cells which appeared multi-nucleated from our counts and be reasonably confident that we were only counting macrophages. Therefore, macrophages were counted on the basis of being calprotectin-positive as photographed and observed via immunofluorescent staining and appropriate nuclear morphology.

We began the immunofluorscence protocol with deparaffinizatin of the tissue in a xylene substitute (VWR, Radnor, PA). The tissue was then rehydrated in a graded series of ethanol: 1 minute in two changes of 100%, 2 minutes in 95% and 2 minutes in 70% before being placed in distilled water for 5 minutes. Sections were then outlined on the slides following rehydration, and a trypsin digest (ultravision trypsin, Thermo Fisher scientific, Suwanee, GA) was used for antigen retrieval. We used the trypsin at the manufacturer recommended dilution (1 drop trypsin solution in 3 drops buffer) and covered each tissue section with 75-100μL. The slides were then incubated in a 37°C dry oven for 10 minutes. The enzymatic digestion was the recommended antigen retrieval method for this antibody, however we did also try some heat induced antigen retrieval methods (citrate buffer and Tris-EDTA buffer) which did not work as well with

this primary antibody. Following incubation, the trypsin solution was vacuumed off the sections and all sections were washed 3 times with 1x Phosphate Buffered Saline (PBS). Following the wash, sections were blocked by covering each section with Cas-Block™ (Invitrogen, Grand Island, NY), then incubating 30 min at room temperature. After the incubation, the blocking solution was removed from all planned primary antibody-positive sections. A primary antibody solution was prepared at a dilution of 1:100. We tried multiple different macrophage antibodies, and, as mentioned above, settled on a mouse anti-human macrophage monoclonal antibody (Abcam, Cambridge, MA) . This primary antibody was diluted in Cas-Block™, then applied to the sections, with 100µL per section. The slides were placed in a humidified chamber and allowed to incubate overnight at 4°C.

The second day of the immunofluorescence procedure began with vacuuming off the primary antibody dilution from all sections, then washing sections 3 times in 1x PBS. The secondary antibody solution was then prepared. We used a goat anti-mouse secondary antibody conjugated to Alexa Fluor® 488 (Invitrogen, Grand Island, NY). The secondary antibody was used at a dilution of 1:200, and 100µL was used for each section, including negative controls. The secondary antibody was centrifuged at 10,000g to precipitate out aggregates before use. Once the secondary antibody (goat anti-mouse IgG conjugated to Alexa Fluor® 488, Invitrogen, Grand Island, NY) was placed on the sections, they were allowed to incubate at room temperature for one hour. The secondary antibody was then vacuumed off and the sections were washed 3 times in 1x PBS. The PAP pen outlines around the sections were removed using xylene substitute and the slides were cover-slipped using Slow-Fade Gold with DAPI

(Invitrogen, Grand Island, NY). Slides were allowed to sit for at least 20 minutes before being visualized.

A4 Discussion of stains

I did find that all three stains allowed me to adequately identify the respective cell types. However, though they did work, I would possibly change some things. I switched from using the toluidine blue stain to the May-Grunwald Giemsa stain to identify mast cells for a couple reasons. First, the May-Grunwald Giemsa stain when executed properly can allow identification of eosinophils as well. However, I found that the colors that provided easiest identification of mast cells and eosinophils differed, and often that eosinophils were not readily visible when I could easily identify mast cells as mast cell identification required a more blue section while mast cells were somewhat less visible when eosinophils were very visible as eosinophil identification required a more red section. The second reason I chose the May-Grunwald Giemsa stain over the toluidine blue was that this stain appeared in testing to yield more easily visible mast cells which showed up better in photomicrographs. However, in larger batches of slides I found the stain to be a bit inconsistent, and I believe the ease of use of the toluidine blue would have made it more consistent, and as I ended up using the Luna stain to identify eosinophils, the co-identification of the cells became unimportant.

I also struggled finding an antibody to identify bovine macrophages. For all of my antibody and stain testing I used bovine tonsil and lymph tissue which was formalin fixed and paraffin embedded. These tissues allowed me to test antibodies and stains in

tissue that I could be reasonably confident would contain large enough numbers of the immune cell types I sought to identify. The first antibody I tried was a mouse monoclonal anti-CD68 (ABD Serotec, clone ED1, cat# MCA341GA), which unfortunately did not yield consistent results. The next antibodies I tested to identify macrophages were a pair of rabbit polyclonal anti-CD68 antibodies (Abbiotec cat# 250594 and 252281). These antibodies were suggested to work based on sequence homology between the epitope to which they were raised and the bovine CD68 protein. Though they did appear to identify macrophages in lymph and tonsil tissue sections, both antibodies had extremely high background and non-specific binding which made it difficult for the camera to differentiate between signal and noise. Therefore we continued searching for a primary antibody which would work. The one we finally settled on, the mouse anti-human macrophage, is a mouse monoclonal to the calprotectin molecule (Abcam, cat# ab22506). As mentioned above, calprotectin is a cytoplasmic molecule found in granulocytes and monocytes. Though not exactly specific to macrophages, this antibody had a good signal to noise ratio and was consistent across the tissue types I tried it with and also worked reliably from batch to batch.

The one caveat with the anti-calprotectin antibody was the required use of a protease digestion for antigen retrieval. Though proteases are an accepted and widely used method of antigen retrieval, we tend to prefer heat induced antigen retrieval as it can be easy to leave a protease on too long and suddenly digest too much of your tissue. Along with many antibodies, I tested many antigen retrieval methods. Between a citrate buffer, a Tris-EDTA and an EDTA buffer, I found that the Tris-EDTA buffer worked best with the rabbit polyclonal antibodies. The citrate buffer and EDTA buffer

also worked. However, with the anti-calprotectin, the heat induced antigen retrieval methods did not work well and gave a high background, resulting in the choice to use a trypsin digest. We also tried several of the antibodies with a Horseradish peroxidase detection system. This worked best for the anti-calprotectin antibody, and did not work well for any of the others tested.

In summary we chose to use the anti-calprotectin antibody because it gave the most consistent results in staining. The DAPI counterstaining allowed us to differentiate macrophages from the granulocytes. We were also able to successfully identify eosinophils and macrophages in tissue using specific chemical stains. Overall, the staining protocols were successful, though there are potentially some changes I would make in the future.

APPENDIX B:

Table 1: Summary of Antibody Testing Results[1]

		Antigen retrieval method: signal/background			
Antibody	Catalog Number	Citrate Buffer	Tris-EDTA buffer pH 9	EDTA buffer pH 8	Trypsin digest
CD 68 (mouse anti-rat)	ABD serotec: MCA341GA	-/+	NA	NA	-/+
CD 68 (rabbit polyclonal anti mouse, rat)	Abbiotec: 250594	++/++	NA	++/+	+/+
CD 68(rabbit poly, anti human)	Abbiotec: 252281	++/++	+/+	++/+++	+/+
Anti-macrophage	Abcam: ab22506	NA	NA	+/+++	+++/-

[1] Results of antibodies against macrophages tested in bovine tissue. All antibodies were tested in bovine lymph and tonsil tissue prior to use in bovine mammary tissue. - indicates did not work, +, ++, and +++ indicate signal: fair, good and best. NA indicates the antibody was not tested with this antigen retrieval method. Symbol before the slash indicates signal, symbol after the slash indicates background. This is important to note as there were some antibodies which had good signal but which had such high background that computer processing was not practical.