

Characterization of Dendritic Cells in the Bovine Mammary Gland

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ABSTRACT

Bacterial mastitis is a significant problem for the dairy industry. A vaccine against mastitis pathogens could potentially target dendritic cells (DC). While there has been some research describing bovine DC populations *in-vitro*, little is known about DC in mammary tissue. In this study, immunohistofluorescence was used to identify and localize bovine mammary DC. DC were found in alveoli, in epithelia, and in interalveolar tissue. Fluorescence-activated cell sorting (FACS) was used to characterize mammary DC as expressing CD11c, MHC-II, CD205, CD11b, and CD8 α . FACS allowed us to distinguish DC (CD14^{lo}) from macrophages (CD14^{hi}). Two DC subsets, CD11a⁻, CD11a^{lo}, were evident in the mammary gland while an additional CD11a^{hi} population was identified in the supramammary lymph node. After phagocytosis of bacterial components such as lipopolysaccharide (LPS), DC undergo a maturation process, in which they upregulate homing receptors, such as CCR7, and antigen presentation markers, including MHCII and CD80. A primary cell culture model was used to evaluate changes in transcription of CD80 and CCR7 after LPS stimulation. Cell cultures contained digested and Ficoll separated mammary tissue or supramammary lymph node tissue. While the presence of CCR7 and CD80 was confirmed, CD80 and CCR7 transcripts were not upregulated after LPS stimulation. Further, CD11c, CD14, MHCII, CD11b, CD11a, and CD205 protein levels, as assessed by FACS, were similar in LPS stimulated cultures and unstimulated controls. Overall, these studies provide a better understanding of mammary gland immunology, while potentially aiding in the development of novel DC based vaccines.

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List of Abbreviations

4',6-Diamidino-2-Phenylindole	DAPI
Afferent Lymph Veiled Cells	ALVC
Antigen Presenting Cells	APC
C-C Chemokine Receptor Type 7	CCR7
Cluster of Differentiation	CD
Dendritic Cells	DC
Dermal Dendritic Cells	dDC
Fluorescent Activated Cell Sorting	FACS
Follicular Dendritic cell	FDC
Interleukin	IL
Langerhans Cell	LC
Lipopolysaccharide	LPS
Lipoteichoic Acid	LTA
Mean Fluorescent Intensity	MFI
Monocyte Derived Dendritic Cell	MoDC
Myeloid DC	mDC
Natural Killer Dendritic Cell	NKDC
Peptidoglycan	PGN
Plasmacytoid Dendritic Cell	pDC
<i>Staphylococcus aureus</i> Enterotoxin A	SEA
Toll-like Receptor	TLR

Chapter 1. Literature Review

Overview and Objectives

Dendritic cells (DC) play a pivotal role in the initiation of an adaptive immune response. They are professional phagocytes and are resident in a wide variety of mucosal and epidermal tissue. DC also possess the ability to stimulate lymphocytes, and are particularly noted for their ability to stimulate naïve T-cells. These properties make them attractive for use in novel vaccines. As an adjuvant, DC could be cultured *in-vitro* or targeted *in-vivo*. Targeting *in-vivo* could potentially be used to treat mastitis, a severe problem in agriculture. However, there is little previous research describing DC in mammary tissue, primarily because of the difficulty distinguishing DC from macrophages, which possess a similar phenotype.

The goals of this project were to localize DC in the mammary gland and supramammary lymph node using immunohistofluorescence, and to further characterize and quantify them using flow cytometry, immunocytochemistry, and RT-PCR.

Mastitis Overview

Bacterial mastitis is a significant problem in the dairy industry. Costs associated with mastitis include money spent for prevention, treatment, discarded milk, reduced milk production, and culling. The average cost of clinical mastitis alone is \$71 per case [1]. Mastitis is also an important human health concern as well, and occurs in 9.5% of lactations in the U.S. [2].

The species or strain of bacteria can influence bovine mastitis severity, duration, and treatment and prevention strategies. Some bacteria are contagious (*Streptococcus agalactiae* and *Staphylococcus aureus*), while others are primarily environmental. Environmental pathogens are diverse, and include *Streptococcus uberis*, *Streptococcus dysgalactiae*, coliforms, and coagulase

negative *Staphylococcus*. Clinical mastitis is typically caused by environmental coliforms, including *Escherichia coli*, *Enterobacter* spp., and *Klebsiella* spp. Infected udders are not the primary reservoirs for environmental pathogens. Rather, environmental contamination from manure on dirty bedding, etc, constitutes the primary source of these pathogens. While many of treatment and prevention strategies overlap in their effectiveness against diverse types of pathogens, some techniques are successful against a few specific pathogens.

There are two principle contagious mastitis pathogens. *S. agalactiae* is a gram-positive obligate aerobic pathogen of the mammary gland that has been successfully controlled in recent years by proper management techniques [3]. *S. aureus* is a gram-positive bacterium that can infect a variety of tissues, and utilizes a wide range of toxins while avoiding immune defense mechanisms. In the bovine, *S. aureus* can cause chronic, subclinical, and acute mastitis [4]. While *S. aureus* is considered an extracellular pathogen, it can survive and replicate *in-vivo* in non-professional phagocytic cells (endothelial, epithelial, fibroblasts, and others) as well as phagocytic cells, including neutrophils and macrophages [5-7]. In addition, *S. aureus* can reside in the mammary gland and periodically shed, making it difficult to detect. Antibiotic treatment of *S. aureus* mastitis has poor cure rates and preventative therapy has moderate success [8].

Cows with clinical mastitis produce milk not fit for consumption. In subclinical mastitis, outward symptoms are masked and confirmation can only be achieved by culturing. Therefore, many cows may have a mastitis infection, but, as they present no outward symptoms, treatment is not offered. Preventing mastitis outbreaks requires utilizing good management practices to prevent new outbreaks, proper antibiotic treatment of clinical cases, and culling chronically infected cows [8].

Vaccines

There have been few successful vaccines against mastitis pathogens. The J5 vaccine is derived from a mutant *E. coli*, 0111:B4 and it provides some protection against clinical coliform mastitis [9, 10]. A Re-17 mutant *Salmonella typhimurium* bacterin vaccine reduces coliform mastitis severity and associated mortality rates [11].

While there have been several vaccination trials against *S. aureus*, these have been of limited success. Most studies have utilized bacterins or combinations of bacterins and toxins. A vaccine cocktail, consisting of formalin killed bacteria (*S. aureus* and coagulase-negative *Staphylococcus simulans*), *S. aureus* toxoids and lipopolypeptides contained in liposomes, and a dextran sulfate adjuvant, conferred protection against a single strain *S. aureus* challenge in sheep [12]. A combination of *S. aureus* bacterin and purified protein A offered no protection against an infectious challenge, but did provide a higher recovery rate [12]. A field trial with a vaccine consisting of whole, inactivated *S. aureus* with pseudocapsule, α and β toxoids and a mineral oil as adjuvant, did not offer significant protection against clinical mastitis, but did produce specific antibodies for pseudocapsule and α toxin in both milk and serum [13, 14]. Heifers vaccinated with a *S. aureus* enterotoxin type C mutant bacterin were protected against a challenge with *S. aureus* isolated from a case of subclinical mastitis [15]. A polyvalent *S. aureus* bacterin, when combined with antimicrobial therapy, was more successful than a control at facilitating resolution of induced *S. aureus* mastitis [16]. Vaccination with an avirulent live *S. aureus* RC122 caused an increase in the production of antibodies against RC122, but long term field trials have not been conducted [17]. These *S. aureus* vaccination studies were of a short duration, and often used an intrammary challenge consisting of a single pathogen. None of these vaccines provided robust and long-lived immunization against multiple strains.

A new vaccine strategy utilizes activated DC to elicit T-cell mediated memory and immunity. DC play a key role in the initiation of an immune response. DC are phagocytic and can facilitate immunological memory by presenting antigens to naïve T-cells. Thus, DC bridge the gap between the innate and adaptive immune system.

DC can be generated from genetically modified cultures, derived from monocytes or bone marrow *in-vitro* or DC can be targeted *in-vivo*. CD11c, a β integrin and marker for DC, was targeted with an antibody that was conjugated to tumor specific antigens to elicit protection from transplantable and spontaneous mammary tumors [18]. The C-type lectin CD205 can also be targeted and, when an additional maturation signal is included, a potent CD4 and CD8 memory response can be induced [19-21]. Antibodies to CD205 have been conjugated to HIV gag protein in order to target lymph node DC for vaccination against HIV [22]. As there is no universal DC marker, highly specific targeting may be warranted, as related cells, such as macrophages, might compete with DC for the vaccine. However, DC possess a high phagocytic capacity, and their high surface area, might make highly specific targeting unnecessary [21]. Other DC markers also have been successfully targeted in tumor vaccines, including Clec9A, DCIR2, Dectin-1, and MHCII [21, 23-27].

While there are several studies using DC vaccines to prevent tumors or viral infection, little has been done to investigate their potential in protecting against microbial pathogens, notably *S. aureus*. A DC vaccine against microbial pathogens would be similar in design to DC vaccines against tumors or HIV. An antibody specific for a DC marker, such as a CD205, could be fused with a microbial antigen. This conjugate could bind the DC target, become phagocytized or endocytized, and ultimately be presented to T-cells (Fig. 1-1).

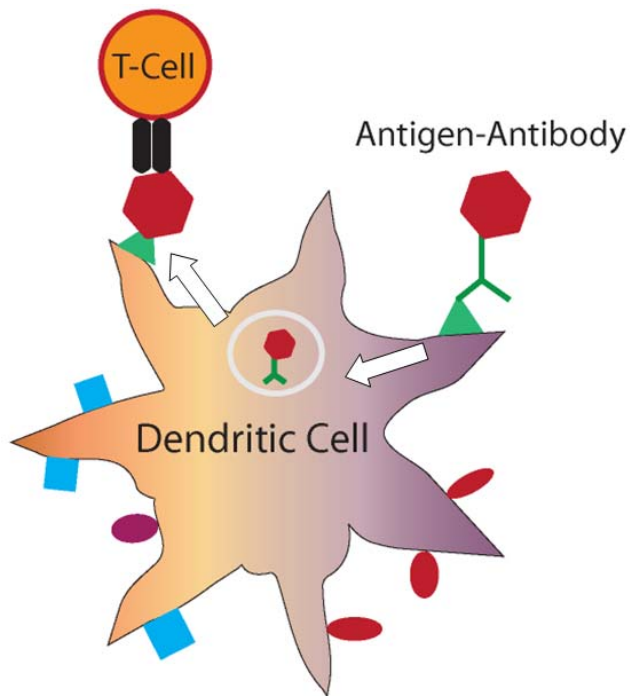


Figure 1-1 Dendritic cell vaccination strategy.

An antigenic component of a pathogen (red hexagon) is conjugated to an antibody specific for a dendritic cell marker (green triangle). This complex could be endocytosed, processed, and presented to T-cells.

Vaccination can potentially target both lymph node resident DC and tissue DC, which could then mature and home to the lymph nodes. However, DC are a heterogeneous population, and specifically targeting a particular subset might be an efficacious vaccine strategy.

DC Populations

DC subsets are described primarily in the mouse model. In the bovine, the functions and phenotypes of DC are not well understood. The following is a summary of the major subsets of DC. It should be noted that expression profiles for any subset of DC vary depending on the species, source tissue, and assay. It is often difficult to discern whether two populations of DC are unique, represent a single cell type at different stages of maturation or reacting to different stimuli.

Myeloid DC

Much of the literature regarding DC focus on DC of myeloid origin, though the exact lineage of this heterogeneous population remains to be fully described [28]. Many studies of DC, including work done in bovine, use DC derived from cultures of blood monocytes cultured with the cytokines interleukin (IL)-4 and granulocyte colony stimulating factor (GM-CSF) to generate monocyte-derived DC (MoDC) [29]. While MoDC possess a profile similar to macrophages, they can be distinguished by relatively high expression of antigen capture and presentation proteins, including major histocompatibility complex II (MHC-II), CD80, CD86, and CD205 [30]. Immature DC can be found in a wide variety of tissues, where they serve as sentries to pathogens. Upon stimulation by foreign particles, such as viral or bacterial components, DC expand their expression of proteins associated with lymphocyte stimulation. For example, bovine MoDC mature by upregulating CD1, CD80, MHCII, IL-12, tumor necrosis

factor- α (TNF- α), and interferon- γ (IFN- γ) when exposed to LPS [31]. Mature or stimulated DC then migrate to secondary lymphoid tissues. In this process C-C chemokine receptor 7 (CCR7), upregulated after maturation, is known as an important regulator of chemotaxis and migration speed [32, 33]. In the lymph node, they present antigens and stimulate lymphocytes [34, 35]. DC are particularly noted for their ability to present antigen and stimulate naïve T-cells [36].

Langerhans Cells

Langerhans cells (LC), which reside in skin and mucosa, were originally described in 1868 by Paul Langerhans, who believed them to be a subset of nerve cells, as they were characterized by long dendritic extensions. However, these cells are actually of a hematopoietic, or of bone marrow origin. LC function to serve as peripheral sentries – they are immature DC. LC can be characterized by tennis racket shaped Birbeck granules, which have an elusive function. They are also noted for their expression of langerin (CD207), a C-type lectin used in a region capture [37]. They can be generated *in-vitro* by culturing CD34 progenitors with GM-CSF and TNF α [38]. The importance of these cells is unclear. It is also uncertain whether unique subsets of LC exist [39]. Bryan et al., [40] used a combination of antibody for MHC-II staining and electron micrography to identify LC in bovine skin. The MHC-II⁺ cells identified all possessed Birbeck granules [40].

Dermal DC

Dermal DC (dDC) arise from a myeloid progenitor and express common DC markers: CD11b, CD1a, CD1d, CD83, CD205, and MHC-II[30]. These cells also share a similar morphology with LC [41, 42]. However, they can be differentiated from LC by their lack or low expression of langerin (CD207), by the occupation of separate tissue regions, and by the absence of Birbeck granules. However, there is no unique marker for dermal DC. Dermal DC fulfill all

of the general DC duties: they capture and present antigen and stimulate the proliferation of T cells [41, 43].

CD8 α DC

CD8 α ⁺ DC have previously been identified in the mouse spleen, lymph node, and thymus [44, 45]. They have not been identified in human or bovine DC populations. It has been suggested that CD8 α ⁺ DC originate from a CD8 α ⁻ DC subset [46, 47]. CD8 α ⁺ is present as a heterodimer and is believed to facilitate the presentation of uptaken antigen to CD8 cytotoxic T-lymphocytes (CTL), via MHCI [48-50]. In the mouse, CD8 α ⁺ DC can facilitate a strong TH1 response, facilitated by production of IL12p70, stimulation of CTL, and to a lesser extent, CD4 T-cells [26, 51-53]. They also have been shown to be better at inducing differentiation of Foxp3⁻ T-regulatory cells (Treg) to Foxp3⁺ via TGF- β [54]. CD8 α has not been identified on either DC or macrophages.

DC with Natural Killer Cell Markers

A unique subset of DC includes natural killer DC (NKDC). They are generally described as possessing a mixture of dendritic (CD11c) and natural killer (NK1.1) markers and possess the ability to kill transformed cells [55-57]. The exact origin and functional capacity (antigen presentation or tumor surveillance) remains unclear, and there are potentially plasmacytoid and myeloid subsets [58, 59]. Caminschi et al., described NKDC as being more like NK cells, in that they were unable to stimulate T cell proliferation after exposure to protein antigens [60]. Like NK cells, they can be expanded in culture when exposed to IL-15 [61]. These cells have been isolated from lymphoid tissue or from bone marrow and can lose their NK1.1 expression while up-regulating MHC-II expression [62]. Almost all of the research on NKDC has been done in mice, but bipotential thymic cells that possess a mixture of NK and DC markers have been

identified in man [63]. A subset of these has been further described as interferon producing killer DC (IKDC) [64-67]. It has been suggested however, that NKDC and IKDC are merely activated NK cells [68-70]. Recently, however, IKDC have been shown to cross present antigens via MHCI to CD8 T-cells, a trait seen in DC, but not NK cells [71]. Given their ability to secrete large amounts of IFN- γ , these cells would be especially attractive candidates for vaccine targeting. Neither population has been identified in the bovine or in mammary tissue.

Plasmacytoid DC

Humans and mice have been shown to possess plasmacytoid DC (pDC), which represent a distinct lineage from myeloid DC. A review by McKenna et al., (2004) summarizes the current research [72]. These cells can be found in blood, lymphoid, and some inflammatory tissues. Pre-pDC express CD4, but not CD3, and lack T-cell receptor alpha (TCR α), TCR β , and CD3. B-cell markers (CD19, CD21) are absent, as are myeloid markers (CD13, CD14, CD33). Human pDC are CD4⁺CD45RA⁻IL⁻3R α (CD123)⁺ILT3⁺ILT1⁻CD11c⁻. Murine pDC differ from their human counterparts because of their moderate CD11c expression; also, murine pDC from lymphoid tissue can express CD4 and/or CD8 α . After exposure to bacteria or viruses, pre-pDC mature and can then produce large amounts of interferon (both IFN- α/γ). Plasmacytoid DC have been shown to present antigens, stimulate T cell proliferation, and facilitate maturation of other pDC, NK, monocytes, mDC, and B and T-cells. Interestingly, murine pDC have been shown to express Toll-like Receptor 7 (TLR-7) and TLR-9 with only moderate expression of TLR-1, 2, 4, 5, and 8 – indicating that the pDC subset is primed for handling viral infections [73]. The role of these cells in maintaining immunity is currently unknown, but their limited presence in non-lymphoid tissue suggests that they may serve to regulate the immune response [74]. Currently, no subpopulations of pDC are or have been identified in the bovine.

Follicular DC

It should be noted that follicular DC (FDC), which reside in lymphoid follicles and assist in B-cell maturation, are not considered true DC. Morphologically, they resemble DC in that they possess dendritic morphology. Some evidence suggests that they are of a fibroblast origin [75, 76]. However, FDC-like cells have been obtained from monocyte precursors [77]. FDC can be identified by morphology (dendrites), an interaction with B-cells, and by antibodies to the complement receptors CD21 (CR2) and CD35 (CR1), and markers for FDC-M1 and FDC-M2 [78].

Bovine DC In-Vivo

In peripheral blood, DC make up less than 1% of the population, and it is therefore difficult to isolate such cells [79]. It is likewise difficult to isolate immature DC from non-lymphoid tissue. However, mature bovine DC can be isolated from afferent lymph, yielding afferent lymph veiled cells (ALVC) [80]. Using this procedure, DC can be identified from other leukocytes by their irregular morphology and by their expression of CD205 [81]. However, this cell population is believed to be mixed, with some members expressing different (or different amounts) of markers, including CD1b, CD11c, and CD206 (a mannose receptor) [82]. There also seem to be at least two cytokine expression profiles common to bovine DC. Stephens, et al., [83] described two major populations as SIRP α ⁺ CC81-Ag DC and SIRP α ⁺CC81Ag⁺ DC, which produced relatively high amounts of IL-12 and IL-1/IL-6, respectively. ALVC can stimulate the proliferation of CD4⁺ and CD8⁺ T-cells, but not gamma delta TCR⁺ T cells [84].

Miyazawa et al., [79] attempted to isolate peripheral blood DC in the bovine by a combination of negative and positive column chromatography selection. The identifying phenotypic expression of the cells was CD11c⁺/CD172a⁺. In addition, these possibly myeloid

DC were CD1 negative, a characteristic that may be acquired later in DC development.

Corresponding cells were identified in the medulla of the thymus by immunohistochemistry using antibodies specific for CD11C, CD172a, and CD1 [79].

DC and Pathogen Interaction

Toll-Like Receptors and Related Pattern Recognition Receptors

The immune system can recognize a number of different pathogens because many pathogens share highly conserved regions. Pathogen-associated molecular patterns (PAMP) are small molecular motifs consistently found on pathogens. They are recognized by toll-like receptors (TLR) and other pattern recognition receptors (PRR). In general, bovine TLR have been shown to share similar functions to their human and murine counterparts. Specific TLR bind to specific PAMP. For example, TLR-4 binds lipopolysaccharide (LPS), TLR5 binds bacterial flagellin, TLR-3 binds double stranded (ds)RNA and TLR-9 binds bacterial DNA [85]. After pathogen recognition, TLR can facilitate the production of reactive oxygen species (ROS), inflammatory and regulatory cytokines, and antigen presenting components, which help drive cell mediated and humoral immunity.

The role of TLR-4 in mediating *E. coli* recognition by innate immune cells has been widely studied. The binding and processing of *E. coli* LPS requires multiple receptors. Myeloid differentiation protein 2 (MD-2), CD14, LPS-binding protein, and TLR-4 are all required. MD-2 has been shown to bind LPS and TLR-4 [86]. When TLR-4 is inactivated by a blocking antibody, LPS induced DC maturation is reduced [87]. Also, CD14 knockout mice are insensitive to LPS; however, antibody inactivation of CD14 in human macrophages fails to completely block LPS recognition [88]. After LPS is recognized, an internal signal is generated,

ultimately leading to activation of the transcription factor NF- κ B. This then leads to cytokine production, particularly IL-12, TNF- α , IFN- γ , and ROS synthesis [87, 89].

S. aureus, a gram-positive coccus, is often said to trigger an immune response by binding of TLR-2. The reality, however, is much more complex. *S. aureus* possesses a multilayered cell wall, where teichoic acids are linked to the peptidoglycan (PGN) and lipoteichoic acid (LTA) is linked to the cytoplasmic membrane [90]. LTA by itself has been shown to be moderately effective at triggering DC maturation. Alone, it causes a moderate increase in CD80, CD86, TNF- α , and IL-12 in murine DC [91]. In human MoDC it causes a similar effect [92]. There is a synergistic effect on DC maturation when LTA and PGN are administered together *in-vivo* [91]. Both LTA and PGN have been shown to facilitate DC maturation via TLR-2 [93]. However, recognition of PGN via TLR2 remains controversial, as it is difficult to obtain pure PGN, without contaminating LTA [94].

S. aureus may utilize TLR-2 as a means of facilitating intracellular infection. In a study in mice, TLR-2 deficient macrophages were less susceptible to intracellular infection, though rates of phagocytosis were similar [95]. Survival was attributed to diminished superoxide production after TLR-2 activation.

Other cellular receptors are critical in recognizing and processing *S. aureus* antigens. Mannose binding lectins have been shown to coordinate TLR-2 and TLR-6 binding to *S. aureus* [96]. TLR-1 and TLR-6 were shown to complex with TLR-2 in macrophages and facilitate recognition of PGN [97]. Similarly, CD36 helps facilitate a macrophage TNF- α response to PGN (60). CD14 binds both PGN and LTA [98]. Intracellular PGN can be recognized by the intracellular PRR nucleotide-binding oligomerization domain containing 2 (NOD2) [96].

The recognition of other *S. aureus* virulence factors such as hemolysins and adhesins in mediating infection in immunity are less well studied. *S. aureus* enterotoxin B (SEB) is a secreted exotoxin that is associated with food poisoning. SEB has been shown to promote TH2 cell polarization via TLR-2 in human MoDC, characterized by high expression of IL-2 [99].

DC and T-Cell Response

When exposed to *S. aureus*, DC mature and are capable of stimulating naïve T cells to produce a TH1 response. This is mediated by DC production IL-12 and other cytokines. A similar response is seen with LPS stimulation of DC [100]. Stimulated T-cells can then produce IFN- γ to enhance the response [101]. Purified toxins have also been shown to elicit DC maturation and subsequent stimulation of other T-cell subsets. CD4+CD25+ regulatory T cell populations can be expanded by exposure to DC primed with *S. aureus* enterotoxin A (SEA) [102]. SEA, SEB, and toxic shock syndrome type-1 mature MoDC and facilitate WC1+ $\gamma\delta$ T cells [103]. Such T-cells are not well understood, but are believed to regulate the immune response.

While DC are considered to promote a TH1 response, this is not always the case. The ratio of DC to T cells has been shown to be important in determining whether a TH1 or TH2 response occurs. In a study using MoDC, a low ratio of DC:T (1:300) stimulated DC facilitated a TH2 response in naïve T cells. In contrast, a higher DC:T ratio (1:4) produced a mixed TH1 and TH2 response [36]. The type of pathogen may also affect the type of response. SEB has been shown to cause promote TH2 cell polarization via TLR-2 in human MoDC, characterized by high expression of IL-2 [99].

A further description of DC and T-cell interactions will provide a better understanding of the immune response, while potentially aiding the development of novel vaccines. For example, eliciting a CD8 T-cell response might alleviate intracellular infections via cytotoxic killing.

Localization of DC

Lymph node DC, often referred to as interdigitating DC (iDC), have been described in the spleen, thymus, tonsils, as well as a variety of other secondary lymphoid tissues. DC have also been identified in a wide variety of non-lymphoid tissue, including the skin, lungs, liver, kidney [104, 105]. DC have yet to be definitively identified in the mammary gland or its supramammary lymph nodes.

Mammary Overview

The gross anatomy of the mammary gland varies between species, though physiological function is largely similar. In the bovine, there are four separate mammary glands. Milk is produced in specialized secretory epithelial cells, which are organized into alveoli, similar to the structure of the lung. These alveoli are clustered into lobes surrounded by lobular epithelia. Milk is secreted into the alveoli, drains into the ducts and flows down to the gland cistern, then through the teat canal, and out of the teat opening. The mammary gland also contains a variety of other specialized cells and tissues, including supportive connective tissue, lymphatics, blood vessels and capillaries, and fat.

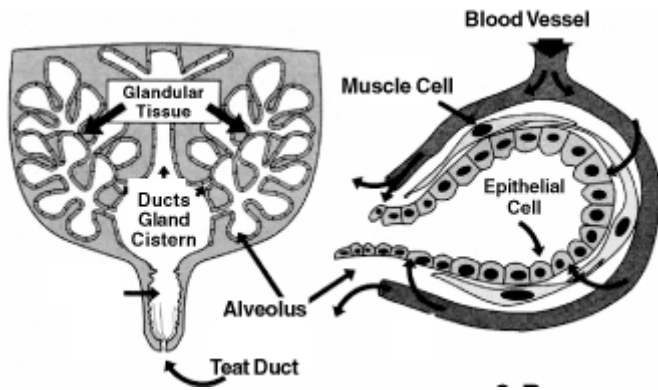


Figure 1-2 Microanatomy of the mammary gland [125].

[Fair use]

This illustration shows the microanatomy of the mammary gland. Epithelial cells in the alveoli secrete milk, which flows out into collecting ducts and eventually out of the teat. Alveoli are supported by a wide variety of other tissues, including lymphatic tissue, adipose tissue, veins and venules, and other connective tissue.

There have been few studies describing antigen-presenting cells (APC) in the mammary gland. Typically, a single and relatively broad marker, such as CD14 or MHCII is used. Thus, it remains unknown whether the APC populations described represent DC or macrophages.

It has been known for some time that mastitic infections induce leukocyte migration to the mammary gland. These cells are often used as part of a diagnostic profile, termed somatic cell count (SCC). Leitner et al., [106] characterized this migration in the udders of cows infected with *S. aureus*. These authors used immunohistochemistry and specific antibodies against CD4, CD8, CD21, CD18, and CD14. T-cells bearing the CD4 antigen were identified in interalveolar connective tissues. Cytotoxic T-cells (CD8+) were found around the alveoli and in the alveolar lumen. B-lymphocytes (CD21+) were found in small clusters (2-5 cells) in connective tissue or areas with no alveoli. Macrophages (CD14+) were found in epithelial cells and around alveoli. The authors also describe a CD21+ positive cell in the alveoli with a roughly dendritic form. With the exception of CD21 B-lymphocytes, all leukocyte numbers increased with *S. aureus* mastitis, as evidenced by an increased number of positive cells (including CD4, CD8, CD18, and CD14) in histological slides and in corresponding flow cytometry analysis [106].

Joshi et al., [107] used a combination of electron microscopy and antibody staining specifically to the Ia antigen – a subunit of MHC-II, to localize mammary DC. Another study used antibodies to MHCII to identify cells with a dendritic morphology among mammary alveoli [108]. In the rat mammary gland, cells with a dendritic morphology that stained positively for MHC-II were identified. There were no true Birbeck granules visible, only plasma-lammal projections ending in bulbous ends, so the nature of these DC (LC and dermal) was unclear [107]. Other evidence suggests that mammary DC are, in fact, LC. Tatarczuch et al., [109] evaluated sheep leukocytes in mammary secretions taken after weaning. Analysis was

performed using electron microscopy and antibodies to MHC-II. The authors identified Birbeck-like granules in cells cellular projections, which could be called either dendrites or pseudo-podia [109].

CD14+ cells, which can mark macrophages, neutrophils, and DC, were found to be recruited to the mammary gland upon *S. aureus* and *S. uberis* infection [110]. Human breast milk macrophages were CD14+, CD11b+, and CD80+; these cells could be driven to become DC by the addition of IL-4 alone [111].

MHCII+ cells were isolated from milk and described as macrophages, though this marker is also found on B-cells and DC. Isolated MHCII+ cells induced T-cell proliferation after *S. aureus* induced maturation. However, this induction was less than that induced by peripheral blood mononuclear cells [112].

CD11b and CD11c are β -integrins that are involved in chemotaxis and complement activation. Both are found on DC, macrophages, and to a lesser extent, neutrophils, and B-cells. These two markers were identified in milk from cows challenged with *E. coli* using flow cytometry. Younger cows typically had greater CD11c and less CD11b expression. [113]. CD11a, another β -integrin, has also been identified in cows before and after calving, and its presence was believed to be from monocytes and neutrophils [114].

These limited studies do not provide an adequate description of mammary DC phenotype or activity. A combination of DC markers could be used to identify this population and characterize it.

Supramammary Lymph Node Overview

The structure of secondary lymph nodes is similar among different animals and in different regions of the body (Fig. 1-3). Afferent lymphatics drain from surrounding tissues and

pass through the capsule. The outer layer of the lymph node, the cortex, contains B-cell follicles and germinal centers, where B-cells mature into plasma cells. Beyond this region lies the paracortex, which contains many T-cells, as well as some secondary follicles. The medulla, which can be distinguished by medullary cords, also contains efferent vessels. The lymph node is also compartmentalized by segments called trabiculae.

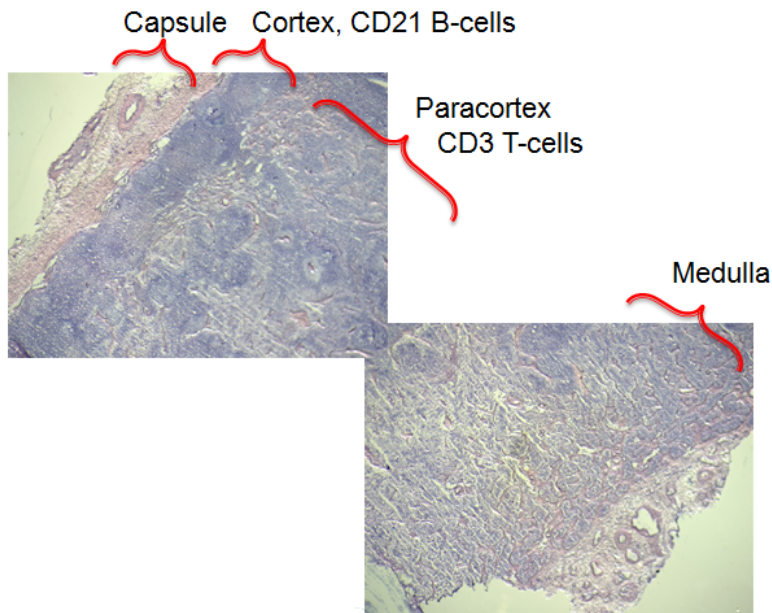


Figure 1-3 Cross-section of supramammary lymph node.

This figure shows a 15 μM thick cross-section of supramammary lymph node stained with haematoxylin and eosin. The capsule, cortex, paracortex, and medulla are indicated with brackets. CD21 B-cells are found in follicles of the paracortex, while CD3 T-cells can be found primarily in the paracortex. Magnification is 10X.

In the bovine mammary gland, the lymphatics drain to the supramammary lymph nodes. Although there can be variation in the arrangement and number, typically there are two lymph nodes for each side, right and left. In each pair, one is larger and to it, drains superficial vessels. The other is smaller, and it receives vessels from deep within the mammary gland. These nodes lie dorsal and caudal to the udder. The lymph nodes are typically encapsulated in fat. Drainage, however, is not necessarily side restricted: 23% of superficial supramammary lymph nodes can drain from the opposite side [115]. The size of the lymph node can vary widely between individual animals, as a result of lactation, age, immune experience, and pregnancy [116].

Leitner et al, [106] previously described the distribution of lymphocytes types in the bovine supramammary lymph node prior to and after *S. aureus* infection. In their study, CD3 cells were found in the paracortex, sparsely scattered in the medulla, whereas CD21 B-cells were seen in the follicles [106]. Similar findings appear in the bovine temporal lymph node and hemal node [117]. MHCII+ cells, either macrophages, DC, or B-cells enter the supramammary lymph node at a higher frequency after *E. coli* LPS challenge [118].

While DC have not been studied in the supramammary lymph node, they have been described in other bovine secondary lymph nodes. It is hypothesized that the populations and activities in different secondary lymph nodes would be similar. CD11c+CD172a+ DC were present in the interfollicular regions and reticular epithelium of the bovine palatine tonsil [119]. CD11c+CD86+ DC were identified in the bovine palatine tonsil [120]. MHCII has been quantified in Ficoll separated supramammary lymph node cells as well as localized in the cortex and paracortex of bovine distal jejunal lymph nodes [118, 121]. CD205 has been identified in cortex and paracortex of the prescapular lymph node [81]. These findings are consistent with mouse and human lymph nodes. ALVC, which are considered DC, possess both CD11a+ and

CD11a- subsets, of which only the CD11a- subset can induce allogeneic proliferation of CD8 lymphocytes, possibly because of diminished IL1 α production by the CD11a+ population [122].

Further phenotypic and functional analysis of the bovine supramammary lymph node could reveal important immunological functions, provide insight into the pathology of mastitis, and aid in the development of novel treatments.

Conclusion

DC could be targeted *in-vivo* as part of a vaccine against *S. aureus* mastitis. DC could be targeted by infusing an antigen-antibody complex into the mammary gland or injecting it near the supramammary lymph nodes. However, DC have not been definitively identified in the mammary gland or the supramammary lymph nodes. The goals of this thesis were to localize DC in the mammary gland and supramammary lymph node using immunohistofluorescence and to characterize and quantify them using flow cytometry, immunocytochemistry, and RT-PCR.

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Chapter 2. Characterization of Bovine Mammary Dendritic Cells

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Summary Sentence: Bovine mammary DC are a heterogeneous population, distinct from macrophages and similar to afferent lymph veiled cells.

Running title: Bovine Mammary DC

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Key words: macrophages, supramammary lymph node, mastitis

Abstract

Dendritic cells (DC) are a heterogeneous population of professional antigen presenting cells and are potent stimulators of naïve T-cells. However, there is little previous research describing DC in mammary tissue, primarily because of the difficulty distinguishing these cells from macrophages, which possess a similar phenotype. Using immunohistofluorescence and a combination of markers (MHC-II, CD205, CD11c) DC were localized in the bovine mammary gland and supramammary lymph node. In mammary tissue, DC were found within the alveolar epithelium and within the intralobular connective tissue. In the lymph node, DC were found on the periphery of B-cell areas in the cortex, and among T-cells in the paracortex and medulla. DC in mammary parenchyma and supramammary lymph nodes were quantified and further characterized using fluorescence-activated cell sorting (FACS). DC were CD11c^{hi}, CD14^{lo/-} cells expressed high levels of MHC-II, CD205, and CD8α⁺. Two DC subsets, CD11a⁻, CD11a^{lo}, were evident in the mammary gland while an additional CD11a^{hi} population was identified in the supramammary lymph node. DC could be distinguished from macrophages based on their expression of CD14. Macrophages also possessed a single CD11a^{lo} population. This research provides a better understanding of the mammary gland immunology, while potentially aiding in the development of novel DC based vaccines.

Introduction

Dendritic cells (DC) play a pivotal role in the initiation of an adaptive immune response. They are professional phagocytes and are resident in a wide variety of mucosal and epidermal tissues. DC also possess the ability to stimulate lymphocytes, and are particularly noted for their ability to stimulate naïve T cells. These properties make them attractive for use in vaccines – either cultured *in-vitro* or targeted *in-vivo*. Targeting could potentially be used to treat mastitis, a severe problem in dairy production and a human health concern. However, there is little previous research describing DC populations in mammary tissue, primarily because of the difficulty distinguishing these cells from macrophages, which possess a similar phenotype.

Previous studies have characterized bovine DC obtained through various means. Bovine DC have been derived from monocytes in culture by incubating with IL-4 and GM-CSF (MoDC). Similar to human and mouse, bovine MoDC express CD11c, MHCII, CD80, and CD11a and differ from monocytes in their reduced expression of CD14 and CD11b [29, 31, 123]. In addition, Miyazawa and collaborators [79] characterized ex-vivo bovine DC in peripheral blood by removing lymphocytes and CD14⁺ cells while selecting for CD11c⁺, CD172a⁺ cells. Several studies have focused on afferent lymph veiled DC (ALVC) that were CD14^{lo/-}, CD11b^{lo}, MHCII^{high}, CD11c^{high}. These cells also contained at least two subsets, expressing differing levels of CD172a and CD11a [83, 124, 125]. ALVC were later found to express CD205 and DC-LAMP [81]. These studies have provided valuable information about bovine DC but have not detailed the existence of characteristics of mammary DC specifically.

Previous studies have identified MHCII⁺ cells with a dendritic morphology in the mammary gland and in milk of various species [107, 108, 126]. These cells were located among alveoli and in ducts. A mixed population of adherent MHCII⁺ cells from milk, which include

macrophages and DC, were shown to present antigens and respond to LPS and *Staphylococcus aureus* by stimulating T-cell proliferation, presenting antigen, and by secreting IL-1 [112].

In this study DC were identified and characterized in the bovine mammary gland using two-color immunohistofluorescence and fluorescence-activated cell sorting (FACS). This study is the first to characterized mammary DC and provides a better understanding of mammary gland immunology, while aiding in the development of novel DC based vaccines.

Materials and Methods

Animals. Tissue from 6 lactating cows (*Bos taurus*) was harvested at the time of slaughter. Brown Packing Co, Gaffney, SC and the Virginia Tech Dairy Facility provided the animals. Protocols were in accordance with Virginia Tech's Institutional Animal Care and Use Committee.

Immunohistofluorescence. Small, sections of mammary and supra-mammary lymph nodes were covered in optimal cutting temperature compound (OCT), frozen in liquid nitrogen, and stored at -80°C until sectioning. Mammary tissue was sectioned at 8 µm thickness and lymph nodes at 14 µm. Sections were mounted onto SuperFrost Plus microscope slides (Fisher Scientific, Loughborough, UK) and stored at -80°C. For analysis, tissue sections were warmed to room temperature, and then fixed in ice-cold acetone for 10 min. After washing (0.05% TWEEN-20 in phosphate buffered saline (PBS), 3× for 2 min each), sections were blocked with 10% goat serum for 5 min and washed. Sections were incubated with primary antibodies or isotype controls overnight at 4°C, washed, and incubated with secondary antibodies for 30 min at room temperature. Sections were then washed and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 2 min at room temperature, washed, and mounted with (Fluoromount-G, SouthernBiotech, Birmingham, AL, USA). Slide images were viewed using a Nikon Eclipse

E600 (Nikon, Melville, NY, USA), photographed with a Zeiss AxioCam MRm camera (Thornwood, NY, USA), and composited using Q-Capture Pro 6.0 (Surrey, BC Canada). Isotype controls were used in place of primary antibodies to establish background.

Isolation of low density Cells. Mammary and supramammary lymph tissue was collected at time of slaughter and transported on ice in PBS containing 5% heat-inactivated Fetal Bovine Serum (FBS) and 10 µg/mL gentamicin (Invitrogen, Carlsbad, CA, USA).

Mammary tissue was minced, and then digested overnight in 0.5 U/mL elastase (CalBioChem, Darmstadt, Germany) and 1300 U/mg type 3 collagenase (Worthington Biochemica, Lakewood, NJ, USA) in 37°C in Krebs's Ringer Bicarbonate, supplemented with 5% FBS and gentamicin at 10 µg/mL. Cells were passed through a 70 µm nylon mesh and washed in 45 mL Hank's balanced salt solution (HBSS). To isolate low-density cells, including DC, mammary digests were then separated using Ficoll (BD Falcon, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Briefly, cells were suspended in 25 mL of HBSS and layered over 12.5 mL of Ficoll. This was then centrifuged at 367 g for 30 minutes at 25°C. A roughly 10 mL middle layer containing low-density cells, lying directly above the Ficoll layer, was removed. These cells were washed in 45 mL HBSS and counted using a haemocytometer.

Single supramammary lymph nodes were minced with a razor blade and grated with a wire mesh, producing pieces of about 3 mm³. This tissue was then passed through a 70 µm cell strainer (BD Falcon) and washed in HBSS. Cells were separated by density centrifugation using Ficoll, washed in HBSS, and counted using a haemocytometer.

Fluorescence Activated Cell Sorting (FACS). Ficoll separate cells from the mammary gland or the supramammary lymph node were washed in HBSS, resuspended in PBS containing 0.05% sodium azide, and 10⁶ cells were used for each antibody combination. Cells were incubated with

appropriate primary antibodies at 4°C for 1 h. Primary antibodies used were specific for CD11a, CD11b, CD11c, CD14, MHCII (VMRD, Pullman, WA, USA), NKp46-Alexa Flour 488 conjugated and CD205 (Serotec, Raleigh, NC, USA) and used according to manufacturer's instructions. CD21 and CD3 specific antibodies (VMRD) were used to exclude lymphocytes. This was followed by the addition of corresponding secondary antibodies conjugated to FITC, PE, APC, or Alexa Fluor 594 (Invitrogen) at 4°C for 30 min. Cells were then washed and analyzed with a FACS Aria flow cytometer (BD, San Jose, California, USA) and FlowJo software (Tree Star, Ashland, OR, USA). This analysis was performed on tissues collected from 4 animals.

Immunocytochemistry. Mammary and lymph node DC (CD14^{lo/mid}, CD11c^{hi}) or macrophages (CD14^{hi}, CD11c^{hi}) were sorted from a mixed population using a FACS Aria. Approximately 1000 cells per sample were stained with DAPI and then placed onto a microscope slide via a cytopsin. Cells were examined using a Nikon Eclipse E600 microscope (Melville, NY, USA) and photographed using Zeiss AxioCam MRm camera (Carl Zeiss Light Microscopy, Göttingen, Germany). Composite images were created showing the expression of MHCII, CD205, DAPI, and a phase contrast image at 400X. An unstained and sorted population was used as a control to determine background fluorescence. This characterization was performed on tissues collected from 3 animals.

Results

Localization of Mammary and Supramammary Lymph Node DC.

Using immunohistofluorescence, cells bearing the markers CD11c, MHCII, and CD205 were identified in the supramammary lymph node. Tissue sections were costained with antibodies against either CD3 (marking T-cell areas, top row Fig. 2-1) or CD21 (marking B-cell areas and follicles,

bottom row Fig. 2-1). Sections were counterstained with DAPI. DC ($CD11c^+$, $MHCII^+$, or $CD205^+$) were diffusely scattered throughout the paracortex. DC were also evident in the periphery of follicles. Note that some cells were double positive for CD21 and a DC marker. These could be B-cells or the result of overlapping cells. Few DC were seen in the capsule or the medulla (data not shown).

Two-color immunohistofluorescence was used to identify DC in the bovine mammary gland. $MHCII^+$, $CD11c^+$ cells could be identified among alveoli (Fig. 2-2A) and were regularly scattered in the ductal epithelia (Fig. 2-2D). Likewise, $MHCII^+$, $CD205^+$ DC (Fig. 2-2B) and $CD11c^+$ $CD205^+$ DC (Fig. 2-2C) were evident among the alveoli and connective tissue. Some of these cells exhibited a dendritic morphology; others may be macrophages or B-cells. Likewise, some cells were positive for only a single marker, and these cells may be B-cells, monocytes, or macrophages. Such cells may also be DC that express the missing marker, but at a level below the limit of detection.

Confirmation of mammary DC by flow cytometry. FACS was used to analyze Ficoll separated lymph nodes and collagenase and elastase digested and Ficoll separated mammary glands. CD21 and CD3 staining were used separately to demarcate lymphocytes and electronic gating was used to exclude these cells and debris based on forward and side scatter (Fig. 2-3 A, C). The remaining large, granular cells were then analyzed for their expression of CD11c and CD14. This differentiated two populations, $CD14^{mid/lo}$, $CD11c^{hi}$ DC and $CD14^{hi}$, $CD11c^{hi}$ macrophages in the supramammary lymph node (Fig. 2-3B) and mammary gland (Fig. 2-3C). Note that there is some degree of overlap in these populations. Other cell populations were also evident. These included a mixture of autofluorescent cells, contaminating neutrophils and lymphocytes, and some $CD14^{lo}CD11c^{lo}$ cells. The later of these may be immature myeloid cells

– either DC or macrophages, as they expressed low levels of MHCII, CD205, and CD11b (data not shown).

Other markers were evaluated on the DC gate in both the mammary gland and lymph node – illustrated in Fig. 2-1E. The level of CD11b expression was similar in both tissues. In the lymph node, three different populations of DC were indicated based on their expression of CD11a, including CD11a⁻, CD11a^{mid}, and CD11a^{high}. In the mammary gland, only the CD11a⁻ and CD11a^{mid} populations were evident. Both macrophages and DC expressed CD8α. In both tissues, DC expressed no or low levels of NKp46.

Mammary and supramammary lymph node DC and macrophages were compared based on their expression of surface makers (Fig. 2-4 A, B). In the lymph node, DC expressed higher levels of CD205 and MHCII, while expressing lower levels of CD11b. Macrophages were CD11a^{mid}, while LN DC possessed 3 populations: CD11a⁻, CD11a^{mid}, and CD11a^{high}. Both DC and macrophages were negative for NKp46. In the mammary gland, both DC and macrophages expressed similar levels of CD205 and MHCII, while mammary DC expressed slightly less CD11b. Mammary macrophages were CD11a^{mid}, while DC possessed 2 populations: CD11a⁻ and CD11a^{mid}. Both mammary DC and macrophages were negative for NKp46.

Morphology of Mammary DC. Mammary and supramammary lymph node DC were sorted and placed onto a slide via a cytospin; they were then counterstained with DAPI. Composite images were generated showing expression of CD205 (red), MHCII (green), and a DAPI counterstain (blue). The harsh treatment of DC in this experiment, which included physical disruption of tissue, enzymatic digestion (in the case of mammary tissue), Ficoll separation, and cell sorting left few sorted cells with intact dendrites. However, some of the sorted DC did possess a dendritic morphology (Fig. 2-5).

Discussion

These studies describe the location and phenotype of mammary and supramammary lymph node DC. CD3 and CD21 were used to help delineate T and B cell areas of the supramammary lymph node. Leitner et al., [106] previously described the distribution of these two cell types in the bovine supramammary lymph node prior to and after *S. aureus* infection. In their study, CD3 cells were found in the paracortex and sparsely scattered in the medulla whereas CD21 B-cells were seen in the follicles. Similar findings appear in the bovine temporal lymph node and hemal node [117]. Results are consistent with these findings, though CD21 cells also were localized in clusters outside well-defined follicles and T-cells were found in the intrafollicular space as well as the paracortex and medulla.

In the supramammary lymph node, cells bearing the markers MHCII, CD11c, or CD205 were found both intrafollicular space and in T-cell areas of the paracortex and medulla. Although this is the first such study done in bovine supramammary lymph nodes, similar findings in other secondary lymphoid tissues have been documented. CD11c/CD172a DC were present in the interfollicular regions and reticular epithelium of the bovine palatine tonsil [119]. CD11c/CD86 DC were identified in the bovine palatine tonsil [120]. MHCII has been quantified in Ficoll separated supramammary lymph node cells as well as localized in the cortex and paracortex of bovine distal jejunal lymph nodes [118, 121]. CD205 has been identified in cortex and paracortex of the prescapular lymph node [81]. Similar findings have been found in mouse and human lymph nodes.

Macrophages of the mammary gland have often been described in literature based on the expression of a sole marker, either CD14, MHCII, or based on their scatter properties as accessed by flow cytometry [106, 114]. Two-color immunohistofluorescence was used to identify

mammary DC. In mammary tissue, DC were morphologically identifiable, and were found among alveoli, in epithelia, and in interalveolar tissue.

Consistent with MoDC, mammary and supramammary lymph DC (CD11c^{hi}, CD141^{lo/-} cells) expressed high levels of MHCII, CD11b, and CD205. These cells expressed little or no NKp46. These expression patterns were similar in both the mammary gland and supramammary lymph node. While it might be expected that mammary DC express higher levels of CD205, a mannose-like receptor known for its scavenger activity, mammary DC also expressed slightly higher levels of MHCII. The general paradigm for DC maturation has been that DC upregulate MHCII and home to the lymph node, where they can present antigen to T-cells. *In-vitro*, bovine MoDC stimulated with LPS upregulate maturation markers such as CD80, MHCII, and produce IL-12 [31]. It may be that antigen presentation to localized T-cells is also important. Functional assays are needed to discern if this phenotype corresponds with different physiological functions.

These results indicate the presence of distinct DC populations, based on their expression of CD11a. These mammary and supramammary lymph DC subsets correspond with the ALVC subsets. ALVC possess both CD11a^{lo} and CD11a⁻ subsets, of which only the CD11a⁻ subset can induce allogeneic proliferation of CD8 lymphocytes, possibly because of diminished IL1 α production by the CD11a⁺ population [122]. Both subsets can present OVA to memory CD4⁺ T cells, but the CD11a⁻ subset was better in presenting respiratory syncytial virus antigen [124]. Culturing of monocytes with staphylococcal enterotoxin C1 (SEC1) induces a transient rise in CD11a as the cell transitions to a dendritic phenotype. Blocking CD11a DC matured with SEC1 limits the proliferation of autologous CD8 T-cells [127]. Both CD11a⁻ and CD11a^{lo} subsets were evident in the mammary gland. Additionally, a CD11a^{hi} population was also apparent in the lymph node. It is unknown what stimuli cause the differentiation of these two subsets, and what

additional physiological differences they might impart. In the lymph node, these subsets may be the result of different populations homing to the lymph from the skin (Langerhans cells and dermal DC), mucosal and other tissue types, or directly from the blood (blood-derived inflammatory DC).

It is important to note that bovine MoDC have been reported to be singularly CD11a^{lo} [29, 128]. This is supported by our research (unpublished data). Thus, inferring the function of mixed DC populations, whether from mammary tissue or afferent lymph, based on *in-vitro* MoDC studies, should be done cautiously. More research is necessary to compare these, and many of the other naturally occurring DC subsets. The results were consistent with the conclusion of Howard, et al., that these CD11a subsets are not representative of the DC1 and DC2 paradigm (MoDC and plasmacytoid DC (pDC), respectively) [82]. In both the mammary gland and supramammary lymph node, both CD11a⁻ and CD11a⁺ DC possess high levels CD11c, CD11b, CD205, and low levels of CD14. The pDC remains to be described in the bovine.

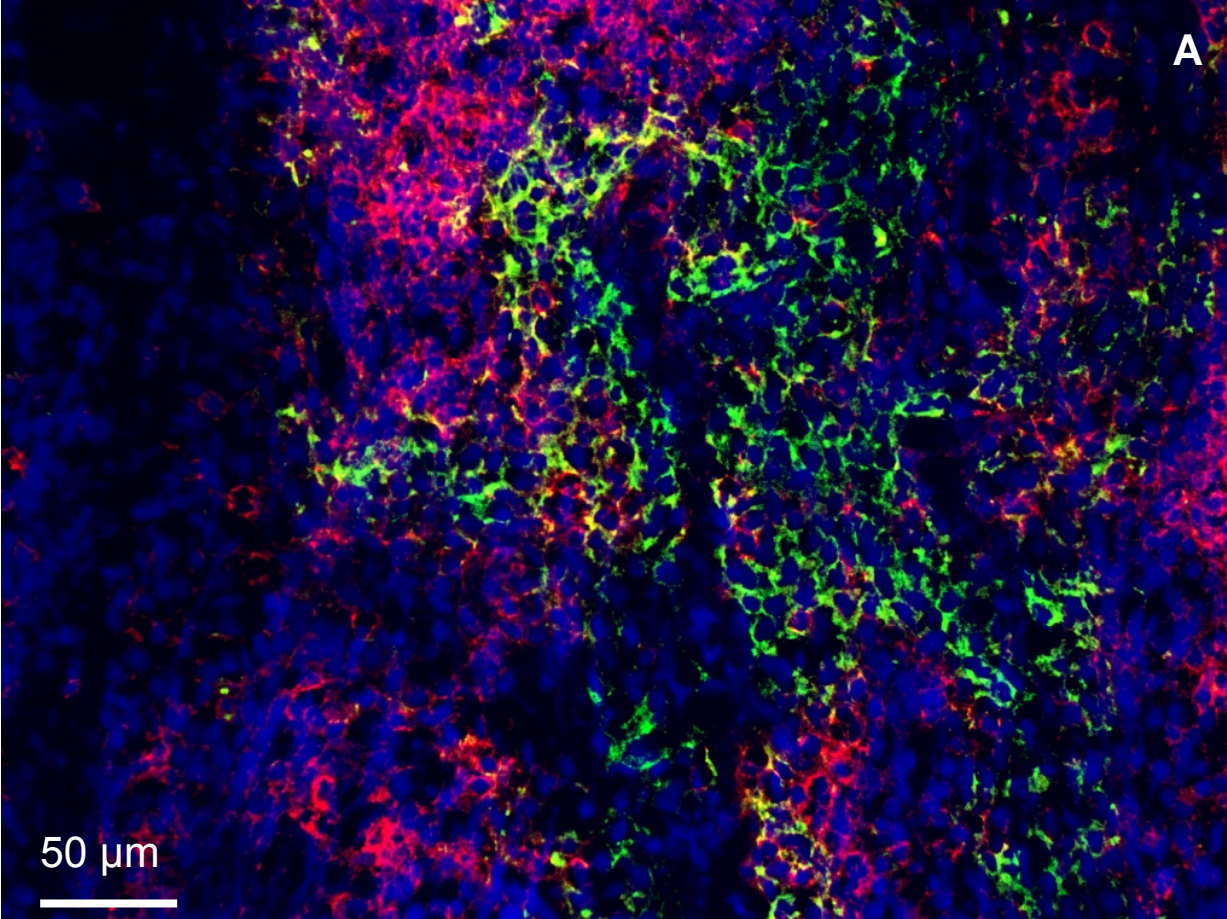
Another DC subset includes CD8 α ⁺ DC. CD8 α is believed to facilitate the presentation of uptaken antigen to CD8 CTL via MHC I [48-50]. In the mouse, CD8 α ⁺ DC can facilitate a strong TH1 response, facilitated by production IL12p70, stimulation of CD8 cytotoxic T-lymphocytes (CTL), and to a lesser extent, CD4 T-cells [26, 51-53]. Results indicate that both mammary and supramammary lymph node DC and macrophages express moderate amounts of CD8 α . If they possess similar activities in the mouse counterpart, this feature makes them attractive candidates to target in a vaccine.

Another DC subset has been described in mouse having expression patterns similar to natural killer cells (NKDC) [56, 61, 62, 129]. These cells are described as nonlymphocytic

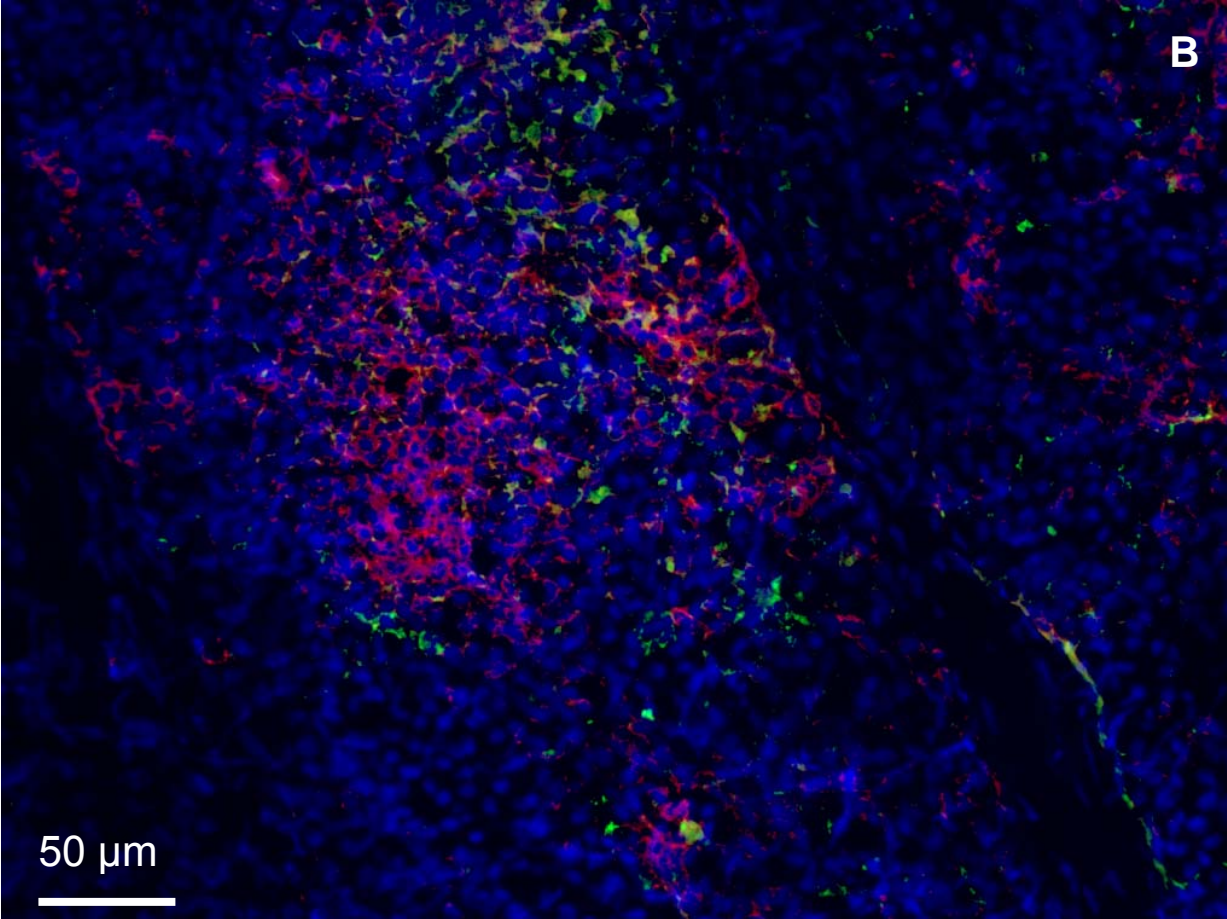
CD11c⁺ and NK1.1⁺. A subset of these has been described by their interferon production, termed interferon-producing killer DC (IKDC) [55, 58, 59, 130]. While some results indicate these cells have special phenotypic activities, it has also been suggested that these cells are merely activated NK cells [60, 70]. In the mammary gland and supramammary lymph node, no significant populations of CD11c⁺ cells expressing the NK marker NKp46 were identified (data not shown). Studies of other bovine tissues, and using other NK markers, coupled with functional assays, could determine whether these unique populations exist in the bovine.

Mammary DC and supramammary lymph node DC likely play an important role in protecting the mammary gland against mastitic pathogens. It has also been shown that the mouse mammary tumor virus can spread via mammary DC [131]. This study opens the door for novel strategy for DC targeting. This research provides the important first step of definitively identifying the DC population. Future research, using additional markers and purified populations of mammary DC is warranted.

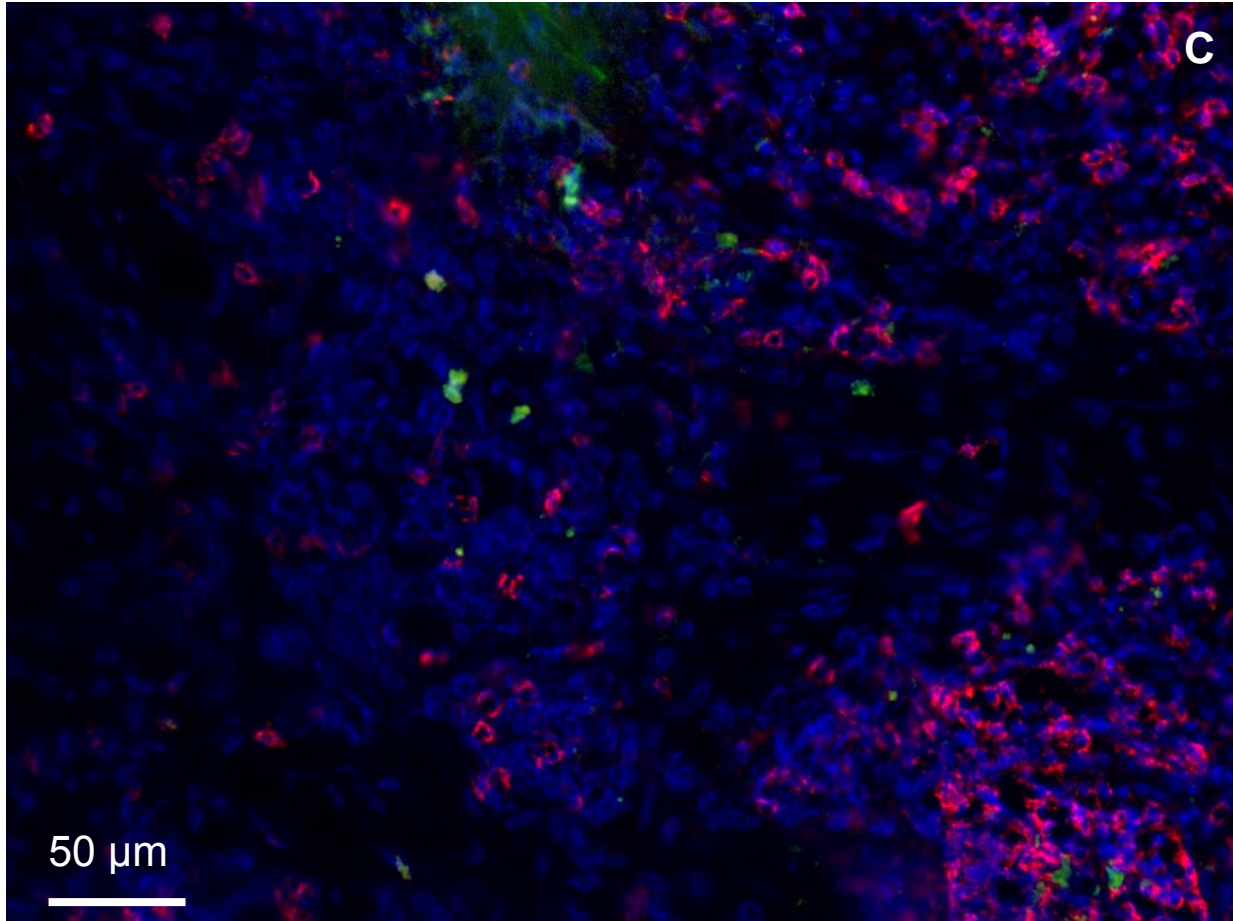
CD3 / CD11c



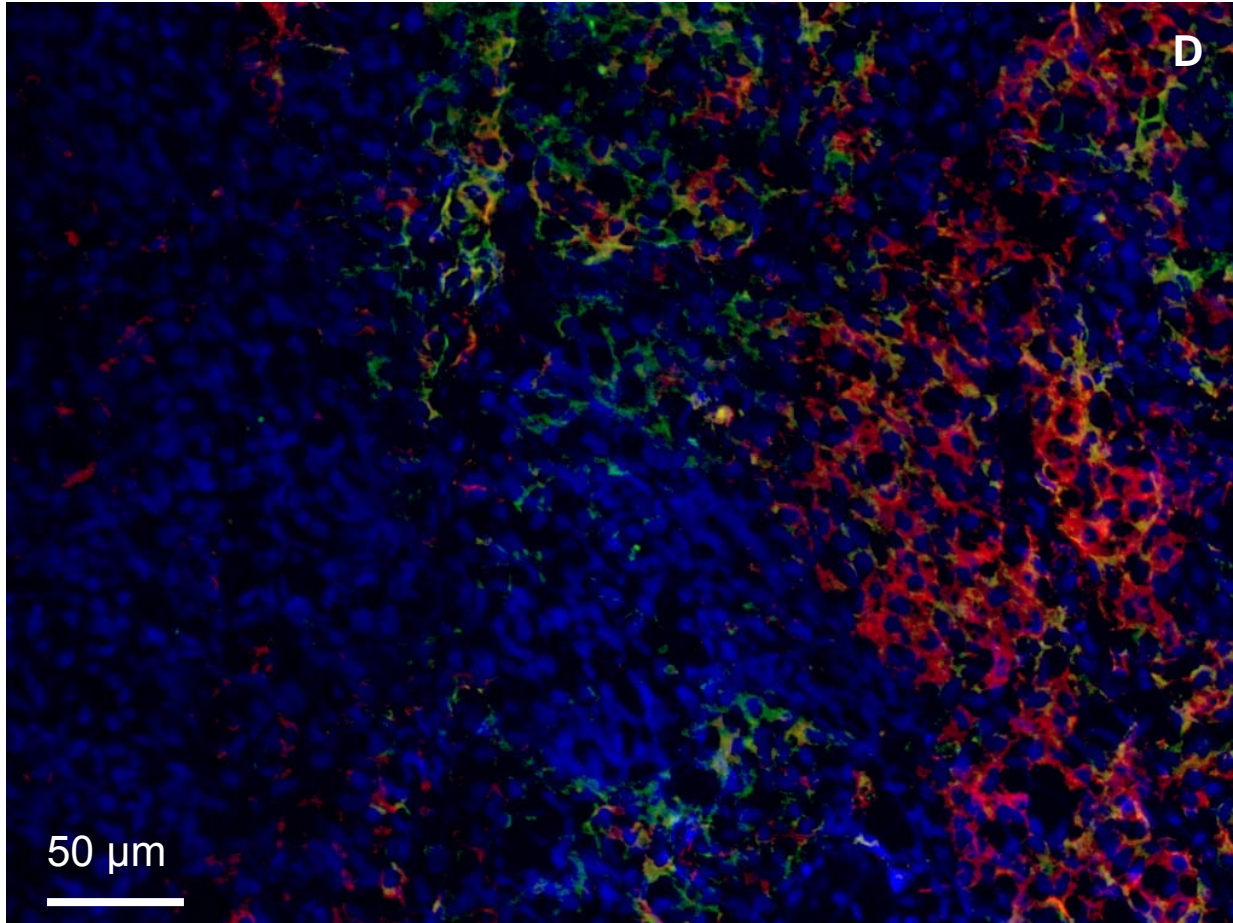
CD3 / MHCII



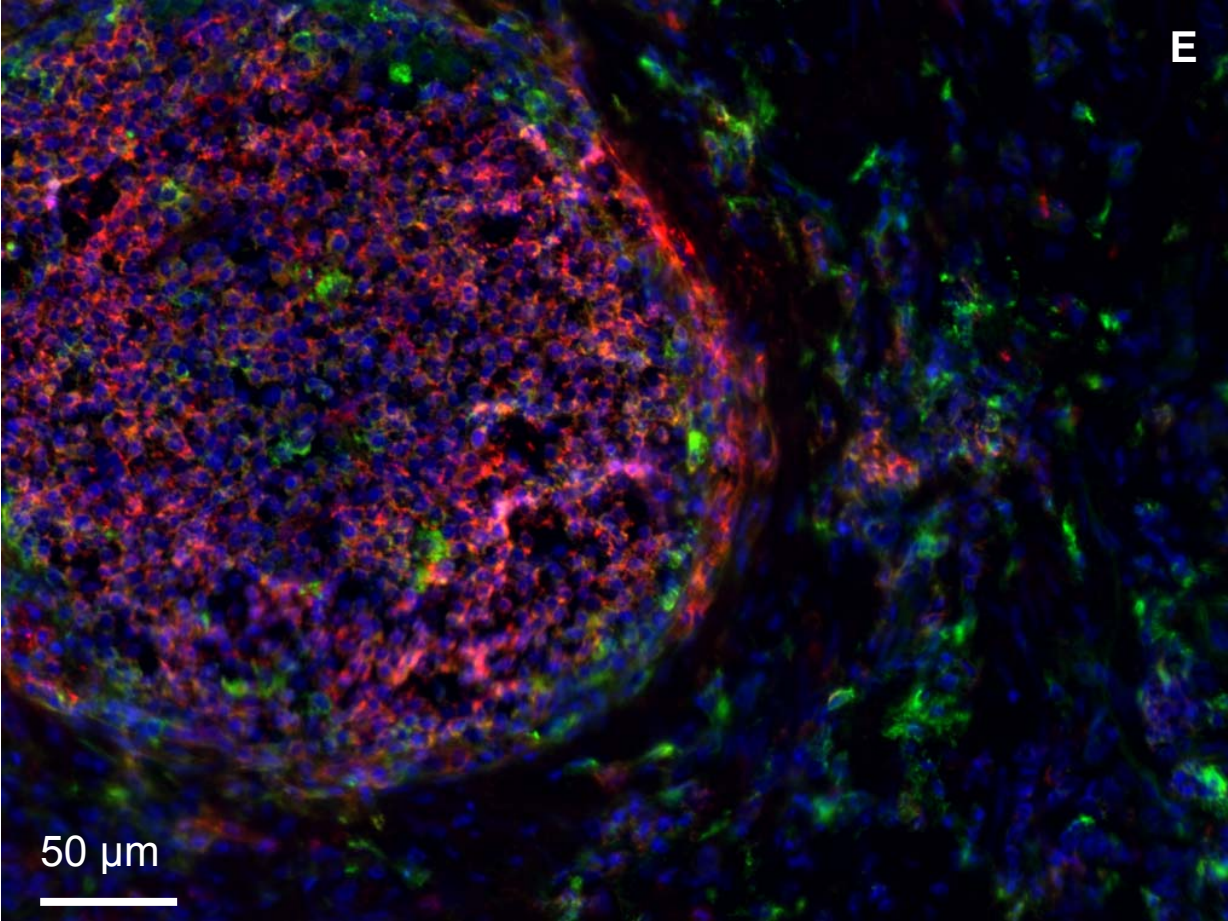
CD3 / CD205



CD21 / CD11c



CD21 / MHCII



CD21 / CD205

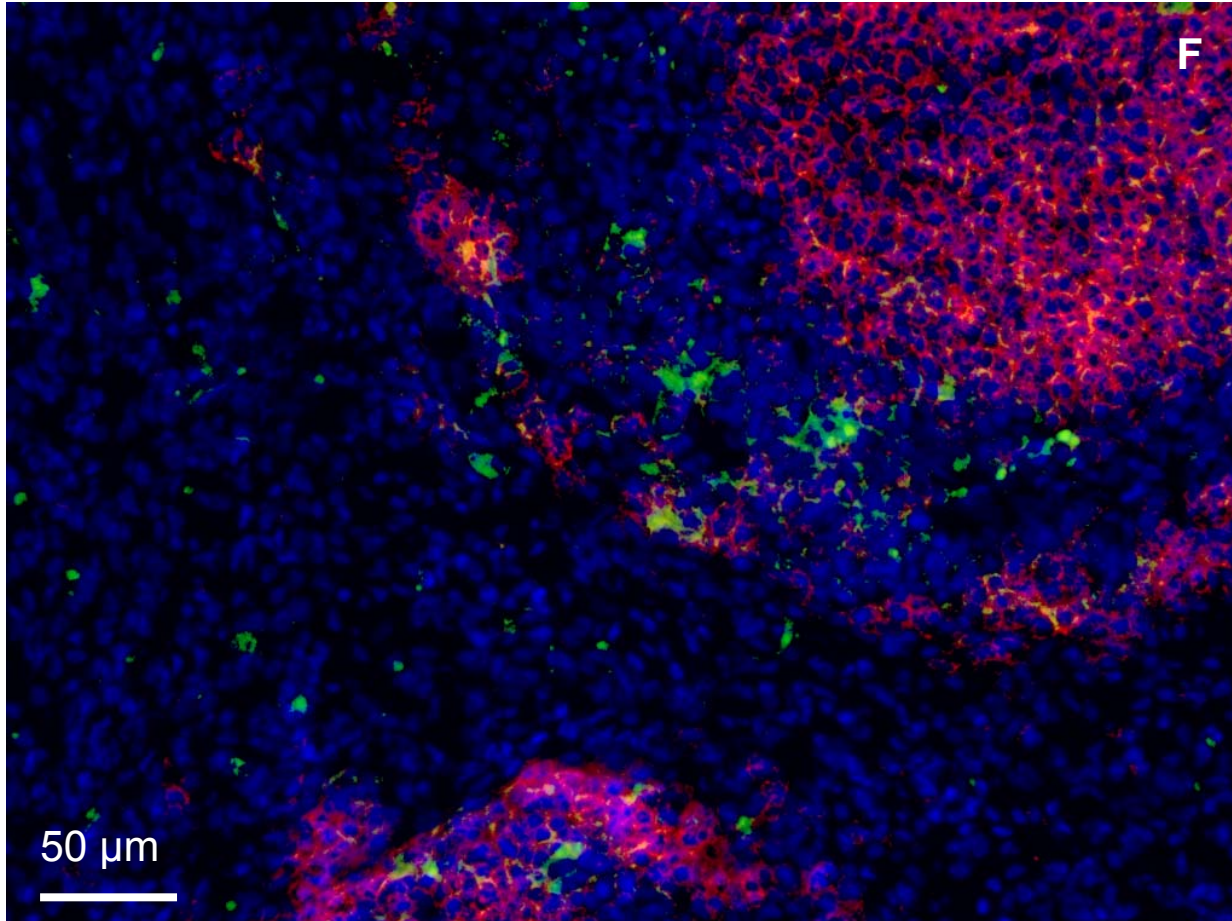
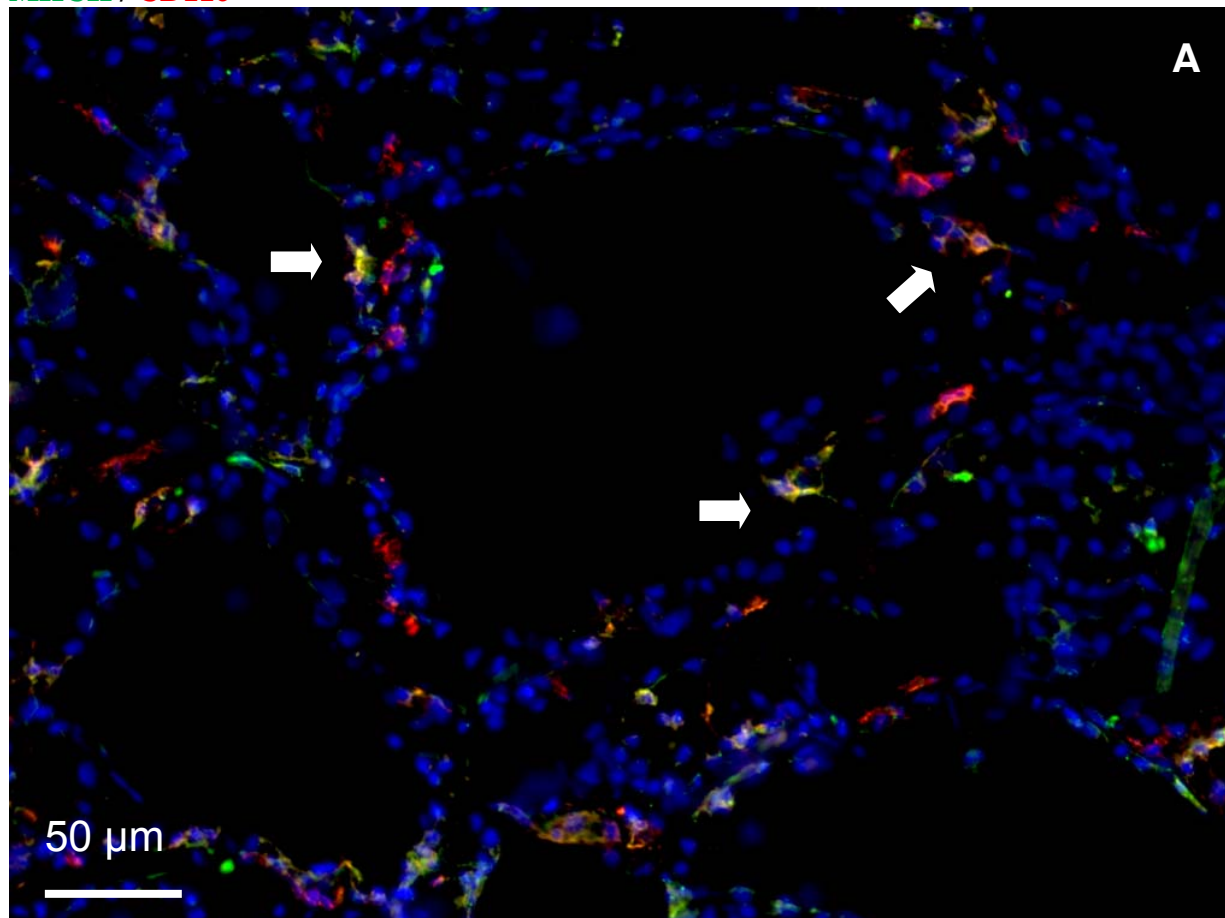


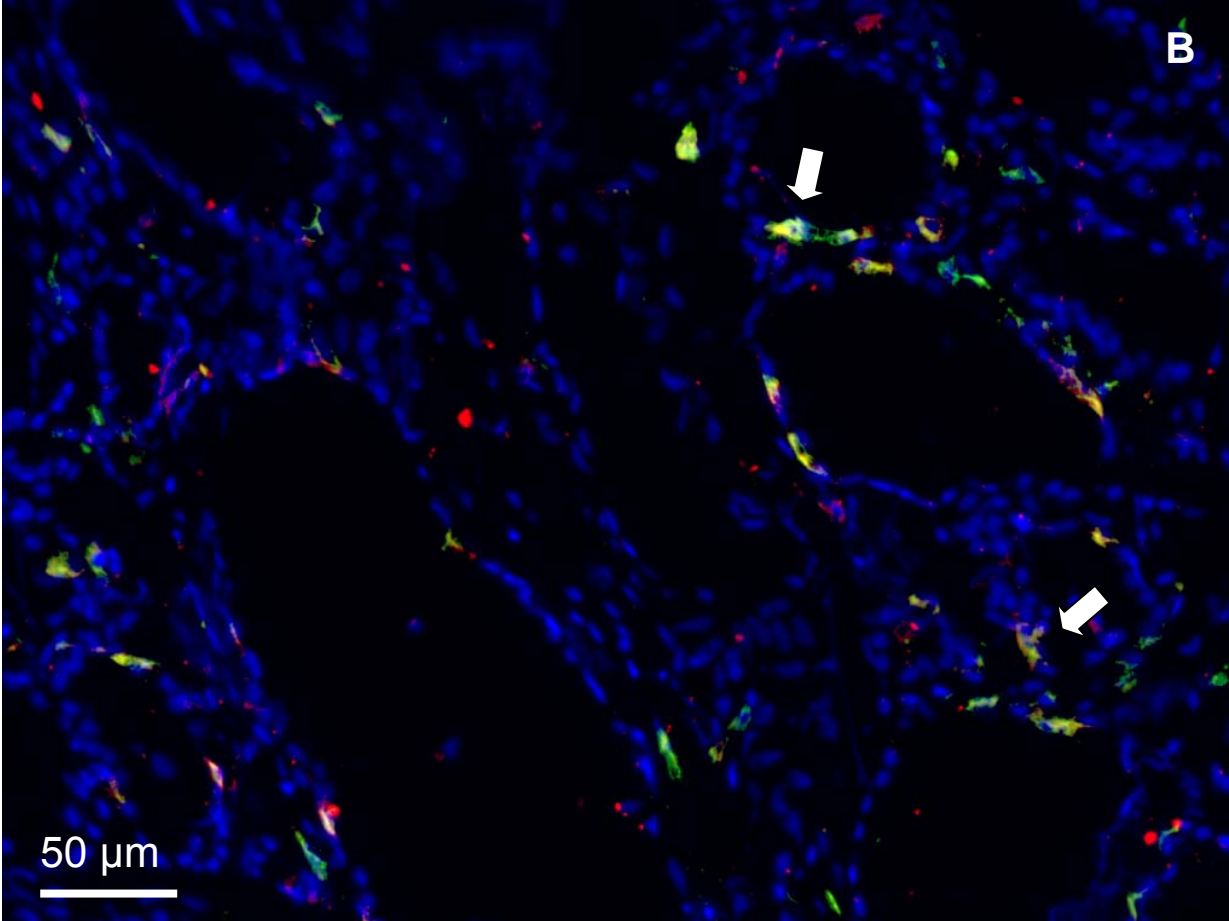
Figure 2-1 DC in Supramammary Lymph Nodes.

Images shown are composites of immunofluorescently labeled sections of supramammary lymph node. DAPI (blue) is used as a counterstain. Cells bearing the DC markers CD11c (A and D), MHCII (B and E), and CD205 (C and F) in green are scattered throughout the CD3 T-cell (red) areas of the paracortex (A-C) and on the periphery of follicles, labeled with CD21 (red) (D-F). Images are from one representative experiment of three. Magnification is at 200X.

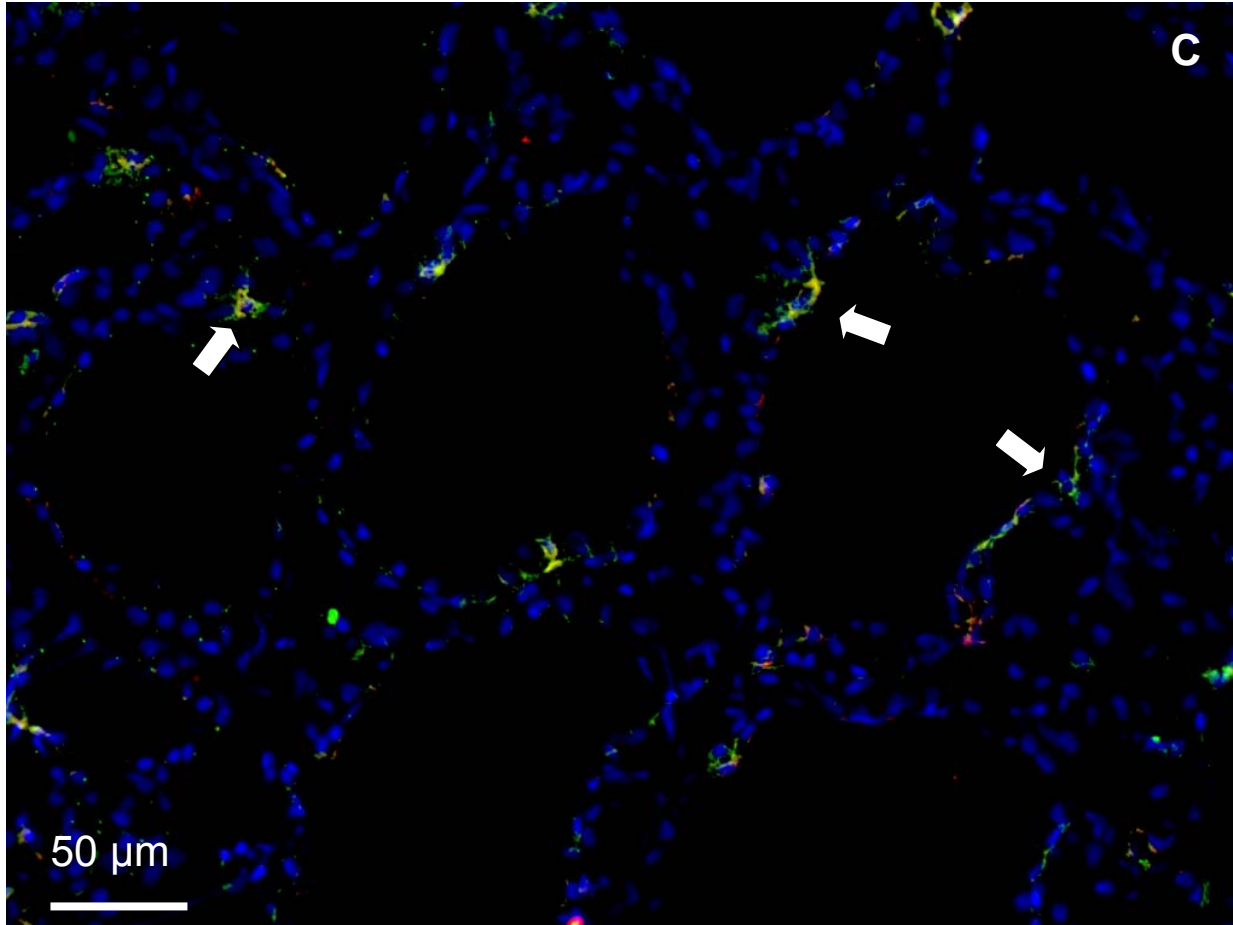
MHCII / CD11c



MHCII / CD205



CD11c / CD205



MHCII / CD11c

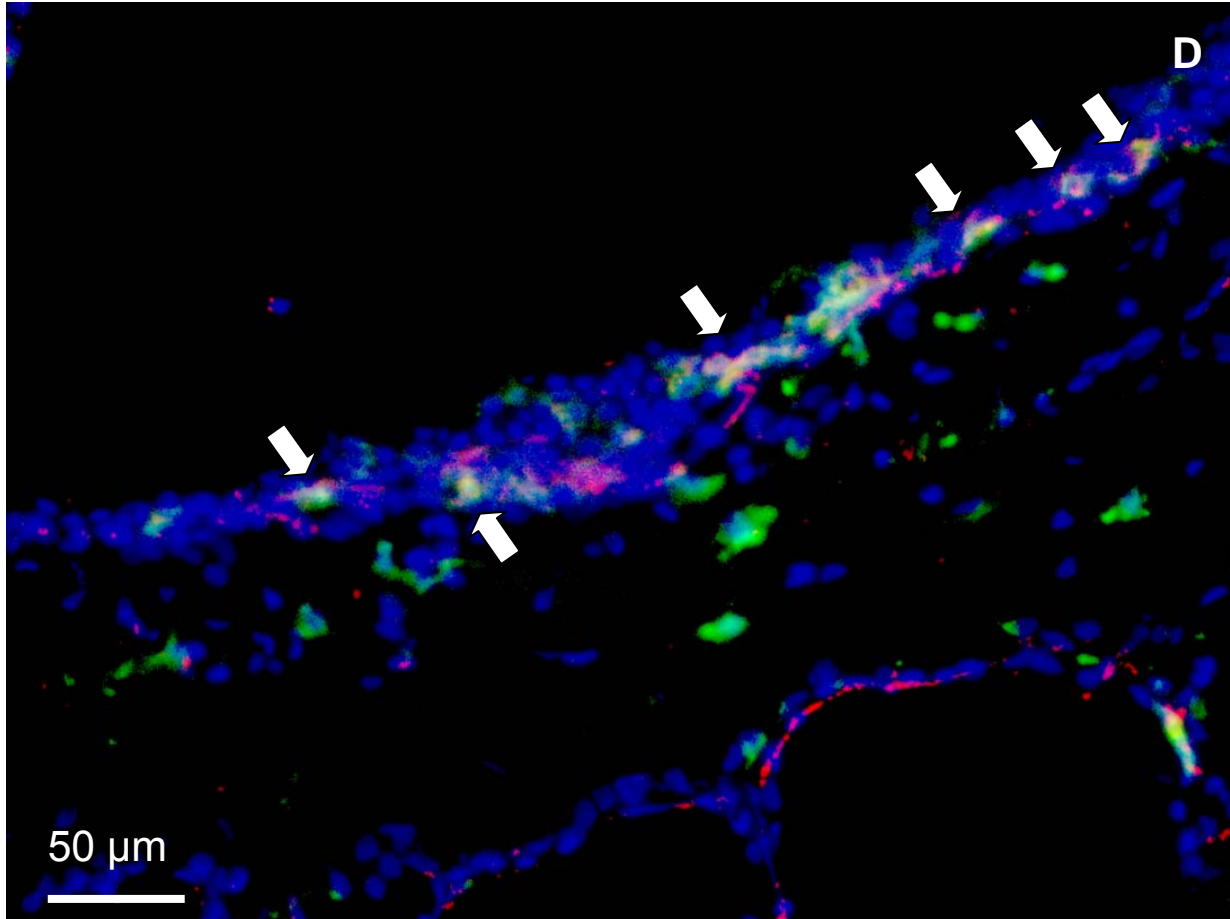


Figure 2-2 DC in the Mammary Gland.

Images shown are composites of immunofluorescently labeled mammary tissue. All samples were counterstained with DAPI (blue). Compositing images show cells showing the presence of MHCII (green in A, B, D), CD11c (red in A, C, D) and CD205 (red in B and green in C).

Double positive cells expressing two of these markers are yellow (arrows). MHCII⁺ CD11c⁺ cells (arrows) can be seen among alveoli (A) and regularly spaced in mammary ductular epithelia (D). Similarly, MHCII⁺ CD205⁺ cells are found among alveoli (B). CD11c⁺CD205⁺ are also found among alveoli (C). Images are at 200X magnification. Images are from one representative experiment of three.

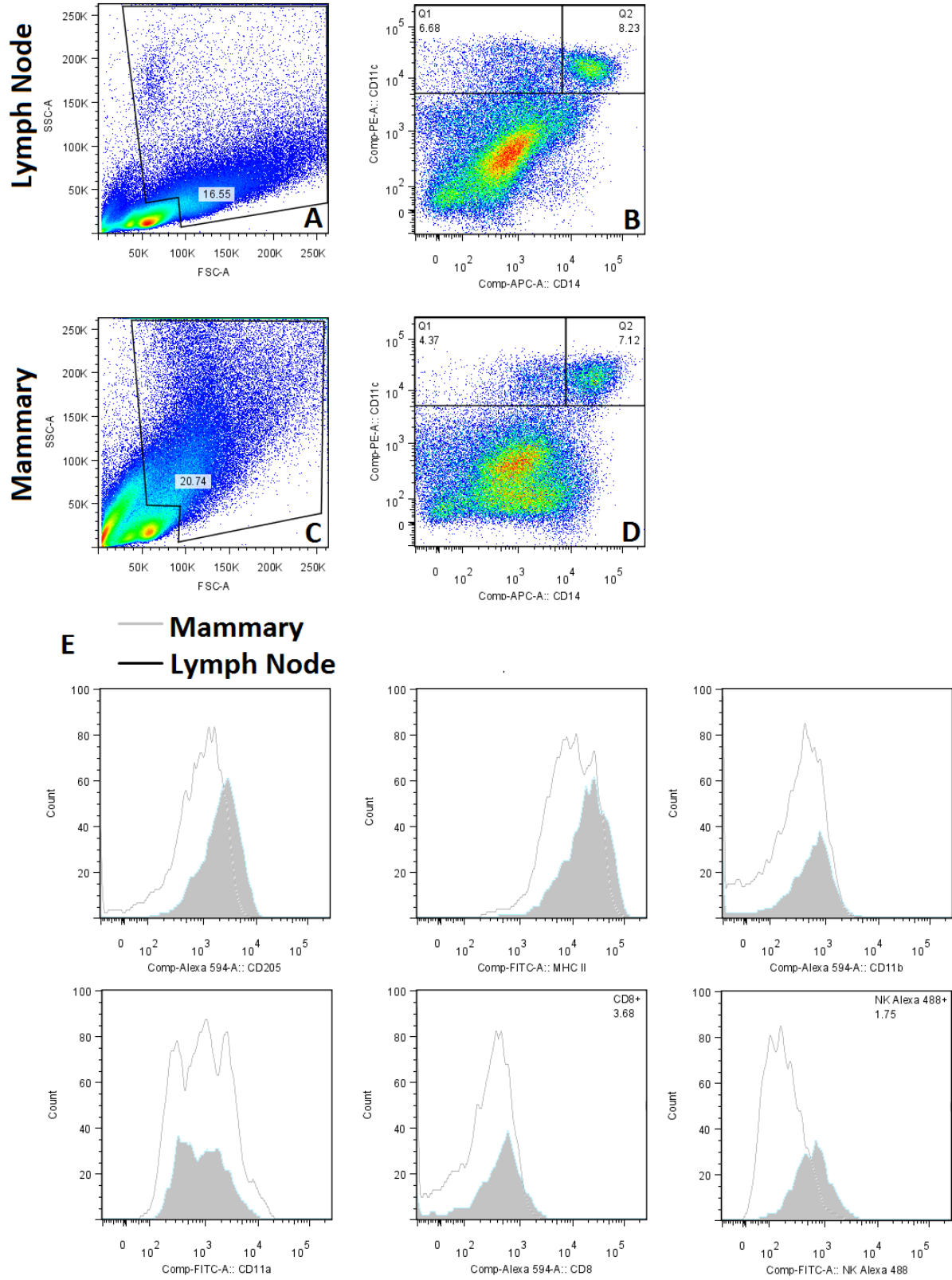


Figure 2-3 Comparison of supramammary lymph node and mammary DC.

Using FACS, large granular cells were electronically gated based on their FSC and SSC (A and C). From this gate DC, CD14^{mid/lo}, CD11c^{hi} (quadrant 1 in B and D), were clearly distinguishable from macrophages, which were CD14^{hi}, CD11c^{hi} (quadrant 2 in B and D). Flow cytometry histograms compare expression of CD205, MHCII, CD11b, CD11a, CD8 α , and NKp46 on DC (from quadrant 1 in B and D) from the mammary gland (filled area) and the supramammary lymph node (unfilled area) (E). Images are from one representative experiment of three.

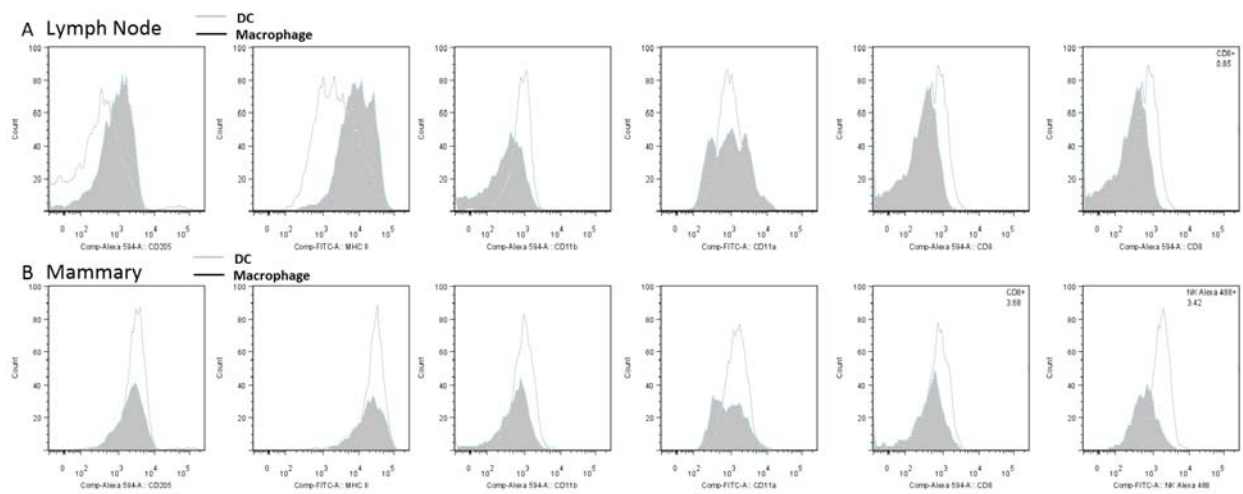


Figure 2-4 Comparison of markers in mammary and lymph node DC and macrophages. Flow cytometry histograms of CD205, MHCII, CD11b, CD11a, CD8 α , and NKp46 are compared on DC (CD14^{mid/lo}, CD11c^{hi}, filled area) and macrophages (CD14^{hi}, CD11c^{hi}, unfilled area). Images are from one representative experiment of three.

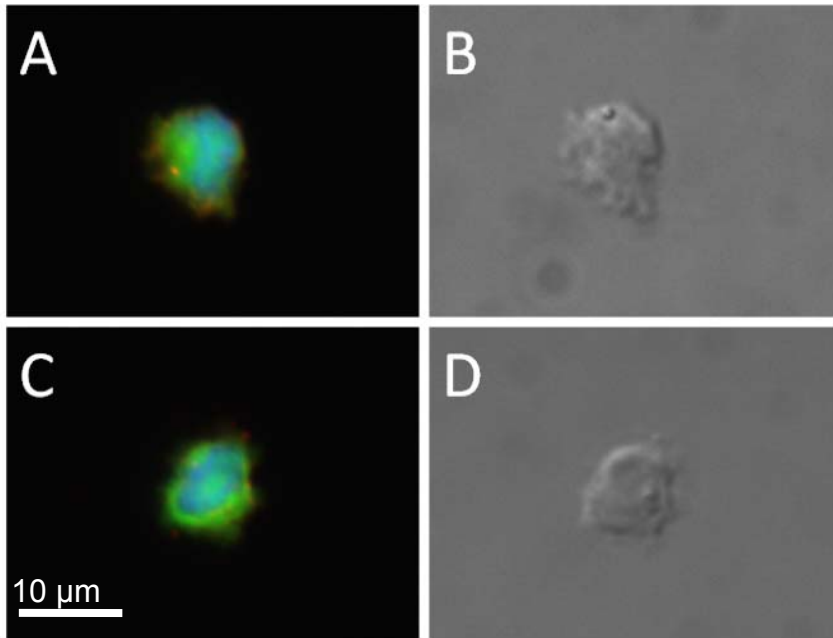


Figure 2-5 Immunocytochemistry of mammary and lymph node DC.

Mammary (A and B) and lymph node (C and D) DC ($CD14^{mid/lo}$, $CD11chi$) were sorted using FACS, labeled with DAPI (blue) and placed onto a slide via a cytopspin. Composited images (A and C) show DC expressing MHCII (green) and CD205 (red). Phase contrast images of the same slides (B and D) illustrate the dendritic morphology of these cells. Images shown were captured at 200X. Images shown are representative of 3 experiments.

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Chapter 3. Response of Bovine Mammary Dendritic Cells to Lipopolysaccharide

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Summary Sentence: Supramammary lymph node dendritic cells survive in mixed cultures but are unresponsive to LPS stimulation.

Running title:

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Key words: bovine, dendritic cells, LPS, supramammary lymph node

Abstract

Dendritic cells (DC) are often described as bridging the gap between the innate and adaptive immune system. In mucosal tissues, they serve as guardians against microbial pathogens, phagocytosing the invaders. After phagocytosis of superantigens, including lipopolysaccharide (LPS) and lipoteichoic acid (LTA), DC undergo a maturation process, where they upregulate markers related to antigen presentation, notably MHCII and CD80. They also home to the regional lymph node, upregulating homing markers such as C-C chemokine receptor type 7 (CCR7). The phenotype of mammary and supramammary lymph node DC was previously described (our unpublished data). In this study, Ficoll separated mammary and supramammary lymph node cultures were evaluated for changes in DC populations and markers after 12h of exposure to LPS. These cultures contained a mixture of DC, lymphocytes, and other cell types. DC did not survive well in LPS stimulated cultures and unstimulated control cultures. No apparent differences in phenotype (CD11c, CD14, MHCII, CD11b, CD11a, and CD205) could be identified by fluorescent activated cell sorting (FACS), between stimulated cultures and controls. The presence of CD80 and CCR7 mRNA was confirmed in these cultures, which is consistent with presence of DC. However, no significant differences in these cultures were apparent in cultures stimulated with LPS.

Introduction

Mastitis caused by coliforms and *S. aureus* is a significant problem for the dairy industry. A novel vaccine could potentially target mammary or supramammary lymph node dendritic cells (DC) to elicit a T-cell mediated memory response. The phenotype of mammary and supramammary lymph node DC was previously described (our unpublished data). In mammary tissue, DC were found among alveoli, in epithelia, and in interalveolar tissue. In the lymph node, DC were found on the periphery of B cell areas in the cortex, and among T-cells in the paracortex and medulla. DC were also quantified in mammary parenchyma and supramammary lymph nodes using fluorescence-activated cell sorting (FACS). CD11c^{hi}, CD14^{lo/-} cells (DC) expressed high levels of MHCII, CD11b, and CD205. CD11c^{hi}, CD14^{lo/-} cells (DC) expressed MHC-II, CD205, CD8 α but did not express NKp46. These expression patterns were similar in both the mammary gland and supramammary lymph node. These cells could be distinguished from macrophages based on their lower expression level of CD14 and reduced frequency of CD11b expression.

Upon stimulation by foreign particles, such as viral or bacterial components, immature DC expand their expression of proteins associated with lymphocyte stimulation. For example, bovine monocyte-derived DC mature by upregulating expression of CD1, CD80, MHCII, IL-12, TNF, and IFN- γ when exposed to LPS [31]. Mature, or stimulated DC, then migrate to secondary lymphoid tissues where they present antigens and stimulate lymphocytes [34, 35]. DC are particularly noted for their ability to present antigen and stimulate naïve T-cells [36]. In this process CCR7 (upregulated after maturation) has been shown to be an important regulator of chemotaxis and migration speed [32, 33].

In this study, mixed cultures of low-density cells (including macrophages and DC) were stimulated with LPS and changes in DC phenotype measured by flow cytometry. The mixed cultures were used to evaluate changes in gene expression of two DC markers, CD80 and CCR7.

Materials and Methods

Animals. Mammary tissue from lactating cows (*Bos taurus*) was harvested at the time of slaughter. Brown Packing Co, Gaffney, SC and the Virginia Tech Dairy facility provided the animals. Protocols were in accordance with Virginia Tech's Institutional Animal Care and Use Committee.

Isolation of low-density cells. Mammary and supramammary lymph tissue was collected at time of slaughter and transported on ice in PBS containing 5% heat-inactivated Fetal Bovine Serum (FBS) and 10 µg/mL gentamicin (Invitrogen, Carlsbad, CA, USA).

Mammary tissue was minced, and then digested overnight in 0.5 U/mL Elastase (CalBioChem, Darmstadt, Germany) and 1300 U/mg type 3 collagenase (Worthington Biochemical, Lakewood, NJ, USA) in 37°C in Krebs's Ringer Bicarbonate, supplemented with 5% FBS and 10 µg/mL gentamicin. Cells were passed through a 70 µm nylon mesh and washed in Hank's balanced salt solution (HBSS). To isolate low-density cells, including DC, mammary digests were then separated by density using Ficoll (BD Falcon, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Briefly, cells suspended in 25 mL of HBSS and layered over 12.5 mL of Ficoll. This was then centrifuged at 367 x g for 30 minutes at 25°C. A roughly 10 mL middle layer containing low density cells, lying directly above the Ficoll layer, was removed. These cells were washed in 45 mL HBSS and counted using a haemocytometer.

Single supramammary lymph nodes were minced with a razor blade and grated with a wire mesh, producing pieces of about 3 mm³. This tissue was then passed through a 70 µM cell

strainer (BD Falcon), and washed in HBSS. Cells were separated by density centrifugation using Ficoll (see above), washed in HBSS, and counted using a haemocytometer.

***In-vitro* Stimulation.** Isolated mononuclear cells from both mammary glands and lymph nodes were adjusted to a concentration of 10^7 cells/mL in RPMI-1640 medium (Gibco, Carlsbad, California, USA) supplemented with 10% heat-inactivated FBS (HyClone, Waltham, MA, USA), 4mM L-glutamine (Gibco), gentamicin, 10 mM HEPES, 50 μ M 2-2-mercaptoethanol, 4mM L-glutamine (Gibco). Isolated cells were cultured in 6-well tissue-culture polystyrene Petri dishes and incubated at 37°C, 5% CO₂ for 4 h. Cells were then stimulated with either 1 μ g/mL LPS (*E. coli* 055B5, Sigma-Aldrich, St. Louis, USA) for 12h. Treatments were performed in duplicate, using unstimulated samples as negative controls.

RT-PCR. RNA was extracted from cells using Qiagen's Applied Biosystems RNeasy Mini Kit and DNA was removed with DNase (Qiagen, Valencia, CA, USA). cDNA was prepared from 1 μ g RNA using Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen, Carlsbad, California, USA). Primers for QRT-PCR were designed with Primer Express Version 2.0 (Applied Biosystems) based on GenBank sequences. The RT-PCR reactions were conducted using the SYBR Green I dye master mix, and an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold change as calculated by the $2^{-\Delta\Delta Ct}$ method. Briefly, $\Delta Ct = Ct$ of the target subtracted from the Ct of GAPDH, and $\Delta\Delta Ct = \Delta Ct$ of samples for target subtracted from the ΔCt of corresponding control samples.

Fluorescence Activated Cell Sorting. Low-density cells were washed in HBSS, resuspended in PBS containing 0.05% sodium azide, and 10^6 cells were used for each antibody combination. Cells were incubated with appropriate primary antibody at 4°C for 1 h. Primary antibodies used

were specific for CD11a, CD11b, CD11c, CD14, MHCII (VMRD, Pullman, WA, USA) and NKp46-Alexa Flour 488 conjugated and CD205 (Serotec, Raleigh, NC, USA). This was followed by corresponding secondary antibodies conjugated to FITC, PE, APC, or Alexa Fluor 594 (Invitrogen) at 4°C for 30 min. CD21 and CD3 specific antibodies (VMRD) were used to help delineate lymphocytes. Cells were then washed and analyzed with a BD FACSAria flow cytometer and FlowJo software (Tree Star, Ashland, OR, USA). This was performed on tissue isolated from 3 cows.

Statistics. Graphs were analyzed and plotted using Microsoft Excel 2007 and its analysis tool pack (Redmond, Washington, USA). The unpaired Student's t-test was used to evaluate significance.

Results

In lymph node cultures, adherent cells bearing a dendritic morphology were evident (Figure 3-1). Adherent cells with this morphology were rare in mammary cultures (data not shown).

FACS was used to electronically gate out lymphocytes and debris; gates were set to include large and granular cells based on forward and side scatter properties. In the mammary gland and supramammary lymph node, a reduction in the DC population (CD11c^{hi}, CD14^{lo/-} cells) was observed after culturing for 12h (Table 3-1). The percent decrease was similar for both cell tissues, with an average reduction of 54 % ± 0.12. Both unstimulated and LPS stimulated treatments possessed similar percentages of DC.

Surprisingly, stimulation did not alter the DC phenotype. Cultured cells, whether LPS stimulated or unstimulated, possessed DC marker expression levels similar to fresh or uncultured cells (Fig. 3-2). No significant changes were observed in CD14, CD11c, CD205, MHCII,

CD11a, or CD11b (Fig. 3-2). This occurred in both mammary and supramammary lymph node cultures.

CD80 and CCR7 mRNA was identified in the bovine mammary gland and lymph node. This is consistent, but not definitive of the presence of DC. However, no significant up-regulation of either of these maturation markers was observed when stimulating mixed cultures for 12h with LPS (Fig. 3-4).

Discussion

Monocyte derived DC (MoDC) significantly change expression of maturation markers such as MHCII, CD80, and CD86 after stimulation [31]. This has been confirmed by our lab, and a similar response is seen when MoDC are stimulated with LTA and *S. aureus* supernatants (unpublished data). However, no changes in maturation markers (CD11a, CD11b, MHCII, and CD80) in mixed primary cultures of mammary tissues and supramammary lymph node were identified.

This confounding result may have several explanations. The cultures included other cell types, which may have altered the response of the DC. Purified cultures of DC could provide a better model for investigation.

Future experiments using primary mammary DC may require finely tuned media. DC did not survive well in the DC culture media, which was RPMI based. Purification, through either magnetic beads or FACS, could provide a pure population of primary DC. This might potentially remove influence of other cell types on DC responses to stimuli.

CD80 and CCR7 was measured using RT-PCR to evaluate changes in gene expression after LPS stimulation, but no significant changes were observed after 12 h. This may not be surprising in primary lymph node cultures, as CCR7 is upregulated after maturation when DC

begin to transit to the lymph node [32, 33]. A wider variety of DC markers is needed to describe changes in transcription. Likewise, additional time points and other stimulants (such as LTA, PGN, or whole bacteria) would provide more information. MoDC upregulate a variety of cytokines, including IL-12p70, when exposed to LPS [31]. ELISAs could be used to evaluate the production of IL-12p70 and other cytokines, including inflammatory cytokines (IFN, TNF, IL-1) and T-cell stimulating cytokines (IL-12, IL-23, IL-27), in primary and stimulated DC cell cultures. Additionally *in-vivo* infection or stimulation followed by DC isolation and characterization could provide a description of infection and response at the animal level.

Percent DC

Supramammary Lymph Node				Mammary Gland			
Cow	Fresh	Unstimulated	LPS	Cow	Fresh	Unstimulated	LPS
A	4.41	1.83	1.92	A	6.68	3.66	4.82
B	4.91	2.20	2.34	B	7.97	4.96	6.77
C	1.89	1.22	1.05	C	2.47	0.89	0.91

Table 3-1 Survival of DC in mixed cultures after LPS stimulation.

Mixed cultures of cells from either supramammary lymph node or the mammary gland were exposed to 1 μ M LPS for 12h. Unstimulated cultures were used as negative controls, while fresh, uncultured cells are presented for comparison. Data shows the percent of DC (CD11c^{hi}, CD14^{lo/-} cells) as analyzed by FACS (see Figure 3-2).



Figure 3-1 Supramammary lymph node DC in culture.

Adherent cells with a dendritic morphology were abundant in cultures of supramammary lymph nodes and occurred with a frequency of about 1:100 cells. Images are from one representative experiment of three. This image was taken at 400X in bright field. Such cells were rarely observed in mammary cultures (data not shown).

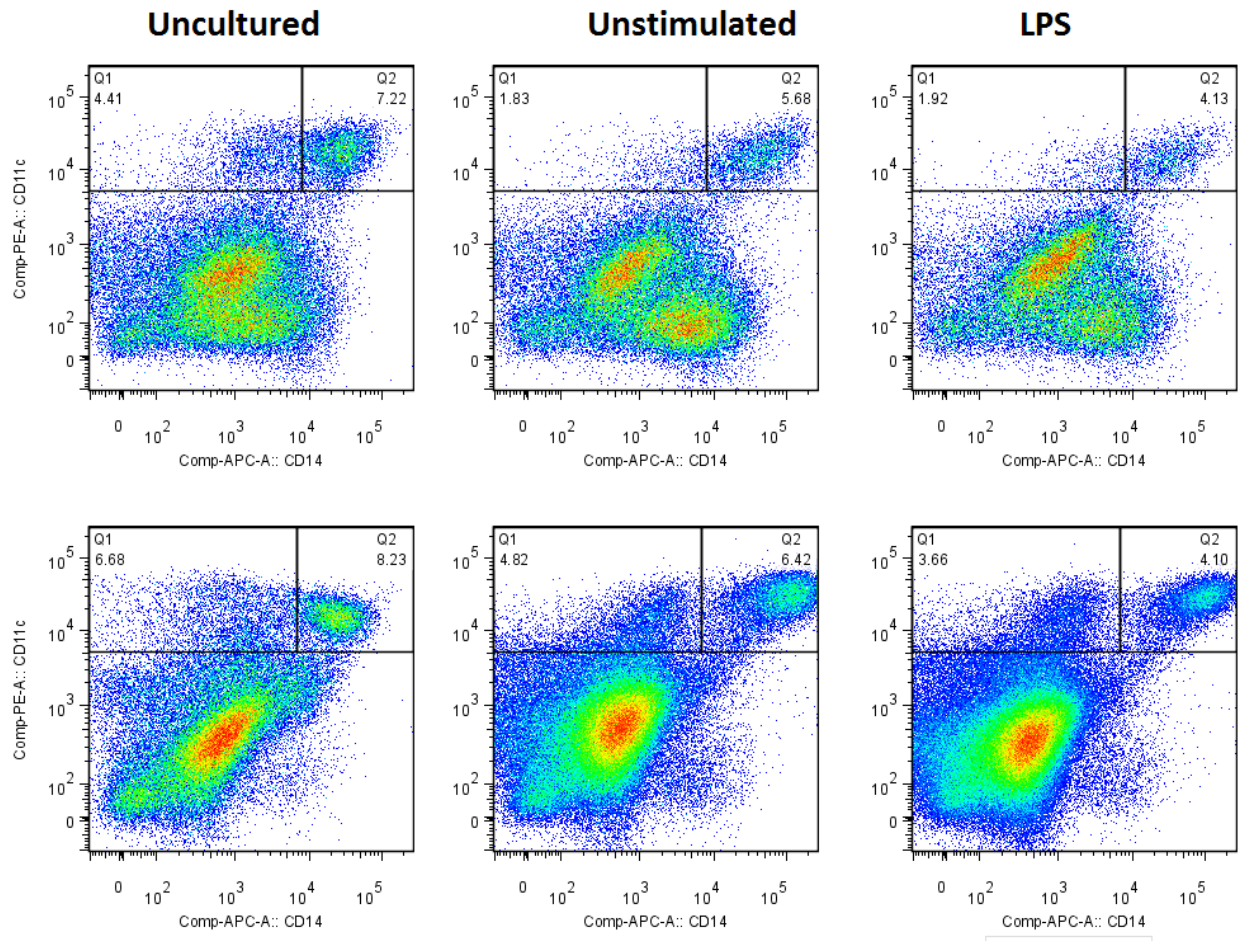


Figure 3-2 LPS stimulation of mixed cell cultures from mammary and supramammary lymph node.

LPS was added to mixed cultures of low density cells isolated from the bovine mammary gland (top row) and supramammary lymph node (bottom row). Flow cytometric electronic gating was used to exclude lymphocytes and debris based on forward and side scatter properties (not shown). Changes in the percent and distribution of DC ($CD11c^{hi}$, $CD14^{lo/-}$ cells) are illustrated (Q1). Uncultured and unstimulated cells were analyzed using the same method for comparison. Data is representative from analysis performed on 3 different cows.

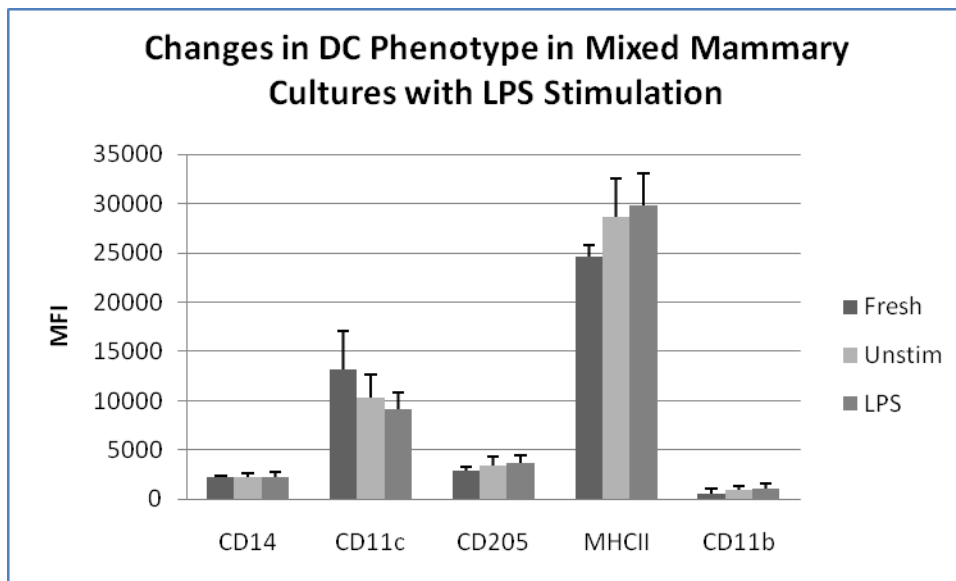
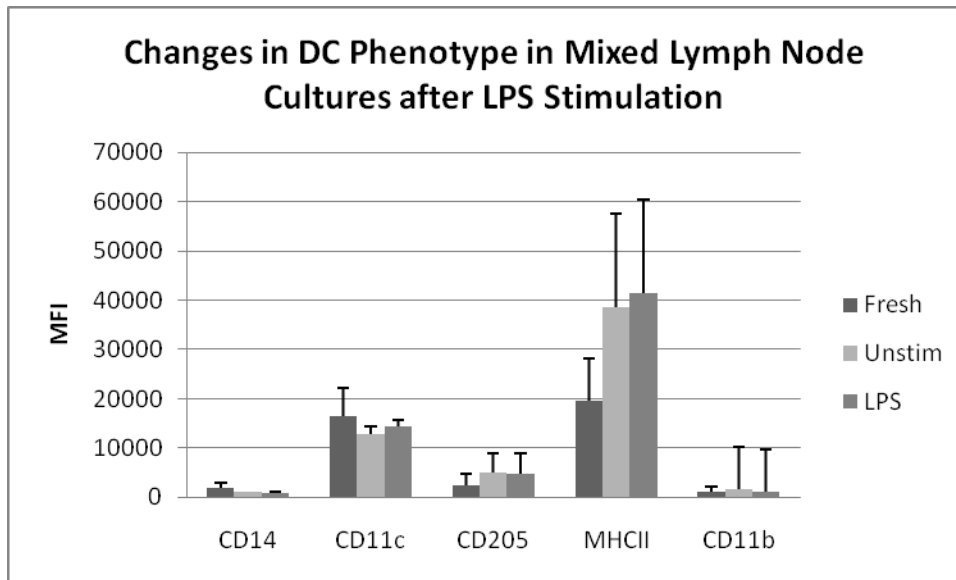


Figure 3-3 Changes in DC phenotypes after LPS stimulation of mixed cultures.

Mean fluorescent intensity (MFI) of DC markers in mixed cultures of cells isolated from supramammary lymph node tissue (top) and mammary tissue (bottom) and stimulated with LPS for 12h (LPS). These markers included CD14, CD11c, CD205, MHCII, and CD11b. An unstimulated (unstim) culture was used as a control. Freshly isolated DC (Fresh) are also

included for reference. Bars indicate standard deviation. There were no significant differences between treatments. Images are from one representative experiment of three.

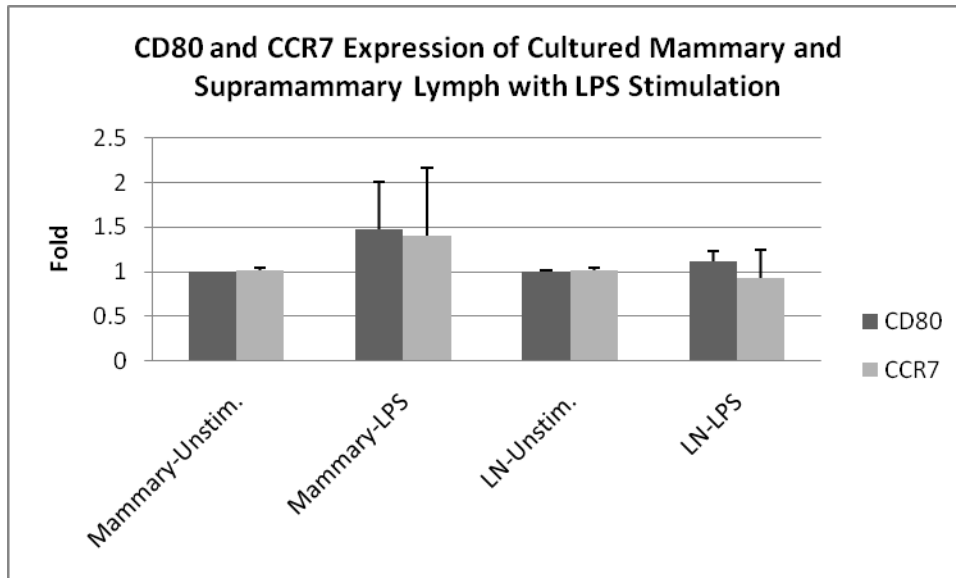


Figure 3-4 CD80 and CCR7 Gene Expression in Cultured Mammary and Supramammary Lymph Node after LPS stimulation.

Mixed cultures of cells isolated from supramammary lymph node tissue (LN) and mammary tissue were stimulated with LPS for 12h. RT-PCR was used to evaluate the expression of CD80 and CCR7. Expression levels are represented as a fold change. Changes in expression were not significantly different across treatments. Bars indicate standard deviation. There were no significant differences between treatments. Images are from one representative experiment of three, each performed in duplicate.

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Chapter 4. Conclusions

Using two-color immunohistofluorescence, DC were localized in the bovine mammary gland and supramammary lymph node. In addition to MHCII, CD11c (a β -integrin) and CD205 (a C-type lectin) were used as markers for DC. In mammary tissue, DC were found among alveoli, in epithelia, and in interalveolar tissue. In the lymph node, DC were found on the periphery of B-cell areas in the cortex, and among T-cells in the paracortex and medulla.

The DC in mammary parenchyma and supramammary lymph nodes were quantified using fluorescence-activated cell sorting (FACS). CD11c^{hi}, CD14^{lo/-} cells (DC) expressed high levels of MHCII, CD11b, and CD205. CD11c^{hi}, CD14^{lo/-} cells (DC) expressed MHC-II, CD205, CD11b, and CD8 α but did not express NKp46. DC also possessed at least two distinct phenotypes, defined by the presence or absence of CD11a. These DC expression patterns were similar in both the mammary gland and supramammary lymph node. DC could be distinguished from macrophages based on their lower level expression of CD14 and a reduced frequency of CD11b expression.

In order to view the morphology of these cells, FACS was used to obtain a pure population of DC (CD11c^{hi}, CD14^{lo/-}). Some of these sorted DC possessed a dendritic morphology. Other cells may have been more spherical in appearance due to the harsh digestion and treatment conditions. A lower pressure and wider sorting nozzle might provide gentler sorting techniques. Using magnetic beads to separate DC from other cell types might also limit morphological changes.

Distinguishing macrophages from DC currently requires the quantification of several markers. A dendritic morphology can be seen in monocyte-derived cultures; however, this is not

always apparent in whole tissue. A universal marker that clearly differentiates DC from myeloid relatives would aid in the detection, targeting, and purification, of DC in and from tissue.

Putative populations of DC bearing natural killer cell markers (either NK1.1 or NKp46) and CD11c have been identified – hereafter NKDC [61, 62, 129]. A subset of these has been further described as interferon producing killer dendritic cells (IKDC) [64, 65, 69, 132]. Given their ability to secrete large amounts of IFN- γ , these cells would be especially attractive candidates for vaccine targeting. It has been suggested, however, that this population represents activated natural killer cells, which can also express CD11c [68-70]. Significant populations of cells bearing the DC markers MHCII and CD11c along with the NK marker NKp46 were not identified. However, previous research has relied heavily on the marker NK1.1 to identify NKDC, and NKp46 may not be expressed on bovine mammary NKDC. Further research is needed to determine whether bovine DC subsets, such as NKDC or plasmacytoid DC exist in the bovine.

This research provides phenotypic description of mammary DC. It may be hypothesized that these cells share similar functional activities of other DC populations; particularly well studied monocyte derived DC. This hypothesis, however, remains to be validated in the mammary gland. Comparing mammary DC to monocyte or bone marrow derived DC, or DC from other tissues would require a large sample of processed tissue and a gentle cell sorter. The high pressure and other forces that DC experience on being sorted has been reported to be activating, thus limiting the extent on which functional analysis can be achieved on sorted DC.

Current DC vaccines that target tissue DC use antibodies specific for CD11c or CD205 that are conjugated to an immunogenic component [22, 133-135]. Theoretically, this may be any number of microbial components, including cell wall components (LPS, LTA, and PGN),

individual toxins, methylated DNA, or other antigens. Further experimentation is needed to assess which of these components would produce the best response. Dose, developmental timing, and long-term immunity will all need to be evaluated. Adjuvants may be necessary to enhance the immune response.

The exact origin and differentiation of mammary and supramammary gland DC remains uncertain, though they share a close lineage with macrophages. DC have been generated *in-vivo* by culturing either monocytes or macrophages with IL-4 and GM-CSF [29]. Other cytokines may be important in regulating DC survival and differentiation *in-vivo*. In the mixed cultures used herein, the percent of cells bearing DC markers was reduced by an average of 54%, indicating cell death. A similar reduction was seen in the lymph node.

There are a myriad of variables that could affect the number and functional activity of DC in the bovine mammary gland and supramammary lymph node. These include breed, stage of lactation, pregnancy status and number, age, diet, as well as immune status and history. By comparing the DC populations among the 4 quarters of an udder, no major differences were found. This study, however, was performed on a single cow and further investigation is needed to draw valid conclusions. It has been previously shown that CD14 macrophage populations do not expand during LPS induced mastitis. This may not be true of mammary DC [136]. Infection might sequester blood derived inflammatory DC to home to the supramammary lymph node. Expanded analysis to include one or more of these variables regarding mammary DC populations and activities would further refine our understanding of mammary gland immunology and DC biology.

References

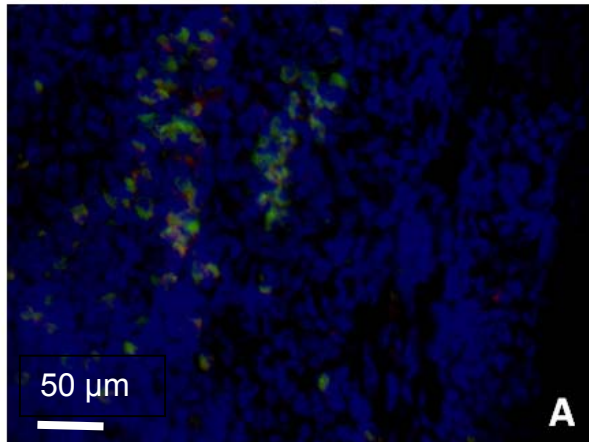
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Appendix A. Supporting Data

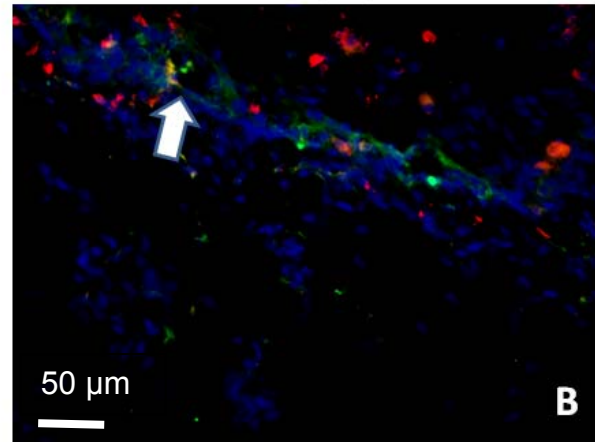
CD11b MHCII

Supramammary Lymph Node



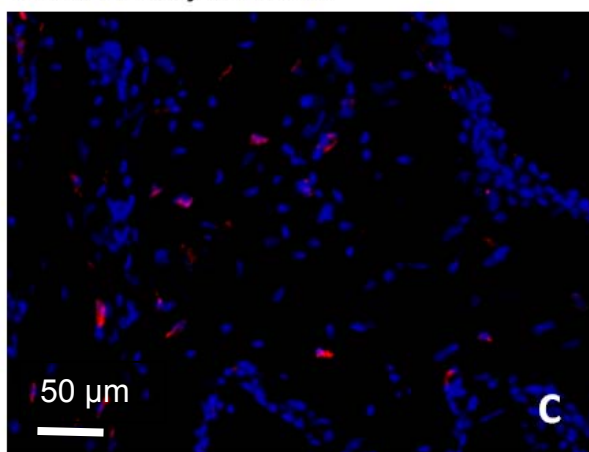
CD11b MHCII

Mammary Epithelia



CD11b

Mammary Alveoli



CD14 MHCII

Mammary Alveoli

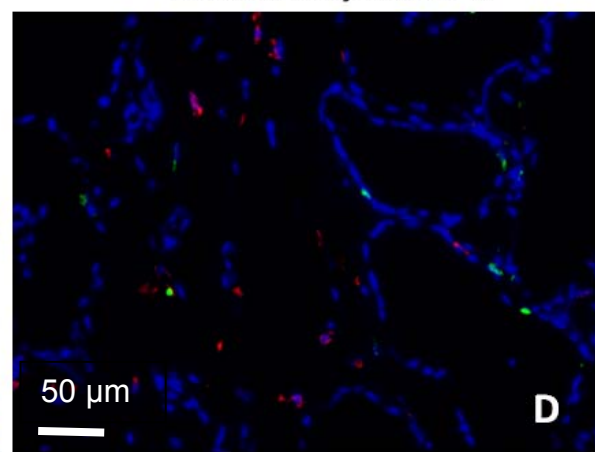


Figure A-1 CD11b, CD14, and MHCII in Supramammary Lymph Node and Mammary Gland. Images shown are composites of immunofluorescently labeled sections of supramammary lymph node and mammary gland. DAPI (blue) is used as a nuclear counterstain. Cells bearing both CD11b (red) and MHCII (green) were found primarily in the T-cell areas of the paracortex (A). CD11b cells were found among mammary epithelia, with only a few expressing MHCII as well

(arrow, B). CD11b was expressed in the mammary alveoli (C). CD14 was also expressed in mammary alveoli, but these cells also expressed low or no MHCII (D). Images are from a single experiment.

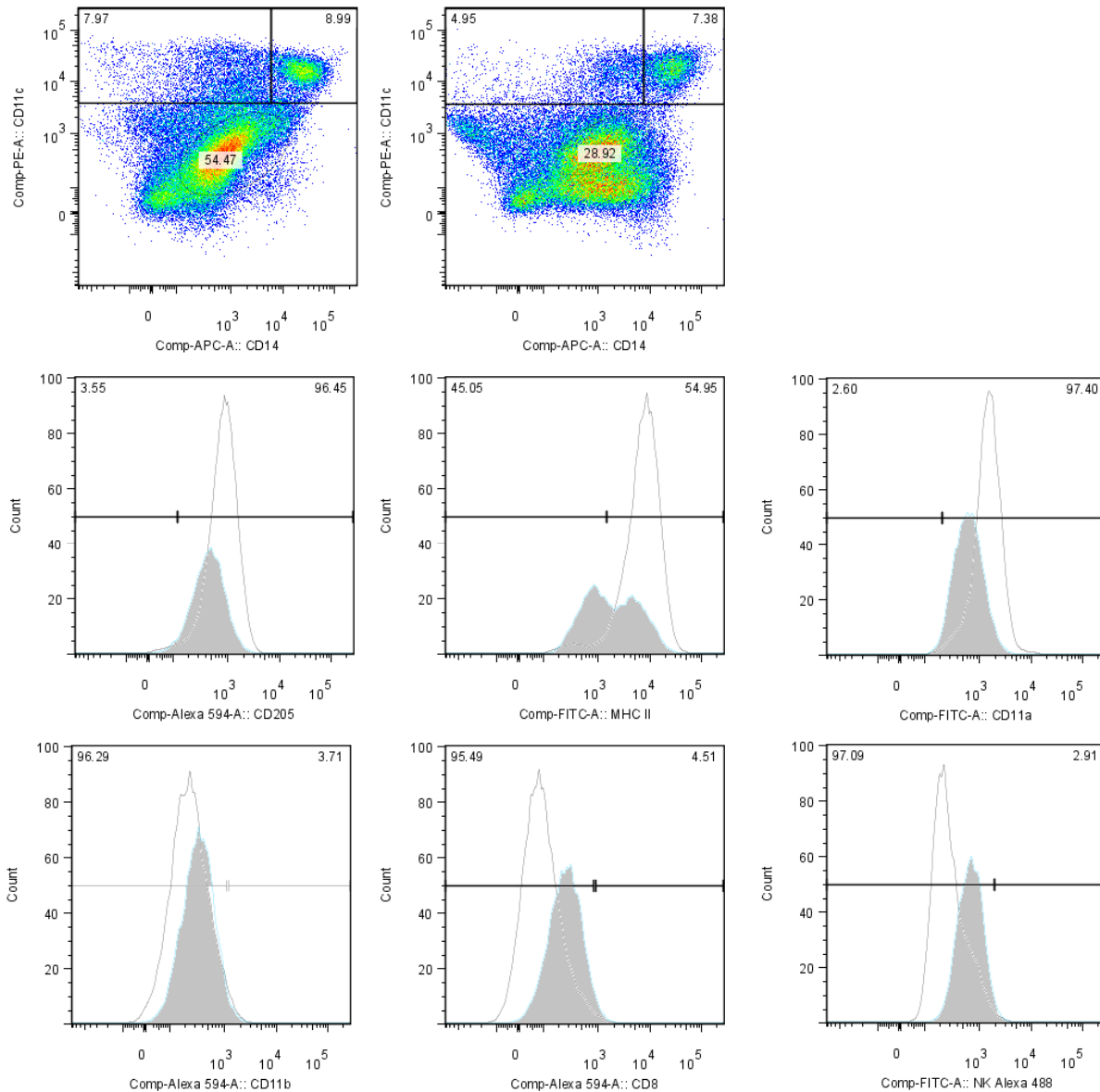


Figure A-2 Comparison of mammary and supramammary lymph node that are CD14^{mid/lo}, CD11c^{mid/lo}.

A large population of CD14^{mid/lo}, CD11c^{mid/lo} cells could be identified in both the mammary gland (top right) and lymph node (top left). These populations were electronically gated using flow cytometry (top row) and analyzed for common DC markers: CD205, MHCII, CD11a, CD11b, CD8 α , and NKp46. Histograms of mammary gland (shaded area) and supramammary lymph node (solid line) show the expression of these markers (middle and bottom rows). These

myeloid cells may be a mixture immature macrophages, immature DC, or monocytes. Images are from one representative experiment of three.

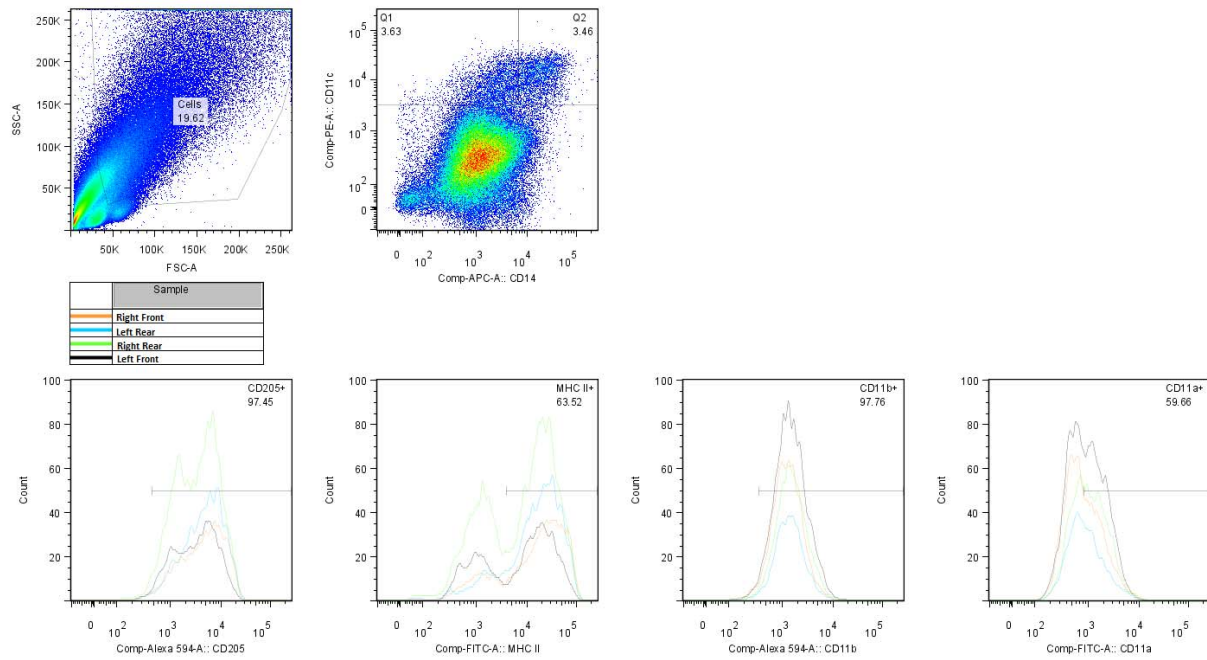


Figure A-3 Comparison of DC in quarters.

Mammary DC were isolated from the four quarters of an udder and analyzed using FACS. First, large and granular cells were electronically gated (top left). Analysis was made on CD14^{lo}, CD11c^{high} DC (Q1, top right). Histograms show the expression of CD205, MHCII, CD11b, and CD11a (bottom row) in each quarter. The udders possessed similar expression of DC markers. This analysis was performed on a single cow.

Gene	Forward primer	Reverse primer	Probe
GAPDH	GACCCCTTCATTGACCTTCA	GATCTCGCTCCTGGAAGATG	TTCCAGTATGATTCCACCCACGGCA
CCR7	TTCAGCCGCCGAGAGAGT	TCGTCCGTGACCTCATCTTG	CCTTCTCGTCATTTTCCAGGTGTGCC T
CD80	GGTGCTCACTGGTCTTTTTTA CTTC	AGTGGATGTGTTGTAATCACAGGAT AG	TTCAGGCATCACCCCAAAGAGTGTG A
CD86	TCTCTGTTTCCACTGTTCTTT TTC	TGGCAGTTCTCCAGTCTCGTT	TGCTGCTTCTTGAAAAGTCATGCC TTC
NK1.1	TGTCCCAAATGATCTTTGTC A	TGTCCCAAATGATCTTTGTCA	CCACGTTGGCATAACTTTCCCTGA AA
TLR6	TTTGCAACTTGACCCAACT	TGCAAGTGAGCAATGGGTAG	GGATTAAGTGCTACAAAGTTACAAC AA
TLR1	GATGCCGAGAGCCTTCAAGA	ACACGTCCAAAATAAAATGGAATT C	AGTCTGCACATTGTTTTCCCACAG GA
NOD2	CACGGATCAGGAGCGTCACT	CACCTTGCGGGCATTCTT	CCGCCCCACGTCAGTCCAG

Table A-1 RT-PCR Primers.

Bovine primer and probe sequences from 5' to 3' end were designed using Primer Express 3

(Applied BioSystems, Foster City, CA, USA).

Appendix B. Detailed Protocols

Isolation of Macrophages and Dendritic Cells from Mammary Tissue

Reagents / Materials

- Liquid Nitrogen
- Optimal Cutting Temperature Compound
- Labeled Plastic Bags
- Long Tongs
- Razor blades
- Knives
- Tin foil
- Weigh boats
- 50 mL Tubes
- PBS + Gentamicin
- Ice
- Gloves
- Coveralls
- Paper towels
- Sharpie
- Notepad and Pen
- Cutting Board
- Bleach
- DMEM + 10% FBS + Antibiotic and antimycotic (GIBCO)
- FACs Wash
- HBSS
- Gentamicin
- DC Media (No Cytokines)
- Nylon Filter Cloths
- Ficoll
- Formaldehyde
- Sterile H₂O
- 2X MEM

- 70% Ethanol
- Digestion Solution (200 mL)
 - 192 mL Kreb's Ringer Bicarbonate
 - 0.1 g MgCl
 - 7.0 g NaCl
 - 0.1 g Sodium Phosphate (dibasic)
 - 0.18 g Sodium Phosphate (monobasic)
 - 1.26 g Sodium Bicarbonate
 - 0.54 g D-glucose
 - Bring volume up to 1L with ddH₂O and filter through a 0.22-micron bottle top filter.
 - 8 mL of heat inactivated bovine serum albumin
 - 400 µL of Elastase (0.5 U/mL)- Elastase: CalBioChem #324682
 - Add 2mL of HBSS to lyophilized powder
 - Aliquot to 50uL tubes
 - Store at -20 °C
 - 697.6 mg of Collagenase type 3 (1300 U/mg) - Worthington Biochemical Collagenase Type 3 4180
 - Lyophilized at 4°C
 - Filter sterilize and store at 4°C.

Protocol

1. Clean cutting board and any other surfaces with bleach or 70 % ethanol prior to chopping tissue.
2. Clean udder with 70 % ethanol. Strip milk into a 50 mL tube. Keep on Ice.
3. Collect small (3 cm³ pieces) of lymph node tissue (try to remove obvious fat or connective tissue) at time of slaughter.
4. Transport in DMEM+10% FBS supplemented with antibiotics/antimycotic on ice.
5. Rinse tissue in HBSS
6. Cut small sections of tissue to about 1 mm³; place in a 500 mL bottle. For good yield, 20 grams or more of tissue
7. Digest with 5 mL/g of tissue. Incubate at 37°C for 6-8 hours. Shake at 120 strokes/min, with a loose or non-air tight cap.
8. Pass digest through nylon filter cloth in a funnel.
9. Collect into a 50 mL tube and gently squeeze to help cells through.
10. Top off with HBSS
11. Spin at 10 minutes, 1000 RPM (138 g), 15°C. Decant and resuspended in 10 mL HBSS.
12. If yellow fat is still abundant, repeat the spin.
13. Resuspend in 25 mL HBSS
14. Layer over 12.5 mL of Ficoll
15. Spin at 25°C, 1350 RPM (285 g), 30 minutes
16. Take the middle layer (PBMC) (~ 10 mL) to separate tubes 50 mL tubes containing 20 mL HBSS. The bottom layer contains neutrophils and myoepithelial cells and others.
17. Top off to 50 mL HBSS
18. Spin at 15°C, 10 minutes, 1000 RPM (138 g). Decant.
19. If red blood cells are present in pellet:
20. Combine like samples to 5 mL HBSS. Add 10 mL ddH₂O, pipette for 18 sec., and then add 10 mL 2 X RPMI and mix.
21. Top off tube with HBSS, then spin 10 min. at 1000 RPM (138 g), 4°C. Repeat if necessary. Wash with 10 mL HBSS (take sample for counting).
22. Spin at 15°C, 10 minutes, 1000 RPM (138 g) – Decant
23. Seed 96 well V bottom plate to go directly to flow with (200 µL of ~ 2x10⁶ cells) or culture as necessary.

Isolation of Macrophages and Dendritic Cells from Lymph Nodes

Materials / Reagents

- HBSS
- Gentamicin
- DC Media (No Cytokines)
- Filter Cloths
- Ficoll
- Sterile H₂O
- DMEM+10%FBS + Antibiotic/ Antimycotic (GIBCO)

Protocol

1. Clean cutting board and any other surfaces with bleach or 70 % Ethanol prior to chopping tissue.
2. Collect small 2cm³ pieces of lymph node tissue (try to remove obvious fat or connective tissue) at time of slaughter.
3. Transport in DMEM+10%FBS + Antibiotic/ Antimycotic (Gibco) on ice.
4. Rinse tissue with HBSS + Gentamicin (10 μM).
5. Mince tissue and grate with filter.
6. Pass through cheese cloth. 50 mL Tube.
7. Fill to 45 mL with HBSS--.
8. Centrifuge in 50 mL tubes at 2000 rpm (276 g), 30 min, and 15°C. Turn off the brake.
9. Resuspend pellet in 20 mL HBSS.
10. Layer 25 mL on to 12.5 Ficoll.
11. Centrifuge 1350 rpm (285 g), 30 min 15°C, with the brake off.
12. Take off mononuclear cell band and add to 20 mL HBSS in a 50 mL centrifuge tube with a 10 mL pipette. Try not to pick up much Ficoll.
13. Fill tube with HBSS to 50 mL
14. Spin for 10 min at 1,000 RPM (138 g), 15°C.
15. If red blood cells are present in pellet:
 - a. Resuspend cells in 5 mL HBSS. Combine like samples to 5mL HBSS. Add 10 mL ddH₂O, pipette for 18 sec., and then add 10 mL 2 X RPMI and mix.
 - b. Top off tube with HBSS, then spin 10 min. at 1000 RPM (138 g), 4°C.
16. Resuspend cells in 10 mL HBSS and take an aliquot for counting. Spin at 10 min. 1000 RPM (138 g) 4°C.

17. Resuspend in DC media. Want 10^6 cells per 100 μL

18. D0 Flow: Seed 96-well V bottom plate and go directly to flow with (200 μL of $\sim 2 \times 10^6$ cells) or culture as necessary.

Flow Cytometry

Protocol

1. Add 200 μ L of cells per well in a 96 well plate
2. Centrifuge: 1000g, 2 min, 4°C, brake = 1. Flick out liquid.
3. Resuspend in 50 μ L of FACS wash (unstained control) or 50 μ L of primary antibody.
4. Incubate for 1 hour at 4°C
5. Centrifuge: 1000 RPM (138 g), 2 min, 4°C, brake = 1. Flick out liquid
6. Wash 3 with 200 μ L of FACS wash
7. Add secondary antibody in 50 μ L
8. Incubate for 30 minutes at 4°C
9. Wash 3X in FACS wash
10. Resuspend in 1% PBS formaldehyde (20 mL PBS + 0.55mL of 36.5% Formaldehyde)
11. Analyze by flow. Sort cells if applicable.

Immunohistochemistry Tissue Collection and Slide Preparation

Materials / Reagents

- Scalpel/Razor
- OCT Compound
- Cryostat Blades
- Microscope slides
- Liquid Nitrogen
- Weigh boats
- Tissue blocks
- Fine paint brush
- Fine tweezers

Protocol

1. Collect tissues sections 0.5 cm³.
2. Place in tissue block and complete cover with OCT compound. Deeper blocks work better.
3. Place this block in a weigh boat and float over liquid nitrogen for 3-5 minutes.
4. Tissue can be submerged in liquid nitrogen for transport or short-term storage.
5. Store frozen sections in a plastic bag at -80°C.
6. Use fresh OCT to glue tissue blocks to the cryostat chuck. Allow it to harden at -25°C for 5 minutes.
7. Mount the chuck and make 50 μM thick slices until you have entered the middle of the tissue or the area of interest.
8. Allow tweezers and paint brush to cool to -25°C.
9. To cut sections on cryostat, adjust depth to appropriate level (5 – 10 μM mammary; 10-20 μM lymph node), and make a few test sections.
10. If using the plastic cover (directly over the section area), tissue will slide in between the metal surface and this plastic after slicing. After making a slice, keep this plastic cover over the tissue to hold it flat for 20 seconds. This will keep the tissue from curling.
11. Use tweezers to place it on cold slide. Use the fine paint brush to brush out curled up tissue.
12. Place slide on top of the cryostat to warm it and cause it to stick to the glass.
13. Allow sections to air dry, 5 minutes at RT. If making many sections, put them in a slide box in the -25°C cryostat until transport.

14. Store slides at -80°C .

Ultravision / DAB Immunohistochemistry Protocol

Materials / Reagents

- PBS
- PBS + 0.5% TWEEN
- UltraVision LP Value Detection System HRP Polymer and DAB
- 0.3% Hydrogen Peroxide
- Haematoxylin
- Primary antibody
- Acetone
- Paramount

Protocol

1. Fix tissue in cold acetone for 8 min.
2. Aspirate slide (but not to dryness) then was in PBS; 3 X for 1 minute.
3. Label slides and circle sections with a pap pen. This is done at this step because if done beforehand, acetone can interfere.
4. Inactivation of endogenous peroxidase: Incubate in 0.3% H₂O₂/methanol for 30 minutes.
5. Aspirate slide (but not to dryness) then was in PBS+0.05% TWEEN; 3 X for 1 minute.
6. Cover sections with UltraV Blocking Reagent or appropriate alternative, for example 10% v/v normal goat serum in PBS (no TWEEN) for 5 minutes.
7. Aspirate slide (but not to dryness) then was in PBS+0.05% TWEEN; 3 X for 1 minute.
8. Add primary antibody and incubate at overnight at 4 C.
9. Aspirate slide (but not to dryness) then wash in PBS+0.05% TWEEN; 3 X for 1 minute.
10. Apply Value Primary Antibody Enhancer and incubate for 20 min at room temperature.
11. Aspirate slide (but not to dryness) then was in PBS+0.05% TWEEN; 3 X for 1 minute.
12. Apply Value HRP Polymer and incubate for 30 minutes at room temperature. (NOTE: HRP Polymer is light sensitive.
13. Aspirate slide (but not to dryness) then was in PBS+0.05% TWEEN; 3 X for 1 minute.
14. Add 1 drop (40 µL) DAB Plus Chromogen to 2 mL of DAB Plus Substrate, mix by swirling and apply to tissue. Incubate for 5 minutes. This mixture can be stored for 1 week. Please avoid unnecessary light exposure and store in opaque vial.
15. Aspirate slide (but not to dryness) then was in PBS+0.05% TWEEN; 3 X for 1 minute.

16. Counterstain in haematoxilin and/or eosin for 2 - 5 minutes.
17. Aspirate slides and rinse with distilled water, 3 X for 1 minute.
18. Mount with Paramount (or other mountant) and cover slip.
19. Allow slides to dry for a few hours.
20. Seal with nail polish.
21. Store at room temperature.
22. Photograph or evaluate.

Fluorescent Immunohistochemistry Protocol

Materials / Reagents

- PBS
- PBS + 0.05% TWEEN
- Primary antibody
- Appropriate fluorescent secondary antibody
- Acetone
- Southern Mount (anti-fade mounting media)
- 10 % Goat Serum
- DAPI

Protocol

1. Allow frozen sections to acclimate at room temperature for 20 minutes.
2. Fix tissue in cold acetone for 5 min.
3. Aspirate slide (but not to dryness) then wash in PBS pH7.6; 3 X for 2 minutes.
4. Circle sections with a Pap Pen, label slides according to treatment.
5. Cover sections with 10 % goat serum (in PBS with No TWEEN) or appropriate alternative for 5 minutes.
6. Aspirate slide (but not to dryness) then wash in PBS+0.05%TWEEN; 3 x 1 minutes.
7. Add primary antibody (or isotype control) and incubate at overnight at 4 C. Keep the slides in a covered box bedded with a moist paper towel. This cushions the slides and keeps them moist.
Note that IHC typically needs more antibody than flow.
8. Aspirate slide (but not to dryness) then was in PBS+0.05%TWEEN; 3 x 1 minutes.
9. Apply appropriate secondary antibody. Secondary dilutions are usually in FACs wash. Note these are light sensitive, so slides should be covered in foil or in a covered box or closed drawer.
10. Aspirate slide (but not to dryness) then wash in PBS+0.05%TWEEN; 3 X for 1 minute.
11. Add DAPI (999uL PBS (no TWEEN) and 1 μ L of stock DAPI). Incubate for 5 minutes. Keep covered.
12. Aspirate slide (but not to dryness) then wash in PBS+0.05%TWEEN; 3 X for 1 minute.
13. Mount with fluorescent mounting media and coverslip.
14. Keep covered until examination. After slides have dried (usually 24h) seal with nail polish.

15. Examine slides within a few days. Even with the anti-fade mounting media, fluorescence fades over time.

Cytospin

Materials / Reagents

- Slides
- Coverslips
- Mounting media
- Cytospin with chambers, cardboard covers

Methods

1. Cover slides with cardboard covers.
2. Load slides in chamber and place in cytospin centrifuge.
3. Add cells to the chamber, in general, it is quarter million cells per 150-200 μL and 500-1000 RPM (138 g) for 5 minutes.
4. Carefully remove the chambers and cardboard (do not drag across the surface, as it will wipe away your cells).
5. Allow these cells to dry for about 5 minutes.
6. Mount with a coverslip. Dry overnight; cover if using fluorescent labels.
7. Seal with nail polish the next day.

Photographing fluorescent slides

Protocol

1. Look at your isotype controls (you should have each combination of secondary antibodies). Find the exposure time that keeps sections almost completely black. Take pictures with these exposure times and save them with names that indicate the tissue and color.
2. Photograph stained tissue samples at these same exposure times.
3. Use QCapture or Image pro to adjust images and make composites. These instructions are for Q Capture, though the methods are similar.
 - a. Open your isotype controls in QCapture
 - b. Adjust the range (Display Range) of black (Z- left bar) and white (Z+ right bar) so that no background is visible (a few tiny spots of background (either debris or RBC) is ok. For me this was Z+ = 20, Z >80.
 - c. If applicable, merge images (Processing → Color Composite). You can also use this technique to false color single color stains. Assign colors, typically FITC is green and Texas Red is red. DAPI is blue, but these assignments are arbitrary
 - d. Still using your isotype controls adjust the B/C/G (brightness, contrast, gain) controls in the composite window and find the maximum values before high background appears. Do this for each color separately.
 - e. Repeat this with tissue samples.

Native Agarose Gel Electrophoresis of RNA

Reagents / Materials

- TBE (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA, made from DEPC H₂O or nuclease free water)
- Nuclease or DEPC Water (Add 0.5 mL DEPC to 500 mL of water. Shake vigorously, open bottle to vent gas. Autoclave on liquid cycle.)
- Agarose
- Ethidium Bromide
- RNase Away or RNAzap
- 10X native agarose gel loading buffer (15% Ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue)
- RNA + controls or DNA ladder

Protocol

1. Clean all equipment (flasks, electrophoresis chamber) with RNase Away or RNAzap. Rinse with DEPC H₂O or nuclease free water.
2. Make a 1% RNA gel. 2 g agarose, 200 mL. Heat gel in microwave for a minute, mix well, heat again until solution is liquid.
3. To the molten agarose, add either 0.5 µg/mL ethidium bromide or 20 µL Sybr Stain gel stain. Mix well.
4. Pour gel into chamber, add comb, and use a pipette tip to remove bubbles.
5. Load gel into electrophoresis chamber, submerge with 1X TBE. Add 5 µL of ethidium bromide or Sybr stain to this buffer for a brighter stain.
6. Mix 1 µg of RNA samples with 10 µL nuclease free H₂O. Note that if your technique is good, you can approximate with 1 µL of RNA from either Qiagen or Trizol prep.
7. Add 10X native agarose gel loading buffer (15% Ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue) to the RNA dilution to a final concentration of 1X.
8. If possible, use positive RNA controls (available from Ambion). However, you can use a DNA ladder for a control instead.
9. Run the gel at 5-6 V/cm measured between the electrodes. I used 120mV for 1.5 hours.
10. Evaluate under U.V. at 254 nm wavelength.
11. Look for two big bands: 28S and 18S rRNA in eukaryotic samples. A pronounced smear and no clear bands indicates degraded sample. Bands are generally not as sharp as in denaturing gels, and a single RNA species may migrate as multiple bands representing different structures.