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Endocrine and Other Influences on the Trafficking of Immune Cells

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Riva Dill

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Dissertation Committee:
Dr. Ameae M. Walker, Chairperson
Dr. Christian Y. Lytle
Dr. Frances M. Sladek
Dr. Djurdjica Coss

The Dissertation of Riva Dill is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Endocrine and Other Influences on the Trafficking of Immune Cells

by

Riva Dill

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This dissertation examines endocrine and other influences on the trafficking of immune cells. This work aimed to increase the understanding of underlying mechanisms pertaining to immune cell entry into breast milk, and prolactin's involvement in this important physiological phenomenon. Because of observations made during this study, the work also examined various aspects of sexual dimorphism in lymph nodes and thymus.

Luminal mammary epithelial cell secretions act as chemoattractants, inducing migration of a variety of lymphocytes, phagocytes, and granulocytes. Prolactin treatment of mammary epithelial cells increases production of chemoattractants and migration of most of these cell types. This supports a role for luminal mammary epithelial cells in immune cell concentration into milk. CCL2 and CXCL1 were two chemoattractants identified responsible for a significant degree of migration of monocytes and neutrophils, respectively. In-vivo prolactin treatment increased immune cell flux through the

mammary tissue and increased expression of CCL2 and CXCL1 in a mammary cell line in vitro.

Female mice have more T-cells in their popliteal lymph nodes than males, while males have a greater proportion of T-cells that are suppressive in function. This combination supports a rationale for females having a higher delayed-type hypersensitivity response in a region drained by this lymph node. The sex difference in T-cells is present prior to puberty and is to some extent dependent on *Sry* expression as males overexpressing *Sry* have even fewer T-cells. In addition, male gonadal secretions amplified the sex difference as mice aged.

Adult female mice with two X chromosomes exhibit a greater delayed-type hypersensitivity response to *Candida albicans* at proestrus than at metestrus or diestrus, while females with an X and a Y chromosome, the latter minus the *Sry* gene, did not exhibit different responses across these stages of the estrous cycle. There is therefore a combined effect of the sex chromosomal complement and hormonal changes occurring at proestrus that permits maximal response.

These results demonstrate significant interactions between the endocrine and immune systems both in regions where one might expect (the mammary gland during lactation) and in regions (the popliteal lymph node) where gene and endocrine sex effects were not anticipated.

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INTRODUCTION

The immune system protects individuals from invading pathogens as well as functions in other physiologic processes such as tissue repair. Sex differences in immunity have led to the exploration of gonadal-derived hormones, and more recently, the sex chromosomal complement, in relation to immune function in a variety of physiologic contexts. Immune cell trafficking into breast milk, gonadal and gene-related regulation of lymphoid tissue, and chromosomal complement-dependent roles in regards to T-cell mediated immune response, have been examined in this dissertation.

Lymph Node

Lymph, derived from interstitial fluid, flows through the lymphatic system, bringing antigenic material into lymph nodes situated all over the body to elicit an immune response. Lymph nodes are secondary lymphoid tissue containing a variety of lymphocytes, antigen-presenting cells such as macrophages and dendritic cells **[von Andrian and Mempel 2003]**. Lymph nodes are divided into cortex and medulla. The cortex can be sub-divided into the T-cell rich paracortex, and primary B-cell follicles. The medulla contains draining sinuses and some immune cells, but is not as well defined in function **[Fossum and Ford 1985]**. Specialized high endothelial venules, composed of cuboidal endothelial cells, permit entry of circulating immune cells into the lymph nodes **[Tohya et al. 2010]**. Once an experienced immune

cell, such as a memory T-cell, re-enters a lymph node and comes into contact with an antigen it recognizes, it subsequently engages in necessary signaling with the antigen-presenting cell, and becomes activated. This results in proliferation and the production of cytokines/chemokines that attract additional immune cells, sequelae that result in local inflammation [Sallusto et al. 1999 and Weninger et al. 2001, Stoll et al. 2002].

Immune Cell Migration

Immune cells are caused to migrate into, out of, and through tissues, the circulation, and lymph nodes, through responses to cytokines, chemokines, and adhesion molecules [Yoshie 2000]. The common process of immune cell exit from the circulation involves 3 major events, while the specific molecules involved for each immune cell and target exit site can differ. The first is tethering of the immune cell through interactions with adhesion molecules, such as L-selectin, which can allow the cell to start rolling and slowing in the circulation. The second is chemokine engagement with receptors on immune cells which leads to signaling and cell "sticking". The third is final extravasation through the layer of endothelial cells and into the target tissue [Springer 1994 and von Andrian and Mackay 2000].

Sex Differences in Immunity

Females are generally thought to have stronger immune responses, as is evidenced by increased responses to immunization [Davis et al. 1993] and greater overall risk of developing many autoimmune diseases [Verthelyi 2001]. Immune cells express several hormone receptors such as those for estrogen [Greene et al. 1984], testosterone [Lai et al. 2012], progesterone [Dosiou et al. 2008], and prolactin [Leite et al. 1995].

Sry Gene Function

Sry, sex determining region on the Y chromosome, is responsible for male gonadal development [Berta et al. 1990]. Testes formation is dependent on *Sry* expression during a critical period in development [Wu et al. 2012]. In mice, *Sry*'s expression in the gonadal ridge is generally limited to embryonic day 10.5 with a peak at embryonic day 11 and disappearance by embryonic day 12.5 [Hacker et al. 1995]. In humans, *SRY* expression is still indicative of male gonadal development, however its duration of expression differs from that of the mouse in that *SRY* is expressed in the gonadal ridge around 41 days post ovulation, peaks at 44 days post ovulation, and subsequently reduces to low levels, where it remains throughout the remainder of development and beyond [Hanley et al. 2000].

Sex chromosome complement

Sex chromosome complement is referring to an individual having either an X and Y sex chromosome or two X chromosomes, with the first being gonadal male, and the latter being gonadal female. Sry and other Y chromosome gene expression permit proper development of the testes and sperm production, while retention of two X chromosomes allows for proper ovary development and egg production [Arnold 2004]. Not as many genes are coded for on the Y chromosome as compared to the X chromosome and in order to attain more equivalent expression levels between XY and XX individuals, XX individuals undergo silencing of one X chromosome through the function of Xist, a gene coded for on the X chromosome [Plath et al. 2002]. However, some genes on the "silenced" X chromosome are able to escape the process of X inactivation, and contribute to the higher expression levels of some genes in females than males. The consequences of this higher expression have been suggested to contribute to some physiological functions as well as to autoimmune disease [Brooks 2010].

Four Core Genotypes Mouse Model

The Four Core Genotypes (FCG) mouse model is a novel tool for exploring the independent effects of Sry expression, and subsequent gonadal development, and the sex chromosomal complement. The Sry gene was

deleted from the mouse Y chromosome [Lovell-Badge and Robertson 1990], and reinserted onto an autosome, allowing for independent segregation of the Sry gene and the Y chromosome [Mahadevaiah et al. 1998]. Recently, it was determined that 12-14 copies of the Sry transgene were reinserted onto chromosome 3 [Itoh et al. 2015]. Breeding an FCG XY^{-} $^{+Sry}$ male mouse with wildtype C57Bl/6 female mice produces 4 genotypes of offspring XY^{-} $^{+Sry}$ and XX $^{+Sry}$, both gonadal males, and XX and XY^{-}, both gonadal females. This model allows for exploration of the sex chromosomal complement and gonadal hormone production independently as well as of their combined effects [Arnold and Chen 2009].

Flow cytometry and immune cell markers

Flow cytometry is a wonderful tool to use for examination of the proportions of different cell types within a given tissue. Analysis of cell types is dependent on the properties of cells as determined by size, granularity, and markers present on the cell surface or found intracellularly. Size is reflected as forward angle light scatter (Forward scatter) with lower and higher values correlating to smaller or larger cells, respectively. Granularity is reflected as right angle scatter (Side scatter) with lower or higher values correlating to less or more granular cells, respectively. Cell types are identified by cell surface or intracellular markers that are commonly labeled with

fluorescently-conjugated antibodies, the fluorophore regions of which can be excited with different lasers. Emission from these fluorophores is captured in wavelength restricted channels. It is important to include appropriate controls to calculate compensations for spectral overlap between channels, and fluorescently labeled isotype controls to account for non-specific binding of antibodies [Brown and Wittwer 2000].

With the ability to identify several markers on a single cell, we are able to more reliably classify a cell type. Rapid analysis of multiple markers simultaneously on a large number of cells originating from a fully dissociated tissue permits more thorough evaluation of that tissue than if fewer, potentially non-representative sections, of a tissue were histologically analyzed for the same purpose. This benefit comes at the cost of knowledge of the localization of cell types and the ability to save a tissue for countless years and allow for later analysis by different markers of interest.

Many use flow cytometry to identify the proportions of cells in a tissue and their manipulation with treatments or variations between groups. However, when a population increases in proportion in a tissue, then we may not know if it has increased in number or if another population has decreased in number. Also, if total numbers of all cell types are higher or lower in a tissue for a specific group, then upon analysis, the proportions of cells may be identical between groups, however, one group actually has more of all cell

types. For this reason, experiments with flow cytometry should first determine the number of cells in a given tissue if possible, and use that information in conjunction with proportions of cell types to determine if there are truly differences in cell types between groups.

Lymphocytes

CD19 is a B-cell Receptor co-receptor, and is commonly used as a marker to identify B-cells in mice **[Lai et al. 1998]**. It is expressed on B-cells at early stages of development as well as on mature B-cells. However, its expression is lost upon terminal differentiation into a plasma cell **[Stamenkovic and Seed 1988]**. CD19 is also expressed on follicular dendritic cells **[Baban et al. 2005]**.

CD3 is a T-cell receptor subunit and a commonly used pan-T-cell marker in mice **[Lai et al. 1998]**. CD4 and CD8 are T-cell co-receptors that bind to MHCII and MHCI, respectively **[Doyle and Strominger 1987 and Gao and Jakobsen 2000]**. These markers are mainly found on T-cells, but can also be found on dendritic cells in mice **[Winkel et al. 1994, Vremec et al. 2000, and De la Mata et al. 2001]**. CD44, a hyaluronate receptor, and CD127, an Interleukin-7 receptor subunit, are markers that can be used to identify memory T-cells **[Lai et al. 1998 and Huster et al. 2004]**. CD25, the alpha subunit of the IL-2 receptor, and FOXP3, forkhead box P3, in combination

with CD4 can be used to identify T-regulatory cells in mice **[Hori et al. 2003 and Fontenot et al. 2005]**.

Macrophage/Monocytes, Neutrophils, and Eosinophils

CD45, protein tyrosine phosphatase, receptor type, C **[Kaplan et al. 1990]**, is expressed on all leukocytes **[Nakano et al. 1990]**. CD11b, or integrin alpha M, is the alpha subunit of the MAC-1 antigen **[Solovjov et al.2005]**. F4/80 is a commonly used marker for macrophages **[Zwadlo et al. 1985 and Leenen et al. 1994]**, however it is also found on Langerhans cells **[Hume et al. 1983]** and eosinophils **[McGarry and Stewart 1991]**. Siglec F is a marker used to identify eosinophils by flow cytometry **[Stevens et al. 2007]**. Ly6-C and Ly6-G can be used to distinguish monocytes and neutrophils, with Ly6-G being more restricted to neutrophils **[Daley et al. 2008]**.

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Chapter 1

Immune Cell Flux Through the Mammary Gland: Implications for Trafficking of Immune Cells into Breast Milk.

ABSTRACT

The lactating mammary gland is the site at which the concentration of immune cells into breast milk occurs. Because of the important functions some of these cells have on early mucosal protection of neonates, it is important to understand the mechanisms leading to their specific concentration into breastmilk. We found that prolactin, a hormone necessary for lactation, increased immune cell flux through the mammary gland as reflected by increased numbers of immune cells in mammary gland-draining, but not other lymph nodes. Using transwell systems and conditioned media from luminal mammary epithelial cell cultures, we demonstrate that secretions from luminal mammary epithelial cells induced migration of a number of immune cell types; B-Cells, CD4+ T-cells, CD8+ T-cells, CD4+ memory T-cells, CD8+ memory T-cells, macrophages, monocytes, eosinophils, and neutrophils. Prolactin did not act as a direct chemoattractant, but by effects on luminal mammary epithelial cells, increased the chemoattractant properties of conditioned media. Neutrophil and macrophage/monocyte migration showed the largest difference with prolactin treatment. Analysis of potential mediators of neutrophil and monocyte chemoattraction showed involvement of CXCL1 and CCL2. Thus, antibody depletion of CXCL1 and CCL2 from conditioned medium reduced chemoattraction and mRNA expression of both chemoattractants was increased by prolactin. We conclude

that prolactin could facilitate immune cell concentration into breast milk by its activities on luminal mammary epithelial cell production and/or secretion of chemoattractants.

INTRODUCTION

The presence of cells in breast milk was first observed centuries ago [Anthony van Leewenhoek 1695]. Upon histologic analysis, these were determined to be epithelial and immune cells [Simon C et al. 1970 and Breborowicz D. et al. 1971]. The proportions of immune cell types present in the milk of normal women during the first four days of lactation were originally determined to be; 30-47% macrophages, 40-60% polymorphonuclear leukocytes, and 5.2-8.9% lymphocytes [Crago et al. 1979]. Of the lymphocyte subset present during the first few days of lactation in the milk of healthy female donors, 83% were identified as T-cells and 6% as B-cells [Wirt et al. 1992].

Neutrophils are suggested to be important to protection of both the mammary gland during lactation when the ducts are open, and the neonatal gut [Robinson et al. 1978]. The macrophages in milk are adept to contributing to immune defense of the neonate in a number of ways; phagocytosis of invading pathogens, production of oxygen intermediates, like superoxide anion, which has microbicidal activities, and production of lysozyme, which is able to break down parasitic cell wall [Cummings et al. 1985, Speer et al. 1985 and 1986, Pittard et al. 1977]. Additionally, other leukocytes from milk have been shown to enter the neonate in several different species; rats [Sheldrake and Husband 1985, Seelig and Head 1987], lambs [Sheldrake and

Husband 1985 and Tuboly et al. 1995], pigs [Tuboly et al. 1988], baboons [Jain et al. 1989], and mice [Zhouh et al. 2000, Arvola et al. 2000, and Ma et al. 2008]. Antibody secreting cells have been found in mice made B-cell deficient, due to a targeted mutation of an exon for the IgM heavy chain, when fostered and breastfed by wildtype dams [Arvola et al. 2000]. Although many of these studies demonstrating this phenomenon have been questioned due to the experimental design not reflecting physiologic delivery of the milk derived immune cells, others have studied the phenomenon in a more physiologic context and provided stronger evidence to show that maternal CD4+ and CD8+ T-cells from milk can cross the intestine of neonatal mice and eventually home to their spleen and thymus respectively [Ma et al. 2008].

Although the presence of immune cells in milk was recognized centuries ago, and increasing knowledge has been gained in regards to the benefits these cells can offer neonates, the processes by which some of these cells are concentrated into breast milk are still largely unresolved.

What we do know is that prolactin contributes to the increased numbers of IgA+ cells in the mammary gland at the start of lactation [Weisz-Carrington P. et al. 1978]. These cells situate themselves near the alveolar epithelial cells and their presence is suggested to contribute to the large amounts of IgA present in breast milk [Tanneau et al. 1999]. There is

evidence that prolactin may mediate the homing of IgA+ cells to the lactating mammary gland by increasing Chemokine Ligand 28 (CCL28) expression in that tissue **[Hyde 2007]**. Through studies utilizing in vivo antibody blockade and knockout mice for CCL28 and its receptor CCR10, respectively, both have been demonstrated to be critical to homing of IgA+ cells to the mammary gland during pregnancy and lactation **[Wang et al. 2000, Morteau et al. 2008, and Wilson and Butcher 2004]**. Other studies demonstrated that Vascular adhesion molecule 1 and alpha 4 integrin, but not Mucosal vascular adhesion molecule 1, Beta 7 integrin, or alpha4beta7 integrin, are also important to IgA+ cell concentration in mammary tissue during lactation with use of in-vivo antibody blockade studies for each **[Low et al. 2010]**.

Near the end of pregnancy there is a gradual increase in the T-cell populations present in the mammary gland, followed by a decrease at the start of lactation **[Tanneau et al. 1999]**. This pattern allows for the speculation that the T-cells that once populated the mammary gland decrease in number for that tissue because of their pooling into milk. It has been suggested that prolactin to some extent regulates the T-cell populations in the mammary tissue during lactation as rats, deficient of prolactin secondary to a partial deletion of the Desmoglein 4 gene, experience an impairment of entry of T cells during early lactation **[Mackern-Oberti et al. 2013].** Although correlative, it does not conclusively demonstrate that the reduced T-cell entry

during early lactation is due to prolactin and not Desmoglein 4 or purely due to the accompanied lactation impairment.

There is an increased concentration of activated and memory T cells present in milk compared to the general circulation of lactating mothers that has been observed when samples were collected during the first few days of lactation through to about the fourth month, thereby supporting the rationale of the existence of a mechanism allowing for the specific concentration **[Wirt et al. 1992 and Sabbaj et al. 2005].**

In order for an immune cell to enter milk it must navigate across an endothelial cell layer, two basement membranes, around a myoepithelial cell layer, and finally, across a luminal epithelial cell layer. Endothelial cells initiate extravasation by slowing immune cells in the circulation through tethering and initiating rolling through use of chemokines and selectins **[Butcher 1991 and Ley et al. 2007]**. After crossing out of the circulation, immune cells must work through two basement membranes, and while some mechanisms permitting this have been elucidated, many others still remain misunderstood. Some have shown that there are regions near to where cells would exit the circulation that represent a low expression of components that make up the basement membrane. Monocytes can migrate through the ~ 1.8 micrometer sized low expression regions without affecting the basement membrane's structure, whereas neutrophils enlarge these regions in order to

pass through [Wang et al. 2006 and Voisin et al. 2009, 2010]. Navigation through the basement membrane is also suggested to be dependent on proteolytic cleavage, although this conclusion is questioned due to the nature of experimentation to determine this, which is confounded by off-target effects of inhibitors of proteolytic enzymes that were tested [Young et al. 2007, Lin et al. 2008, Reichel et al. 2008, and Voisin et al. 2009]. Myoepithelial cells contribute to the production of basement membrane surrounding the epithelial cells layers, the polarity of other epithelial cells, and milk motility during lactation by contractile actions [Deugnier et al. 2002]. During lactation, the myoepithelial cell layer is not continuous, a serendipitous re-discovery attributed to inadvertent silver staining of mammary sections [Linzell 1952], which allowed for the first visualization and distinction of the mammary myopepithelial cells histologically [Richardson 1949]. Luminal epithelial cells secrete maternally derived nutrients and milk proteins that are either directly synthesized by the cell or trafficked across the cell into the milk [Hennighausen and Robinson 2005]. This is the final barrier before entry into the ducts where milk is to be pooled prior to ingestion by the neonate.

Some have assessed migration of neutrophils across mammary endothelium and epithelium in response to a pathogen or known stimulus [Lin et al. 1995 and Smits et al. 1996]. Although that work contributes to the

understanding of neutrophil migration during a time of infection, it does not necessarily reflect the signals provided by mammary epithelial cells in the context of non-mastitic lactation.

Not a lot of research has examined the ability of luminal epithelial cells to induce migration of immune cells, especially various lymphocytes such as T-cells and B-cells. As these cells are the final barrier which immune cells must cross prior to entry into milk, we hypothesized that luminal mammary epithelial cells are capable of producing and secreting chemoattractants that could contribute to homing of a diverse set of lymphocytes, phagocytes, and granulocytes, all of which have been found present in milk. Secondly, we hypothesized that prolactin would increase immune cell entry into the mammary tissue in-vivo. Luminal mammary epithelial cells express the prolactin receptor on the apical membrane during lactation and are responsive to prolactin **[Ueda et al. 2011]**. These cells are exposed to the concentrated amounts of prolactin that are secreted into milk **[Grosvenor and Whitworth 1976]**, and so we also assessed if prolactin would increase luminal mammary epithelial cell production and/or secretion of chemoattractants, thus allowing for increased homing of immune cells towards their secretions. This manuscript presents the findings that arose from testing these hypotheses.

MATERIALS AND METHODS

Compliance with Ethical Standards

The authors have no conflict of interest regarding any of the work presented in this manuscript. All animal procedures were approved by the University of California, Riverside, institutional animal care and use committee and were in accordance with guidelines from the American Association for Laboratory Animal Care, the United States Department of Agriculture, and the National Institutes of Health.

Animals

Intact 8 week old female mice were obtained from Jackson Laboratories. Animals were housed in a specific pathogen free facility on a 12-hour light dark cycle.

In vivo continuous prolactin treatment

At 8 weeks of age, mice were anesthetized with isofluorane and osmotic mini pumps (Model 2004 Alzet, Cupertino CA) were implanted subcutaneously. Pumps either contained prolactin or Dulbecco's phosphate buffered saline as vehicle control. Mice were treated for 28 days and pumps continuously released their contents, allowing for a total of 2.4 ug of prolactin to be

administered per day. Prolactin utilized for experiments described in this manuscript was prepared as previously described **[Chen et al. 1998]**.

Vaginal Smears

Vaginal smears were collected daily for the last two weeks of treatment to determine the pattern of estrous cycling for each animal. Slides of smears were stained with 1% Toluidine blue, washed with deionized water, and left to air dry overnight. Estrous cycle stage was determined based on cell types present, as described by **Caligioni, 2009**.

HC11 cell culture and in vitro prolactin treatment

HC11 cells were a gift from Nancy Hynes (Friedrich Miescher Institutes, Basel, Switzerland). Cultures were maintained in Growth Media (RPMI 1640, 10% FBS, 5 ug/mL insulin, 10 ng/mL Epidermal growth factor, 100 Units/mL Penicillin, 100 ug/mL Streptomycin). HC11 cells were plated in growth media for experiments at 1 X 10^6 cells/well in a 6 well plate. 48 hours after plating, cell media were changed to Priming Media (RPMI 1640, 10% charcoal stripped Horse serum, 10 ug/mL Insulin, 1 ug/mL Hydrocortisone, 100 Units/mL Penicillin, 100 ug/mL Streptomycin). Twenty-four hours later, media were changed to priming media plus Dulbecco's phosphate buffered saline or 100 ng/mL prolactin. Forty-eight hours later, medium was removed and cells were washed with Serum free priming

medium and given either serum free priming media with Dulbecco's phosphate buffered saline or 100 ng/mL prolactin. Twenty-four hours later, conditioned media were collected for transwell experiments and in some experiments, cells were collected for RNA extraction.

Chemokine Depletion of Conditioned Media

The conditioned media of HC11 cells were collected and subsequently depleted of CXCL1 or CCL2. Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology sc-2003, Dallas TX) 10 uL/1ug antibody used, were washed three times with PBS. Each was followed by 10 minutes of centrifugation at 1,000 x g at 4°C. The beads were then incubated with purified Anti-CXCL1 (R and D systems MAB453, Minneapolis MN) 2 ug/mL, Anti-CCL2 (R and D systems AF-479) 5 ug/mL, Rat IgG2a Isotype Control (R and D systems MAB006) 2 ug/mL, or Goat IgG (R and D systems AB-108) 5 ug/mL, vortexed, and rocked for 30 minutes at room temperature. Beads were again washed three times with PBS and finally incubated with 1 mL of Conditioned media from HC11 cells, vortexed and left rocking overnight at 4°C. The beads were pelleted and the depleted conditioned media was collected and used for downstream applications.

RNA extraction and Reverse transcription PCR

RNA was extracted from cells with Trizol/Chloroform (15596-018 Life Technologies, Carlsbad CA/ C2432 Sigma Aldrich, St. Louis MO) separation and further purification with columns (74104 Qiagen, Valencia CA) as per manufacturer recommended protocol. RNase-Free DNase (79254 Qiagen) treatments were used to eliminate any residual genomic DNA. RNA was quantified by nanodrop and equivalent amounts of RNA were reverse transcribed using Oligo$(dT)_{12–18}$ Primer (18418-012 Invitrogen, Carlsbad, CA), M-MLV Reverse Transcriptase (28025-013 Invitrogen), RNase OUT (10777-019 Invitrogen), and 10 mM DNTP mix (18427-088 Invitrogen) for 1 hour at 37°C. Gene expression was analyzed by Quantitative PCR and Classical PCR using 2x SYBR Green (Biorad, Hercules CA) or 2x Taq Mastermix (TP01-01 Bioland Scientific, Paramount CA) and primers for GAPDH, CCL2, and CXCL1. Primer sequences were: GAPDH (forward)- TGCACCACCAACTGCTTAG, (reverse)- GGATGCAGGGATGATGTTC, CCL2 (forward)- AGTTAACGCCCCACTCACCT (reverse)- GAGCTTGGTGACAAAAACTACAGC , CXCL1 (forward)- ACCCAAACCGAAGTCATAGCC (reverse)- TTGTCAGAAGCCAGCGTTCA. Quantitative PCR was performed with Initialization at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C 10 seconds and annealing 30 seconds. After each run, a melt curve was run to assess appropriately

amplified products. For classical PCR, the Initialization step was set at 94°C for 5 minutes, followed by 30 cycles of denaturation at 95°C 30 seconds, annealing at 55°C 30 seconds, and extension at 72°C 30 seconds, and a final elongation at 72°C for 7 minutes. Products were run on a 2% agarose, ethidium bromide gel and imaged on an ultraviolet light box. Relative band intensity was measured using Image J software.

Transwell experiments

Serum free conditioned media from HC11 cells treated with Dulbecco's phosphate buffered saline or prolactin (described in methods for HC11 cell culture) was collected and aliquoted into the bottom well of 5 uM pore size transwells (Corning 3421, Corning CA). 1.5 x 10^6 splenocytes in 200 uL RPMI1640 were loaded onto the tops of transwells. Plates were covered and incubated at 37 °C with 5% CO2. After 2 hours, transwells were carefully removed and samples in the bottom well were mixed by pipetting. 400 uL of each sample was utilized for staining with fluorescent-labeled antibodies to assess cell type by Flow Cytometry. 200 uL of each sample was aliquoted into tubes containing 10 uL of fluorescent count beads (Polysciences 19096, Warrington PA). After mixing, the sample/bead suspensions were run on the flow cytometer and 5,000 gated bead events were recorded for each. The Flow Cytometry Standard files were then analyzed by gating on immune cells and

the total number of gated cell events was calculated for each sample. This value provides a rapid, standardized means to quantify the number of cells that migrate. Percentage of the Cell type of interest present in Input that crossed into the bottom well was calculated by the following equation. Total # Cell Type of Interest Input / Total # Cell Type of Interest in bottom well * 100. Values were then normalized to the CM group or Media only group, setting this at a value of 1.

Flow cytometric analysis

All antibodies were diluted in 0.2% BSA in Dulbecco's phosphate buffered saline (FACS Buffer). Fc receptors were blocked with purified anti-CD16/32 (eBioscience 14-0161, San Diego CA) at the dilution 1:50 for 15 minutes at 4 °C. Cells were washed with FACS buffer, and resuspended in a mixture of fluorescently-labeled antibodies at the following dilutions, 1:100 rat anti-mouse CD3 (eBioscience 11-0031), 0.5:100 rat anti-mouse CD4 (eBioscience 47-0041), 0.5:100 rat anti-mouse CD8a (eBioscience 17-0081), 0.5:100 rat anti-human/mouse CD11b (Tonbo Biosciences 11-0112, San Diego CA), 0.5:100 rat anti-mouse CD19 (eBioscience 25-0193), 0.5:100 rat anti-mouse CD44 (eBioscience 25-0441), 0.25:100 rat anti-mouse CD45 (eBioscience 11-0451), 1:100 rat anti-mouse CD127 (eBioscience 12-1271), 1:100 rat anti-mouse F4/80 (eBioscience 50-4801), 0.3:100 rat anti-mouse Ly6c (eBioscience

12-5932), 0.25:100 rat anti-mouse Ly6g (eBioscience 45-5931), and 1:100 rat anti-mouse Siglec F (BD Biosciences 562680, San Jose CA), or appropriate isotype controls for 1 hour at 4 °C in the dark. Cells were washed and re-suspended in FACS buffer and run live on the BD Facs Canto II. FLOJO software was used for analysis.

Statistical Analyses

Comparisons for in vivo experiments were analyzed by Student t-test and all other experiments utilized the Mann-Whitney test. Data were presented as mean + Standard Error. P-values were assigned as follows;

* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

RESULTS

Estrous Cycle Monitoring

Mice were treated with prolactin for 28 days in order to mimic exposure to prolactin/placenta lactogens for the duration of rodent pregnancy, plus the first week of lactation.

Mice continuously treated with prolactin remain in a pseudopregnant state for two weeks and subsequently return to normal cycling after that time period **[Alloiteau and Vignal 1958]**. To determine whether prolactin treatment caused any abnormal estrous cycling later than 2 weeks, we assessed the number of days the mice experienced proestrus, estrus, metestrus, and diestrus by cytology from daily vaginal smears through the last two weeks of treatment. No difference in cycling during the monitored period was observed with prolactin treatment when compared to the control animals **(Figure 1.1)**.

Prolactin specifically increased lymphocyte numbers in the axillary and inguinal lymph nodes.

Our initial interest was on prolactin's effects on the numbers of T-cells and B-cells in the major mammary gland draining lymph nodes, as these were the cell types that have been observed to enter milk and migrate across the pup's intestinal epithelium and home to lymphoid tissue of pups with

breastfeeding [Ma et al. 2008 and Arvola et al. 2000]. We counted and determined the total number of cells within lymph nodes and used these numbers in combination with percentages of each cell type of interest that were generated by flow cytometric analysis to calculate the total number of each cell type present in each tissue.

With prolactin treatment, there were increased numbers of T-cells (CD4+ and CD8+), and B-cells (CD19+) present in the axillary lymph node by 60%, 50%, and 140%, respectively (Figure 1.2a). There were also increased numbers of these cell types present in the inguinal lymph nodes by 50%, 50%, and 100%, respectively (Figure 1.2b). To determine if prolactin's effects were specific to the lymph nodes draining the mammary gland, rather than a more global effect on the animal, we assessed a more distal lymph node, the popliteal lymph node. Although many fewer cells were present in this lymph node compared to the axillary and inguinal lymph nodes for both groups, prolactin treatment had no effect on cell numbers of B-cells or either T-cells. (Figure 1.2c).

Media conditioned by mammary epithelial cells increased the migration of lymphocytes

In order for immune cells to enter milk, these cells must appropriately migrate towards mammary epithelial cells. Given the complexity of the in

vivo mammary gland, we assessed the ability of specifically luminal mammary epithelial cells to attract CD4+ T-cells(CD3+/CD4+), CD8+ T-cells(CD3+/CD8+), and B-cells(CD19+) by using an in vitro transwell system with either non-conditioned serum free media or conditioned serum free media from HC11 cells, an established non-malignant luminal mammary epithelial cell line.

Conditioned serum free media increased the migration of CD4+ T-cells, CD8+ T-cells, and B-cells by 26, 7, and 7 fold, respectively, in comparison with the non-conditioned serum free media **(Figure 1.3a, 1.3b, and 1.3c)**.

Because of the increased concentration of memory T-cells in milk that others have observed **[Wirt et al. 1992 and Sabbaj et al. 2005]**, we also examined memory T-cell migration towards conditioned serum free media and found that it increased migration of CD4+ memory T-cells(CD3+/CD4+/IL7R+/CD44+) and CD8+ memory T-cells(CD3+/CD8+/IL7R+/CD44+) by 14 and 5 fold, respectively, in comparison with non-conditioned serum free media **(Figure 1.3d and 1.3e).**

Media conditioned by mammary epithelial cells increased migration of phagocytes and granulocytes

Due to the presence and protective functions that phagocytes and granulocytes have in milk, we assessed the ability of mammary epithelial

cells to cause migration of these cell types. Conditioned serum free media increased the migration of monocytes (CD45+/CD11b+/Ly6c+/Ly6g-) and macrophages (CD11b+/F4/80+) by 4 and 30 fold, respectively, in comparison to non-conditioned serum free media **(Figure 1.4a and 1.4b).** Additionally, conditioned serum free media increased migration of eosinophils (CD11b+/SiglecF+) and neutrophils (CD45+/CD11b+/Ly6c+/Ly6g+) by 23 and 13 fold, respectively, in comparison to non-conditioned serum free media **(Figure 1.4c and 1.4d).** Others have reported that neutrophils in milk display increased CD11b expression due to prior activation **[Keeney et al. 1993 and Goldman et al. 1998]**. To determine whether mammary epithelial cells may to some degree be responsible for the homing of neutrophils with increased CD11b expression and their concentration into breast milk we compared the mean fluorescent intensity of CD11b on neutrophils that migrated towards non-conditioned serum free media with neutrophils that migrated towards conditioned serum free media and found that those that migrated towards the latter exhibited a 2.1 fold increased expression of CD11b **(Figure 1.5a)**.

CCL2 and CXCL1 depletion from conditioned serum free media

Macrophages/monocytes and polymorphonuclear cells, like neutrophils, compose a great majority of immune cells present in milk **[Crago et al. 1979]**. Given that these cell types also demonstrated substantial migration towards

media conditioned by mammary epithelial cells, we attempted to elucidate what was present in conditioned media that would allow for their migration.

Previously, others have examined the presence of Interleukin-8 (IL-8) in mammary secretions and determined that mammary epithelial cell production of IL-8 somewhat contributed to neutrophil migration. It had a greater contribution to neutrophil migration in the context of infection compared to that of health of mammary epithelial cells as demonstrated by in vitro antibody neutralization experiments **[Barber and Yang 1998 and Barber et al. 1999]**. Chemokine (C-X-C motif) ligand 1 (CXCL1) is another strong chemoattractant for neutrophils and is known to be a functional homologue of IL-8, making it an attractive candidate for our studies **[Sekido et al. 1993 and Miura et al. 2001]**.

Chemokine (C-C motif) ligand 2 (CCL2) is known to be an important mediator of monocyte migration, and their entry into various tissues and is necessary to allow their subsequent differentiation into other cell types such as macrophages **[Shi and Pamer 2011]**. There are increased concentrations of both CCL2 and CXCL1 in milk as compared to the general circulation of lactating women **[Kverka et al. 2007]**. Others have shown that both CCL2 and CXCL1 are produced and secreted by HC11 cells **[Csanaky et al. 2014]**, and given their respective functions as chemoattractants for monocytes and neutrophils, we assesed the degree migration that was dependent on their

production by mammary epithelial cells. To assess their respective roles in the homing of monocytes and neutrophils towards mammary epithelial cells, we employed cytokine depletion experiments.

We depleted CCL2 from conditioned serum free media of HC11 cells with use of antibody coated beads. We then placed either anti-CCL2 treated conditioned serum free or isotype control treated conditioned serum free media in the bottoms of transwells to assess migration of monocytes. When CCL2 was depleted, there was a 35% reduction of monocyte migration towards the conditioned serum free media, demonstrating that CCL2 is in part responsible for the monocyte migration **(Figure 1.6a)**.

We depleted CXCL1 from conditioned serum free media in the same manner as we had for CCL2. When CXCL1 was depleted from the conditioned serum free media, there was a 60% reduction of neutrophil migration towards conditioned serum free media, demonstrating that CXCL1 is in part responsible for the neutrophil migration **(Figure 1.6b)**.

Prolactin treatment of mammary epithelial cells increased migration of most immune cells

We next determined if conditioned serum free media collected from prolactin treated luminal mammary epithelial cells could increase migration of the immune cells over and above that assessed thus far. Prolactin

receptors have been found on T-cells, B-cells, monocytes, macrophages, and granulocytes **[Pellegrini et al. 1992, Gala and Shevach 1993, and Dogusan et al. 2001]** and others have shown that prolactin itself can act as a chemoattractant **[Maus et al. 1999]**. To determine if prolactin at the concentration that would be present in conditioned media from mammary epithelial cells treated with prolactin could independently act as a chemoattractant, we placed non-conditioned serum free media with or without prolactin (100 ng/mL) in the bottoms of transwells and determined immune cell migration to the bottom of the wells. We used this concentration for treatment as it would best reflect the physiologic amounts present during lactation in the mouse **[Parkening et al. 1980]**.

Prolactin itself at 100 ng/mL did not prove to act as a chemoattractant for B-cells, CD4+ T-cells,CD8+ T-cells, CD4+ memory T-cells, CD8+ memory T-cells, macrophages, monocytes, eosinophils, or neutrophils **(Figure 1.7 and 1.8)**. This allows us to conclude that any observed increases in migration towards conditioned serum free media from HC11 cells treated with prolactin would be due to prolactin's effects on mammary epithelial cell production of chemokines and/or their release into the media.

Although not large, prolactin produced a consistent increase in migration of most immune cells. In comparison to conditioned serum free

media, conditioned serum free media collected from HC11 cells treated with prolactin increased migration of B-cells by 23%, and migration of CD4+ T-cells, CD4+ memory T-cells, and CD8+ memory T-cells each by 20%, but had no effect on migration of overall CD8+ T-cells **(Figure 1.9).**

Conditioned serum free media from HC11 cells treated with prolactin also increased migration of monocytes and macrophages by 30% and 40%, respectively **(Figure 1.10a and 1.10b)**, and migration of eosinophils and neutrophils by 30% and 40%, respectively **(Figure 1.10c and 1.10d)**.

Prolactin regulates mammary epithelial cell cytokine expression

Since at least 35% of monocyte migration and 60% of neutrophil migration was dependent on CCL2 and CXCL1, respectively and prolactin increased the monocyte and neutrophil chemoattractant properties of conditioned medium by 30 and 40% respectively, we analyzed mRNA expression by the HC11 cells. After normalization to GAPDH, mRNA expression of both CCL2 and CXCL1 increased with prolactin treatment by 40% and 20%, respectively **(Figure 1.11a and 1.11b)**. GAPDH expression is an indirect measure of the metabolic activity of cells and can potentially be altered by prolactin treatment if cells were proliferating. To address this concern and determine if expression changes were due to increased

expression per cell, rather than due to increased cell number, we counted cell numbers with each condition and found no difference in cell number with prolactin treatment **(Figure 1.12)**.

DISCUSSION

There is evidence that breastfeeding provides immunologic benefit to infants and even contributes to long-lasting immune education **[Verhasselt et al. 2008, Aoyama et al. 2009, and Ma et al. 2008].** Infants who breastfed have been shown to have larger thymi than those who were bottle fed **[Hasselbalch et al. 1996]**. There is also an observed positive correlation between amount of breastfeeding, infant thymus size, and the numbers of CD4+ and CD8+ cells in the infant's circulation **[Jeppesen et al. 2004]**. Others have suggested that some functional immunologic benefits are dependent on certain factors present in milk **[Ngom et al. 2004]**. Aside from nutrients, there are also many immune cells present in breast milk, including T -cells, B-cells, macrophages, eosinophils, and neutrophils and some of these cells have been shown to functionally benefit the neonate by immediate defense **[Goldman 1993 and Hanson 1998]**. Given the potential contributions of these maternal cells at a time of immunologic weakness for infants, it is important to understand the mechanisms allowing for immune cell concentration into breast milk.'

Prolactin, a hormone necessary for lactation, has been demonstrated to increase the migration of antibody secreting cells to the mammary epithelium **[Weisz-Carrington P. et al. 1978]**. It is also suggested to contribute to T-cell entry into the mammary gland during early lactation **[Mackern-Oberti et al. 2013].** The latter study based their suggestion on flow cytometric analysis of

the mammary tissue of rats deficient in prolactin, and therefore, lactation, as a consequence of a Desmoglein 4 defect. The evidence is correlative and therefore, it is difficult to conclusively state that the reduced T-cell entry during early lactation is due to reduced prolactin and not the defect in Desmoglein 4 or purely due to insufficient lactation.

To more directly approach the function of prolactin to increase immune cell concentration to the mammary gland as it might during lactation, we directly treated female mice continuously with prolactin and assessed immune cell presence in the mammary gland by analysis of two of the major mammary gland draining lymph nodes. Because mice in our experiments were not infected or challenged with any specific antigen, we can conclude that the increased T and B cell numbers in the axillary and inguinal lymph nodes with prolactin treatment was due to increased migration through the mammary gland rather than activation induced proliferation of immune cells already within that lymph node. Others have demonstrated that although prolactin can increase activation of certain cell surface antigens that are necessary for T-cell activation and proliferation **[Ziegler et al.1994 and Takizawa et al. 2005]**, it cannot do so unless in the context of other stimuli **[Gagnerault et al. 1993 and Dugan et al. 2002]**.

Immune cells must undergo extravasation followed by migration through two basement membranes, a myoepithelial cell layer and luminal

epithelium to gain access to milk. We assessed the specific ability of luminal mammary epithelial cells to produce and secrete chemoattractants that could contribute to homing of immune cells towards the final barrier before entrance into milk. Interestingly, we found that secretions produced by mammary epithelial cells induced migration at varying degrees for all immune cell types analyzed. Prolactin to some extent further increased migration for most cell types analyzed, however we did not find that prolactin itself acted as a chemoattractant for any immune cell types analyzed. Others have reported that prolactin was able to act as a chemoattractant at the concentration we used, however those studies assessed migration 48-96 hours later, unlike our experiments, which were assessed after 2 hours of cell migration [Maus et al. 1999]. Given that in the time-frame of our experiments, prolactin had no direct effect on immune cell migration through activity as a chemoattractant, we can conclude that its effects on immune cell migration are indirect by its effects on mammary epithelial production and/or release of chemoattractants. The effects of exogenous prolactin treatment on immune cell migration in our experiments were not substantial for several cell types, however it is important to note that HC11 cells produce prolactin [Wu et al. 2006]. This autocrine source of prolactin may nearly maximize prolactin's contribution to mammary epithelial cell production and/or release

of chemoattractants, diminishing the effect of the exogenous prolactin treatment.

One way by which mammary epithelial cells can increase migration of immune cells through the mammary gland and potentially direct immune cells into milk is by increasing chemokine expression. Although some efforts have been made to understand neutrophil migration to mammary epithelial cell secretions, little has been explored about monocyte migration in this context. Others have found that IL-8 is present in mammary secretions and is responsible for some amount of neutrophil migration to healthy mammary epithelial cells **[Barber and Yang 1998]**. Still, a large amount of neutrophil migration to mammary epithelial cell secretions remained unexplained. We found that CXCL1, a known neutrophil chemoattractant, and CCL2, a known monocyte chemoattractant were important to migration of these cell types towards mammary epithelial cells. Paralleling the increased migration of these cell types with prolactin treatment of mammary epithelial cells, their expression was also increased.

It is possible that these chemokines aid neutrophil and monocyte migration into milk. CCl2 may contribute to monocyte entry into the mammary tissue through two mechanisms; we have demonstrated that mammary epithelial cells can directly attract monocytes by producing CCL2 and others have shown that CCL2 produced by epithelial cells can be loaded

onto endothelial cells and contribute to monocyte rolling and slowing in the circulation in preparation to enter tissue [Palframan et al. 2001]. Once in mammary tissue, monocytes would differentiate into macrophages that would subsequently concentrate into the milk. Macrophages in the milk can provide more of an immediate protection to the neonate by phagocytosing and killing pathogens such as Candida Albicans [Cummings et al. 1985]. The macrophages present in milk are also able to initiate T-cell activation and proliferation, showing that they can contribute to T-cell mediated immune responses [Ichikawa et al. 2003]. Given that milk derived maternal T-cells have been found to enter the neonate, it is possible that macrophages could contribute to activation of these cells against encountered pathogens prior to entry into the neonate, allowing for passive T-cell mediated immune protection [Mat et al. 2008].

CD11b and CD18 form the alpha and beta subunits, respectively, of the Macrophage 1 Antigen, an integrin that is important for neutrophil adhesion, migration, and phagocytosis. CD18 is important for neutrophil migration across mammary endothelial cells and CD11b is important for its migration across mammary epithelial cells [Smits et al. 2000]. Others have reported finding neutrophils with elevated CD11b in breast milk compared to neutrophils in the peripheral blood and suggested that they may not contribute to immune function because the increased CD11b is an indicator

that they have already been activated. [Keeney et al. 1993, Michie et al. 1998, and Goldman 1998]. We found that neutrophils that migrated to conditioned serum free media had increased CD11b expression compared to those that migrated to non-conditioned serum free media. Our data suggest that CXCL1 and potentially other products made and secreted by mammary epithelial cells induce migration of neutrophils retaining greater CD11b expression. This subset may be selected to allow for more efficient CD11b dependent migration across the mammary epithelium and into the breast milk. The increased CD11b that others have observed on breast milk neutrophils may not be reflective of their activation status, but rather a functional advantage to permit their entry into breast milk.

All together, we have shown that luminal mammary epithelial cells are able to induce homing of a variety of immune cell types that are also found to be concentrated into breast milk. Prolactin permits an increase in homing of many immune cells to the luminal mammary epithelial cells in vitro and is able to increase immune cell transit to and through the mammary gland in vivo. This work highlights yet another important role that prolactin may play through the process of lactation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Figure 1.1

Estrous cycle is not altered by prolactin treatment for the latter two weeks of treatment.

Vaginal smears were collected every morning from 8:00AM-10:00AM for the last two weeks of treatment. Slides were stained with 1% Toluidine blue and analyzed by light microscope to determine estrous cycle stage on the basis of ratios of cell types present. Data are presented as the average number of days that mice in each group experienced (A) Diestrus (B) Proestrus (C) Estrus (D) Metestrus.P-values were assigned as follows;

* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

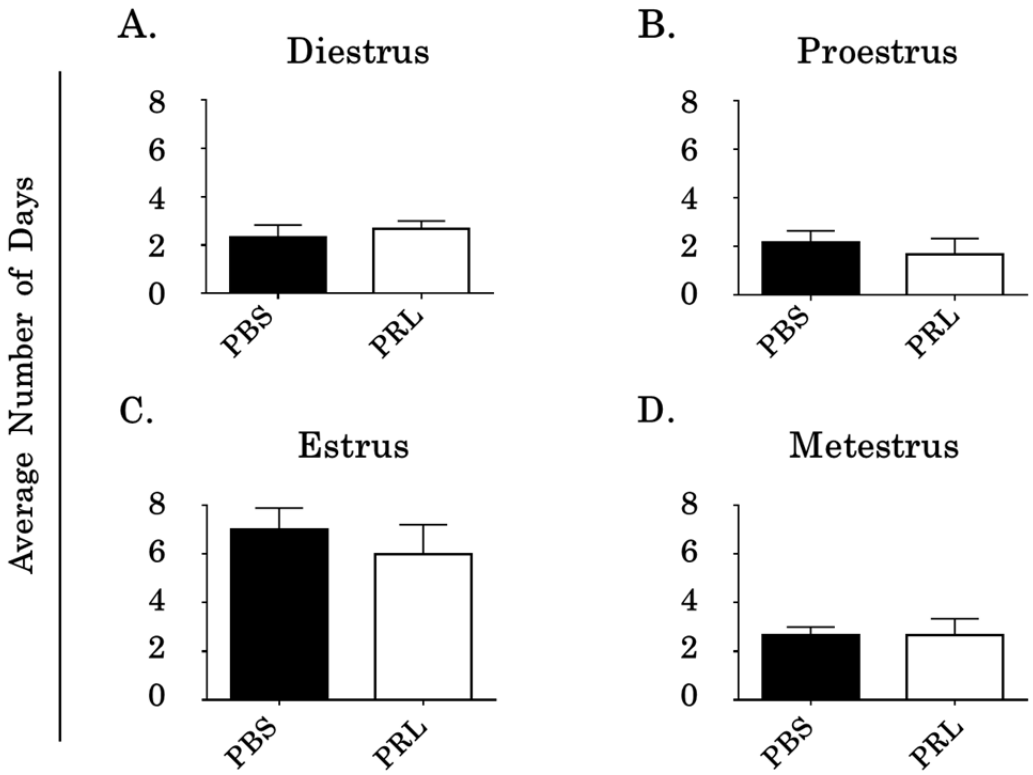

A.
Diestrus
B.
Proestrus
C.
Estrus
D.
Metestrus
Average Number of Days
8
6
4
2
0
PBS
PRL

Figure 1.2
Prolactin increased CD4+, CD8+, and CD19+ cell numbers in mammary gland draining, but not other lymph nodes
Numbers of indicated cell types were determined for each animal by total cell counts per lymph node and flow cytometric analysis of the markers, CD4, CD8, and CD19 for (A) axillary lymph node, (B) inguinal lymph node, and (C) popliteal lymph node. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

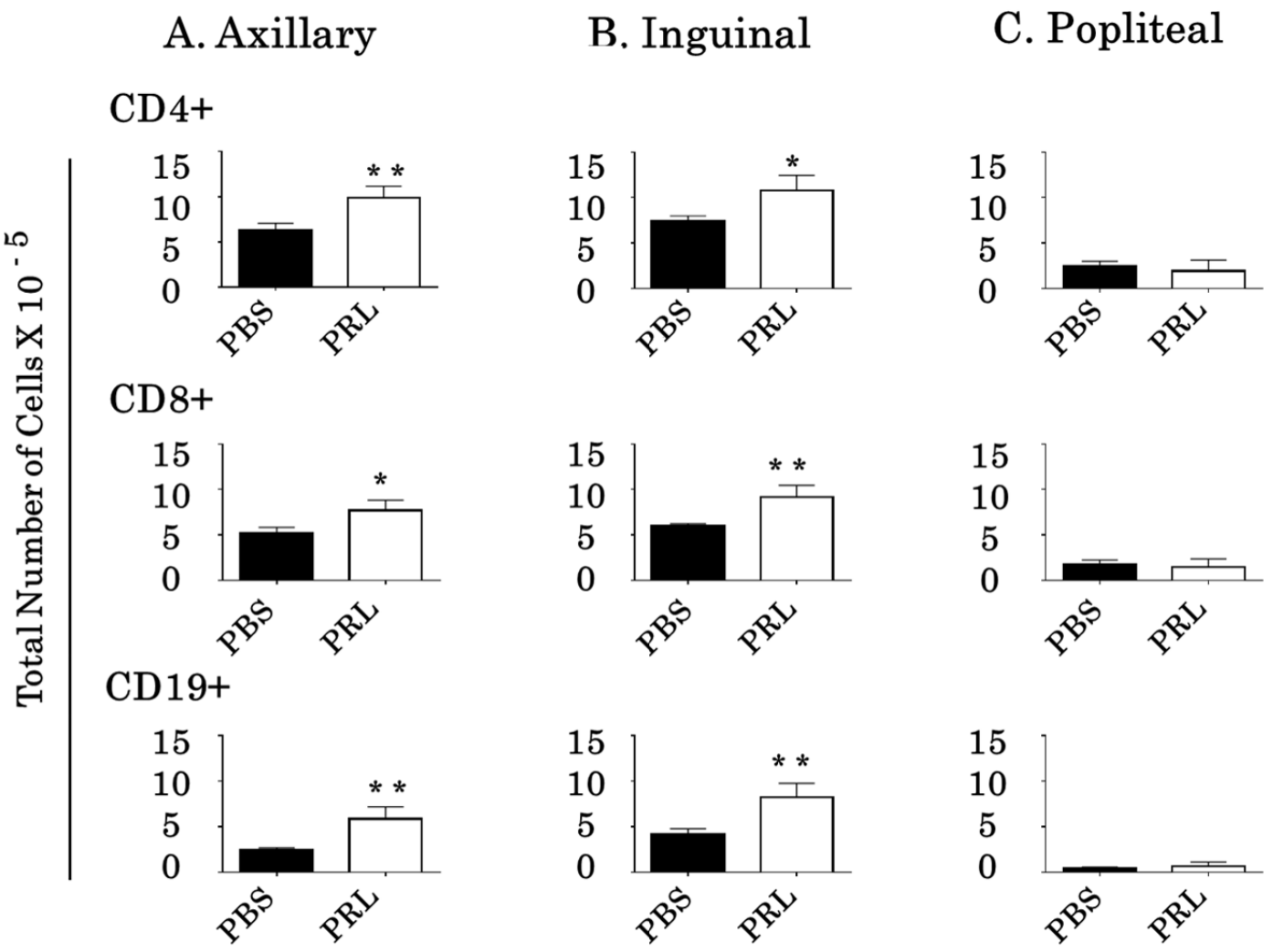

A. Axillary
B. Inguinal
C. Popliteal
CD4+
CD8+
CD19+
Total Number of Cells X 10 - 5
15
10
5
0
* *
*
PBS
PRL

Figure 1.3
Media conditioned by mammary epithelial cells increased migration of lymphocytes.
Splenocytes were loaded into the top of transwells with either serum free media (M) or mammary epithelial cell conditioned serum free media (CM) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) CD4+ T-cells, (B) CD8+ T-cells (C) B-cells, (D) CD4+ T-memory cells, and (E) CD8+ T-memory cells to the bottoms of transwells with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

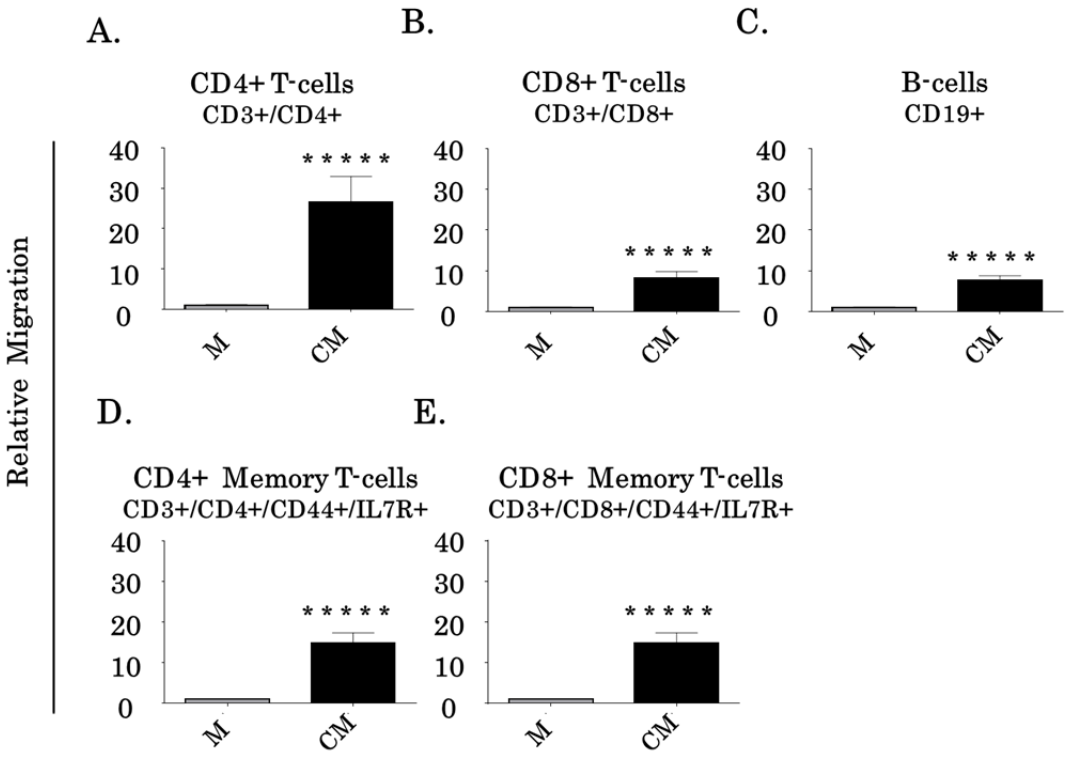

A.
B.
C.
CD4+ T-cells
CD3+/CD4+
CD8+ T-cells
CD3+/CD8+
B-cells
CD19+
Relative Migration
40
30
20
10
0
* * * * *
M
CM
D.
E.
CD4+ Memory T-cells
CD3+/CD4+/CD44+/IL7R+
CD8+ Memory T-cells
CD3+/CD8+/CD44+/IL7R+

Figure 1.4
Media conditioned by mammary epithelial cells increased migration of phagocytes and granulocytes
Splenocytes were loaded into the top of transwells with either serum free media (M) or mammary epithelial cell conditioned serum free media (CM) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) monocytes, (B) macrophages (C) neutrophils, and (D) eosinophils to the bottoms of transwells with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

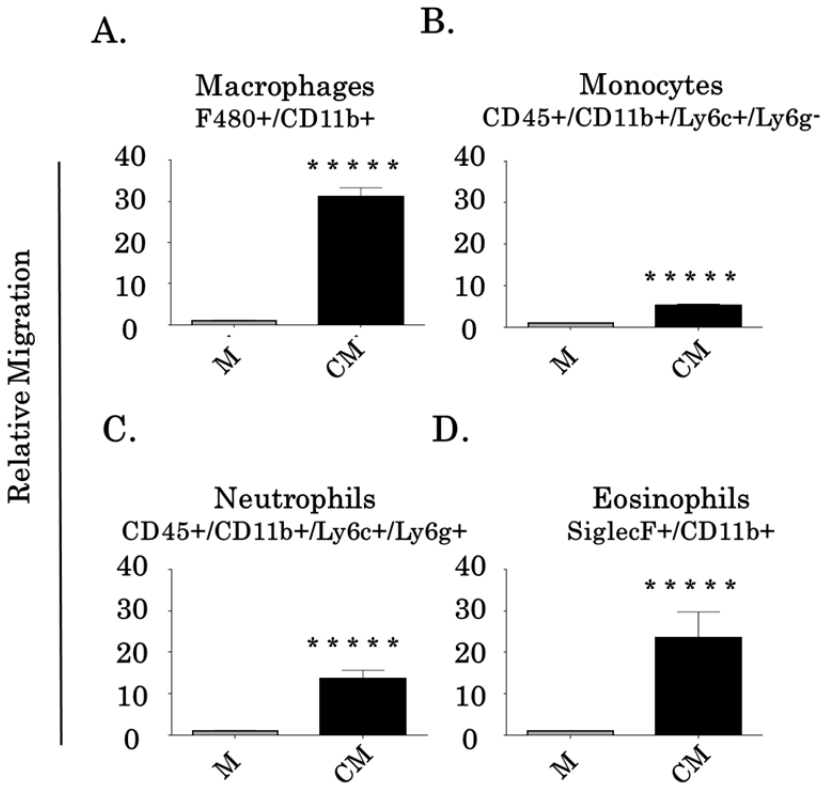

A.
B.
Macrophages
F480+/CD11b+
Monocytes
CD45+/CD11b+/Ly6c+/Ly6g-
Relative Migration
40
30
20
10
0
* * * * *
M
CM
C.
D.
Neutrophils
CD45+/CD11b+/Ly6c+/Ly6g+
Eosinophils
SiglecF+/CD11b+

Figure 1.5
Neutrophils that migrated towards media conditioned by mammary epithelial cells expressed more CD11b
Relative mean fluorescent intensity (MFI) of CD11b on neutrophils that migrated to the bottoms of transwells containing either serum free media (M) or mammary epithelial cell conditioned serum free media (CM) with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

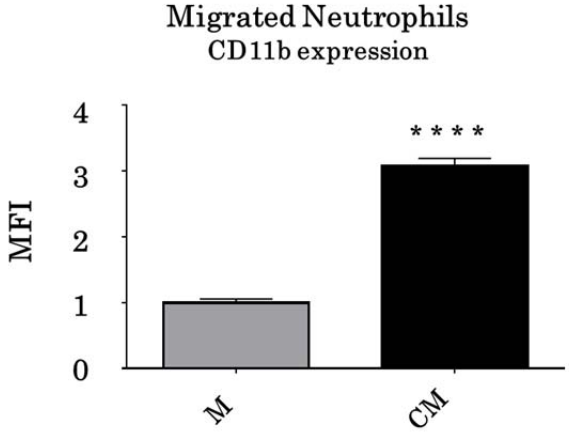

Migrated Neutrophils
CD11b expression
MFI
4
3
2
1
0
* * * *
M
CM

Figure 1.6
CCL2 and CXCL1 depletion of mammary epithelial cell conditioned media reduces monocyte and neutrophil migration
Splenocytes were loaded into the top of transwells with either mammary epithelial cell conditioned serum free media treated with isotype control antibody or chemokine specific antibody (Anti-CCL2 or Anti-CXCL1) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) monocytes with CCL2 depletion, and (B) neutrophils with CXCL1 depletion to the bottoms of transwells with Isotype control = 1. Migration of each cell type in response to serum free media is subtracted from each condition.P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

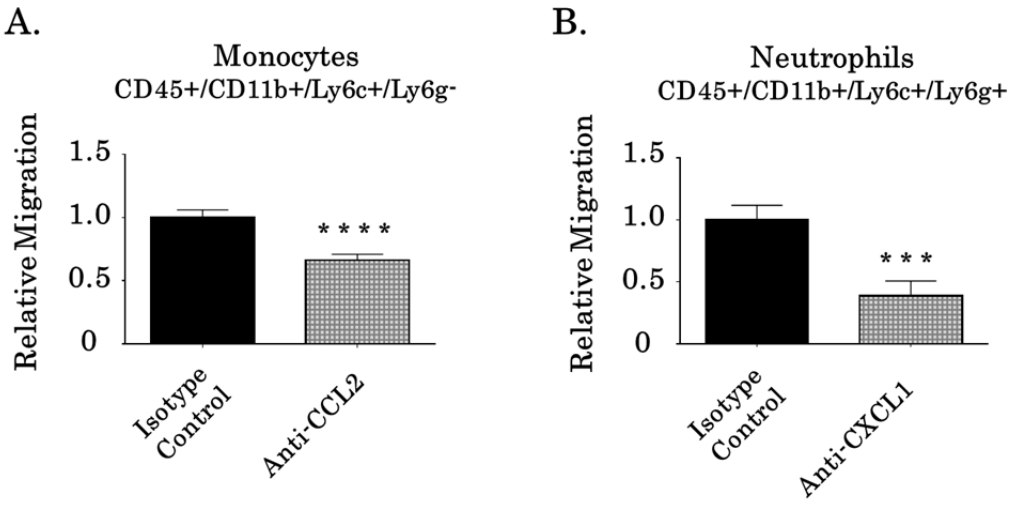

A.
Monocytes
CD45+/CD11b+/Ly6c+/Ly6g-
Relative Migration
1.5
1.0
0.5
0
* * * *
Isotype Control
Anti-CCL2
B.
Neutrophils
CD45+/CD11b+/Ly6c+/Ly6g+
Relative Migration
1.5
1.0
0.5
0
* * *
Isotype Control
Anti-CXCL1

Figure 1.7
Prolactin does not act as a chemoattractant for lymphocytes
Splenocytes were loaded into the top of transwells with either serum free media (M) or serum free media containing 100 ng/mL prolactin (PRL) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) CD4+ T-cells, (B) CD8+ T-cells (C) B-cells, (D) CD4+ T-memory cells, and (E) CD8+ T-memory cells to the bottoms of transwells with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

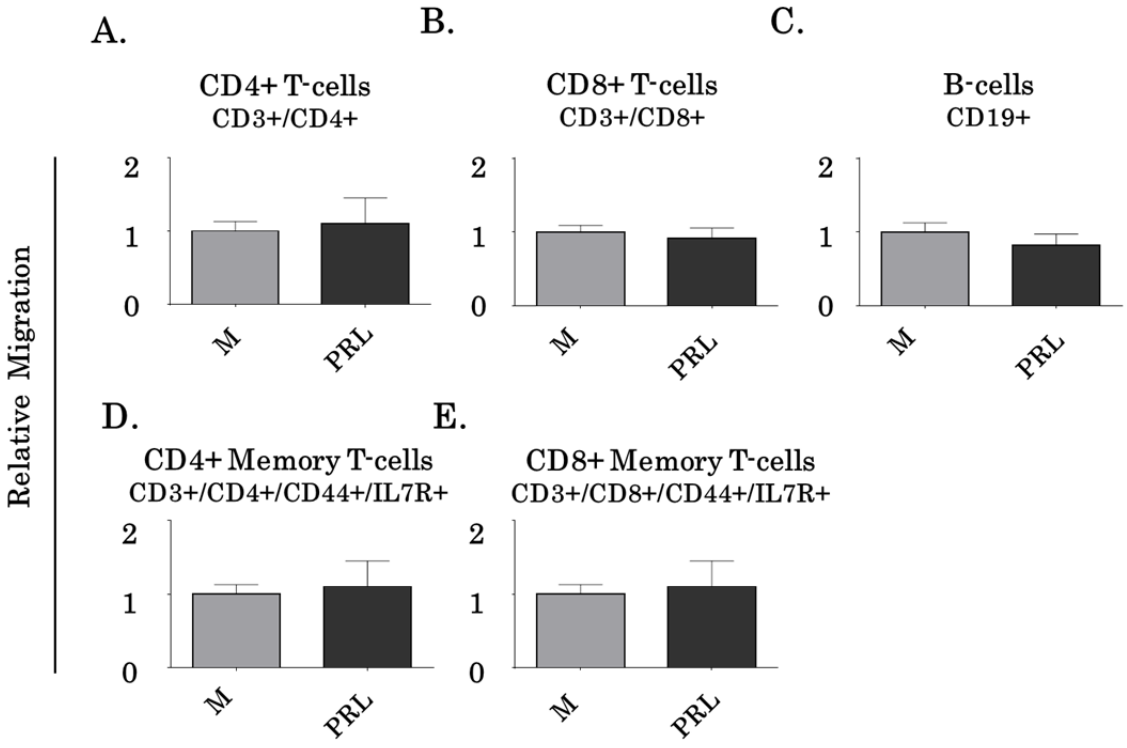

A.
B.
C.
CD4+ T-cells
CD3+/CD4+
CD8+ T-cells
CD3+/CD8+
B-cells
CD19+
Relative Migration
2
1
0
M
PRL
D.
E.
CD4+ Memory T-cells
CD3+/CD4+/CD44+/IL7R+
CD8+ Memory T-cells
CD3+/CD8+/CD44+/IL7R+

Figure 1.8
Prolactin does not act as a chemoattractant for phagocytes or granulocytes
Splenocytes were loaded into the top of transwells with either serum free media (M) or serum free media containing 100 ng/mL prolactin (PRL) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) monocytes, (B) macrophages (C) neutrophils, and (D) eosinophils to the bottoms of transwells with M = 1. P-values were assigned as follows;
* $P< 0.05$, * * $P< 0.02$, * * * $P< 0.01$, * * * * $P< 0.001$, * * * * * $P< 0.0001$.

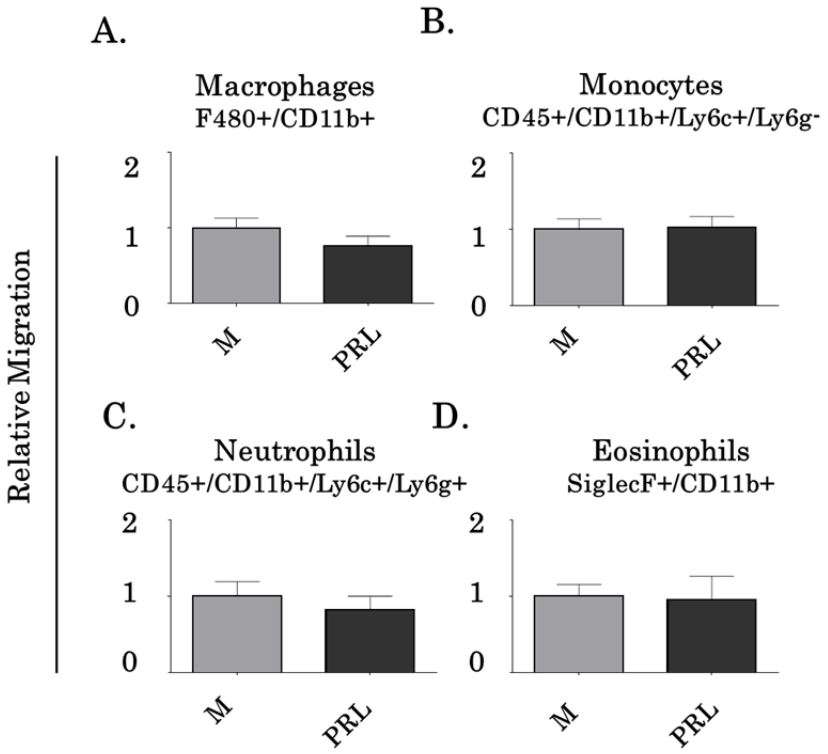

A.
B.
Macrophages
F480+/CD11b+
Monocytes
CD45+/CD11b+/Ly6c+/Ly6g-
Relative Migration
2
1
0
M
PRL
C.
D.
Neutrophils
CD45+/CD11b+/Ly6c+/Ly6g+
Eosinophils
SiglecF+/CD11b+

Figure 1.9
Prolactin treatment of mammary epithelial cells increased migration of most lymphocytes
Splenocytes were loaded into the top of transwells with either mammary epithelial cell conditioned serum free media (CM) or mammary epithelial cell conditioned serum free media from HC11 cells treated with 100 ng/mL prolactin (PRL) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) CD4+ T-cells, (B) CD8+ T-cells (C) B-cells, (D) CD4+ T-memory cells, and (E) CD8+ T-memory cells to the bottoms of transwells with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

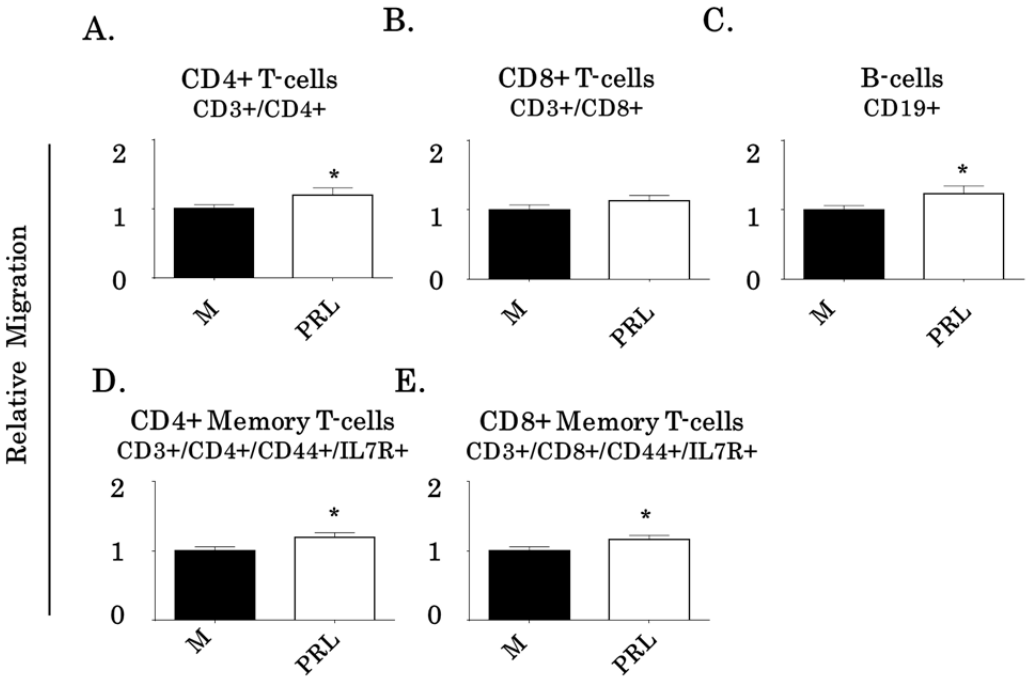

A.
B.
C.
CD4+ T-cells
CD3+/CD4+
CD8+ T-cells
CD3+/CD8+
B-cells
CD19+
Relative Migration
2
1
0
*
M
PRL
D.
E.
CD4+ Memory T-cells
CD3+/CD4+/CD44+/IL7R+
CD8+ Memory T-cells
CD3+/CD8+/CD44+/IL7R+

Figure 1.10
Prolactin treatment of mammary epithelial cells increased migration of phagocytes and granulocytes
Splenocytes were loaded into the top of transwells with either mammary epithelial cell conditioned serum free media (CM) or mammary epithelial cell conditioned serum free media from HC11 cells treated with 100 ng/mL prolactin (PRL) in the bottoms of the transwells. Data are presented as relative migration of immune cells (A) monocytes, (B) macrophages (C) neutrophils, and (D) eosinophils to the bottoms of transwells with M = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

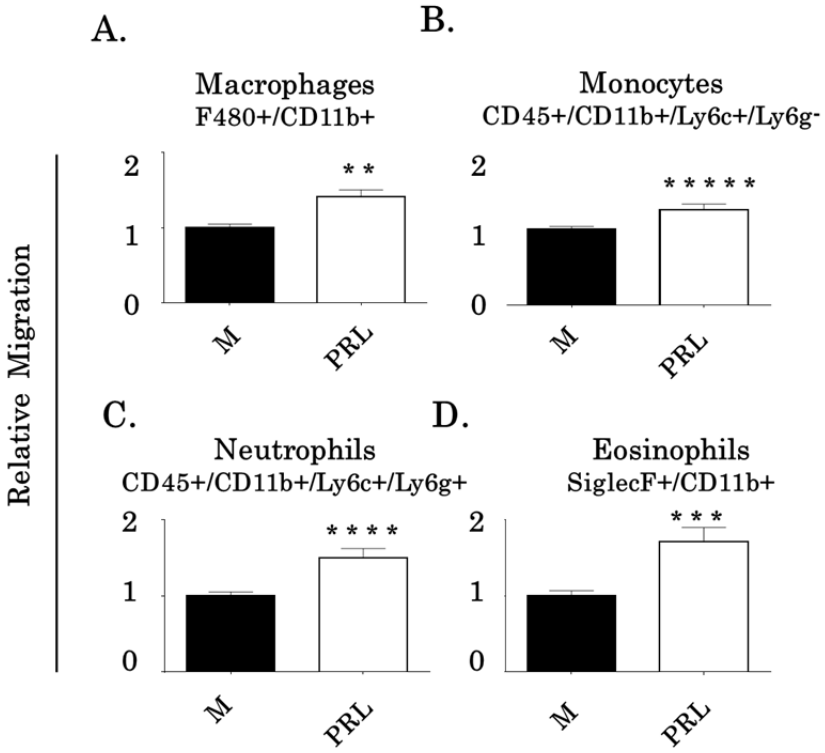

A.
B.
Macrophages
F480+/CD11b+
Monocytes
CD45+/CD11b+/Ly6c+/Ly6g-
Relative Migration
2
1
0
* *
* * * * *
M
PRL
C.
D.
Neutrophils
CD45+/CD11b+/Ly6c+/Ly6g+
Eosinophils
SiglecF+/CD11b+
* * * *
* * *

Figure 1.11
Prolactin regulates mammary epithelial cell cytokine expression.
Relative HC11 mRNA expression of (A) CXCL1 and (B) CCL2 normalized to GAPDH without (PBS) or with prolactin (100ng/mL) treatment (PRL). Data are presented with PBS = 1. P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.

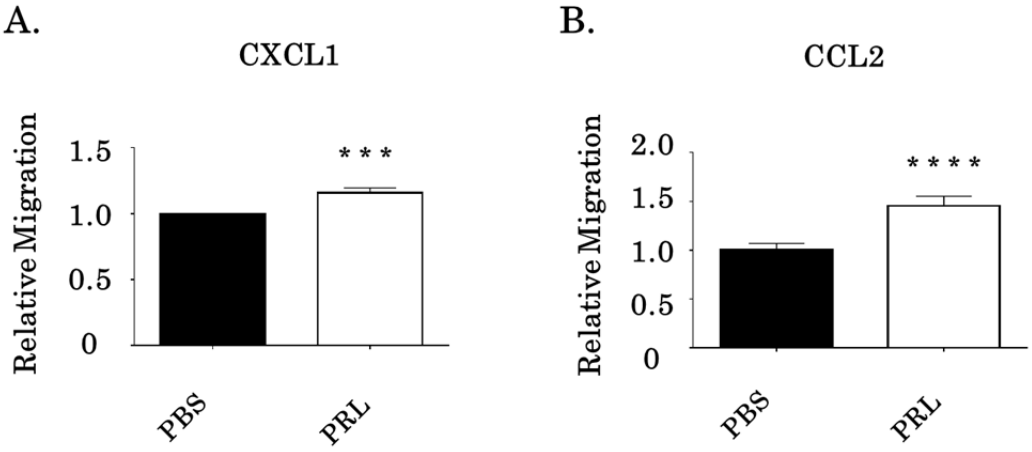
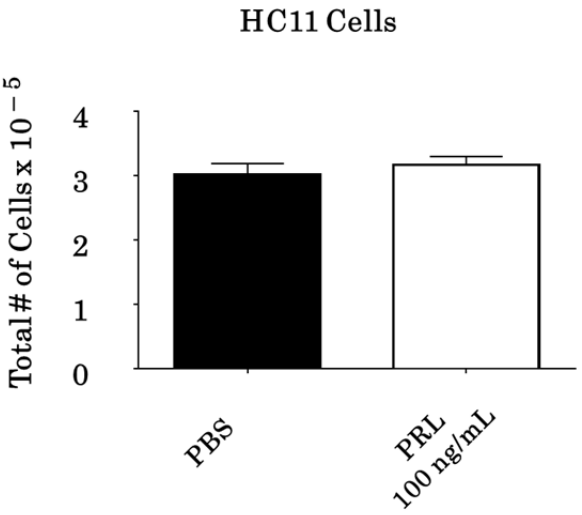

A.
CXCL1
Relative Migration
1.5
1.0
0.5
0
* * *
PBS
PRL
B.
CCL2
Relative Migration
2.0
1.5
1.0
0.5
0
* * * *
PBS
PRL

Figure 1.12
Prolactin treatment did not change HC11 cell numbers
HC11 cell numbers were determined by counts on a hemocytometer for those without (PBS) or with prolactin (100ng/mL) treatment (PRL). P-values were assigned as follows;
* P< 0.05, * * P< 0.02, * * * P< 0.01, * * * * P< 0.001, * * * * * P< 0.0001.


HC11 Cells
Total # of Cells x 10 − 5
4
3
2
1
0
PBS
PRL
100 ng/mL

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Chapter 2

Sexual Dimorphism of the Popliteal Lymph Nodes

ABSTRACT

Adult female mice exhibit stronger delayed type hypersensitivity responses to *Candida albicans*. We have discovered the existence of a sexual dimorphism in the popliteal lymph nodes of male and female mice. While females have more CD4+ and CD8+ T-cells in this lymph node, males have a higher percentage of CD4+ T-cells that are T-regulatory cells. The initial differences develop prior to the onset of puberty and the degree of difference in CD4+ and CD8+ T-cell numbers continues to grow during development into adulthood. At the same time, the difference in percentage of CD4+ T-cells that are T-regulatory cells is maintained at the level first observed prior to the onset of puberty. In this study, we established that the pre-pubertal sex differences are dependent on *Sry*, a gene necessary for male development, by assessing a model of its overexpression. Overexpression of *Sry* led to an exaggeration of the sex difference, but the increasing difference in CD4+ and CD8+ T-cells that occurs into adulthood is dependent on testosterone.

INTRODUCTION

Generally, females are considered to have stronger cell-mediated immune responses when compared to males. Several have reported that in humans the mortality rate of tuberculosis is twice as high in males [Clarke et al. 1956, Frieden et al. 2000]. Experimentally, others have shown that female mice exhibit longer survival than males when infected with *Mycobacterium marinum*, although the duration of survival was variable for males dependent on their strain. They further examined hormonal contribution to this sex difference, and found that testosterone was responsible for poor survival rates of males through castration and hormone replacement studies [Yamamoto et al. 1991]. Upon examination of individuals who spontaneously cleared Hepatitis C Virus during acute infection, a process known to be reliant on cytotoxic CD8+ T-cells [Sung et al. 2014], women were found to be more likely to do so [Grebely et al. 2014]. Although many of these studies show strong correlation between the sex of an individual and the degree of disease burden, evidence for what underlies the stronger immune responses in females is very limited.

Our lab previously demonstrated that female mice exhibit a larger delayed-type hypersensitivity response to *Candida albicans* when compared to male mice [Ma et al. 2007]. This is a T-cell mediated immune response model [Janeway et al. 2001] accomplished by sensitization of the mouse by

intradermal injection of *Candida albicans*, and challenge with *Candida albicans* purified protein injected directly into the footpad of the mouse. The major draining lymph node most relevant to the mouse footpad is the popliteal lymph node, and so we examined the potential existence of an inherent sexual dimorphism of relevance to the sex difference in immune responses previously observed.

Two factors that could contribute to the development of a sexually-dimorphic phenotype are gonadal hormone production and *Sry* expression. It is difficult to independently determine contributions of male sex hormones and *Sry* expression because of their connection to one another. The *Sry* gene is located on the Y chromosome and is responsible for testicular development and ultimately the production of male sex steroids. In the mouse, *Sry* is normally expressed during a very small window of time, embryonic day 10.5 through embryonic day 12.5 **[Hacker et al. 1995]**, and is critical for development as a male **[Berta et al. 1990]**. It is not possible to knock down or out this gene during this critical period without confounding the results due to feminization of the animals **[Wu et al. 2012]**.

An alternative approach to studying other functions of *Sry* is in a context of its overexpression and thus potential exaggeration of a phenotype of interest. In the Four Core Genotypes (FCG) mouse model, the *Sry* gene

was deleted from the Y chromosome and reintroduced as a transgene onto an autosome, allowing it to segregate independently from the Y chromosome. Upon more recent assessment of the model, it was noted that there were 12-14 copies of the *Sry* gene inserted onto chromosome 3 **[Itoh et al. 2015]**. As a result of reinsertion, the *Sry* gene became overexpressed in FCG male tissues when compared to wildtype male mice of the same strain background. Although *Sry* is overexpressed, there are no differences in circulating testosterone levels in adult FCG male mice compared to wild type male mice **[Manwani et al. 2015]**.The increased copy number and expression of the gene permitted a means for studying effects of *Sry* through "overexpression".

We hypothesized that there may be an inherent sexual dimorphism of T-cell populations in the popliteal lymph nodes and upon discovering that such a difference in fact exists; we examined the relative contributions of *Sry* expression and male and female gonadal secretions to its development.

MATERIALS AND METHODS

Animals

Adult (8-19 weeks of age) male and female C57Bl/6J mice were obtained from Jackson Laboratories. Mice were either used for experiments shortly after obtaining the animals from Jackson Laboratories, or bred for additional experiments I order to use mice of various ages. Additionally, 8 week old Four Core Genotype (FCG) XY^{-} $^{+Sry}$ (C57Bl6/J background) male mice were obtained from Jackson Laboratories. FCG XY^{-} $^{+Sry}$ male mice were bred to C57/Bl6 female mice and FCG (*Sry* overexpressing) male mice derived from this breeding scheme were utilized for experiments described herein. Animals were housed in a specific pathogen free facility on a 12-hour light dark cycle. All animal procedures were approved by the University of California, Riverside, institutional animal care and use committee and were in accordance with guidelines from the American Association for Laboratory Animal Care, the United States Department of Agriculture, and the National Institutes of Health.

DNA extraction and Genotyping

DNA was extracted from tail clips using the Qiagen DNEasy kit per manufacturer instructions. Classical PCR was performed using the genotyping protocol recommended by Jackson Mice for the FCG mouse.

Gonadectomies

C57Bl/6 male and female mice were either castrated or ovariectomized at 21 days of age. Surgeries were performed with isoflurane anesthetic and mice were kept on a heating pad for the duration of the procedure. Hair was removed from surgical site and the site was wiped with Providone Iodine solution and subsequently wiped with 70% ethanol. Ovaries or testes were ligated prior to removal and final closure was made with surgical staples. Mice were kept under surveillance until they awoke and demonstrated the ability to be motile. After 7 days, surgical staples were carefully removed if the incision site was completely healed. Experiments were performed at 6 weeks of age.

Tissue collection

Mice were euthanized by CO2 asphyxiation. Popliteal lymph nodes were collected from both legs of each mouse and combined to be gently pressed

through sterile 70 uM cell strainers (Corning 352350 Manassas, VA) in RPMI1640 (Corning 10-040). Medium with cells was then transferred to a conical tube and samples were all brought to the same volume with fresh RMPI 1640. Cells were counted with an automated cell counter Cell Scepter (EMD Millipore PHCC20040 Billerica, MA) with a 40 uM sensor (EMD Millipore PHCC40050). Cell counts were determined as number of cells per milliliter, and this number was multiplied to the total number of mL to which samples were brought in order to calculate total number of cells per tissue for each animal. Cells were kept on ice and cell counts were used for downstream applications.

Flow cytometric analysis

All antibodies were diluted in 0.2% BSA in Dulbecco's phosphate buffered saline (FACS Buffer). Fc receptors were blocked with purified anti-CD16/32 (eBioscience 14-0161, San Diego CA) diluted 1:50 for 15 minutes at 4 °C. Cells were washed with FACS buffer, and resuspended in a mixture of fluorescently-labeled antibodies at the following dilutions, 0.5:100 rat anti-mouse CD3 (eBioscience 11-0031), 0.5:100 rat anti-mouse CD4 (eBioscience 47-0041), 0.5:100 rat anti-mouse CD8 (eBioscience 17-0081), 0.5:100 rat anti-mouse CD19 (eBioscience 25-0193), 0.5:100 rat anti-mouse CD25 (eBioscience

12-0251), 0.5:100 rat anti-mouse CD62L (eBioscience 25-0621), 1:100 rat anti-mouse CD11a (eBioscience 17-0111), or appropriate isotype controls for 1 hour at 4 °C in the dark. For T-regulatory cell staining, after Fc block and extracellular staining with CD4 and CD25, intracellular staining for 0.6:100 rat anti-mouse FoxP3 (eBioscience 45-5773) was performed using an intracellular staining set (eBioscience 00-5523) per manufacturer's instructions. Cells were washed and re-suspended in FACS buffer and run live on the BD Facs Canto II, with exception of samples with T-regulatory cell staining, which were fixed and permeabilized, and thus dead at the time of running samples. FlowJo software was used for analysis. Total numbers of cell types per tissue were determined by multiplying percentage of cell type of interest present in total gated immune cells by the total number of cells per tissue for the animal from which it originated.

Statistical Analyses

Comparisons were analyzed by Student t-test. Data were presented as mean + Standard Error. P-values were assigned as follows;

* $P< 0.05$, * * $P< 0.01$, * * * $P< 0.001$, * * * * $P< 0.0001$.

RESULTS

Sexual dimorphism in mature adults

Given the increased T-cell mediated immune response to *Candida albicans* in female compared to male mice previously described, we assessed T-cell numbers in the popliteal lymph node, the lymph node most relevant to generation of the observed immune response. We found that female mice had twice as many cells as males **(Figure 2.1 a)**. Females had 105% T-cells (CD3+), 90% more CD4+ T-cells (CD3+/CD4+) and 120% more CD8+ T-cells (CD3+/CD8+), than males (**Figure 2.2 a, 2.2 b, and 2.2 c)**. Males had 20% higher percentages of CD4+ cells that were T-regulatory cells (CD25+/FOXP3+) **(Figure 2.2 d)**.

Sexual dimorphism prior to puberty

Although in mice the first vaginal cornification, an indication of ovulation, may not occur until 34-36 days of age **[Nelson et al. 1982]**, female mice are physiologically affected by circulating estrogen as early as 24 to 26 days of age as is evidenced by vaginal opening **[Ojeda and Urbanski et al. 1994]**. To avoid potential influences of early estrogen or pubertal levels of hormones, we chose 21 days of age to assess the existence of any pre-pubertal

sex differences in the popliteal lymph nodes. Even at this age, we found that mice exhibited immune sex differences. Female mice had 25% more cells, than males **(Figure 2.1 b)**. Females had 20% more T-cells, 15% more CD4+ T-cells and 30% more CD8+ T-cells, than males (**Figure 2.3 a, 2.3 b, and 2.3 c)**. Males already had 20% higher percentages of CD4+ cells that were T-regulatory cells **(Figure 2.3 d)**.

Sexual dimorphism in young adults

At 6 weeks of age, mice exhibited greater sex differences of the popliteal lymph nodes. Female mice had 25% more cells in their popliteal lymph node when compared to males **(Figure 2.1 c)**. Females had 40% more T-cells, 45% more CD4+ T-cells, and 40% more CD8+ T-cells **(Figure 2.4 a, 2.4 b, and 2.4 c)**. Males had an even higher percentage (40%) of CD4+ cells that were T-regulatory cells **(Figure 2.4 d)**.

Pre-pubertal sexual dimorphism is regulated by Sry

To determine whether *Sry* expression contributed to the pre-pubertal establishment of sexual dimorphism in the popliteal lymph node, we compared wild type males with males overexpressing *Sry* at 3 weeks of age.

At this age, wildtype males had twice as many cells in their popliteal lymph node when compared to *Sry* overexpressing males **(Figure 2.5)**. Wildtype males had 4 times as many T-cells, 3 times as many CD4+ T-cells, and 15 times as many CD8+ T-cells **(Figure 2.6 a, 2.6 b, and 2.6 c)**. Sry over expressing males also had 65% higher percentages of CD4+ cells that were T-regulatory cells **(Figure 2.6 d)**.

Young adult sexual dimorphism is regulated by male gonadal secretions

The degree of sexual dimorphism increased from 3 to 6 weeks of age. During this period, mice enter puberty and thus are exposed to adult levels of gonadal hormones **[Szauter et al. 2016]**. To determine whether male gonadal hormones or female gonadal hormones were responsible for the increasing dimorphism gonadectomies or sham surgeries were performed on mice at 3 weeks of age and the cellular makeup of the popliteal lymph nodes determined at 6 weeks of age. CD4+ and CD8+ T-cell numbers in the popliteal lymph nodes of ovariectomized mice were not different from sham female mice. However, cell numbers increased in castrated mice to attain female levels **[Figure 2.7 a and 2.7 b]**.

Lymphocyte homing and adhesion molecules

Differential numbers of cells in the popliteal lymph node could have been related to differential expression of lymphocyte homing and adhesion molecules. Of particular relevance to CD4+ and CD8+ T-cells are CD62L and CD11a.

CD62L, also known as L-Selectin, is an adhesion molecule expressed on lymphocytes that is important for their entrance into secondary lymphoid tissue. CD62L binds to Glycosylation dependent cell adhesion molecule 1 (GLyCAM-1) **[Lasky et al. 1992]** and the mucosal addressin, cell adhesion molecule 1 (MAdCAM-1) **[Berg et al. 1993]**. CD11a, also known as Integrin alpha L, is the alpha chain of the lymphocyte function-associated antigen – 1, which mediates intracellular adhesion molecule (I-CAM)-mediated cell attachment to endothelial cells and entry into sub-endothelial connective tissue **[Rothlein et al. 2011]**.

As was true for total cells, females had more CD4+ and CD8+ T-cells that were CD11a+ and CD62L+ in their popliteal lymph nodes than males at both 3 **(Figure 2.8 a, 2.8 b, 2.8 c, and 2.8 d)** and 6 weeks of age **(Figure 2.9 a, 2.9 b, 2.9 c, and 2.9 d)**. However, at either 3 or 6 weeks of age, females and males did not differ in the amount of CD11a or CD62L expressed per cell for CD4+ T-cells **(Figure 2.10 a, 2.10 c, and 2.11 a, 2.11 c)**, as determined by

mean fluorescence intensity. While, at 3 weeks of age, males and females did not differ in the amount of CD11a or CD62L expressed per cell for CD8+ T-cells **(Figure 2.10 b, 2.10 d, and 2.11 b)**, at 6 weeks of age there was a slight, but significantly higher expression of CD62L on male CD8+ T-cells **(Figure 2.11 d)**

DISCUSSION

Delayed-type hypersensitivity responses to *Candida albicans* are T-cell mediated immune responses. Mice are sensitized by intradermal injection and antigen presenting cells digest and display peptides of *Candida albicans* on MHC molecules to the animal's T-cells. This, in combination with induction of co-stimulatory molecules activates T-cells and leads to the development of specific immunologic memory in T-cells that circulate and are distributed around the body. Upon challenge, in this case through footpad injection with *Candida albicans* purified protein, various antigen presenting cells such as macrophages and dendritic cells take peptides to the tissue-draining popliteal lymph node where they may encounter appropriate, antigen-specific memory T-cells, thereby precipitating an immune response. T-cells proliferate and travel to the site of challenge where they produce inflammatory cytokines and signal to local endothelial cells to increase adhesion molecule expression and blood vessel permeability. This allows plasma and circulating cells to enter and respond, resulting in swelling of the tissue, measurement of which can determine the degree of immune response **[Janeway et al. 2001]**.

Although from previous work in the laboratory we knew that females exhibited greater swelling of their footpads than males in this assay **[Ma et al. 2007]**, our goal in this study was determine the basis of this difference by

examining potential sex differences in the most relevant lymph node. Upon examination, we discovered that females maintained larger numbers of CD4+ and CD8+ T-cells in their lymph nodes than males. Having higher numbers of effector cells could well result in more robust responses.

T-regulatory cells suppress immune responses **[Takahashi et al. 1991 and Thornton and Shevach 1998]**. We assessed the percentage of CD4+ cells that were T-regulatory cells and found that males had a higher percentage of CD4+ T-cells that were T-regulatory cells. Therefore, not only do males have fewer effector cells, but they also have a larger proportion of T-cells that can dampen any response.

Prior to the onset of puberty, sexual dimorphism of the popliteal lymph node is already in place, albeit at a smaller degree for CD4+ and CD8+ T-cell numbers than is found in adult mice. This was a surprising discovery as we first predicted that the sexual dimorphism would be entirely dependent on adult circulating gonadal secretions. From our results, we can conclude that this pre-pubertal sexual dimorphism is to some extent dependent on *Sry* expression as its overexpression produces an exaggeration of the normal dimorphism. To our knowledge, no one has previously demonstrated *Sry* to have a role in establishment of lymph node populations. Although one associates *Sry* with the development of the testes and therefore the production of testosterone, it is important to reiterate that testosterone levels

are not different between the wildtype and *Sry* overexpressing mice. There must be some other result of *Sry* expression. *Sry* expression in the developing mouse from embryonic day 10.5 to embryonic day 12.5 is essential to proper gonadal development of the male **[Berta et al. 1990]**. Development of the structure of the lymph node is initiated around embryonic day 10 and completes around embryonic day 15.5 **[Van de Pavert and Mebius 2014]**. The coincidence in timing makes it tempting to speculate that *Sry* expression may play a role in development of the lymph node, but this remains to be examined by further experimentation. Alternatively, *Sry* expression may influence thymic output of T-cells, as discussed in chapter 5.

Although we have demonstrated that *Sry* contributes to pre-pubertal sexual dimorphism of the popliteal lymph node, we must still consider the potential contribution of the perinatal testosterone surge. Around birth, male mice experience a large surge in testosterone, which, through its conversion to estradiol, is responsible for masculinization of the brain **[Wu et al. 2009]**. Future experiments will be performed to determine if the perinatal testosterone surge contributes to the sexual dimorphism of the popliteal lymph nodes by function of either testosterone or its conversion to estradiol. This will be accomplished through treatment of male pups with flutamide or letrozole, respectively. Flutamide functions to prevent testosterone from

signaling through the androgen receptor, and letrozole acts by inhibiting aromatase, and thus testosterone's conversion to estradiol.

Adult male gonadal secretions amplify sexual dimorphism of the popliteal lymphnode. Only one other study has found any sexual dimorphism by measuring T-cell numbers, in this case in the peritoneal and pleural cavities. They found adult females had more T-cells in the peritoneal and pleural cavities than adult males [Scotland et al. 2011]. They did not however assess pre-pubertal sex differences or the relative role of male gonadal secretions. Instead, they focused on the function of female gonadal hormones, but were unable to explain the sex differences in T-cell numbers by the presence or absence of female gonadal hormones, as was true in our study. It is very possible that the elements we describe herein were responsible.

Although we found fewer T-cells expressing certain adhesion molecules in males, this was a consequence of the smaller number of cells overall in males. Contrary to expectations, we did not find any substantial differences in their expression per cell and thus no explanation inherent to the T-cells themselves for lower accumulation in the lymph node. It seems more likely therefore that the difference lies in the structural components of the lymph node. One possibility is that high endothelial venules of females express more GLyCAM-1, MaDCAM-1, and I-CAM than males, allowing for the entry of more CD11a+ and CD62L+ T-cells. However, it is also possible that female

T-cells could express more of these adhesion molecules in the circulation and quickly downregulate their expression after entry into the lymph node.

All together we have shown that there is an inherent sex difference in the number of T-cells in the popliteal lymph nodes, and suggest that this may underlie the sex differences observed in at least one T-cell mediated immune response. In the process of examination, we have also uncovered a potential new role for the *Sry* gene product in development of secondary lymphoid tissue. This work highlights the importance of examining sex as a variable in immunologic experiments since by overlooking this factor one could lose valuable information regarding sex differences in disease progression or treatment responsiveness.

Figure 2.1
Total number of cells the popliteal lymph nodes of C57Bl/6 mice.
Data represent the total number of cells counted in popliteal lymph nodes of C57Bl/6 males and females at (A) 5 months of age (B) 3 weeks of age, and (C) 6 weeks of age. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

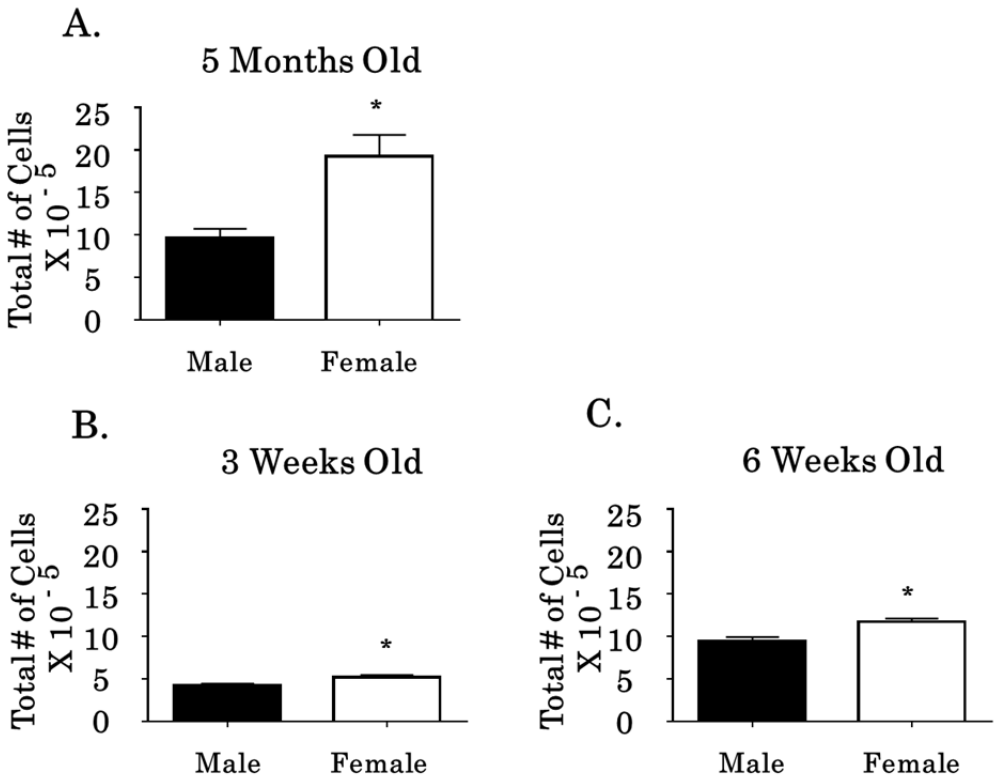

A.
5 Months Old
Total # of Cells X 10 - 5
25
20
15
10
5
0
*
Male
Female
B.
3 Weeks Old
Total # of Cells X 10 - 5
25
20
15
10
5
0
*
Male
Female
C.
6 Weeks Old
Total # of Cells X 10 - 5
25
20
15
10
5
0
*
Male
Female

Figure 2.2
T-cells in the popliteal lymph nodes of 5 month old C57Bl/6 mice
Data are shown for total numbers of (A) T-cells, (B) CD4+ T-cells (C) CD8+ T-cells, and (D) % of CD4+ T-cells that are T-regulatory (CD25+/FOXP3+) in the popliteal lymph nodes of 5 month old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

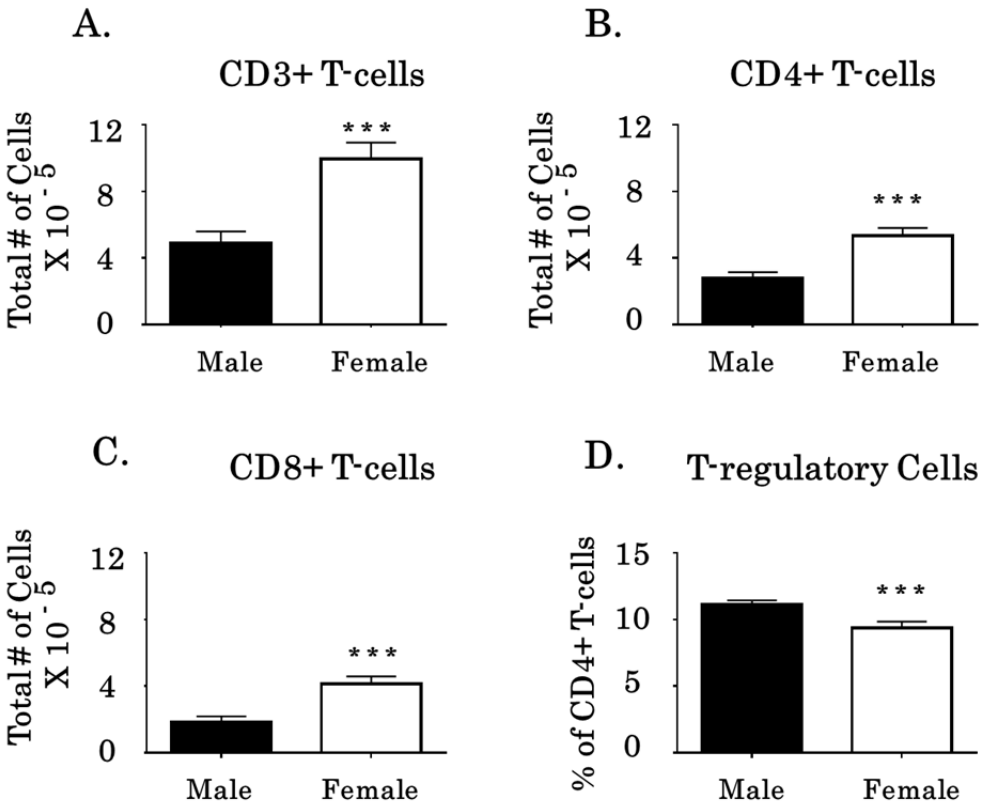

A.
CD3+ T-cells
Total # of Cells X 10 - 5
12
8
4
0
***
Male
Female
B.
CD4+ T-cells
Total # of Cells X 10 - 5
12
8
4
0
***
Male
Female
C.
CD8+ T-cells
Total # of Cells X 10 - 5
12
8
4
0
***
Male
Female
D.
T-regulatory Cells
% of CD4+ T-cells
15
10
5
0
***
Male
Female

Figure 2.3
T-cells in the popliteal lymph nodes of 3 week old C57Bl/6 mice
Data are shown for total numbers of (A) T-cells, (B) CD4+ T-cells (C) CD8+ T-cells, and (D) % of CD4+ T-cells that are T-regulatory (CD25+/FOXP3+) in the popliteal lymph nodes of 3 week old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

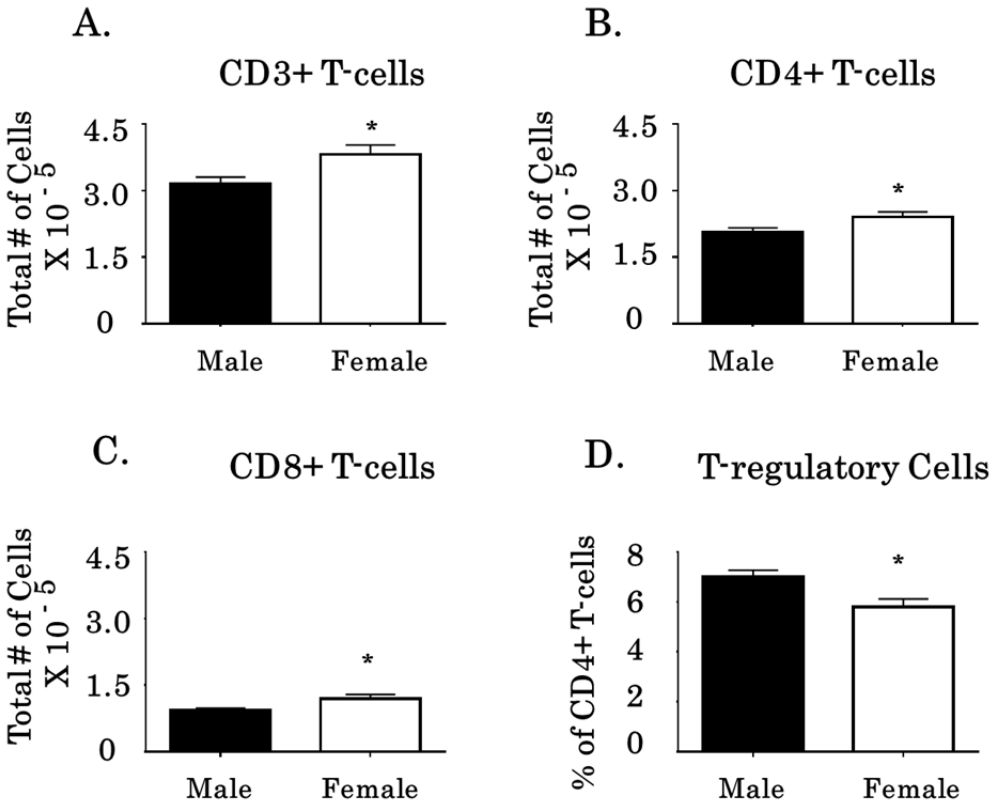

A.
CD3+ T-cells
Total # of Cells X 10 - 5
4.5
3.0
1.5
0
*
Male
Female
B.
CD4+ T-cells
Total # of Cells X 10 - 5
4.5
3.0
1.5
0
*
Male
Female
C.
CD8+ T-cells
Total # of Cells X 10 - 5
4.5
3.0
1.5
0
*
Male
Female
D.
T-regulatory Cells
% of CD4+ T-cells
8
6
4
2
0
*
Male
Female

Figure 2.4
T-cells in the popliteal lymph nodes 6 week old C57Bl/6 mice
Data are shown for total numbers of (A) T-cells, (B) CD4+ T-cells (C) CD8+ T-cells, and (D) % of CD4+ T-cells that are T-regulatory (CD25+/FOXP3+) in the popliteal lymph nodes of 6 week old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

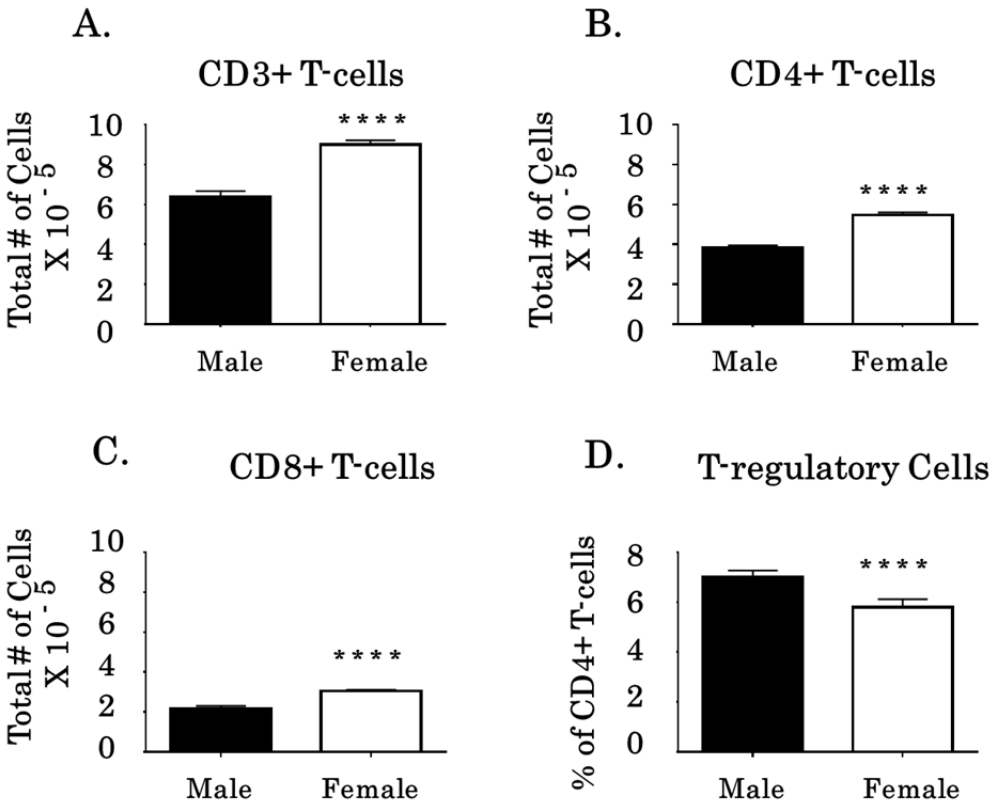

A.
CD3+ T-cells
Total # of Cells X 10 - 5
10
8
6
4
2
0
****
Male
Female
B.
CD4+ T-cells
Total # of Cells X 10 - 5
10
8
6
4
2
0
****
Male
Female
C.
CD8+ T-cells
Total # of Cells X 10 - 5
10
8
6
4
2
0
****
Male
Female
D.
T-regulatory Cells
% of CD4+ T-cells
8
6
4
2
0
****
Male
Female

Figure 2.5
Total number of cells in the popliteal lymph nodes of wildtype C57Bl/6 males and *Sry* overexpressing males at 3 weeks of age
Data are shown for total numbers of cells in the popliteal lymph nodes of wildtype C57Bl/6 males (WT) and *Sry* overexpressing males (*Sry* Hi) at 3 weeks of age. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

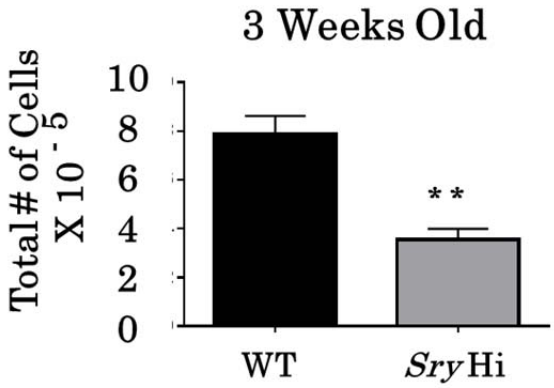

3 Weeks Old
Total # of Cells
X 10 - 5
10
8
6
4
2
0
* *
WT
Sry Hi

Figure 2.6
T-cells in the popliteal lymph nodes of 3 week old wild type C57Bl/6 males and *Sry* overexpressing males
Data are shown for total numbers of (A) T-cells, (B) CD4+ T-cells (C) CD8+ T-cells, and (D) % of CD4+ T-cells that are T-regulatory (CD25+/FOXP3+) in the popliteal lymph nodes of 3 week old wild type C57Bl/6 males (WT) and *Sry* overexpressing males (*Sry* Hi). P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

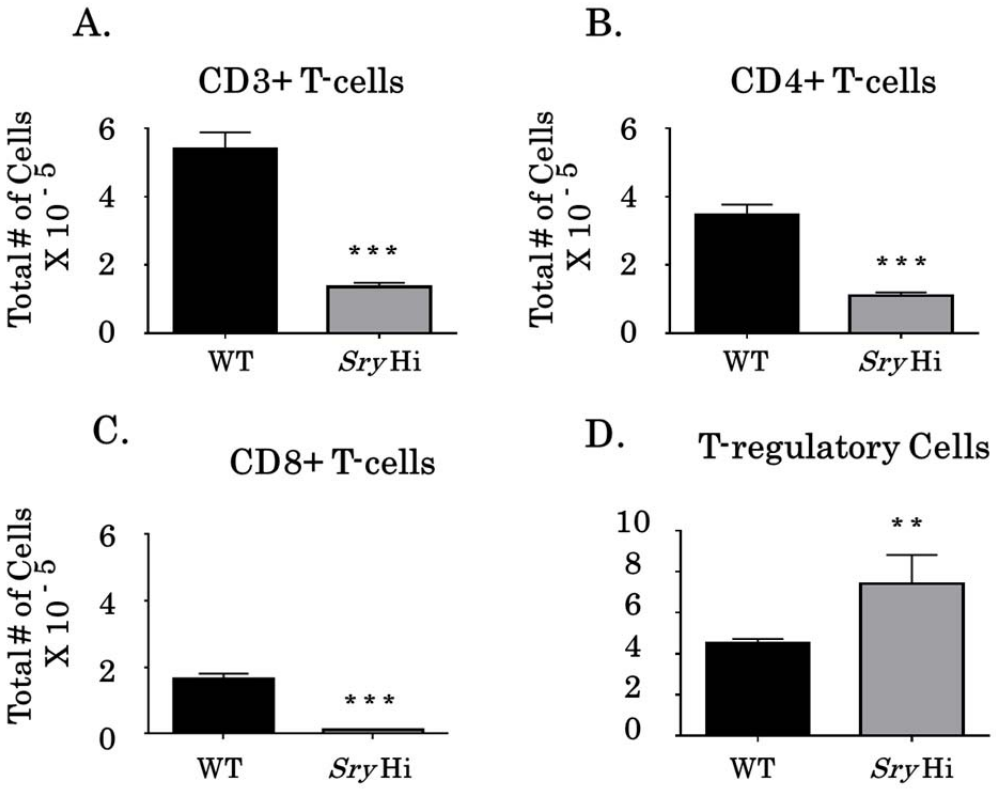

A.
CD3+ T-cells
Total # of Cells X 10 - 5
6
4
2
0
***
WT
Sry Hi
B.
CD4+ T-cells
Total # of Cells X 10 - 5
6
4
2
0
***
WT
Sry Hi
C.
CD8+ T-cells
Total # of Cells X 10 - 5
6
4
2
0
***
WT
Sry Hi
D.
T-regulatory Cells
10
8
6
4
2
0
**
WT
Sry Hi

Figure 2.7
T-cells numbers in the popliteal lymph node 6 week old C57/Bl6 mice with either gonadectomy or sham surgery
Data are shown for total numbers of (A) CD4+ T-cells, (B) CD8+ T-cells in the popliteal lymph nodes of 6 week old males and females that had were female with sham surgery (F SHAM), female with ovariectomy (F OVX), male with sham surgery (M SHAM), or male with castration (M CAST) . P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

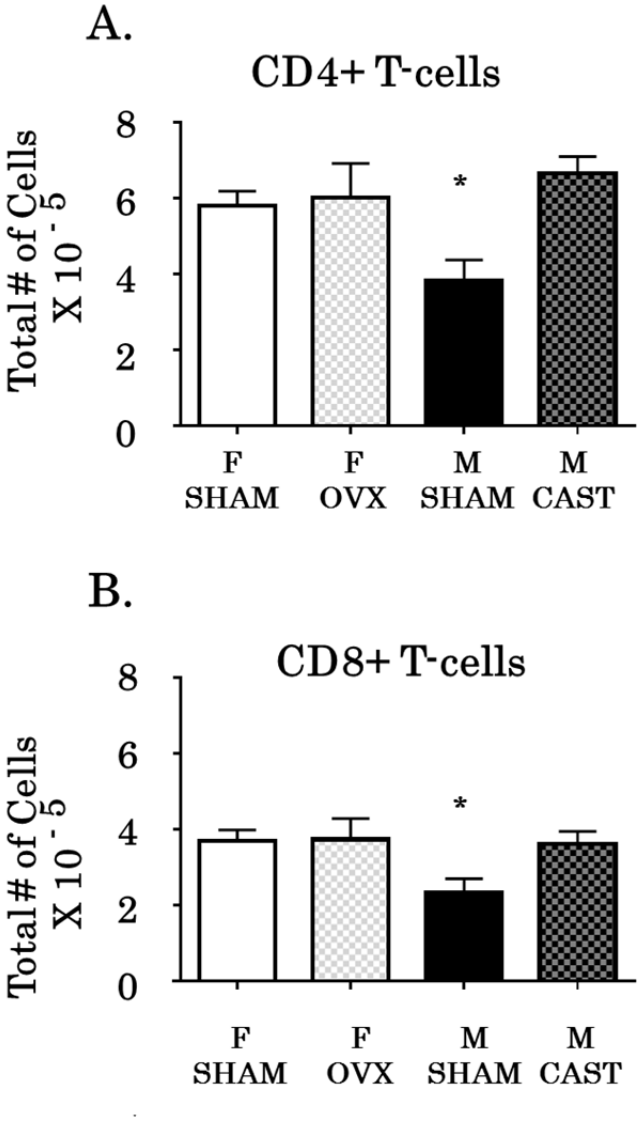

A.
CD4+ T-cells
Total # of Cells
X 10 - 5
8
6
4
2
0
*
F
SHAM
F
OVX
M
SHAM
M
CAST
B.
CD8+ T-cells
Total # of Cells
X 10 - 5
8
6
4
2
0
*
F
SHAM
F
OVX
M
SHAM
M
CAST

Figure 2.8
CD11a+ and CD62L + T-cells in the popliteal lymph nodes of 3 week old C57Bl/6 mice
Data are shown for total numbers of (A) CD4+/CD11a+ T-cells, (B) CD8+/CD11a+ T-cells, (C) CD4+/CD62L+ T-cells, and (D) CD8+/CD62L+ T-cells in the popliteal lymph nodes of 3 week old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

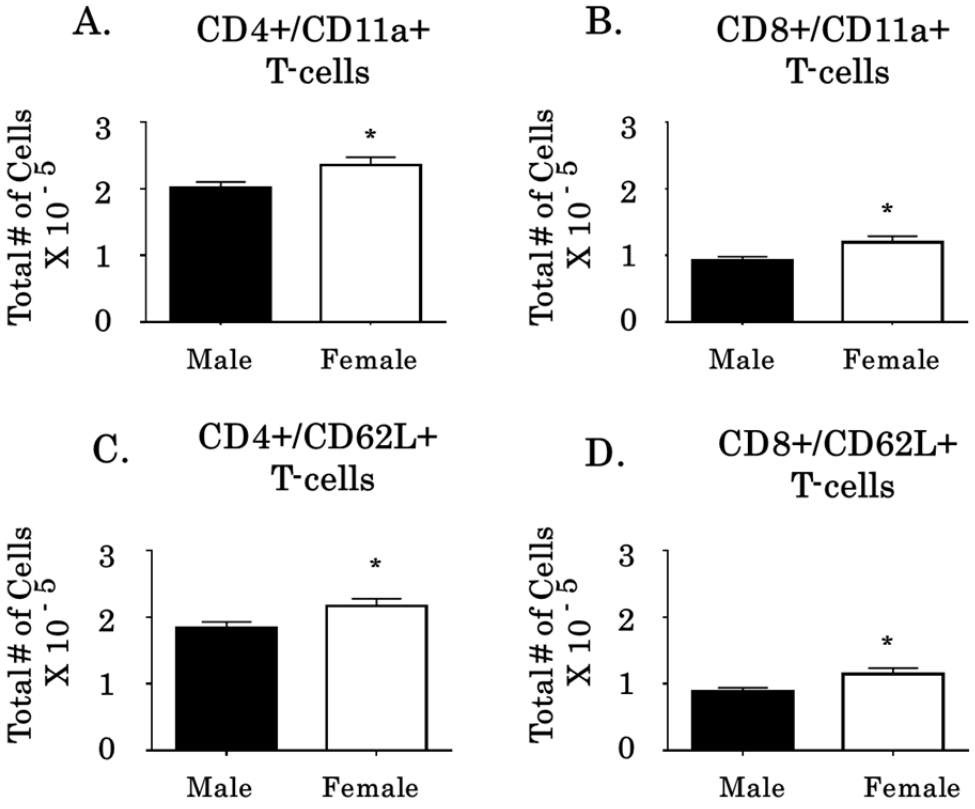

A.
CD4+/CD11a+
T-cells
Total # of Cells
X 10 - 5
3
2
1
0
*
Male
Female
B.
CD8+/CD11a+
T-cells
Total # of Cells
X 10 - 5
3
2
1
0
*
Male
Female
C.
CD4+/CD62L+
T-cells
Total # of Cells
X 10 - 5
3
2
1
0
*
Male
Female
D.
CD8+/CD62L+
T-cells
Total # of Cells
X 10 - 5
3
2
1
0
*
Male
Female

Figure 2.9
CD11a+ and CD62L + T-cells in the popliteal lymph nodes of 6 week old C57Bl/6 mice
Data are shown for total numbers of (A) CD4+/CD11a+ T-cells, (B) CD8+/CD11a+ T-cells, (C) CD4+/CD62L+ T-cells, and (D) CD8+/CD62L+ T-cells in the popliteal lymph nodes of 6 week old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.

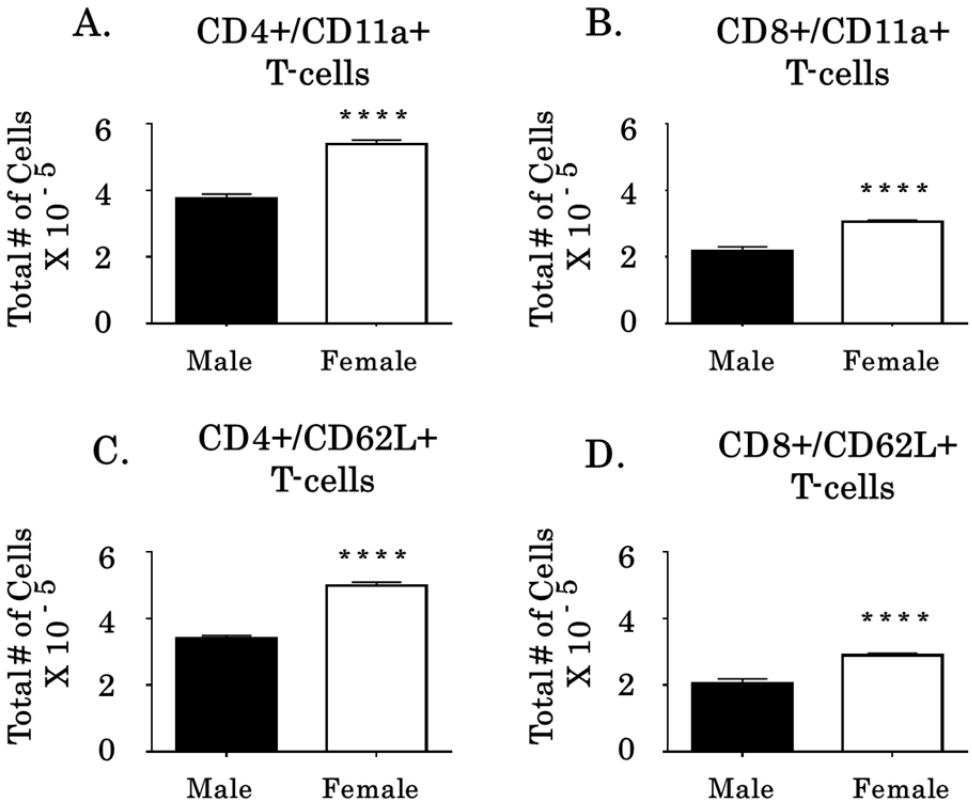

A.
CD4+/CD11a+
T-cells
Total # of Cells
X 10 - 5
6
4
2
0
****
Male
Female
B.
CD8+/CD11a+
T-cells
Total # of Cells
X 10 - 5
6
4
2
0
****
Male
Female
C.
CD4+/CD62L+
T-cells
Total # of Cells
X 10 - 5
6
4
2
0
****
Male
Female
D.
CD8+/CD62L+
T-cells
Total # of Cells
X 10 - 5
6
4
2
0
****
Male
Female

Figure 2.10

CD11a and CD62L MFI on T-cells positive for these markers in the popliteal lymph nodes of 3 week old C57Bl/6 mice

Data are shown for CD11a MFI on (A) CD4+/CD11a+ T-cells, (B) CD8+/CD11a+ T-cells, and CD62L MFI on (C) CD4+/CD62L+ T-cells, and (D) CD8+/CD62L+ T-cells in the popliteal lymph nodes of 3 week old C57Bl/6 males and females. P-values were assigned as follows;

* $P< 0.05$, * * $P< 0.01$, * * * $P< 0.001$, * * * * $P< 0.0001$.

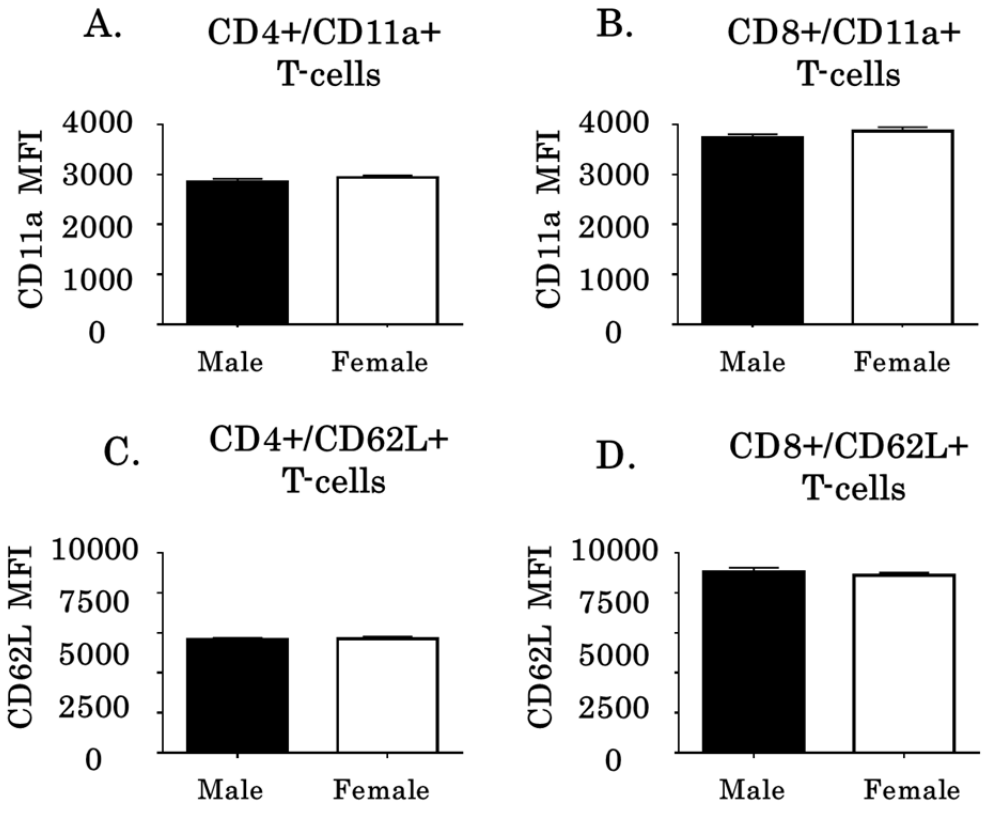
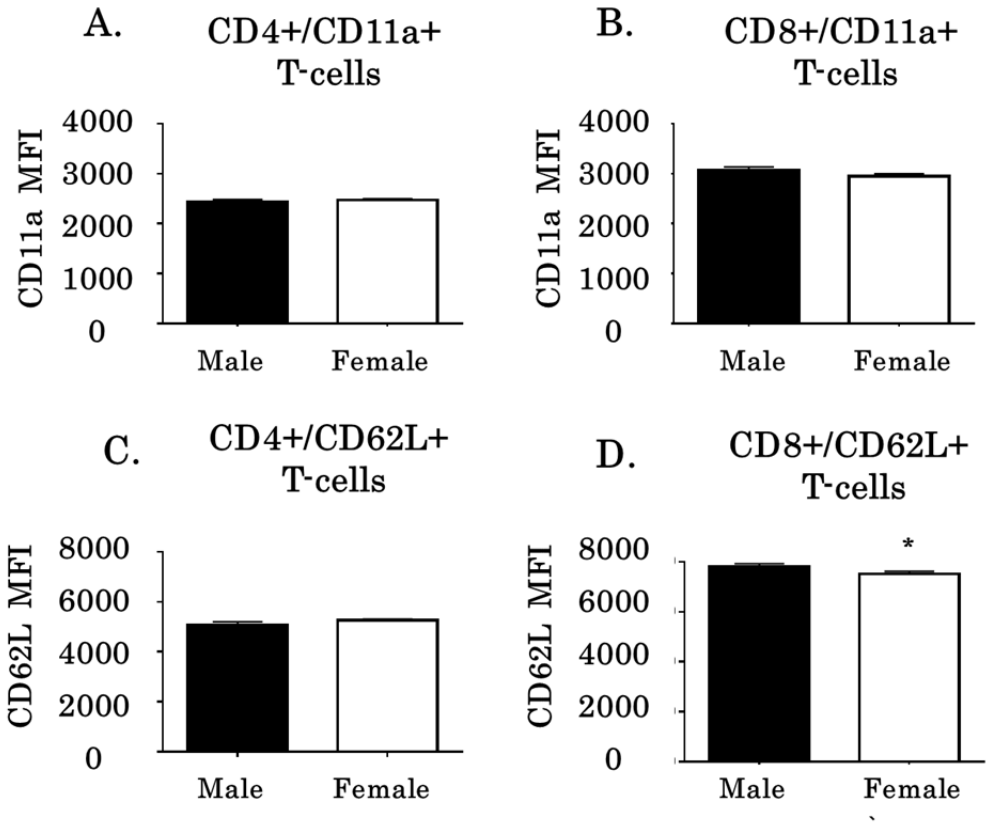

A.
CD4+/CD11a+
T-cells
CD11a MFI
4000
3000
2000
1000
0
Male
Female
B.
CD8+/CD11a+
T-cells
CD11a MFI
4000
3000
2000
1000
0
Male
Female
C.
CD4+/CD62L+
T-cells
CD62L MFI
10000
7500
5000
2500
0
Male
Female
D.
CD8+/CD62L+
T-cells
CD62L MFI
10000
7500
5000
2500
0
Male
Female

Figure 2.11
CD11a and CD62L MFI on T-cells positive for these markers in the popliteal lymph nodes of 6 week old C57Bl/6 mice
Data are shown for CD11a MFI on (A) CD4+/CD11a+ T-cells, (B) CD8+/CD11a+ T-cells, and CD62L MFI on (C) CD4+/CD62L+ T-cells, and (D) CD8+/CD62L+ T-cells in the popliteal lymph nodes of 6 week old C57Bl/6 males and females. P-values were assigned as follows;
* P< 0.05, * * P< 0.01, * * * P< 0.001, * * * * P< 0.0001.


A.
CD4+/CD11a+
T-cells
CD11a MFI
4000
3000
2000
1000
0
Male
Female
B.
CD8+/CD11a+
T-cells
CD11a MFI
4000
3000
2000
1000
0
Male
Female
C.
CD4+/CD62L+
T-cells
CD62L MFI
8000
6000
4000
2000
0
Male
Female
D.
CD8+/CD62L+
T-cells
CD62L MFI
8000
6000
4000
2000
0
*
Male
Female

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Chapter 3

Estrous Cycle Stage in Combination with Sex Chromosomal Complement Influence Delayed Type Hypersensitivity Responses to *Candida Albicans*

ABSTRACT

Delayed type hypersensitivity responses can vary depending on the gonadal sex of an individual. However, little has been done to explore how the estrous cycle may affect female immune responses. Using gonadal female mice with the XX chromosomal complement (XXF) and gonadal female mice with the XY chromosomal complement (XYF), we examined delayed type hypersensitivity responses to *Candida albicans* at proestrus, estrus, and diestrus. We found that XXFs exhibited the greatest response on the morning of proestrus. XYF mice at proestrus exhibited the same degree of response as both XXF and XYF mice at both estrus and diestrus. Ovariectomy removed the differences between XXF and XYF. Thus, there are interactive effects of the sex chromosomal complement and hormones that contribute to the sex differences in this T-cell mediated immune response.

INTRODUCTION

Studies have shown that immunologic parameters may fluctuate across the estrous cycle, both in the context of normal physiology as well as in the presence of disease.

Myocarditis models, for example, have provided some insight. For instance, more CD4+ T-cells expressing interferon-gamma in response to coxsackievirus B3 (CVB3) infiltrate the myocardium of female mice at proestrus and diestrus as compared to estrus and metestrus **[Schwartz et al. 2004]**. Females infected with CVB3 at estrus displayed increased cardiac viral titres as compared to females infected at the other estrous cycle stages. Also, there was an increase in T-regulatory cells in the peripheral blood and heart in females infected at estrus **[Huber 2008]**. Thus, the ability of animals to mount an initial immune response, as well as maintain a response, can be dependent on the stage of the estrous cycle.

In the mammary gland, there are twice as many macrophages present at diestrus compared to estrus or proestrus. Also, there is increased contact of macrophages with the epithelial cells at proestrus compared to estrus, metestrus, and diestrus **[Chua et al. 2010]**. Additionally, there are changes in macrophage phenotype that occur across the estrous cycle. In the mammary gland, a greater percentage of macrophages expressing MHCII are present at proestrus as compared to metestrus **[Hodson et al. 2013]**. This may have an

influence on the development of an initial immune response or an ability to mount additional responses upon re-exposure as MHCII is important for antigen presentation to T-cells **[Holling et al. 2004]**. Alternatively, the change in macrophage numbers, phenotype, and localization may primarily be related to re-modeling of the mammary gland throughout the estrous cycle. Macrophages have been demonstrated to be necessary for proper mammary gland development **[Gouon-Evans et al. 2000]**. There are changes in epithelial proliferation and apoptosis that occur in relation to estrous cycle stage **[Fata et al. 2001]**, and macrophage populations may vary according to the need for clearance of apoptotic cells.

Not only can estrous cycle stage affect immune function, but there is the possibility that gene products coded for on the X chromosome are also important. Although the second X chromosome is subject to inactivation, this inactivation is incomplete, allowing some genes to be expressed at higher levels in XX versus XY animals. Because the chromosomal complement is tied gonadal sex, this factor is difficult to study. A novel tool to address these issues is the Four Core Genotype (FCG) mouse model **[Arnold et al. 2009]**. In the FCG mouse the *Sry* gene has been deleted from the Y chromosome and reintroduced onto an autosome, allowing it to segregate independently of the rest of the Y chromosome. This permits four different genotypes of offspring; XX (XXF), XY^- (XYF), which develop ovaries and are female and XY^{-+Sry}

(XYM), XX $^{+Sry}$(XXM), which develop testes and are male. XYM and XXM mice maintain the same levels of Testosterone in the circulation, while XXF and XYF both have the same circulating levels of oestradiol levels as adults [Gatewood et al. 2006, Sasidhar et al. 2012].

We utilized this model to determine the influence of estrous cycle stages and a two versus one X chromosomal complement on delayed type hypersensitivity responses to *Candida albicans*.

MATERIALS AND METHODS

Animals

Eight week old Four Core Genotype (FCG) XY^{-} $^{+Sry}$ (Bl6 background) male mice and 8 week old C57/Bl6 female mice were obtained from Jackson Laboratories. FCG XY^{-} $^{+Sry}$ male mice were breed to C57/Bl6 female mice to produce 4 genotypes of offspring XY^{-} $^{+Sry}$ and XX $^{+Sry}$, both gonadal males, and XX and XY^{-}, both gonadal females. All animal procedures were approved by the University of California, Riverside, institutional animal care and use committee and were in accordance with guidelines from the American Association for Laboratory Animal Care, the United States Department of Agriculture, and the National Institutes of Health. Animals were housed in a barrier facility on a 12-hour light dark cycle.

Delayed Type Hypersensitivity Responses to *Candida albicans*

Mice were shaved on both flanks and put under 1.5% isoflurane anesthesia. Mice were sensitized with fixed *Candida albicans* by intradermal injection in both flanks with either 10 x 10^6 cells in a volume of 100 uL Dulbeccos phosphate buffered saline (PBS) or PBS as vehicle control. 7 days later, footpads were measured by caliper for baseline values. Mice were

anesthetized with 1.5% isoflurane and 50 uL of *Candida albicans* purified antigen (CAPA) (1.4 mg/mL) (Alerchek) were injected into each footpad. Twenty-four hours later, footpads were again measured by caliper. PBS challenged mice allowed for controls to demonstrate that any observed swelling in purified protein challenged mice was a result of immune response and not a lack of clearance of the fluid injected at the time of challenge. Foot swelling was calculated by subtracting before challenge footpad measurements from 24 hours after challenge footpad measurements.

Ovariectomy

Adult XXF and XYF mice were anesthetized with 2% isoflurane and placed on a heating pad throughout the entire procedure. Hair was removed from the surgical site and the site was wiped with Providone Iodine solution and subsequently wiped with 70% ethanol. A dorsal incision was made with sterilized surgical scissors. A small incision was made on both sides to access the ovaries through the abdominal muscles. The ovaries were each ligated with sterilized suture and cut to be removed. Incisions were bathed in Bupivacaine 2.5 mg/kg as an analgesic. The original dorsal incision was closed with surgical staples. Antibiotic ointment with local anesthetic was applied to the surgery site. Mice were kept under surveillance until they awoke and demonstrated the ability to be motile. Each mouse was

individually housed for 7 days after surgery. After 7 days, surgical staples were carefully removed if the incision site was completely healed and mice were re-housed with other females. Experiments were performed 4 weeks after ovariectomy.

Estrous Cycle Stage Determination

Vaginal smears were collected the morning (8:00 A.M.) of the final foot pad measurement after challenge to assess estrous cycle stage. Slides of smears were stained with 1% Toluidine blue, washed with deionized water, and left to air dry overnight. Estrous cycle stage was determined based on cell types present as previously described **[Caligioni 2009]**.

Statistical Analyses

Comparisons were analyzed by Student t-test or ANOVA with post-hoc tests using Dunn. Data were presented as mean + Standard Error. P-values were determined to be significant if, * $P < 0.05$.

RESULTS

Estrous cycle stage and the chromosomal complement-dependent DTH Responses.

Intact adult XXF and XYF mice were sensitized and challenged with *Candida albicans* **(Figure 3.1 a)** as described and vaginal smears were collected at the time of final footpad measurement. We included data for females classified as in estrus, proestrus, and diestrus on the morning of final footpad swelling measurements.

We found that XXF mice in proestrus exhibited greater foot swelling than XXF mice in diestrus. Although the comparison between XXF mice in proestrus and XXF mice in estrus did not reach statistical significance, XXF mice in estrus appeared to have a lower response, more similar to that observed in XXF at diestrus **(Figure 3.2)**. XYF mice did not exhibit any differences in foot swelling between any stages of the estrus cycle and were most similar to the XXF mice at estrus and diestrus **(Figure 3.3)**.

Ovariectomy of XXF and XYF mice and DTH responses

To determine if there are inherent differences in immune responses of XXF and XYF mice without the presence of circulating gonadally derived hormones, we performed the same experiment on gonadectomized mice at 10

weeks of age **(Figure 3.1 b)**. We found that after ovariectomy, there were no differences in immune response between the XXF and XYF mice.

DISCUSSION

Across the estrous cycle, there are fluctuations of several hormones including estrogen, progesterone, prolactin, follicle stimulating hormone and, luteinizing hormone. I will discuss information in relation to primarily three categorized stages of the rodent estrous cycle; proestrus, estrus, and diestrus. Proestrus, a time for follicle growth and maturation, is hallmarked by increasing levels of estrogen and subsequent increases in prolactin and a peaking of follicle stimulating hormone and luteinizing hormone on the evening of proestrus. Estrus, a time for follicle release follows with maintenance of high estrogen. At the end of estrus, progesterone levels increase by production from the corpus luteum and both estrogen and progesterone return to baseline levels through diestrus. Nearing the end of diestrus, estrogen levels begin to increase again as the mouse prepares to re-enter proestrus **[Parkening et al. 1982 and Walmer et al. 1992]**.

We found that T-cell mediated immune responses to *Candida albicans* were strongest when mounted by the morning of proestrus in XXF mice. Others have demonstrated that immune function can be influenced by either the stage that animals are immunized or the stage experienced at the point of mounting an immune response after the initial exposure **[Schwartz et al. 2004 and Huber 2008]**. Because of this, we have to consider if the proestrus response we observe is driven by hormonal changes experienced at

sensitization, or that after challenge, when the mice would be mounting a response upon re-exposure to the sensitizing antigen.

To address the first concern, we ran a pilot experiment in which we determined the estrous cycle stage experienced at the time of sensitization for XXF mice, and at final measure of footpad swelling. Although we had limited samples, the results suggest a better correlation between the estrous cycle stage experienced at final footpad measurement than at sensitization (data not shown). Regardless, it would be necessary to repeat that experiment with more mice before official conclusions could be drawn.

Our pilot experiment suggests that the controlling factor of the immune response mounted in XXFs by the morning of proestrus was dependent on the hormonal environment experienced for the duration of foot swelling. During this period of time, mice would likely be experiencing, a residual dropping of progesterone levels, and possibly a small rise in estrogen levels. FSH, LH, and prolactin levels would not be expected to rise until later in the evening of proestrus, and so may not be related to the timeframe of interest.

It is possible that rising estrogen levels are important to developing the higher response for XXF. However, our lab previously published experiments comparing DTH responses of ovariectomized mice to those of ovariectomized mice given slow release 17-B estradiol pellets. The

conclusions from this study were that estrogen was inhibitory to the DTH response to *Candida albicans* [Ma et al. 2007]. While it is true that estrogen treatment reduced the DTH response in those experiments, it is important to note that the levels of estrogen attained were that of pregnancy [Jacquet et al. 1977, Parkening et al. 1978, and Ma et al. 2007] and it is known that pregnancy itself is an immunosuppressive state [Clark et al.1986]. It is possible that high estrogen levels may function to dampen the immune response, while levels more reflective of estrogen in the context of the estrous cycle might stimulate an immune response.

Progesterone levels would also be dropping during the timeframe of interest. Progesterone has been shown to be suppressive to immune responses [Pisetsy and Spencer 2011 and Xu et al. 2011], and it is possible that with progesterone levels dropping, the animals immune system is more sensitive to an immune stimulus, allowing for a strong immune response to be mounted. We performed a pilot experiment to address this possibility in which mice were treated with mifepristone, a progesterone antagonist, at the time of challenge to antagonize effects of progesterone on the DTH response. Unfortunately, we did not observe any clear increase in immune response for females that received mifepristone compared to sesame seed oil treated control females (data not shown). Regardless, no definitive conclusions could be drawn due to the limited number of mice.

Alternatively, it could be the combination of dropping progesterone levels with steady rise in estrogen levels that permits the stronger immune response in XXFs measured on the morning of proestrus. This could be tested with a combined mifepristone and low does estrogen treatment at the time of challenge.

XYF mice did not exhibit the same increased DTH response at proestrus as observed in XXF mice. When XYF and XXF mice were ovariectomized, there was no difference in DTH response, demonstrating that a difference in intact XYF and XXF immune response is not only dependent on ovarian secretions, but also on expression from the second X chromosome.

Studies report no differences in estrogen levels for XXF and XYF mice on the NZM2328 background [Sasidhar et al. 2012]. However, they did not determine the cycle stage mice were experiencing at the time of sample collection. It would be necessary to include a more comprehensive study to address the lack or presence of differences in hormone levels between the XXF and XYF mice at different estrous cycle stages.

If hormone levels are indeed identical in XXF and XYF mice at all stages of the estrous cycle, then we would have to conclude that either the XY chromosomal environment prevented or gene expression resulting from two X chromosomes promoted the higher DTH response at proestrus. When assessing potentially differing hormonal functions in the presence of the

same amount of hormone, it is reasonable to question differences in their receptor's expression. It seems unlikely that difference in progesterone receptor expression would explain the difference between XXF and XYF. Others have shown that in neonatal mice progesterone receptor expression was dependent on gonadal sex, but not the chromosomal complement [Wagner et al. 2004]. Of course animals in these experiments were of a much younger age and reproductive status, and there could be differences in the adult intact XXF and XYF mice, that are not evident at an early age.

XXF and XYF also differ in gene expression. Those with an XX chromosomal complement express Xist, the gene responsible for X inactivation, in order to allow for mostly equivalent expression of genes on the X chromosomes in XX and XY individuals [Chow et al. 2005]. Although most genes from one X chromosome are silenced in an individual with the XX chromosomal complement, there are some that can escape from X inactivation. Some genes that escape X inactivation have homologues on the Y chromosome. One such gene is DDX3X, located on the X chromosome, however, DDX3Y, its functional homologue on the Y chromosome, while similarly expressed, is translationally limited to male germ cells. DDX3X has been shown to escape from X inactivation in both mice and humans [Yang et al. 2010]. DDX3X is shown to be critical for Type 1 interferon induction [Soulat et al. 2008]. Type 1 interferon is produced in response to infections to

assist in initiation of an immune response and to interfere with viral replication [Gonzalez-Navajas et al. 2012]. It is possible that increased expression of DDX3X as a result of escape from X inactivation in XXFs might permit increased Interferon induction compared to XYFs, allowing for the stronger immune response observed at proestrus in XXFs.

The female estrous cycle is complex in nature due to the hormonal fluctuations occurring throughout. Many studies solely use male or gonadectomized subjects to limit variable results due to female hormonal changes. For certain studies, this may be appropriate, however doing so for other studies severely limits the amount of valuable information that could be extrapolated by including cycling females and assessing results that may be dependent on cycle stage. Additionally, it is useful to consider effects dependent on the XX or XY chromosomal complement as doing so could allow for the understanding of effects dependent on the unchanging chromosomal complement of an individual, or dependent on hormonal effects. If dependent on hormonal effects that could potentially be manipulated in an individual, it may be possible to take advantage of this to exaggerate or mitigate the observed hormone dependent immunologic effects to improve vaccine effectiveness or minimize a detrimental inflammatory response.

Figure 3.1
Experiment timeline

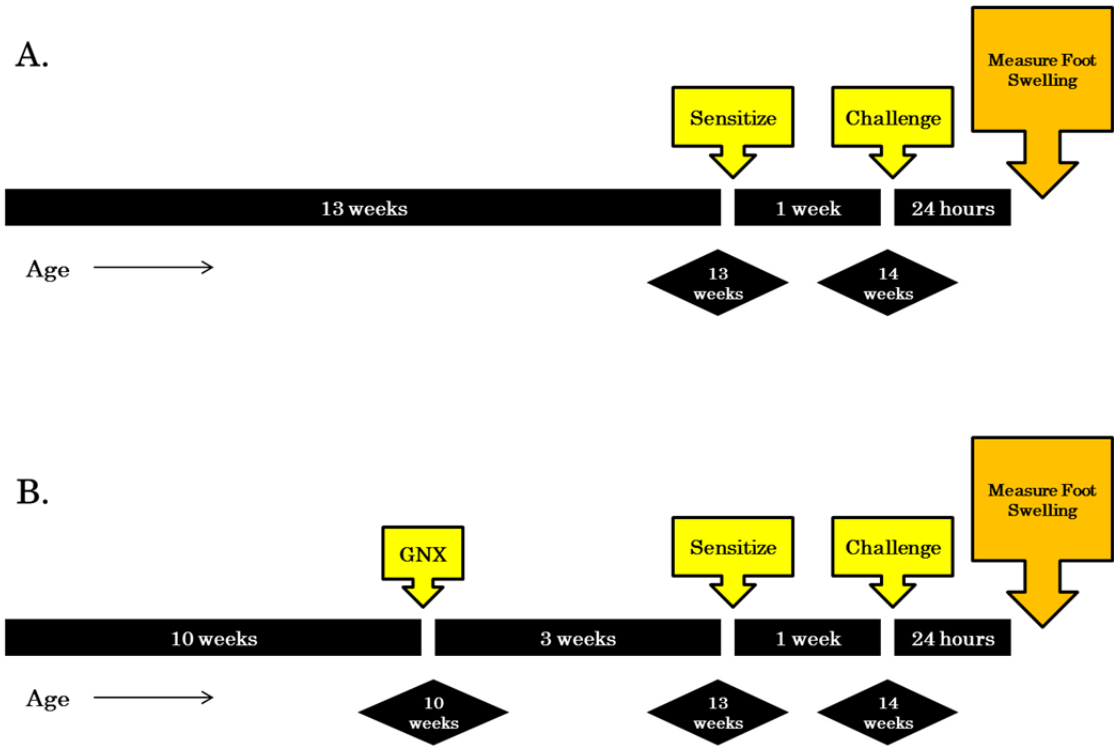

A.
Measure Foot Swelling
Sensitize
Challenge
13 weeks
1 week
24 hours
Age
13 weeks
14 weeks
B.
Measure Foot Swelling
GNX
Sensitize
Challenge
10 weeks
3 weeks
1 week
24 hours
Age
10 weeks
13 weeks
14 weeks

Figure 3.2
XXFs experience highest DTH response to *Candida albicans* at proestrus
Adult intact XXF FCG mice were sensitized with *Candida albicans* or PBS and a week later all mice were challenged in the footpad with *Candida albicans* purified protein. Vaginal smears were used to determine estrous cycle stage at the final footpad measurement and females were classified as proestrus (P), estrus (E), or diestrus (D). Data are presented as change in footpad thickness (footpad thickness 24 hours after challenge subtracted by footpad thickness right before challenge).
P-values were assigned as follows; * $P < 0.05$

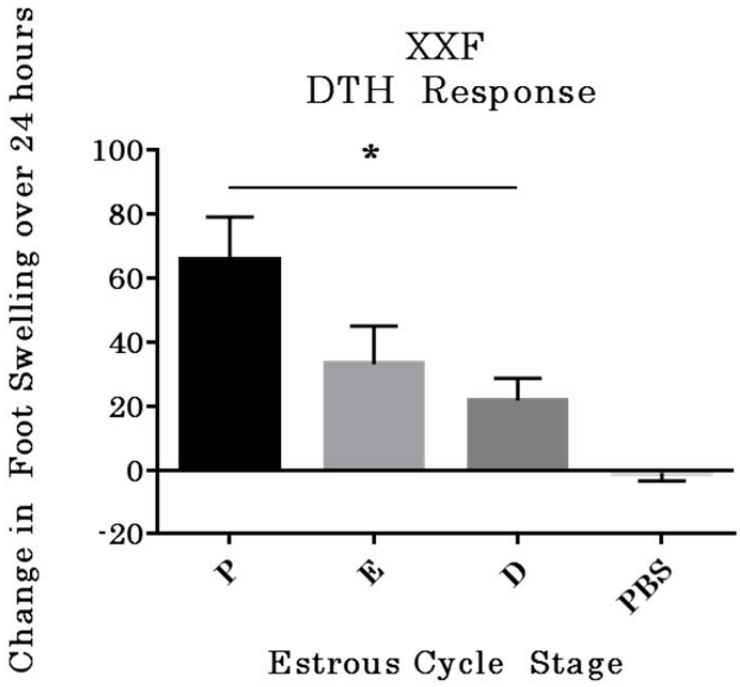

XXF
DTH Response
Change in Foot Swelling over 24 hours
100
80
60
40
20
0
-20
*
P
E
D
PBS
Estrous Cycle Stage

Figure 3.3
XYFs experience similar DTH responses to *Candida albicans* at all estrous cycle stages assessed.
Adult intact XYF FCG mice were sensitized with *Candida albicans* or PBS and a week later all mice were challenged in the footpad with *Candida albicans* purified protein. Vaginal smears were used to determine estrous cycle stage at the final footpad measurement and females were classified as proestrus (P), estrus (E), or diestrus (D). Data are presented as change in footpad thickness (footpad thickness 24 hours after challenge subtracted by footpad thickness right before challenge).
P-values were assigned as follows; * $P < 0.05$

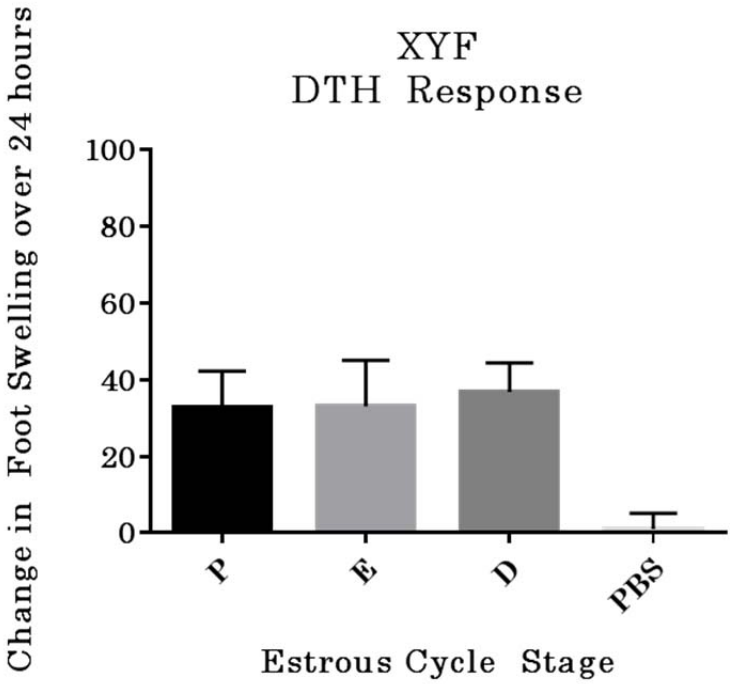
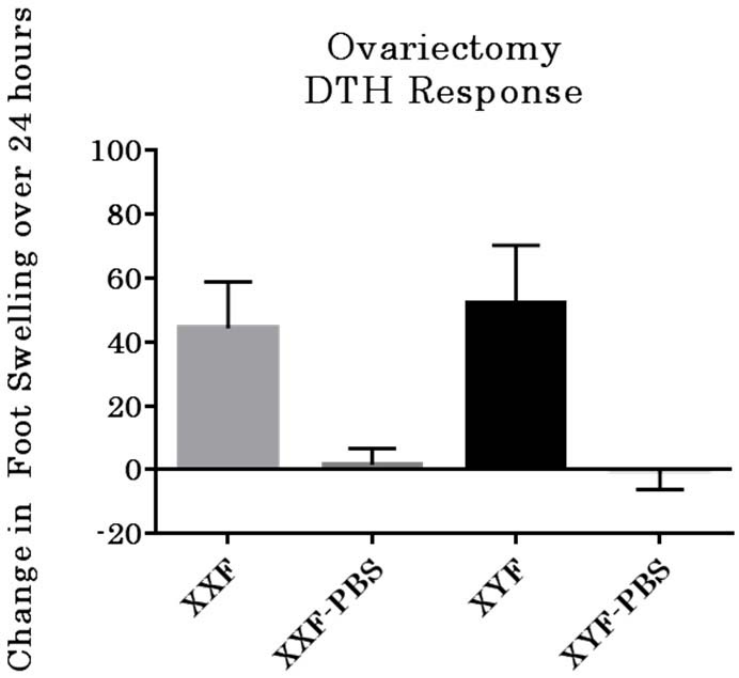

XYF
DTH Response
Change in Foot Swelling over 24 hours
100
80
60
40
20
0
P
E
D
PBS
Estrous Cycle Stage

Figure 3.4
Ovariectomized XXF and XYF mice do not exhibit differences in DTH responses to *Candida albicans*
Adult gonadectomized XXF and XYF FCG mice were sensitized with C*andida albicans* or PBS and a week later all mice were challenged in the footpad with *Candida albicans* purified protein. Data are presented as change in footpad thickness (footpad thickness 24 hours after challenge subtracted by footpad thickness right before challenge).
P-values were assigned as follows; * $P < 0.05$


Ovariectomy
DTH Response
Change in Foot Swelling over 24 hours
100
80
60
40
20
0
-20
XXF
XXF-PBS
XYF
XYF-PBS

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Chapter 4

Early Sexual Dimorphism of Gene Expression in the Thymic Epithelium

INTRODUCTION

Numerous reports have demonstrated sexual dimorphism of the mammalian immune system. Generally, females have a stronger immune response along with a higher susceptibility to certain autoimmune diseases, while males have a weaker immune response and retain a higher susceptibility to infections [Verthelyi 2001]. The basis for many of these differences has been explained as the lack or presence of various "sex hormones", mainly testosterone, estrogen, progesterone, and prolactin [Araneo et al. 1991 and Roberts et al. 2001].

When assessing sex based differences, it is important to consider the relative contributions of gonadal hormone production and the sex chromosomal complement. Until recent years, these effects were made difficult to distinguish due to the tightly bound link between the XY sex chromosomal complement and the *Sry* gene. *Sry* is located on the Y chromosome and is responsible for testicular development and ultimately the production of male sex steroids.

A novel tool called the Four-core-genotype (FCG) mouse model was created to allow research to tease apart the effects of sex chromosomal complement and gonadal steroids. The roots of this model began years ago when the research world first came to discover the existence and function of

the *Sry* gene. From *Sry*'s discovery came a mouse model that lacked the *Sry* gene and developed as a gonadal female **[Lovell-Badge and Robertson 1990]**. Later, researchers used this mouse to insert a transgene containing *Sry,* onto an autosome allowing it to segregate independently from the Y chromosome **[Koopman et al. 1991]**. Finally, when a transgenic $XY^{-\,+Sry}$ male mouse is crossed with a wild type female, the resulting offspring are one of four genotypes; $XY^{-\,+Sry}$ (XYM), XX^{+Sry} (XXM), both with testes, or XY^{-Sry} (XYF), XX (XXF), both with ovaries **[Mahadevaiah et al. 1998 and Arnold and Chen 2009]** .

Previously published work by our lab demonstrated a sex difference in an immune response by showing that intact female mice exhibit higher delayed-type hypersensitivity responses than intact males to *Candida albicans* **[Ma et al. 2007]**. Others have demonstrated that FCG mice on the C57/Bl6 background do not exhibit differences in other immune responses on the basis of their sex chromosomal complement **[Smith-Bouvier et al. 2008]**. To test the influence of the sex chromosomal complement on a T-cell mediated immune response to *Candida albicans* without the confounding influence of adult levels of hormones present in the circulation, we gonadectomized adult FCG mice of all four genotypes and subsequently assessed their immune responses by measurement of footpad swelling. Upon comparison of groups of XYM to XXM and XYF to XXF, we found no

significant differences in responses **(Figure 4.1a and 4.1b)**. This lack of effect from the sex chromosomal complement, allows us to conclude that gonadal sex regulates the delayed type hypersensitivity response to *Candida albicans* in mice on the C57/Bl6 background.

A large proportion of studies on immune sexual dimorphism are focused on adult subjects because of the expectation that adult circulating levels of gonadal hormones are responsible. However, there could be useful information derived from studying the early development of immune systems. In fact, we have shown that pre-pubertal factors are involved in developing the sexual dimorphism of the popliteal lymph node T-cell populations (Chapter 3 unpublished data). Insight into what sexually dimorphic groundwork has been laid out early in development may shed light on important factors that contribute to permanent effects on sex differences observed later in life.

The thymus is a primary lymphoid organ that is composed of a variety of cell types. Endothelial, epithelial, and immune cells all cooperate to allow this organ to successfully develop CD4+ and CD8+ αβT-Cells, as well as others. The high endothelial venules found within this tissue allow for tight control over what cells can enter. This is important because of the sensitive nature of the processes occurring within. It is necessary to maintain tight

control over antigens to be exposed to developing T lymphocytes, whether they are self or non-self, in order to appropriately allow development of functional, non-autoreactive T cells. The epithelial cells, mainly divided between cortical and medullary type, contribute to positive and negative selection of developing T lymphocytes. Also present are thymic nurse cells along with dendritic cells that contribute to development of T lymphocytes through antigen presentation. Permanent early effects on these cell types might contribute to a continuous sexually dimorphic education and selection process of developing T lymphocytes over the lifespan of the animal.

With the FCG model, we explored sex differences in thymic gene expression that were due to gonadal sex. We analyzed samples from each of the four genotypes of the FCG mouse at postnatal day 7, an early time point at which we can reliably explore gene expression permanently altered by the perinatal male testosterone surge without the continued influence of residual maternal hormones.

We performed microarray analysis on a mix of thymic epithelial, stromal, and endothelial cells (TESECs). Thymi were individually pressed through cell strainers and subsequently washed a total of 6 times to remove as many contaminating T cells as possible. Pilot experiments established how many cells were present in media from the washed TESECs with each wash. By wash 5, there were no detectable cells (hemocytometer) **(Figure 4.2)**. The

raw data from the microarray was subjected to a quantile normalization to account for internal variations among probe sets. Following this, the probes were assessed by two-way ANOVA to determine which genes followed the pattern of a sex difference (gonadal males vs. gonadal females) or a chromosomal difference (XX mice vs. XY mice). After ANOVA analysis, the generated differentially expressed genes were subjected to tests for False Discovery Rates (FDR); when thousands of comparisons are being made simultaneously for all the various probes on the microarray, there are increased risks of identifying "false positives", so additional statistical analyses need to be incorporated to address errors of this type.

Originally, we intended to have 3 unique samples for each genotype to allow for statistical analyses to support a comparison of any one genotype against the others, however, there were two issues that arose. One of the samples was incorrectly genotyped as an XYM when it was actually an XXM. Also, a separate XXM sample looked to have cardiac tissue contamination based on the microarray results. With an n=2 for the XYM group and n=3 for the other groups, XXM, XXF, XYF, the only analysis that would allow for statistical significance to be obtained was the two-way ANOVA.

The analysis compared gene expression for all XY animals (XYM and XYF) n=5 against all XX animals (XXM and XXF) n=6, and all gonadal male animals (XYM and XXM) n=5, against all gonadal female animals (XXF and

XYF) n=6. Because of how the analysis was done, if there was a particular genotype that had a greatly increased expression of a gene when compared to all of the other genotypes, it could skew a particular comparison and make it seem like a sex or chromosomal difference was present when in actuality there was a combined effect with only one genotype that was different from the others. For example, if an XXM group had a very high expression of a particular gene and all of the other 3 genotypes had the same low expression for that same gene, the ANOVA might conclude that all gonadal males (both XYM and XXM) have higher expression of that gene compared to all gonadal females (XYF and XXF). This clearly is not true given that only the XXM had a different expression level.

To address this skewing effect, after the list of genes that displayed gonadal sex-based differences or chromosomal complement based differences in expression was produced, further analysis was performed to eliminate genes that reflected an overexpression within one genotype. Only genes that achieved a P-value of <0.01 by ANOVA and that remained after further analysis to exclude false positives were listed in the tables and referenced throughout this chapter. There were a total of 73 genes that achieved this level of stringency. To help focus on genes that had reasonable expression levels, I sorted through the 73 genes to determine which were also expressed

at a probe level greater than 200. To indicate if a gene's expression fulfilled these criteria, the gene name was bolded in the tables.

MATERIALS AND METHODS

Animals

Eight week old Four Core Genotype (FCG) XY^{-} $^{+SRY}$ (Bl6 background) male mice and 8 week old C57/Bl6 female mice were obtained from Jackson Laboratories. FCG XY^{-} $^{+SRY}$ male mice were breed to C57/Bl6 female mice to produce 4 genotypes of offspring XY^{-} $^{+SRY}$ and XX $^{+SRY}$, both gonadal males, and XX and XY^{-}, both gonadal females. FCG mice of these 4 genotypes were used for all experiments. All animal procedures were approved by the University of California, Riverside, institutional animal care and use committee and were in accordance with guidelines from the American Association for Laboratory Animal Care, the United States Department of Agriculture, and the National Institutes of Health. Animals were housed in a barrier facility on a 12-hour light dark cycle.

Delayed Type Hypersensitivity Responses to *Candida albicans*

Mice were shaved on both flanks and put under 1.5% isoflurane anesthesia. Mice were sensitized with fixed *Candida albicans* by intradermal injection in both flanks with either 10 x 10^6 cells in a volume of 100 uL Dulbeccos phosphate buffered saline (PBS) or PBS as vehicle control. 7 days later, footpads were measured by caliper for baseline values. Mice were anesthetized with 1.5% isoflurane and 50 uL of *Candida albicans* Purified

Antigen (CAPA) (1.4 mg/mL) (Alerchek) were injected into each footpad. 24 hours later, footpads were again measured by caliper. Foot swelling was calculated by subtracting before challenge footpad measurements from 24 hours after challenge footpad measurements.

Gonadectomy

Adult FCG mice at 8 weeks of age were anesthetized with 2% isoflurane and placed on a heating pad throughout the entire procedure. Hair was removed from surgical site and the site was wiped with Providone Iodine solution and subsequently wiped with 70% ethanol. A dorsal incision was made with sterilized surgical scissors. A small incision was made on both sides to access the ovaries through the abdominal muscles, or on the scrotum to access testes. The ovaries or testes were each ligated with sterilized suture and cut to be removed. Incisions were bathed in Bupivacaine 2.5 mg/kg as an analgesic. The original incision was closed with surgical staples. Antibiotic ointment with local anesthetic was applied to the surgery site. Mice were kept under surveillance until they awoke and demonstrated the ability to be motile. Each mouse was individually housed for 7 days after surgery. After 7 days, surgical staples were carefully removed if the incision site was completely healed and mice were re-housed with other mice. Experiments were performed 4 weeks after gonadectomy

Tissue Collection

On postnatal day 7, mouse pups were sacrificed by decapitation and the thymus was collected and processed. In order to remove contaminating thymocytes, the thymus was placed in a sterile culture plate containing RPMI1640 and was pressed through a sterile disposable 70 uM cell strainer with a sterile disposable 1 mL syringe plunger. The tissue was washed of potentially contaminating thymocytes in this manner a total of 6 times, with an Rnase OUT treatment at every wash to help reduce degradation of RNA from the RNAses that would be released during the processing of the tissue. The TESECs were then snap frozen in liquid nitrogen and stored at -80 °C until the RNA was extracted for microarray analysis. Tail clips were collected at the time of mouse pup sacrifice and stored at -20 °C until DNA was extracted for genotyping purposes.

DNA extraction and Genotyping

DNA was extracted from tail clips using the Qiagen DNEasy kit per manufacturer instructions. Classical PCR was performed using the genotyping protocol recommended by Jackson Mice for the FCG mouse.

RNA extraction and target preparation/processing for GeneChip

RNA was extracted from the TESECs with the use of the Qiagen RNeasy mini for Microarray Analysis kit as per the manufacturer instructions. An on-column DNAse digestion step was performed as per manufacturer instructions. Target preparation/processing was performed by UCI DNA & Protein MicroArray Facility, University of California, Irvine. Eluted total RNAs were quantified (Nanodrop) with a portion of the recovered total RNA adjusted to a final concentration of 100ng/ul. All starting total RNA samples were quality assessed prior to beginning target preparation/processing steps by running out a small amount of each sample (typically 25-250ng/well) onto a RNA 6000 Nano LabChip that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Single-stranded, then double-stranded cDNA was synthesized from the poly(A)+mRNA present in the isolated total RNA (typically 100ng total RNA starting material each sample reaction) using the GeneChip® WT cDNA synthesis Kit (Affymetrix, Inc., Santa Clara, CA) and random hexamers tagged with a T7 promoter sequence. The double-stranded cDNA was then used as a template to generate many copies of antisense cRNA from an *in vitro* transcription reaction (IVT) of 16hrs in the presence of T7 RNA Polymerase using the Affymetrix Genechip® WT cDNA Amplification Kit. 10 ug of cRNA were input into the second cycle cDNA reaction with random hexamers that are used to reverse transcribe the

cRNA from the first cycle to produce single-stranded DNA in the sense orientation.

The single-stranded DNA sample was fragmented (WT Terminal Labeling Kit, Affymetrix, Inc, Santa Clara, CA) to an average strand length of 60 bases (range 40-70bp) following prescribed protocols (Affymetrix GeneChip® WT Sense Target Labeling Assay Manual). The fragmented single-stranded DNA was subsequently labeled with recombinant terminal deoxynucleotidyl transferase (TdT) and the Affymetrix proprietary DNA Labeling Reagent that was covalently linked to biotin. Following the recommended procedure, 0.54 ug of this fragmented target single-stranded cDNA was hybridized at 45 °C with rotation for 17 hours (Affymetrix GeneChip® Hybridization Oven 640) to probe sets present on an Affymetrix mouse GeneChip1.0 ST array. The GeneChip® arrays were washed and then stained (SAPE, streptavidin-phycoerythrin) on an Affymetrix Fludics Station 450 (Fluidics protocol FS450_007). Arrays were scanned using the GeneChip Scanner 3000 7G and GeneChip Operating Software v. 1.4 to produce .CEL intensity files. These probe cell intensity files (*.CEL) were analyzed in Affymetrix Expression Console software v1.1 using the PLIER algorithm to generate probe level summarization files (*.CHP). (Algorithm: PLIER v 2.0; Quantification Scale: Linear; Quantification Type: Signal and Detection P-Value; Background: PM-GCBG; Normalization Method: Sketch-Quantile)

Microarray Data Analysis

Dr. Yuichiro Itoh from the lab of Dr. Art Arnold at University of California, Los Angeles extracted expression values from .CEL files and performed background normalization followed by quantile normalization on the data.

Fold differences in gene expression presented in tables reflect fold differences of raw expression values.

RT-QPCR

RNA was quantified by nanodrop and equivalent amounts of RNA were reverse transcribed using Oligo$(dT)_{12–18}$ Primer (18418-012 Invitrogen, Carlsbad, CA), M-MLV Reverse Transcriptase (28025-013 Invitrogen), RNase OUT (10777-019 Invitrogen), and 10 mM DNTP mix (18427-088 Invitrogen) for 1 hour at 37°C. Gene expression was analyzed by Quantitative PCR using 2x SYBR Green (Biorad, Hercules CA) and primers for GAPDH, Glcci, and Wisp3. Primer sequences were: GAPDH (forward)-TGCACCACCAACTGCTTAG, (reverse)- GGATGCAGGGATGATGTTC, Glcci1 (forward)-ACACCTAGTTGCTGGGCAGA (reverse)-CTGCGTTGTAGCTGTTGCCT, Wisp3 (forward)-CGCTTCTCCATCTCTCCATCCT (reverse)- GTCTTGTGGTGCACTGCCCT.

Quantitative PCR was performed with Initialization at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C 10 seconds and annealing 30 seconds. After each run, a melt curve was run to assess appropriately amplified products.

Statistical analysis

For microarray data, expression values were submitted to 2-way ANOVA and analysis for false discovery rates. For all other experiments, Student t-test was used and a p-value < 0.05 was considered significant.

David

Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 [Huang et al. 2009] was used to categorize gene functions for genes demonstrating differences in expression after analysis of microarray data.

RESULTS

Genes influenced by strictly gonadal sex:

In order to assess early gene expression differences in thymic epithelium that could contribute to gonadal sex related immune function, we explored genes that exhibited differential expression dependent on gonadal sex (XYM and XXM vs. XXF and XYF).

There was only one sexually dimorphic gene that reached significance after FDR analysis $P<0.05$, Sry. This was expected as only gonadal male mice would have the Sry gene. Other than this, there were a number of genes found to exhibit sexually dimorphic expression; however the fold difference of expression between the sexes ranged mostly from 0.1 to 0.25. Nonetheless, a small difference in a gene's expression could still have the potential to largely alter a phenotype, whether it is through post-translational modifications, increased activity, or by other means.

Chromosome 7 contained the largest number of sexually dimorphic genes, 14 in total, as compared to the other chromosomes. This reflects the large number of olfactory receptor (Olfr) genes expressed more highly in males, with 9/14 of these genes being Olfrs. Others have also observed sexual dimorphism in the expression of many Olfrs [Shiao et al. 2012]. However, it

was interesting to find that the Kegg pathway most represented in all of the sexually dimorphic genes was olfactory transduction **(Table 4.1)**.

Sexually Dimorphic Genes of Interest: Difference of 0.2 fold or higher **(Table 4.2)**:

Of the 73 genes that displayed sexually dimorphic expression, there were only two that exhibited both a fold difference of 0.2 or greater and a probe level expression of 200 or higher. These were Glucocorticoid induced transcript 1 (Glcci1) and Wnt1 Inducible signaling pathway protein 3 (Wisp3), and both were expressed higher in females.

The gene with the highest fold difference of 0.25 was Glcci1. To validate this difference, we collected additional samples of TESECS and analyzed mRNA expression of Glcci1 by quantitative PCR with a larger number of samples (males n=13, females n=8). The analysis was performed by comparing all male samples to all female samples as this was how the comparison was made for the microarray. After expression was normalized to GAPDH, we found that there were no differences in expression between the gonadal males and females **(Figure 4.3)**.

Wisp3 exhibited a fold difference of 0.21 with females expressing it more. To validate this difference we analyzed mRNA expression for Wisp3

normalized to GAPDH by quantitative PCR with increased numbers of samples (males n=8 females n=8) and found that there was a 0.7 fold higher level of expression in females compared to males **(Figure 4.4)**.

Sexually Dimorphic Genes of Interest: Difference of 0.15 – 0.2 fold **(Table 4.3)**:

Only one gene was expressed at the probe level of 200 or higher and demonstrated a 0.15 to 0.2 fold difference, CD7 antigen (CD7), which was expressed higher in males.

Sexually Dimorphic Genes of Interest: Difference of 0.1 - 0.15 fold **(Table 4.4)**:

Five genes were expressed at the probe level of 200 or higher and demonstrated a 0.1-0.15 fold difference in expression. Three were higher in males: PTEN induced putative kinase (Pink1), spark/osteonectin, cwcb and kazal-like domains proteoglycan 1 (Spock1), and survival motor neuron domain containing 1 (SMNDC). Two were higher in females: Opa interacting protein 5 (Oip5) and Ferritin Light chain 1 (Ftl1).

DISCUSSION

These data provide some insight into early TESEC gene expression differences which could contribute to pre-pubertal sexual dimorphism of T-cell populations in the periphery, which are then maintained and enhanced into adulthood (Chapter 3 unpublished data). According to the microarray results, females expressed 4 genes higher than males; Glcci1, Wisp3, Oip5, and Ftl1, however upon tests to confirm expression differences in the genes with the highest fold differences, Glcci1 and Wisp3, only Wisp3 was found to have reproducible sexually dimorphic expression. The microarray showed that males expressed 4 genes higher than females; CD7, Pink1, Spock1, and SMNDC.

Not a lot is known about Wisp3, but it is a member of the CCN (CTGF, CYR61, and NOV) family of connective tissue growth factors. Secreted CCNs regulate cell proliferation, survival, migration, adhesion, differentiation, and extracellular matrix formation **[Moussad and Brigstock 2000, Perbal 2001]**.

It has been demonstrated that Wisp3 can be secreted and may promote superoxide dismutase activity **[Davis et al. 2006]**. In mice with overexpressed superoxide dismutase, there is increased thymic emigration of CD4+ and CD8+ cells and increased numbers of these cells in peripheral lymph nodes **[Laurent et al. 2006].** Although we do not observe a correlative increased expression of superoxide dismutase, it is possible that the higher expression

of Wisp3 in females could still lead to increased superoxide dismutase activity, and subsequent increases in T- cell numbers in peripheral lymphoid tissue.

Based on a mouse model overexpressing Wisp3 and analysis of Wisp3 activity in zebrafish, it binds to and inhibits Bone morphogenic protein (BMP) **[Nakamura et al. 2007 and 2009]**. Some BMPs function to inhibit thymocyte proliferation and progression of thymocytes from the double negative to the double positive stage **[Hager-Theodorides et al.2002]**. If females secreted more Wisp3 that acted on developing thymocytes to inhibit BMP, this may permit increased proliferation and development of single positive T-cells, which would then exit the thymus and increase T-cell numbers in lymph nodes.

Oip5 is important and necessary for normal chromosome segregation of chromosomes during mitosis. Females have a higher level of expression of Oip5 and it is possible that this reflects a more efficient cell division process occurring in the females TESECs.

Ftl1 is important for cellular iron uptake and storage. Iron is necessary for proper cellular metabolism and function. Upon consuming a diet deficient in iron, mice exhibited reduced populations of immunocompetent T-cells in the spleen **[Kuvibidila et al. 1990]**, demonstrating that iron is important to the number of functional T-cells. It is possible that increased Ftl1 in females

allows for increased storage of iron compared to males and this may contribute to the increased numbers of T-cells found in female peripheral lymph nodes.

CD7 is a transmembrane protein suggested to contribute to T-cell development. It has also been found to be expressed by endothelial cells and to behave as an Fc receptor **[Nishimura et al. 2006]**. Fc receptors in the thymus have been implicated in thymocyte development **[Leclercq and Plum 1995]**. It is possible that CD7 binds to the Fc portion of immunoglobulins and presents attached antigens to developing lymphocytes for positive or negative selection, thereby affecting male and female developing thymocytes differently.

When CD7 knockout mice were analyzed to assess the role of CD7 in T-cell development, there were no differences found in the primary and secondary lymphoid organs. However, no analysis was performed to determine the presence or lack of sex-related differences **[Bonilla et al. 1997]**. Even still, when considering gene function with knock out animals, it is difficult to absolutely conclude that with no effect on the primary and secondary lymphoid organs, the gene is not important to development of those tissues. If a gene is truly essential to function of the animal, then there may be compensatory gene expression that substitutes for the knock out gene's function. What would be more convincing is if this were tested in a cre-

animal or with knockdown studies in adult animals, thus allowing the animals to develop normally with the presence and dependence on CD7 gene function. Regardless, the lack of an effect on general lymphoid tissue in knockout mice does not eliminate the potential for CD7 to be important to the sex differences in T cells produced by males and females.

Pink1 is a serine threonine kinase **[Unoki and Nakamura 2001]** and is suggested to act by phosphorylating mitochondrial proteins **[Kim et al. 2008]**. Typically, its activity is linked to autophagy of damaged mitochondria **[Youle and Van der Bliek 2012]**. The function of mitochondrial autophagy may be to present peptide fragments from the mitochondria to developing immune cells in order to contribute to increased self-peptide presentation and negative selection in male thymocyte development.

Pink1has also been shown to be important to trafficking of reactive oxygen species to lysosomes **[McLelland et al. 2014]**. Reactive oxygen species are capable of inducing lysosomal membrane depolarization and inevitably cell death **[Boya et al. 2008]**. Thymic epithelial nurse cells are a unique cell type present in the thymus that can internalize up to 50 thymocytes **[Hendrix et al. 2010]**. Although the function of this cell type is disputed, some have demonstrated their contribution in the thymus to be to lysosomal degradation-dependent removal of apoptotic developing thymocytes **[Samms et al. 1999]**. It is possible that thymic epithelial nurse cells in males produce

more Pink1 and subsequently increase mitochondrial reactive oxygen species transport to lysosomes which may lead to lysosomal membrane depolarization and eventual death of more developing thymocytes that are held within the nurse cell, ultimately leading to decreased thymic emigration to secondary lymphoid tissue.

Spock1 encodes the protein core of a seminal plasma proteoglycan containing chondroitin- and heparin-sulfate chains **[Edgell et al. 2004]**. Because of its similarity to another gene, its function is thought to be related to protease inhibition. It has been shown to inhibit cell attachment and neurite extensions in culture and reduce adhesion of lens epithelial cells **[Marr and Edgell 2003]**. Spock1 also prevents apoptosis of hepatocellular carcinoma cells and was found to be up-regulated with induction of drug-resistance in Her2 positive gastric cancer cells. **[Li et al. 2013]**

Males had a higher expression of Spock1 compared to females. If Spock1 made movement through the thymus more difficult due to reduced adhesion, this migh slow the exit of T cells from the male thymus. If cells moved more slowly through the thymus, this might allow for increased opportunities that they will interact with a dendritic or epithelial cells and undergo negative selection.

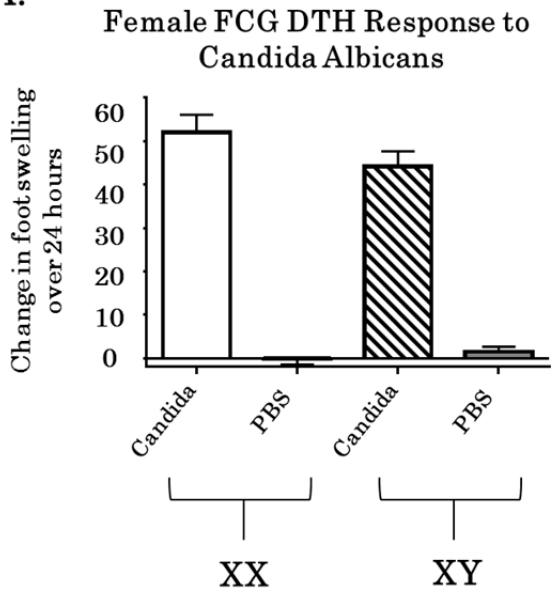
Spock1 is present in tissue of both males and females; however it maintains additional male specific expression as it is found in male ejaculate

secretions [Bonnet et al. 1992]. It is possible that due to male tissue specific increased amounts compared to females, males require more effective negative selection for Spock1 and thus increased levels of its expression by TESECs.

SMNDC is a nuclear protein that is a necessary component of the spliceosome complex [Meister et al. 2001 and Neubauer et al. 1998]. The spliceosome functions to remove introns from pre-messenger RNA and allow the production of messenger RNA along with the different splice variants of genes [Will and Luhrmann 2011]. If increased SMNDC in males led to different splicing patterns compared to females, this may contribute to a more diverse set of splice variants that could be presented to developing thymocytes. This may result in fewer T-cells that are allowed to continue development and be released into the periphery. Although we did not observe any large differences in gene expression between males and females, the microarray is designed to assess overall gene expression, and not splice-variant specific expression. To address sex differences in splice-variant expression, it would be necessary to utilize a technique such as RNA seq.

Figure 4.1
Sex chromosomal complement does not influence delayed type hypersensitivity to *Candida albicans* in adult gonadectomized CD57Bl/6
Adult FCG mice (A) XYM and XXM, (B) XYF and XXF were gonadectomized and sensitized with *Candida albicans* or PBS and a week later all mice were challenged in the footpad with *Candida albicans* purified protein. Data are presented as change in footpad thickness (footpad thickness 24 hours after challenge subtracted by footpad thickness right before challenge).
P-values were assigned as follows; * $P < 0.05$

A.


Female FCG DTH Response to
Candida Albicans
Change in foot swelling
over 24 hours
60
50
40
30
20
10
0
Candida
PBS
Candida
PBS
XX
XY

B.

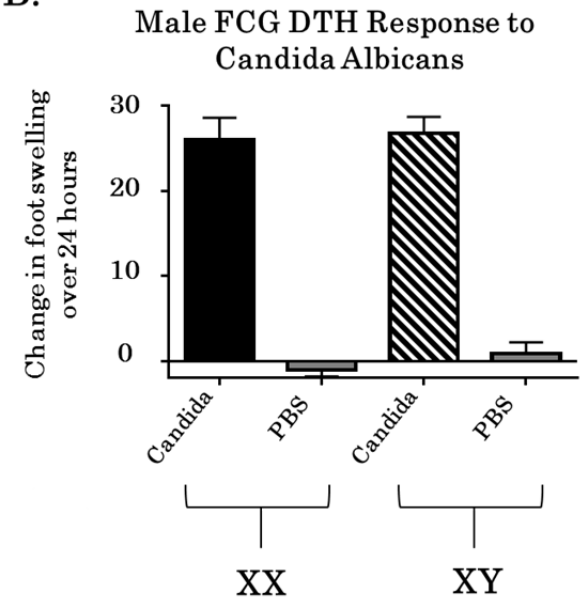

Male FCG DTH Response to
Candida Albicans
Change in foot swelling
over 24 hours
30
20
10
0
Candida
PBS
Candida
PBS
XX
XY

Figure 4.2
Pilot experiment to determine effectiveness of wash protocol to remove thymocytes from TESECs.

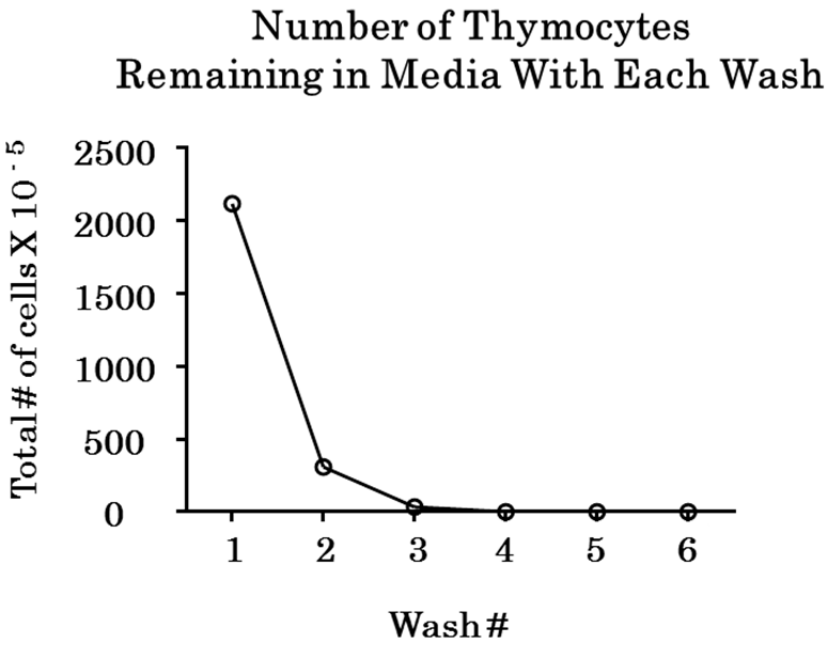

Number of Thymocytes
Remaining in Media With Each Wash
Total # of cells X 10 -5
2500
2000
1500
1000
500
0
1
2
3
4
5
6
Wash #

Figure 4.3
Sexual dimorphism of Glcci1 mRNA expression was not validated upon quantitative PCR assessment.
RNA from TESECs collected from male and female FCG mice at post-natal day 7 was analyzed for Glcci1 mRNA expression by RT-QPCR. Data are presented as relative mRNA expression normalized to GAPDH.
P-values were assigned as follows; * $P < 0.05$

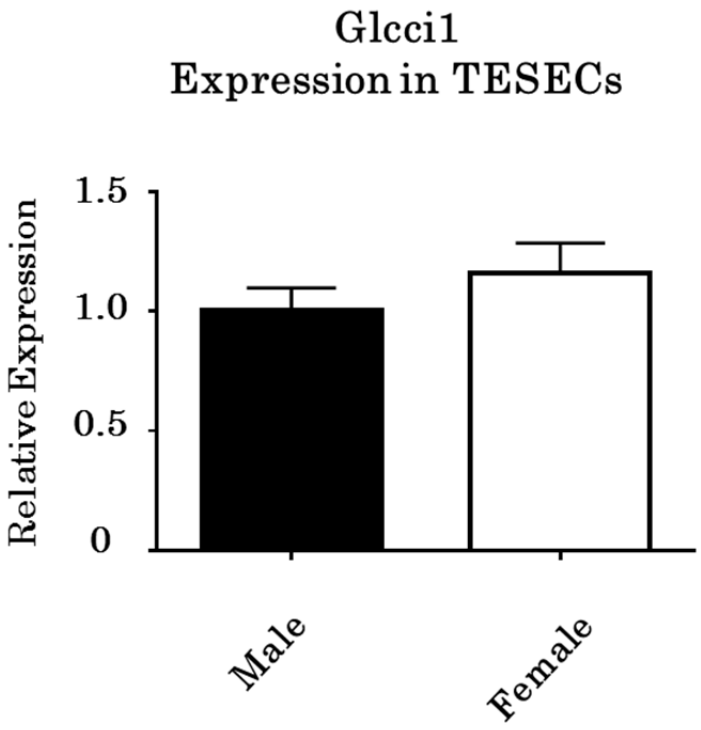

Glcci1
Expression in TESECs
Relative Expression
1.5
1.0
0.5
0
Male
Female

Figure 4
Sexual dimorphism of Wisp3 mRNA expression was validated upon quantitative PCR assessment.
RNA from TESECs collected from male and female FCG mice at post-natal day 7 was analyzed for Wisp3 mRNA expression by RT-QPCR. Data are presented as relative mRNA expression normalized to GAPDH.
P-values were assigned as follows; * $P < 0.05$

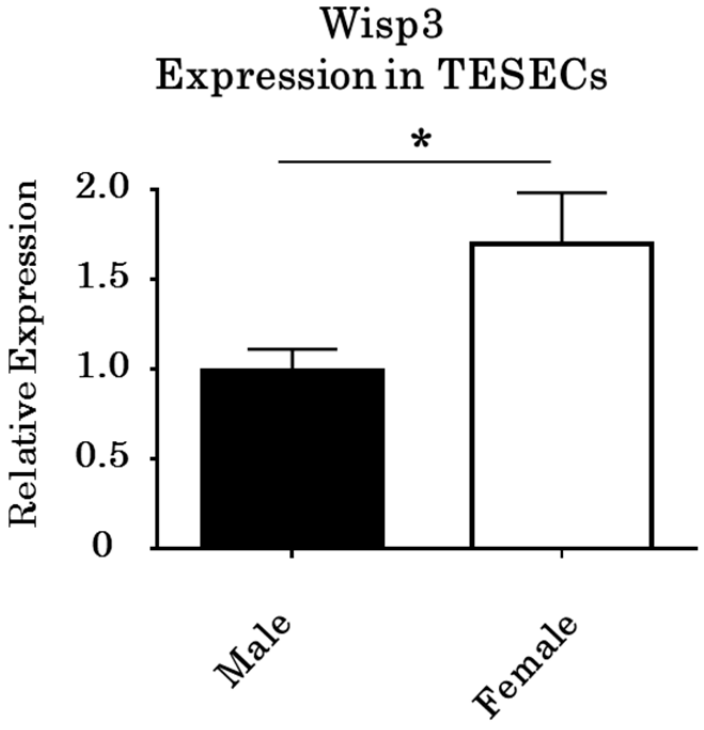

Wisp3
Expression in TESECs
*
Relative Expression
2.0
1.5
1.0
0.5
0
Male
Female

Table 4.1

Kegg pathways over-represented by genes exhibiting gonadal sex-dependent gene expression.

Information obtained through use of DAVID on data collected from a microarray analysis comparing TESECs of 7 day old FCG gonadal males to gonadal females.

Over-represented Kegg - pathways
Olfactory transduction
Spliceosome
Cytokine-cytokine receptor interaction
jak-STAT signaling pathway
Huntington's Disease
Parkinson's disease

Table 4.2
Sexually Dimorphic Genes of Interest: Difference of 0.2 fold or higher
Genes selected from a microarray analysis comparing TESECS of 7 day old FCG gonadal males to gonadal females.

Genes with Fold increased expression by > 0.2		
Expression higher in Males		Fold Increase
Olfr603	olfactory receptor 603	0.23
Gm1524	predicted gene 1524	0.21
Gm5567	predicted gene 5567	0.21
SRY	**sex determining region of Chr Y**	**4.02**

Genes with Fold increased expression by > 0.2		
Expression higher in Females		Fold Increase
Wisp3	**WNT1 inducible signaling pathway protein 3**	**0.21**
H60b	histocompatibility 60b	0.25
glcci1	**similar to glucocorticoid induced transcript 1; predicted gene 5815; glucocorticoid induced transcript 1**	**0.25**

Table 4.3
Sexually Dimorphic Genes of Interest: Difference of 0.15 – 0.2 fold
Genes selected from a microarray analysis comparing TESECS of 7 day old FCG gonadal males to gonadal females.

Genes with Fold increased expression by 0.15-0.2

Expression higher in Males		Fold Increase
Olfr295	olfactory receptor 295	0.16
Olfr975	olfactory receptor 975	0.16
Gm6924	predicted gene 6924	0.15
Gm7444	predicted gene 7444	0.18

Genes with Fold increased expression by 0.15-0.2

Expression higher in Females		Fold Increase
CD7	**CD7 antigen**	**0.17**
C030019I05Rik	RIKEN cDNA C030019I05 gene	0.17
KLRD1	killer cell lectin-like receptor, subfamily D, member 1	0.19
MIR29C	microRNA 29c	0.15
Gm12185	predicted gene, OTTMUSG00000005523	0.18

Table 4.4
Sexually Dimorphic Genes of Interest: Difference of 0.1 - 0.15 fold
Genes selected from a microarray analysis comparing TESECS of 7 day old FCG gonadal males to gonadal females.

Genes with Fold increased expression by 0.1-0.15

Expressed higher in Males		Fold Increase
Pink1	**PTEN induced putative kinase 1**	**0.14**
GRM3	glutamate receptor, metabotropic 3	0.13
ngb	neuroglobin	0.12
Olfr138	olfactory receptor 138	0.12
Olfr402	olfactory receptor 402	0.13
Olfr412	olfactory receptor 412	0.13
Olfr577	olfactory receptor 577	0.14
Olfr669	olfactory receptor 669	0.14
Olfr693	olfactory receptor 693	0.13
Olfr705	olfactory receptor 705	0.12
Spock1	**sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1**	**0.12**
SMNDC1	**survival motor neuron domain containing 1; predicted gene 5871**	**0.11**

Genes with Fold increased expression by 0.1-0.15

Expressed higher in Females		Fold Increase
CLEC4D	C-type lectin domain family 4, member d	0.15
Oip5	**Opa interacting protein 5**	**0.14**
Klk1b4	kallikrein 1-related pepidase b4	0.13
Ftl1	**Ferritin light chain 1**	**0.13**
IL21	similar to interleukin 21; interleukin 21	0.14
TMEM22	transmembrane protein 22	0.12
Vmn2r86	vomeronasal 2, receptor 86; vomeronasal 2	0.12
Zmat1	zinc finger, matrin type 1	0.13

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CONCLUSIONS

This dissertation examined endocrine and other influences on the trafficking of immune cells. Topics included the role of prolactin in immune cell entry into mammary tissue and the potential influence it may have on immune cell concentration into breast milk, the relative contributions of *Sry* expression and male gonadal secretions on the development of the sexual dimorphism of the popliteal lymph nodes, the combined influence of the sex chromosomal complement and estrous cycle stage on the delayed type hypersensitivity response to *Candida albicans*, and early sexual dimorphic gene expression in thymic epithelial and stromal cells.

The work presented in this dissertation demonstrated that luminal mammary epithelial cell secretions induce migration of a variety of lymphocytes, phagocytes, and granulocytes. Prolactin itself did not act as a chemoattractant for these cell types under the experimental conditions used, however mammary epithelial cell treatment with prolactin increased migration of most of these immune cell types, and in vivo it increased immune cell trafficking through the mammary tissue. CCL2 and CXCL1 produced and secreted by mammary epithelial cells are shown to be responsible for varying degrees of migration of monocytes and neutrophils, respectively, as depletion of these cytokines from mammary epithelial cell conditioned media significantly reduced their migration.

This dissertation also presents the first description of sexual dimorphism of the popliteal lymph nodes of C57Bl/6 mice. Female mice have more CD4+ and CD8+ T-cells than male mice; however males have a greater proportion of CD4+ T-cells that are T-regulatory cells. The sexual dimorphism is present prior to the onset of puberty. The pre-pubertal sex difference is to some degree dependent on *Sry* expression because mice with overexpression of *Sry*, had even fewer CD4+ and CD8+ T-cells and an even higher percentage of CD4+ T-cells that were T-regulatory when compared to wildtype males. It was demonstrated that testicular secretions are responsible for an amplification of the sex difference in T-cell numbers from 3 to 6 weeks of age because while ovariectomy of mice had no effect on T-cell numbers, castration increased T-cell numbers to those found in female mice. Since there were no differences in the amount of CD62L or CD11a on T-cells at either 3 or 6 weeks of age, differential expression of these homing markers was not the reason for differential homing to the popliteal lymph node.

Estrous cycle stage and the chromosomal complement were found to influence the delayed-type hypersensitivity response to *Candida albicans*. At proestrus, female mice with the XX chromosomal complement exhibited the greatest immune response, while female mice with the XY (-*Sry*) chromosomal complement did not exhibit any difference in response at proestrus, estrus, or diestrus. When XX and XY female mice were

ovariectomized, there was no difference in immune response. This suggests that the high immune response at proestrus in the XXF mice is dependent on some combined effect of the two X chromosome complement and hormones present at this stage of the estrous cycle.

Finally, a microarray study was performed on epithelium and stroma of the thymus of 7 day old four core genotypes mice. Interestingly, the pathway most sexually dimorphic based on gonadal sex was that of olfactory transduction. Of genes outside of this group, only one, Wisp3, exhibited a greater than 0.2 fold higher expression based on microarray data in all gonadal females compared to all gonadal males. When confirmed by quantitative PCR, expression of this gene was 0.7 fold higher in gonadal females when compared to gonadal males. A number of other genes exhibited differences in expression between gonadal males and gonadal females, however the differences in expression were all less than 0.2 fold.

All together the work in this dissertation has demonstrated influences of hormones, estrous cycle stage, *Sry* expression, and the sex chromosomal complement on both the phenotype and function of the immune system. This work demonstrates the importance of accounting for these factors when performing any immunologic experimentation.