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$\gamma\delta$ T cells are the predominant T cell type in opossum mammary during lactation.

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Abstract

Milk provides mammalian neonates with nutritional support and passive immunity. This is particularly true in marsupials where young are born highly altricial and lacking many components of a fully functional adaptive immune system. Here we investigated the T cell populations in the mammary of a lactating marsupial, the gray short-tailed opossum *Monodelphis domestica*. Immunohistochemistry confirmed the presence of T cells within the opossum mammary throughout lactation. Results of quantifying transcript abundance for lymphocyte markers are consistent with $\gamma\delta$ T cells being the most common T cell type within lactating mammary. Numbers of $\gamma\delta$ T cells appear to peak early during the first postnatal week, and then decline throughout lactation until weaning. In contrast, numbers of $\alpha\beta$ T cells and $\gamma\mu$ T cells appear to be low to non-existent in the lactating mammary. The results support an ancient and conserved role of immune cells in the evolution and function of mammalian mammary tissue.

1. Introduction

A hallmark of mammalian biology is the dependence on milk for immunological and nutritional support during postnatal development. Metatherian mammals, or marsupials, are noteworthy for having a complex scheme of milk production; the nutritional and immunological content of milk changes during the course of lactation to support the progressive maturational stages of the young (Daly et al., 2007; Joss et al., 2009; Fehrenkamp et al., 2018). Much of the work on lymphocytes associated with marsupial lactation has focused on those in the milk itself (Cockson and McNeice, 1980; Young et al., 1997; Young and Deane, 2001). These studies were limited to morphological identification without further classification of lymphocyte types. Less is known regarding lymphocyte subsets within the marsupial mammary tissue throughout lactation.

Investigations into eutherian mammary T cell populations during active lactation are limited as well. Most studies focus on lymphocyte subtypes in milk secretions or during pathological states such as infection or cancer (Sharp et al., 2016; Stanton and Disis, 2016;). Among eutherians, mammary CD4⁺ and CD8⁺ $\alpha\beta$ T cells as well as $\gamma\delta$ T cells were

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Conflicts of interest

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detected, but variation in proportions of these subtypes exist between species (Reardon et al., 1990; Ismail et al., 1996; Chabaudie *et al.*, 1997). For example, in healthy, active goat mammary tissue, CD4⁺ T cells predominated; in contrast, in healthy, active cow mammary tissue CD8⁺ T cells predominated (Ismail et al., 1996; Yamaguchi et al., 1999). In general, mammary T cell numbers in eutherians tend to increase during gestation and decline throughout lactation into weaning and involution (Manning and Parmley, 1980; Salmon and Delouis 1982; Reardon et al., 1990; Ismail et al., 1996; Chabaudie *et al.*, 1997; Yamaguchi et al., 1999; Salmon et al., 2009).

Like all jawed vertebrates, marsupials have both $\alpha\beta$ and $\gamma\delta$ T cells (reviewed in Hansen and Miller, 2015). In addition, the marsupials have a third T cell lineage expressing the T cell receptor (TCR) μ chain. TCR μ is a fifth TCR chain unique to marsupials and monotremes and the phenotype of TCR μ ⁺ T lymphocytes remains unknown (Parra et al, 2007; Wang et al., 2011; Parra et al., 2012). Here we investigated both the presence of T cells in the opossum mammary during lactation as well as the lineages of T cells present using transcript profiles.

2. Materials and methods

2.1 Animal use and tissue collection

Opossums used were from a captive-bred colony housed at the University of New Mexico Department of Biology Animal Research Facility. This study was approved under protocol numbers 16-200407-MC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee.

For immunohistochemistry and histology, mammary tissues from at least three replicates from post-partum (P) days 3, 17, 32, and 44 were collected. For RNA isolation, at least three replicates per time point were collected from the last 24 hours of pregnancy (embryonic (E) day 14) and P1, 2, 3, 5, 7, 10, 13, 16, 17, 20, 22, 26, 31, 32, 33, 36, 38, 44, and 52.

Mammary tissues were also collected from animals 24-48 hours after pups had been removed on day 56 (post weaning) (Supplementary Table 1). Opossum mammary tissues were collected from timed pregnancies as previously described (Hansen et al., 2017). Animals were euthanized by inhaled isoflurane overdose until no evidence of breathing or heartbeat for one minute, followed by decapitation.

2.2 Immunohistochemistry and microscopy

Tissues were preserved in 10% buffered formalin at 4 °C for 24-48 hours, washed repeatedly in 70% ethanol to remove any residual formalin before being dehydrated and embedded in paraffin wax consistent with previously published protocols (Old and Deane, 2003). Embedded tissues were sectioned to 6 microns and mounted on Apex superior adhesive slides (Leica Biosystems, Wetzlar, Germany).

Formalin fixed paraffin embedded sections were de-paraffinized in xylene and rehydrated through graded alcohol solutions to PBS as previously described (Old and Deane, 2001). Antigen retrieval was completed by microwaving submerged sections in pre-warmed Sodium Citrate Buffer (pH = 6) for 20 minutes and allowing to cool to room temperature for

at least 20 minutes. Peroxidase and biotin blocking were completed prior to overnight incubation at 4 °C with rabbit anti-human CD3e polyclonal antibody (DAKO, A045229-2, Agilent, Santa Clara, CA, USA), a pan T cell marker shown to be cross reactive in marsupial species (Kreiss et al., 2009; Hansen and Miller, 2017). Sections were incubated with a polyclonal biotinylated goat anti-rabbit secondary antibody (DAKO, E0432, Agilent, Santa Clara, CA, USA) at room temperature for two hours. Avidin binding (Vectastain, Vector Laboratories, Burlingame, CA, USA) and oxidation via DAB reagent (Abcam, Cambridge, UK) resulted in a brown staining of positive lymphocytes. Sections were counterstained in hematoxylin (Thermo scientific, Waltham, MA, USA) before dehydrating in graded ethanol solutions and coverslipped with DPX (Sigma Aldrich, St. Louis, MO, USA). Isotype, negative, and positive controls were included in optimization of immunohistochemistry conditions. All antibodies were diluted with 0.1% fish gelatin (Sigma Aldrich, St. Louis, MO, USA) 1X PBS solution in 1:500 dilutions. All incubations were performed in a humidified chamber and thorough washings in PBS followed all incubations and blocking procedures. Stained sections were imaged for single field of view on an Eclipse Nikon Ti inverted fluorescence microscope utilizing Nikon ARS (Nikon, Minato, Tokyo, Japan) software.

2.3 RNA extraction and cDNA synthesis

Mammary tissues were preserved in RNALater buffer (Invitrogen, Carlsbad, CA, USA) at 4 °C for 48 hours. The buffer was then removed and tissues were stored at –80 °C until extraction. RNA was extracted from tissues using phenol based extraction methods utilizing Pure Link RNA mini kit (Invitrogen, Carlsbad, CA, USA). Then 10 µg of whole RNA was cleaned of residual DNA using TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA) and cDNA pools were created using 500 ng of DNA cleaned RNA by reverse transcriptase PCR using SuperScript III First Strand Synthesis (Invitrogen, Carlsbad, CA, USA). cDNA synthesis reactions were constructed in triplicate and pooled to reduce bias generated during reverse transcription.

2.4 Quantifying specific gene transcripts

Transcript abundance of specific genes was assessed by quantitative real time PCR (qPCR) using Sso Advanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) according to manufacturer's instructions for 20 µL reactions. qPCR reactions were performed in triplicate on a BioRad CFX96. Primers were designed based on opossum genome sequence according to qPCR manufacturer's parameters (Supplementary Table 2).

Transcript abundances per gene were normalized for each individual by the Vandesompele method of incorporating multiple reference gene expression levels (Vandesompele et al., 2002). Reference genes used in this study were succinate dehydrogenase subunit A (*SDHA*) and actin-related protein 2 (*ACTR2*). These genes were highly expressed at all time points with little variance. They also had M values <1 as defined in Vandesompele and colleagues (Vandesompele et al., 2002).

2.5 Statistical analyses and graphical representation

All statistical analyses were performed using default parameters of Prism 7 software (GraphPad, La Jolla, CA, USA). Normalized expression data were calculated per biological replicate per time point. Mean expression was then calculated per biological set. Grubbs outlier analyses were performed across each biological set. Normalized expression of biological replicates including significant outliers is represented in Supplementary Figure 1. Inclusion of outliers did not significantly alter statistical analyses. Data, not including outliers, were pooled by week (Supplementary Table 1). Mean normalized expression and standard error of the mean (SEM) are reported per gene target for each week.

3. Results

To investigate the presence of T cells within opossum mammarys, immunohistochemistry was performed using an anti-CD3e polyclonal antibody. Mammary sections from each of three individuals with pups at ages P3, 17, 32 and 44 were examined. The presence of mammary tissue CD3e⁺ T cells was evident, albeit not densely, at all time points (Figure 1). Attempts to quantify positive cells across time points were inconclusive due to changing cellular density of the tissue throughout lactation as well as apparent non-homogeneous infiltration. In general, positive cell quantity appeared to decrease throughout the duration of lactation (Figure 1, results not shown). Positive lymphocytes were located in sights of more mature alveoli, the sites of milk production and storage. In addition to being within the mammary tissue, CD3e⁺ T cells were also detected within mammary blood vessels, as well as in the alveolar milk space (Figure 2).

To phenotype and quantify the mammary T cells, the abundance of transcripts encoding various T cell specific markers was used as a proxy for the cell types present. Mammary tissues from at least three individuals per time point for 21 time points were used (Supplementary Table 1). These spanned from the last 24 hours of pregnancy (E14) through 24-48 hours following the removal of offspring at P56. TCR chain identity was determined from amplification using primers specific for the constant region of each chain.

Transcripts encoding the TCR α , β , γ , δ , and μ chains were quantified first. Abundances of TCR α and β chain transcripts were low at all time points (Figure 3). TCR α chain transcript abundance remained consistent throughout lactation with minor increases surrounding birth and weaning. TCR β chain transcripts were partially elevated in week one, however this was due to increased abundance at P5 alone and no other time point (Figure 3, Supplementary Figure 1B). Normalized abundance of either chain transcripts was not significantly different at any time point (Figure 3). Abundance of CD4 and CD8 transcripts were also investigated (Figure 4). These T cell marker transcripts remained low throughout lactation and mostly paralleled TCR α and β chain transcripts.

The most abundant TCR transcripts were those encoding TCR γ chains (Figure 5). In marsupials, TCR γ can pair with either TCR δ or TCR μ , therefore transcripts were quantified for all three chains for comparison (Figure 5). The pattern of TCR γ transcript abundance was most closely overlapping with that of TCR δ . Indeed, there was a coordinated peak in abundance of both TCR γ and TCR δ transcripts following parturition that decreased linearly

throughout the duration of lactation. In contrast, transcripts for the TCR μ chain were detected at only low levels similar to patterns of TCR α and TCR β . Minor increases in TCR μ transcript abundance were seen during weeks one and four, however these were not significant (Figure 5). These results are consistent with the TCR γ transcripts being primarily, if not entirely, from $\gamma\delta$ T cells, and not $\gamma\mu$ T cells.

4. Discussion

An evolutionary innovation that defines mammals is the mammary gland. The mammary glands provide mammalian neonates with nourishment, as well as immune components that regulate the establishment of their microbiome and protect against infectious diseases (Goldman et al., 1998; Goldsmith et al. 2015). Cells of the immune system play a role in both the transmission of maternal immunity to offspring, and fundamentally contribute to the development and remodeling of mammary tissue (Reed and Schwertfeger 2010; Oftedal, 2012).

Marsupials diverged from eutherians at least 165 million years ago and provide a unique comparison for mammary studies (Bininda-Emonds et al. 2007). Marsupials are noteworthy for having a relatively complex and dynamic lactation scheme (Daly et al., 2007; Joss et al., 2009; Fehrenkamp et al., 2018¹). The stage of mammary development at the onset of lactogenesis differs greatly between the marsupial and eutherian lineages. In eutherian mammarys, development is complete by the end of gestation prior to the onset of lactation (Djonov et al., 2001). In contrast, in marsupials, milk production begins before the mammarys have completed their development. Substantial morphological restructuring continues to occur throughout early lactation (Hinds, 1988).

Immunohistochemical analyses suggest a general decline in CD3 ϵ + lymphocytes within the mammarys throughout lactation. This is consistent with gene expression results in which abundance of TCR chain transcription decreased throughout lactation as well. We have shown here that $\gamma\delta$ T cells predominate as the mammary T cell type during lactation in opossums. The $\gamma\delta$ T cells are most abundant in the first five weeks postpartum, corresponding to a time when the mammarys are undergoing their greatest remodeling (Hinds, 1988; Figure 5). It is noteworthy that the stage when $\gamma\delta$ T cells are found in the opossum mammary most resembles the early developmental stages in eutherians when $\gamma\delta$ T cells are also present. In eutherians this stage is during late gestation, whereas in marsupials it is during the early postnatal period when young are suckling (Reardon et al., 1990; Yamaguchi et al., 1999). These observations are consistent with a conserved role of $\gamma\delta$ T cells within the developing mammarys in both marsupials and eutherians.

Recently, we characterized the B cell populations within opossum mammarys throughout lactation. We found there were primarily IgA producing cells. We hypothesized that IgA producing B cells were likely activated in the gut and had trafficked to the mammarys (Fehrenkamp et al., 2018). If the B cells were activated first in the gut as speculated there would be less need for T cell help in the mammarys. The low prevalence of CD4⁺ T cells within opossum mammarys shown here would be consistent with that hypothesis. In addition there are few if any IgG producing B cells within the mammarys and milk IgG is

likely transferred directly from maternal circulation rather than synthesized *in situ*. This would also be consistent with a lack of need for CD4⁺ T cells in the opossum mammary.

Mammarys are a uniquely mammalian system whose origins can be illuminated through comparative approaches. Complex systems generally involve adaptation of preexisting systems to perform novel tasks. Components of the immune system, in particular, have been coopted into non-immune roles multiple times during evolution. For example, recent evidence supports a role of the immune system in the evolution of placentation and parturition in mammals (Hansen et al., 2016; Griffith et al., 2017). Mammary glands are a complex tissue that likely evolved from cutaneous secretory glands (Ofstedal, 2012). Our results using a marsupial, when compared to previous studies in eutherians, supports a role for $\gamma\delta$ T cells in the evolution of mammarys from these secretory glands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Beaudry KL, Parsons CL, Ellis SE, and Akers RM (2016). Localization and quantitation of macrophages, mast cells, and eosinophils in the developing bovine mammary gland. *J Dairy Sci.* 99(1):796–804. 10.3168/jds.2015-9972 [PubMed: 26547646]
- Bininda-Emonds ORP, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Grenyer R, Price SA, Vos RA, Gittleman JL and Purvis A (2007). The delayed rise of present-day mammals. *Nature.* 446 (7135): 507–512. 10.1038/nature05634 [PubMed: 17392779]
- Böttcher MF, Jenmalm MC, Björkstén B, and Garofalo RP (2000). Chemoattractant Factors in Breast Milk from Allergic and Nonallergic Mothers. *Pediatric Research* 47: 592–597 10.1203/00006450-200005000-00006 [PubMed: 10813582]
- Chabaudie N, Le JC, Olivier M, and Salmon H (1993). Lymphocyte subsets in the mammary gland of sows. *Res Vet Sci.* 55 (3): 351–5. 10.1016/0034-5288(93)90106-P [PubMed: 8284500]
- Chollet-Hinton LS, Stuebe AM, Casbas-Hernandez P, Chetwynd E, and Troester MA (2014). Temporal trends in the inflammatory cytokine profile of human breastmilk. *Breastfeed Med.* 9(10): 530–7. 10.1089/bfm.2014.0043 [PubMed: 25380323]
- Cockson A, and McNeice R (1980). Survival in the pouch: the role of macrophages and maternal milk lymphocytes. *Comp biochem and phys.* 66 (2): 221–225; 18 10.1016/0300-9629(80)90155-3
- Cruikshank WW, Berman JS, Theodore AC, Bernardo J, and Center DM (1987) Lymphokine activation of T4⁺ T lymphocytes and monocytes. *J Immunol.* 138 (11): 3817–3823. [PubMed: 3108375]
- Daly KA, Digby M, Lefèvre C, Mailer S, Thomson P, Nicholas K, and Williamson P (2007). Analysis of the expression of immunoglobulins throughout lactation suggests two periods of immune transfer in the tammar wallaby (*Macropus eugenii*). *Vet Immunol Immunop.* 120 (3-4), 187–200. 10.1016/j.vetimm.2007.07.008

- Djonov V, Andres AC, Ziemiecki A (2001). Vascular remodeling during the normal and malignant life cycle of the mammary gland. *Microsc Res Tech*. 52: 182–189.
10.1002/1097-0029(20010115)52:2<182::AID-JEMT1004>3.0.CO;2-M [PubMed: 11169866]
- Fehrenkamp BD, Morrissey KA, and Miller RD. (2018). Opossum milk IgG is from maternal circulation and timing of transfer correlated with neonatal immune development. *Repro Fert Devel*. 10.1071/RD18121
- Goldman AS, Chheda S, and Garofalo R (1998). Evolution of immunologic functions of the mammary gland and the postnatal development of immunity. *Pediatr Res*. 43(2): 155–62.
10.1203/00006450-199804001-00921 [PubMed: 9475278]
- Goldsmith F, O'Sullivan A, Smilowitz JT, and Freeman SL (2015). Lactation and intestine microbiota: How early diet shapes the infant gut. *J Mammary Gland Biol Neoplasia*. 20(3-4):149–58. 10.1007/s10911-015-9335-2 [PubMed: 26227402]
- Gouon-Evan V, Yin EY, and Pollard JW (2002). Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development. *Breast Cancer Research* 4:155
10.1186/bcr441 [PubMed: 12100741]
- Griffith OW, Chavan AR, Protopapas S, Maziarz J, Romero R, and Wagner GP (2017). Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proc Natl Acad Sci* 114(32):E6566–E6575. 10.1073/pnas.1701129114 [PubMed: 28747528]
- Hansen VL, Faber LS, Salehpoor AA, and Miller RD (2017). A pronounced uterine pro-inflammatory response at parturition in a marsupial. *P Roy Soc Lond B Bio*. 10.1098/rspb.2017.1694
- Hansen VL and Miller RD (2017). On the prenatal initiation of T lymphocyte development in the opossum *Monodelphis domestica*. *Jour of Anat*. 230, 596–600. 10.1111/joa.12587 [PubMed: 28052333]
- Hansen VL and Miller RD (2015) The Evolution and Structure of Atypical T Lymphocyte Receptors in Pathogen-Host Interactions: Antigenic Variation v. Somatic Adaptations. Hsu E. and Du Pasquier L. (Eds). Springer.
- Hinds LA (1988) Hormonal Control of Lactation In (Eds Tyndale-Biscoe CH, and Janssens PA) The developing marsupial: models for biomedical research. 55–67. (Berlin: Springer-Verlag).
- Ismail HI, Hashimoto Y, Kon Y, Okada K, Davis WC, and Iwanaga T (1996). Lymphocyte subpopulations in the mammary gland of the goat. *Vet Immun and Immunopath*. 52(3): 201–212.
- Joss JL, Molloy MP, Hinds L, and Deane E (2009). A Longitudinal study of the protein components of marsupial milk from birth to weaning in the *M. eugenii* (*Macropus eugenii*). *Dev Comp Immunol*. 33, 152–161. <https://dx.doi.org/10.1186%2Fs12861-015-0063-z> [PubMed: 18778730]
- Kreiss A, Obendorf DL, Hemsley S, Canfield PJ, and Woods GM (2009). A Histological and Immunohistochemical Analysis of Lymphoid Tissues of the Tasmanian Devil. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*. 292(5): 611–620. 10.1002/ar.20896
- Manning LS, and Parmely. (1980). Lymphocyte determinants of mammary lymphocyte-mediated immunity in the rat. I. The migration of radioisotopically labeled T lymphocytes. *J Immunol*. 125:2508–2514. [PubMed: 6968787]
- Oftedal OT (2012). The evolution of milk secretion and its ancient origins. *Animal*. 6,3: 355–368.
10.1017/S1751731111001935
- Old JM, and Deane EM (2001). Histology and immunohistochemistry of the gut-associated lymphoid tissue of the eastern grey kangaroo, *Macropus giganteus*. *Journal of Anatomy*, 199(6), 657–662.
10.1017/S002187820100872X [PubMed: 11787819]
- Old JM, and Deane EM (2003). The detection of mature T- and B-lymphocytes during development of the lymphoid tissues of the tammar wallaby (*Macropus eugenii*). *J Anat*. 203: 123–131. <https://dx.doi.org/10.1046%2Fj.1469-7580.2003.00207.x> [PubMed: 12892411]
- Parra ZE, Baker ML, Schwartz RS, Deakin JE, Lindblad-Toh K, and Miller RD (2007). A unique T lymphocyte receptor discovered in marsupials. *PNAS*. 104, (23) 9776–9781. 10.1073/0609106104 [PubMed: 17535902]
- Parra ZE, Lillie M, and Miller RD (2012) A model for the evolution of the mammalian T-lymphocyte receptor α/δ and μ loci based on evidence from the duckbill platypus. *Mol Biol Evol* 29(10): 3205–3214. <https://dx.doi.org/10.1093%2Fmolbev%2Fmss128> [PubMed: 22593227]

- Rand TH, Cruikshank WW, Center DM, and Weller PF (1991). CD4-mediated stimulation of human eosinophils: lymphocyte chemoattractant factor and other CD4-binding ligands elicit eosinophil migration. *Jour Exper Med*. 173(6), 1521–1528. 10.1084/jem.173.6.1521 [PubMed: 1851800]
- Reardon C, Lefrancois L, Farr A, Kubo R, O'Brien R, and Born W (1990). Expression of $\gamma\delta$ T lymphocyte receptors on lymphocytes from the lactating mammary gland. *Jour Exper Med*. 172(4) 1263–1266; 10.1084/jem.172.4.1263 [PubMed: 2145390]
- Reed JR and Schwertlfefer. (2010). Immune lymphocyte location and function during post-natal mammary gland development. *J Mammary Gland Biol Neoplasia* 15: 329–339. 10.1007/s10911-010-9188-7 [PubMed: 20730636]
- Salmon H, Berri M, Gerdtz V, and Meurens F (2009). Humoral and lymphocyteular factors of maternal immunity in swine. *Dev and Comp Immun* 33: 384–393. 10.1016/j.dci.2008.07.007
- Salmon H, and Delouis C (1982). Kinetics of lymphocyte sub-populations and plasma lymphocytes in the mammary gland of primiparous sows in relation to gestation and lactation. *Ann Rech Vet*. 13(1):41–9. [PubMed: 7165262]
- Sharp JA, Lefevre C, Watt A, and Nicholas KR (2016). Analysis of human breast milk cells: gene expression profiles during pregnancy, lactation, involution, and mastitic infection. *Functional & Integrative Genomics* 16,3, 297–321. 10.1007/s10142-016-0485-0 [PubMed: 26909879]
- Stanton SE, and Disis ML (2016). Clinical significance of tumor-infiltrating lymphocytes in breast cancer. *Journal for ImmunoTherapy of Cancer* 4:59 10.1186/s40425-016-0165-6 [PubMed: 27777769]
- Vandersompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, 3 (7) 1–12.
- Wang X, Parra ZE, and Miller RD (2011). Platypus TCR μ provides insight into the origins and evolution of a uniquely mammalian TCR locus. *J Immunol* 187(10): 5246–5254 [PubMed: 21976776]
- Yamaguchi T, Hiratsuka M, Asai K, Kai K, and Kumagai K (1999). Differential distribution of T lymphocyte subpopulations in the bovine mammary gland during lactation. *Jour Dairy Sci*. 82(7):1459–64. [PubMed: 10416161]
- Young L, Basden K, Cooper DW, and Deane EM (1997). Lymphocyteular components of the milk of the tammar wallaby (*Macropus eugenii*). *Austr Jour of Zool* 45, 423–433. 10.1071/ZO96063
- Young LJ, and Deane EM, (2001). Lymphocyteular composition of the late milk of the koala (*Phascolarctos cinereus*). *Austr Jour of Zool* 49(2) 195–202. 10.1071/ZO00062

Figure 1: CD3E⁺ T lymphocytes are present within the mammaries throughout lactation. Opossum mammaries sections from P3 (A), P17 (B), P32 (C), and P44 (D) were examined for the presence of CD3E⁺ T lymphocytes. Only lymphocytes with clear membrane staining and identifiable nucleus are indicated by arrow. CD3E⁺ T lymphocytes were present at every time point investigated. Imaged at 40X magnification. Scale bar in lower right = 10 μ m.

Figure 2: CD3E⁺ T lymphocyte within the alveolar space in the mammaryes.
P3 opossum mammary section at 40X magnification. CD3E⁺ T lymphocyte indicated by arrow. Scale bar in lower right = 10 μ m.

Figure 3: Normalized transcript abundance of TCR α and β chains is minimal yet consistently detected.

TCR α (Circles) and TCR β (Squares) abundance appears coordinated throughout lactation. Dotted line denotes normalized expression = 1. For some points the error bar is shorter than the height of the symbol.

Figure 4: CD4 and CD8α transcript abundance is low yet consistently detected.
CD8α (Circles) and CD4 (Squares) transcript abundance throughout lactation. Annotations as in figure 3.

Figure 5: TCRγ and TCRδ chain transcripts are highly abundant early in lactation.

TCRγ (Squares) and TCRδ (Triangles) abundance patterns appear most similar. TCRγ (thick line) and TCRδ (thin line) decrease linearly from week 1 through weaning (slope = -0.1665 , -0.1527 , y-intercept = 1.748 , 1.597 , $r^2 = 0.6731$, 0.8776 , and $p = 0.0067$ and 0.0002 respectively. TCRμ (Circles) abundance is significantly lower than TCRγ during week 1 and 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Annotations as in figure 3.