

Fighting Infections: The Role of Estrogen Beyond Reproductive Health

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This dissertation is submitted in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Molecular Biology, Cell Biology, and Biochemistry in the Division of Biology and Medicine at Brown University.

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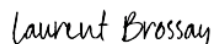
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Preface

The research work for my dissertation is generated in the laboratory of Dr. Lalit Beura, assistant professor in the Department of Molecular Microbiology and Immunology.

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- 1. Mouse Lines:** Mice used in these experiments were generated, managed, genotyped and phenotyped with the help of Corine Perloski, Maria Armillei, Delia Demers, Ying Lin, Jessica Pacia, and Lalit Beura.
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Abstract

Estrogen, the major female sex hormone has been linked with immune response disparity between males and females. However, the exact mechanism by which estrogen impacts our ability to fight viral infection and development of immunologic memory remains unknown. CD8 T cells represent the primary protective mechanism against viruses and cancer. Here we aim to understand how CD8 T cell mediated antiviral immune response is regulated by estrogen. Systemic LCMV infection in ovariectomized female mice generated lower virus-specific memory CD8 T cells compared to sham surgery controls. To check if this reduction in memory response is CD8 T cell intrinsic, I generated a transgenic virus-specific estrogen receptor- α deficient CD8 mouse line (ESR1 KO). Using a co-adoptive transfer model, I compared the differentiation of ESR1 KO and control CD8 T cells in both systemic LCMV and local influenza infection. The lack of estrogen signaling resulted in reduced abundance of effector CD8 T cells in the context of both infections. ESR1 KO CD8 T cells also displayed reduced cytotoxic ability. *In vitro* studies revealed that the CD8 T cell expansion was primarily impacted by the lack of estrogen signaling, whereas activation and cellular apoptosis remain unchanged. Altogether, this suggests a crucial role of sex steroid estrogen in initial effector CD8 T cell differentiation, that has significant ramification for generation of protective immune responses in pathological or physiological estrogen deficiency.

In addition, CD8 T cells in the female reproductive tract are a crucial component of the local antiviral defense. Within the vaginal mucosa, CD8 T cells reside in close contact with vaginal epithelial cells and the CD8-epithelium crosstalk is thought be important

for maintenance and function of CD8 T cells. Estrogen has been shown to influence the epithelial cell proliferation and integrity. However, if this estrogen mediated modulation of epithelial biology could also affect resident CD8 T cells in the vaginal mucosa has not been investigated. This study aims to elucidate how the vaginal epithelium modulates CD8 T cell differentiation in the presence or absence of estrogen, utilizing an in vitro organoid model of mouse vaginal epithelium. To identify the mechanism, biochemical or structural changes of vaginal epithelium by estrogen, I used vaginal epithelium organoid model and co cultured them with CD8 T cells. I found that estrogen treatment promoted the growth and formation of vaginal epithelial organoids, while pharmacological inhibition of estrogen in the same organoid system significantly reduced the overall size and number of these organoids. Overall, our study shows that estrogen plays a significant role in vaginal epithelium proliferation and intact estrogen signaling might be important in differentiation of resident memory CD8 T cells in the vaginal mucosa.

Introduction

Estrogen: History, Function and Therapeutics

Sex Difference and History of Estrogen

Genetic variations, hormonal dissimilarities, followed by sex specific diversity of microbiome abundancy are the major influences for a sexual dimorphism in immune system. Males, assigned at birth, carry an X and an Y chromosome while females, assigned at birth carry two X chromosomes. X chromosome inactivation, a phenomenon first described by Mary F Lyon in 1961, is when one of the x chromosomes in female born mammals gets inactivated during embryonic development to maintain gene dosage compensation(Lyon, 1961). These genes on X chromosomes are called X linked genes. Since the initial discovery, several X linked genes related to immune functions, such CD40L, CD70, and CD11, have been identified where an irregular X chromosome inactivation of these genes is strongly associated with autoimmune disorders, such as systemic lupus erythematosus (SLE) (Bianchi et al., 2012; Pyfrom et al., 2021). In addition to chromosomal differences, women and men, as first assigned at birth, have different sets of dominant sex hormones, not encoded in the sex chromosomes.

Estrogen is the major female sex hormone, and testosterone is the major male sex hormone. The use of estrogen as therapeutics for women has been documented as early as 1987 to manage menopausal hot flushes (Casey & Murray, 2008), however, the term estrogen was not coined until in 1906, deriving from Greek word *oistros* meaning “mad desire” and *gennan* meaning “to produce.” Estrogenic activities in the female reproductive tract were first suggested by Edger Allen and Edward Doisy in 1929 when Edger Allen (Santen & Simpson, 2019). Subsequently, estrogen was first purified independently by Edward Doisy and Adolf Butenandt, both of whom received noble

prize in medicine and chemistry for their work in sex hormones (Santen & Simpson, 2019). In the following years, various pharmaceutical companies rushed to commercially produce estrogen as a source of treatment in women for hot flashes, bone diseases, and ovarian dysfunctions (Albright et al., 1935a; Kohn et al., 2019). Since then, scientists all over the world dedicated decades of research in describing how estrogen impacts reproductive health, aging, menopause, bone density, cardiovascular health, musculoskeletal diseases, cognitive health, and immune health in females, assigned at birth (Albright et al., 1935b; Dubey et al., 2005; Hall & Phillips, 2005; Jacobs et al., 1998; Popat et al., 2009). We still do not fully comprehend the diverse complexity of how estrogen signaling modulates various aspects of women's health.

Estrogen: Genes, Structure, and Antagonists

Estrogen signals through estrogen receptors (ER), member of nuclear hormone receptor family. There are two forms of ER, ER α and ER β , encoded by genes ESR1 and ESR2, respectively. Different ERs are predominated on different tissues performing a diverse array of functions. For example, ER α predominates in mammary gland, uterus, pituitary glands, salivary gland, skeletal muscles, and bones where ER β predominates in ovary, prostate, lung, cardiovascular and central nervous systems (Fuentes & Silveyra, 2019; Yaşar et al., 2017). ESR1 gene, located in chromosome 6, spans about 300kb bases encoding a 66kDa protein. ESR2 gene, located in chromosome 14, spans 254 kb bases encoding a 60 kDa protein (Fuentes & Silveyra, 2019; Yaşar et al., 2017). Each ER contains 6 functionally distinct domains – amino terminal domain (NTD) or A/B domain, DNA binding domain (DBD) or C domain, flexible hinge or D domain containing nuclear localization signal (NLS), carboxy terminal ligand binding domain (LBD) or E domain, extreme carboxy terminal domain or F domain – spanning in 8 exons. Alternative splicing and polymorphism of ER have been reported and linked with various diseases (Deroo & Korach, 2006). Various agonists can also bind to the ER LBD that modulates estrogen binding to ER. Tamoxifen is a mixed regulator of ER. Tamoxifen competes with estrogen to bind with the hydrophobic pocket of LBD and causes conformational changes that inhibits further Estrogen - ER binding. Tamoxifen can act as antagonist in breast cancer tissue whereas as an agonist in most estrogen target tissues, hence the name mixed regulator. This can be attributed to flexible confirmation of LBD in presence of Tamoxifen. On the contrary, fulvestrant, also known as ICI 182,780, is an efficient blocking reagent that blocks ER α – DNA binding. Various

metal ions, such as Al^{3+} , Co^{2+} etc. can also bind with ER to modify ER activities in non-competitive manner by binding with nearby amino acid residues and thus changing ER conformations (Fuentes & Silveyra, 2019).

Estrogen is primarily synthesized in ovary but can also be produced in smaller amount in adrenal gland, osteoblasts, vascular endothelium, pancreas, brain, smooth muscles and adipose tissues (Simpson, 2003). Estradiol is secreted by granulosa cells and theca cells of ovary follicles and corpora lutea. Three structurally variable forms of female sex hormone, Estriol, Estrone, Estretrol are together called estrogen, each of which level rises at different timepoint of female reproductive cycle. 17- β estradiol (simply called estradiol) is the primary estrogen widely studies for understanding ER functions and mechanism. ERs contains DNA binding sequences and as such estradiol directly binds to the ER to translocate to the DNA to activate classical estrogen response dependent signaling pathways (Kovats, 2015). Estrogen-ER complex can also bind to a transcription factor instead and indirectly control cellular pathways which is known as estrogen response independent signaling pathway (Kovats, 2015). Classical pathway can take up to several hours for effects where indirect non genomic signaling pathway is much quicker and can happen within seconds to minutes of estrogen/ligands binding to receptors (Deroo & Korach, 2006). Estrogen can directly enter plasma membrane to bind with $ER\alpha$ or $ER\beta$ to control gene regulation. Once estrogen bound ER goes through conformational changes, the estrogen – ER complex translocates to nucleus. To date, many important gene promoters have been shown to possess ER binding region. A GWAS study has identified over 70,000 ER binding DNA domain in both mouse and human (Bourdeau et al., 2004). Major DNA binding sites of ER involves FOXA1, PBX1,

AP-2 γ , TLE1, and GATA3 where the major transcription factors are NF- κ B, AP1, SP1, C/ERBPb (Kovats, 2015). Unlike direct binding of estrogen-ER to the DNA or estrogen response element dependent pathway, the estrogen response element independent pathway is mostly activated by co-regulators, or phosphorylation of tyrosine or serine residues. These regulatory elements include protein kinases (PKA, PKC), MAPK, cytokines (IL-2), cell cycle regulators (RAS, p21) as well as growth factors (TFG- β , EGF, IGF) (Lee et al., 2009; Liu et al., 2003). The membrane associated ER activation is known to activate ERK/MAPK, PI3/AKT pathway, and nitric oxide synthetase of endothelium (Yaşar et al., 2017). In addition, estrogen – ER binding associated nuclear factors are shown to be directly associated with histone acetylation and transcription initiation, which may explain estrogen's role in chromatin reorganization, post transitional modifications, and epigenetic changes (Yaşar et al., 2017).

Aging in Women, Menopause & Hormone Replacement Therapy

Estrogen differs throughout the life cycle of a woman, assigned at birth. The estrogen level remains low from birth to pre –pubescence in women, rapidly going upwards towards childbearing age, up until mid – 30s, then going downwards steadily towards menopause, up until late 50s (Ferrini & Barrett-Connor, 1998; Lephart & Naftolin, 2021; Shin & Lee, 2016). The level of estrogen post menopause in women is similar to the level of estrogen during pre – pubescence age (Lephart & Naftolin, 2021). This sudden loss of circulating estrogen, among other sex hormones, marked as the start of menopause, has been linked with various age associated diseases in women, including skin disease, cardiovascular diseases, musculoskeletal diseases, mental health issues and autoimmune diseases. Estradiol treatment is associated with increasing cognitive functions and cardiovascular health in women (Maki et al., 2011). Since estrogen is a master regulator of multiple biochemical and developmental pathways, this sudden loss of estrogen during menopause is associated with major physiological and psychological changes in women. Whether the estrogen sensitivity of these estrogen signaling dependent cells changes with the loss of available circulating estrogen, or whether the estrogen receptors expression is compensated with any other nuclear hormone receptor family, is still largely unknown. Even with major scientific discovery over time, scientists are still trying to fully understand menopause and age associated sex hormone derived changes in women.

Hormone replacement therapy, first introduced in the 1960s, replaces the sex hormones, estrogen, and progesterone at a slower level during menopause to treat menopause associated symptoms such as hot flashes (Albright et al., 1935a; Cagnacci &

Venier, 2019). Later studies showed more detrimental effects of the hormone replacement therapy to postmenopausal women, which reduced the usage of estrogen as hormone therapy to treat menopausal treatment over the next few decades (Cagnacci & Venier, 2019; Marjoribanks et al., 2012). Researcher later found that perimenopausal hormonal therapy, referring to starting the hormone therapy with younger women in the 40s, rather than postmenopausal hormonal replacement therapy, referring to starting the hormone replacement therapy during menopause is more beneficial, and can remove the detrimental long-term effects of hormone replacement therapy (Cagnacci & Venier, 2019; Maki et al., 2011; Marjoribanks et al., 2012). In the recent years, hormone replacement therapy as a treatment for postmenopausal symptoms starts at a younger age before the first sign of menopause. In addition to postmenopausal treatment, currently hormone replacement therapy is also widely used as a gender affirming hormone therapy, where estrogen is used as feminizing hormone therapy. More studies are pertinent to understand the effect of gender affirming hormone therapy in younger population to be equipped with providing proper care to transgender and LGBTQ+ community (Connelly et al., 2019).

Loss of circulating estrogen levels during menopause and gender affirming hormone replacement therapy has been associated with altered function of innate and adaptive immune system (Gameiro et al., 2010). In addition, the urogenital structure and composition changes with menopause in women in lack of an estrogen rich environment as estrogen is produced in ovary. Menopause is a risk factor for increased urinary tract and urogenital tract infection, although link between urogenital tract infection and estrogen signaling is not clear. Atrophic changes in uterine mucosa, vaginal microbiota

composition changes, and increased risk of breast cancer are associated with menopause (Geng et al., 2020; Pichigina et al., 2019). Over 80% of all autoimmune disease patients are women, and menopause is a risk factor for increased autoimmune disease severity such as rheumatoid arthritis and SLE, among others (Sammaritano, 2012). Adaptive immune response, such as B cell and T cell response, to influenza vaccination is much higher in premenopausal women than in postmenopausal women, where hormone therapy improved overall response in the postmenopausal women (Engelmann et al., 2016). This phenomenon of lower function of immune system post menopause in women is sometimes known as immune senescence. Although much work has been devoted in finding impact of estrogen in reproductive health, innate immunity, general knowledge of estrogen mediated changes in CD8 T cells of adaptive immune system still lacks a comprehensive understanding.

Figure 1: Pathological Conditions Linked with Estrogen

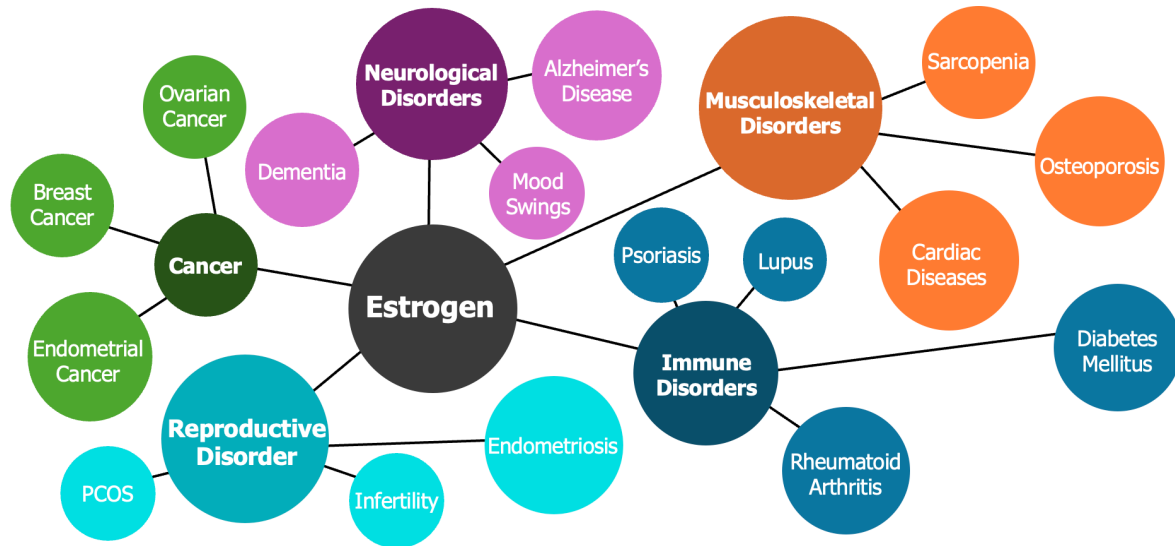


Figure 1: Estrogen is a key cell cycle regulator, and presence of estrogen is linked with increased growth of tumor cells. As such estrogen is associated with a series of cancers and are used as treatment. For example, Letrozole, Arimidex, fulvtrant are used to treat breast cancer which reduces estrogen production/level. Estrogen can be protectant of neurological dysfunction and is associated with multiple neurological disorders. For example, estrogen can block beta amyloid related damages where beta amyloid is linked with Alzheimer diseases. Estrogen increases number of connections with memory, linked with dementia as we age. Estrogen also impacts production of neurotransmitter like serotonin, acetylcholine, and dopamine – linked with mood swings. Estrogen promotes osteoclast progenitor cells and satellite cells proliferation, lack of estrogen signaling is linked with osteoporosis and sarcopenia (loss of muscle). Estrogen is protective of cardiovascular diseases and most women enters cardiovascular diseases risk group post menopause, when the circulating estrogen level is low. Estrogen

promotes fertility and uses a is fertility therapy. Irregular estrogen production is linked with both PCOS, and endometriosis linked with irregular mensuration. In addition, estrogen plays an important role in innate immune system and B cells. Over 80% of all autoimmune disease patients are females, and estrogen is linked with majority of them with immune system.

Pathologically Altered Estrogen Level

Other than menopause, several pathological conditions are linked with altered circulating estrogen level. Polycystic ovary symptom, or PCOS, a condition where women produce excessive amount of circulating testosterone and abnormal expression of estrogen receptors is linked to infertility (Mukerjee, 2018; Xu et al., 2021). In PCOS, elevated testosterone binds with estrogen receptors, albeit at a low affinity, to initiate abnormal estrogen signaling mediated changes through nuclear translocation of estrogen responsive genes (Rochefort & Garcia, 2021). Over 5 – 10 % women suffers from PCOS in the United State. Understanding estrogen signaling mediated immune system changes would clarify infection in this pathological condition in a large population.

Primary ovarian insufficiency (POI) refers to a condition ovarian dysfunction in young women before the age of 40. X chromosome mutations and genetic disposition has been associated with higher changes of POI in women. About 1 in 100 women suffers POI before they reach the age of 40 in the United States (COULAM et al., 1986). POI is associated with autoimmune disease prevalence, altered metabolic function, and other symptoms similar to presented in postmenopausal women. Hormone replacement therapy is traditionally used as a treatment for alleviating symptoms of POI in women (Cox & Liu, 2014). Similar to postmenopausal hormone replacement therapy complications, there are various risk factors associated with this treatment, including increased vulnerability to autoimmune and infectious diseases which needs to be thoroughly investigated.

Over 70% of all breast cancer are sensitive to circulating estrogen, while all ER negative or estrogen unresponsive breast cancer are shown to have reduced estrogen receptor expression, post transcriptionally (Roodi et al., 1995). Increased risk of breast cancer has been linked with estrogen receptor deficiency and circulating overall estrogen concentration in multiple publications (Berrino et al., 1996). In addition, menopausal estrogen therapy and estrogen – progesterone therapy is associated with reduced risk of colorectal cancer and increased risk of breast cancer (Lin et al., 2012; Schairer et al., 2000). This risk is dependent upon the proximity to menopause and the hormone replacement therapy. CD8 T cells and CD4 T cells play in important role in cancer immunity. Understanding how estrogen replacement therapy might affect these adaptive immune cells may give us insights into these risk factors and their associations.

Estrogen Mediated Immunity

Estrogen And Innate Immunity

Estrogen impacts innate immune cells in multiple overlapping pathways. Macrophages are phagocytic innate immune cells that directly clears microorganisms and other immune cells. Impacts of estrogen signaling is well studied in macrophages. Estrogen alters macrophage function, such as phagocytic activities, cytokine production and nitric oxide induction (Khan & Ansar Ahmed, 2016). Studies reported increased MHC II loading, necessary for antigen presentation in CD4 and CD8 T cells, by splenic macrophages lacking estrogen receptor signaling compared to macrophages with intact estrogen signaling after OVA pulsing (Lambert et al., 2005). Burn mouse model showed increased immunity with 17 beta estradiol treatment along with increased IL-6 production by macrophages which was linked to inhibition of NFkB transcription by estrogen signaling (N Messingham et al., 2001). Estradiol treatment also increased immunity, improved survival rates, and improved bacterial infection clearance in these burned mouse model (N Messingham et al., 2001).

Neutrophils are first to be recruited to the site of infection to clear microbial infection and to heal injuries. Multiple mouse studies report altered neutrophil population, function and cytokine production caused by increased estrogen signaling (Dai et al., 2017; Khan & Ansar Ahmed, 2016). Study reports increased neutrophil serine protease and myeloperoxidase upon estradiol treatment in C57BL/6J mice from spleen, bone marrow and blood samples (Dai et al., 2017). The neutrophil serine protease and myeloperoxidases are the two major enzymes secreted by neutrophil for intracellular pathogen killing. Moreover, studies on mammary involution shows increased mammary

inflammation, increased neutrophil infiltration, and increased protumoral microenvironment caused by neutrophil reprogramming with estradiol treatment post ovariectomy (Chung et al., 2017). Studies done on influenza infected lung neutrophils found decreased CCL2 and IFN γ but increase CCL3 and CXCL1 cytokine production with estradiol treatment of ovariectomized mice (Robinson et al., 2014). These cytokines and chemokines functions as chemoattractant for a variety of innate immune cells to the site of infection or injury for healing and viral clearance. Furthermore, increased neutrophil population, enhanced neutrophil mediated CD8 $^{+}$ T cell's viral clearance, and Increased proportion of IFN γ and TNF α producing influenza specific CD8 $^{+}$ T cell numbers have been associated with the estradiol treatment, even though no changes in the overall CD8 T cell population was observed. (Robinson et al., 2014).

Natural Killer (NK) cells play important role in pathogenic protection, cancer, and stress. Study reports increased IFN γ production by activated NK cells in vitro with estradiol treatment (Pierdominici et al., 2010). Another study reports increased NK cell population with decreased NK cytotoxicity with estradiol treatment (Hao et al., 2007). This decreased cytotoxicity is defined by downregulation of NK cell activating receptors CD69, Nkp46, NKG2DL, and CD44 which in turns decreased overall granzyme B and FasL secretion (Hao et al., 2007). The increase in overall NK cell population was attributed to enhanced proliferation in presence of increased estrogen signaling with estradiol treatment (Hao et al., 2007). NK T cells, a subset of NK cells that bridges innate immunity with adaptive immunity induced increased IFN γ in response to estradiol treatment when activated (Gourdy et al., 2005). This phenomenon is ablated in

ovariectomized mice and in estrogen receptor knock out mice where these NKT cells produce overall decreased IFN γ compared to their control groups (Gourdy et al., 2005).

Dendritic cells link innate immune response with adaptive immune response. Dendritic cells are the professional antigen presenting cells (APCs) that can differentially activate T cell lineages and B cells. Two types of dendritic cells, conventional dendritic cells and plasmacytoid dendritic cells have different cytokine profiles and functionalities.

Dendritic cells usually refer to conventional dendritic cells which mostly act on adaptive immune cell activation. On the other hand, plasmacytoid dendritic cells lack major APC capabilities of dendritic cells but plays crucial role in bacterial infection. Estrogens has been known to alter both types of dendritic cells functions, including dendritic cells maturation and development, starting from bone marrow (Douin-Echinard et al., 2008; Khan & Ansar Ahmed, 2016). Increased type I IFN production by plasmacytoid dendritic cells have also been reported in female mice compared to male mice due to estrogen level modulation (Kovats, 2015). This increase in IFN α production in human female plasmacytoid dendritic cells compared to male plasmacytoid dendritic cells are speculated to be in response to viral infection or synthetic TLR induction (Seillet et al., 2013). Studies in estrogen receptor knock out mice shows that plasmacytoid dendritic cells produce less IL-6 in systemic lupus erythematosus (SLE) mouse model (Jiang & Gilkeson, 2014). Overall, estrogen mediated changes in dendritic cells affects both CD4 and CD8 T cell mediated immunity as it related to antigen presentation and cytokine productions.

Estrogen and Adaptive Immunity

B cells and T cells are two major adaptive immune cells. B cells are responsible for cellular immunity while T cells are responsible for humoral immunity. T cell can be further differentiated in CD8 T and CD4 T cells. The CD8 T cells possess cytotoxic capabilities and can differentiate further into cytotoxic T lymphocyte, or CTL upon infections. The CD4⁺ T cells act as helper cells in aiding both CD8 T cells and B cells during infections (Bevan, 2004). There are 6 major lineages of CD4 T cells that are dependent on cytokine signaling by the dendritic cells and their genetic markers, each with different functionalities. Broadly, T Helper 1 or TH1 is involved in intracellular infections, T Helper 2 or TH2 is involved in parasitic infections, T Helper 17 or TH17 is involved in extra and intracellular infections, follicular T helper cells or TFH is involved in B cell activation, and regulatory T cell or Treg is involved immunosuppression post infection or inflammation. Each of these lineages are plastic and can cross activate under different infection scenarios (Zhou et al., 2009). Estrogen regulation of each of these T cell lineages play an essential role in adaptive immune response.

B cells can secrete antibody in response to an antigen and cause the target to either be lysed or undergo apoptosis. Estrogen can alter this apoptosis induced by B cells and promote survival by suppressing apoptotic signals and upregulating survival gene expression such as CD22, shp-1, bcl-2 and vcam-1 (Grimaldi et al., 2002). This enhanced B cell survival against self-receptor mediated by estrogen may be a reason why autoimmune diseases predominate in women compared to men (Grimaldi et al., 2002; Hill et al., 2011). In addition, studies in estrogen receptor 1 knock out mice

increased TFH generation, increased autoantibody production in CD4⁺ T cells, and reduced decrease germinal center B cell production (Kim et al., 2019).

TH1 is characterized by lineage determining factor IFN γ and TNF α and can be induced by IFN α /IFN β as well as transcription factor Tbet. ER α , rather than ER β regulates TH1 response. Estradiol enhances primary antigen specific CD4 T cell responses and Th1 development *in vivo* and ER α expression in hematopoietic cells is essential for a response (Maret et al., 2003). Estradiol treatment can positively regulate T-bet and IFN γ expression, and T cell expansion in ovalbumin antigen presented T cell models (Maret et al., 2003; Navarro & Watkins, 2017). Studies done in ER α deficiency mouse model showed that these splenic macrophages enhanced IFN γ production and proliferative responses on antigen activated TH1 CD4 T cells, however, the addition of estradiol did not alter IFN γ production in cell culture of purified CD4 T cells of these KO mice, suggesting estrogen dependent altered function of splenic CD4 T cells by dendritic cells (Laffont et al., 2017). In addition, Estrogen receptor 1 contributes to CD4 T cell-mediated autoimmune inflammation by promoting CD4 T cell activation and proliferation (Mohammad et al., 2018). Estrogen plays an intricate role in overall TH1 development, signaling, activation, function, and characteristics.

TH2 is characterized by lineage determining factors IL-4, expression of STAT-6, IL-4, IL-5 and IL-13 and transcription factor GATA3. Estradiol treatment increased IL-4 secretion, GATA-3 mRNA levels in CD4⁺ T cells of wild type ovariectomized mice cells when cultured *in vitro*. These markers were already decreased in ER α knock out mice which is independent of IL-2 dependent pathways responsible for Treg lineage specific immunosuppression (Matsuda et al., 2001). *In vitro* studies done in antigen challenged

and estradiol treated TH1 cells show increased IL-4 and TNF α production in female compared to male donors, respectively, pointing to an increased TH1 and TH2 lineages with estrogen signaling (Navarro et al., 2018). In liver cancer, estrogen is shown to promote IL-4 and STAT6 which promotes TH2 cells (Laffont et al., 2017). Airways infection and asthma animal model shows increased TH-2 mediated response with estradiol treatment, wherein female tend to have overall higher prevalence of TH2 cytokine production and airways infection (Chiarella et al., 2023; Fuseini & Newcomb, 2017).

TH17 is characterized by lineage factors IL-6, TGF β , and IL23 along with IL-17, IL-22 secretion, and presence of factors STAT 3, and ROR γ T. Cross relationship has been observed between estrogen and TGF β where E2 suppressed TGF β expression by decreasing Smad expression but TGF β increased ER signaling in cell cultures (Ito et al., 2010; Matsuda et al., 2001). In vitro estradiol treatment is an important enhancer of TH17 cell activation, proliferation and expansion as shown in expense of increased mitochondrial activity (Mohammad et al., 2018). Study also showed decreased IL-17A and IFN γ production in CD4 $^{+}$ T cells in colitis mouse model in spleen and lymphoid organs. ER α activation at the endothelial wall and hematopoietic compartment can increase protection against experimental autoimmune encephalomyelitis by reducing cytokines for encephalitogenic (antigen presented) TH1 and TH17, where the absence of ER α was unable to clear the infection via antigen presented TH1 and TH17 cells even in presence of estradiol treatment eluting that estradiol-estrogen receptor direct binding regulates the estrogen related pathway of TH1 and TH17 cells (Lélu et al., 2011).

TFH is characterized by IL-6 and IL-21 as well as Bcl-6 transcription factor. Estradiol treatment is known to increase TFH response and decreases IL-21 and BCL-6 mRNA expression. Increasing numbers of TFH is also established in estrogen receptor knock out spleen. TFH like cells have inhibitory effect in presence of estradiol only in females, and this is not translated in male mice (Kim et al., 2019). Furthermore, mRNA expression of Bcl-6 and IL-21 is reduced in immunized estradiol treated mice. The presence of estrogen signaling in the system suppresses TFH differentiation and functions such as high affinity antigen specific antibody production and isotype class switching. Peroxisome proliferator-activated receptors γ (PPAR γ), expressed in CD4 T cells, is another nuclear hormone family receptor that plays an important role in adipocyte growth, and are linked with autoimmune disorders, such as type II diabetes mellitus. Lack of PPAR γ signaling induced increased TFH cells in a sex biased manner (Park et al., 2016). Estrogen treatment ameliorated the inhibitory effect when the male mice were treated with a PPAR γ agonist, which only stayed true for female mice in estrus stage of mensuration, showing importance of estrogen signaling and circulating estrogen level dependent characteristics of TFH cells in a gender specific manner.

Treg is characterized by lineage determining factors TGF β and IL-2 as well as with secretion of IL-10 and transcription factor Foxp3. Treg acts as an immunosuppressant and regulates balance between pro and anti-inflammatory responses. Treg activity is highly important against mucosal membrane, autoinflammatory diseases and cancer. For example, estrogen signaling plays a positive role in Tregs in malignant environment. Cell culture studies reports that estrogen expression is significantly higher in cervical cancer tissues and Tregs where inhibiting estrogen signaling reduces overall Foxp3

expression, resulting in reduced Treg infiltrating cervical cancer tissues (Adurthi et al., 2017). In mouse model of Crohn's' disease, genetic lack of estrogen signaling is linked with female specific exacerbation of intestinal inflammation, followed by an overall reduced Treg population, compared to wildtype mice (Goodman et al., 2020). Overall, estrogen signaling in CD4 T cell mediated immunity is understood to be immune protective through multiple intricate pathways in a complex cell dependent system. However, the exact function of estrogen receptor signaling in CD8 T cell mediated immunity is still largely unknown.

CD8 T cell Development and Activation

CD8 T Cell Development

The concept of CD8 T cells as a distinct lymphocyte subset was established a few decades after the establishment of estrogen as a sex hormone. While the concept that lymphocytes are continuously circulating and being replenished regularly was known since 1953, it was not until 1961 when the first mention of T cell and B cell as unique subsets of lymphocytes was coined (Gowans et al., 1964; J Path Bact et al., 1958; Masopust et al., 2007). Over the next few decades, CD8 T cells development into thymus, their activation, function, and characteristics enriched our understanding into the complex CD8 T cell mediated immune network. The role of CD8 T cells has been thoroughly studied in viral immunity, bacterial infection, autoimmunity, inflammation, cancer and mucosal immunity, and scientists are still trying to gain better understanding into the various subsets of differentiating CD8 T cells that occupies the adaptive immunity.

The CD8 T cells arises from the lymphocyte progenitor cells, first generated in the bone marrow. The differentiation of these progenitor cells into distinct CD8 and CD4 T cells are done in thymus as early as in 8 weeks gestation period in humans. Once lymphocyte progenitor cells enter the thymus, they undergo differential expression of CD25 and CD44 followed by the T cell receptor beta (TCR β) rearrangement. These cells neither expresses CD4 nor CD8 and are thus these stages of differentiation and rearrangements are call double negative or DN stages (Takahama, 2006). Only cells that undergoes successful TCR β rearrangement differentially expresses both CD4 and CD8, known as

double positive or DP stages which undergoes further differentiation and rearrangement of TCR alpha chain (Krimpenfort et al., 1989; Takahama, 2006). Followed by positive selection and TCR alpha rearrangement, these cells travel to medulla of the thymus and undergoes negative selection through down regulation of either co receptors CD4 or CD8 T cells. This is a highly regulated process with multiple checkpoints throughout the CD4 and CD8 T cell development in thymus starting from progenitor migration whereas any cell that fails to differentiate, interact with APCs, or reacts strongly with host antigens, undergoes apoptosis, and does not continue in the next step of this developmental process. Only 5 – 10% of these cells successfully completes their journey in thymus and thus expresses either CD4 or CD8s – and only then these naïve CD4 and CD8 T cells are released into the circulations. TGFb has shown to play a crucial role in CD8 T cell development and differentiation process (M. O. Li & Flavell, 2008).

Naïve CD8 T cells are the T cells that was never exposed to their specific antigens. A recent study identified as many as 100 million unique TCR β sequences in human CD8 T cells, encompassing the large diversity of TCRs and the CD8 T cells ability to fight a plethora of unique viral infections (Miconnet et al., 2011). However, the TCR diversity and repertoire richness was inversely dependent on age, whereas distinct naïve and memory TCR repertoire increased with age, significantly reducing diversity of naïve TCRs that are clonally similar to memory TCRs (Qi et al., 2014). CD8 T cells Naïve CD8 T cells express CD62L which allows them to enter and exit lymph nodes. In addition to aiding in circulations, CD62L is also known to play an active role in homeostatic proliferation of these naïve T cells (Schuster et al., 2009). Other than homeostatic

proliferation, Bcl-2 has been shown to play an important role in maintaining naïve CD8 T cell population and diversity with age (Ferreira et al., 2000).

CD8 T Cell Mediated Viral Immunity

Three conditions must be met by antigen presenting cells to CD8 T cells for a complete CD8 T cell activation. First, the CD8 TCRs binding with the APC MHC class II to ensure antigen specific response of adaptive immunity. Second, the costimulatory molecule of APC interacts with CD8 T cells via CD40 – CD40L ligand binding which upregulates CD80 – CD28 mediated costimulatory signaling. Third, cytokines such as IL-12 and other type I/II IFN must bind with their corresponding receptors at the CD8 T cells (Curtsinger & Mescher, 2010). Once all three signaling process is initiated, the CD8 T cells is then primed and undergoes further activation process. These events happen in the lymph nodes, and the location of the lymph nodes with corresponding APCs play a crucial role in the initial priming process of naïve CD8 T cells (Prlic et al., 2007).

Dendritic cells, macrophages and helped CD4 T cells can act as APCs, while dendritic cells are the most common and potent. The presence of CD4 T cells also shown to have importance in the overall activation process where the lack of CD4 T cells is linked with less robust viral clearance and limited ability of CD8 T cell to clear secondary viral infections (Prlic et al., 2007).

Once activated in the lymph nodes, CD8 T cells undergoes differentiation and clonal expansion. The duration of APC – CD8 T cell interaction and the strength of antigen specific interaction can play a role in the CD8 T cells fate in becoming effector cells vs memory precursor cells, while other factors such as maintenance and further differentiation also play equally important role. Effector cells directly signal cytotoxic

death of infected cells while memory precursor cells undergo further differentiation to give rise to immunologic memory. This priming can take anywhere between a few hours to three to seven days, depending on the type of infection (Prlic et al., 2007). Terminally differentiated cells become cytotoxic effector CD8 T cells while less terminally differentiated cells give rise to memory precursor cells that has the ability to undergo further differentiation if presented again with similar antigens (Samji & Khanna, 2017). During the process of priming, CD8 T cells upregulate IL-2 production and IL-2 receptor expression while the APC produces increased IL-12 cytokine (Busselaar et al., 2020). Generally, *in vitro* CD8 T cell activations can be achieved by activating the CD8 T cells with CD3 (TCR signaling) antibody, CD28 (co-stimulatory signaling) antibody in the presence IL-2 and IL-12 cytokines. Once activated and clonally expanded *in vivo* in the lymph nodes, the antigen specific activated CD8 T cells migrate to the site of infection to clear viral infection.

During priming, the CD8 T cells decrease CD62L expression and increase CD69 expression which allows the cells to remain in the lymph nodes throughout the priming process mediated by S1Pr1 pathways. Once the priming is completed, the CD8 T cells then transiently express CD62L, which allows them to travel out of the lymph nodes to the site of infection, through differential inhibition of CD69 expression. Other known activation associated markers are Cd25, CD44, CD11a, along with cytokine expression markers such as IFN γ and TNF α (Schmidt & Varga, 2018). Activated CD8 T cells mediated cytotoxic clearance of viral infected cells are done by secretion of granzyme B and perforin A. Granzymes induce apoptosis of the infected cells while perforins form pores or holes in the target cells to aid apoptosis.

Figure 2: CD8 T Cell Mediated Antiviral Immunity

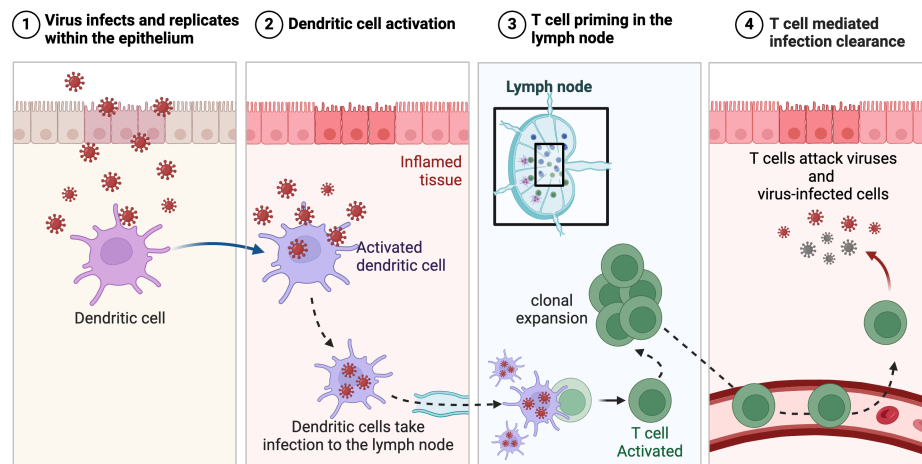


Figure 2: Stages of CD8 T Cell mediated antiviral response adapted from Bio Render.

Upon viral infection, the virus replicates in the epithelium. Antigen presenting cells are then drawn to epithelium to inquire the viral fragments in stage 1. Next, the viral fragments activate the dendritic cells, which then takes this information to the lymph node. In step 3, activated dendritic cells activates naïve CD8 T cells that matches the viral response. Once activated, the CD8 T cells undergoes clonal expansion and differentiates into effector CD8 T cells through signaling from the activated dendritic cells – a process known as priming. In the last stage of response, these effector CD8 T cells migrate to the sites of infections and preferentially clears infected cells.

Memory CD8 T Cells: Differentiation and Function

CD8 T Cell Memory Differentiation

Upon viral clearance, most effector CD8 T cells undergo apoptosis. However, a subset of effector cells remains in the system to provide long term immunologic memory during a secondary infection, known as memory T cells. Memory CD8 T cells are essential in protecting against future infections. While memory CD8 T cells are still activated by APCs, they can bypass the initial priming process and can directly differentiate into effector CD8 T cells upon activation (Wherry & Ahmed, 2004). During early infection, effector cells can be divided into two subsets – short lived effector cells or SLEC and memory precursor effector cells or MPEC. SLEC are more cytotoxic than MPEC, and the major function of MPEC is to give rise to memory cells. The expression of KLRG1, a marker for short lived terminal differentiation, is increased in SLEC compared to their MPEC counterpart (Joshi et al., 2007). Recent studies have also identified IL-7R as a marker of MPEC, which signals through CD127. SLEC are thus characterized as KLRG1^{hi} CD127^{low} and MPEC are thus characterized as KLRG1^{low} CD127^{hi} (Joshi et al., 2007). SLEC undergoes apoptosis upon viral clearance while MPEC further differentiates into different subsets of memory CD8 T cells. There are three major types of memory CD8 T cells – (1) effector memory T cell (2) central memory T cell and (3) resident memory T cells. Each of the subsets have distinct characteristics and functions.

Effector memory T cells of TEM are characterized by CD62L^{low} CCR7^{low}, while central memory T cell or TCM are characterized by CD62L^{high} CCR7^{high}. The TEM resides on both circulation and tissues, while not fully resident. TCM usually recirculates within lymph nodes and thus carries lymph node homing marker, CD62L. Both TCM and TEM

are highly plastic and can give rise to effector CD8 T cells upon reinfection. However, TEM is shown to be more cytotoxic compared to TCM and TCM is shown to have increased plasticity compared to TEM. TCM plays an important role in chronic infection (Martin & Badovinac, 2018). TCM upregulates transcription factors EOMES, TCF1, BCL-6, and STAT3 while downregulating Tbet. In the meantime, TEM upregulates Blimp1 and STAT4 while also upregulating Tbet.

Resident memory T cell or TRM are subsets of memory cells that are tissue resident and does not recirculate (Rosato et al., 2017). Over the course of time, the TRM retained in different tissues increases with age. TRMs are the most abundant and the most heterogenous type of memory CD8 T cell subsets (Jameson & Masopust, 2018; Masopust & Soerens, 2019). TRM is characterized by increased expression of transcription factors Hobit, Klf2, Blimp-1, Tbet and EOMES (Rosato et al., 2017). TRM upregulates CD69 which allows them to reside in a tissue and downregulates CD62L which prevents reentering circulation. TRMs also expresses location specific homing molecule to retain residency. For example, CD49a is a homing molecule for lung and skin specific TRMs while CD103 is a homing molecule for female reproductive tract and small intestine TRMs (Szabo et al., 2019). While TRMs mostly resides on non-lymphoid organs, evidence of long term TRMs in lymphoid organs and secondary lymphoid organs have been reported. Microenvironments are essential for TRM longevity with constant signaling requirements, and thus TRMs are short lived *in vitro*, and is very difficult to study in an *in vitro* system that is possible in other naïve and memory T cells.

Figure 3: Memory CD8 T Cell Differentiations Subsets

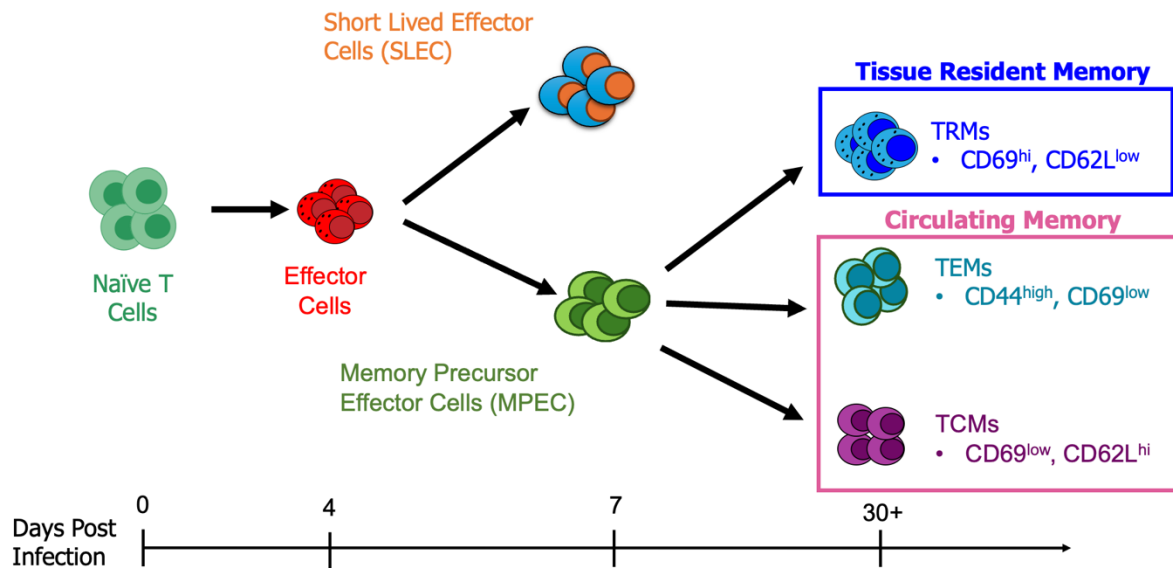


Figure 3: CD8 T cell memory differentiations into sub population in LCVM infections made in Bio Render. First, the naïve CD8 T cells differentiate into effector CD8 T cells upon activation and priming. Throughout the activation process, there are two major subsets of Effector cells that migrates into the site of infections – (1) short lived effector cells or SLEC and (2) memory precursor effector cells or MPEC. SLECs are more cytotoxic and undergoes apoptosis upon the completion of viral clearance. MPEC are slightly less cytotoxic and remains in the organs to give rise further memory subsets for continued immune surveillance. MPECs are further differentiated into three distinct memory subsets, resident memory or TRMs, Effector Memory or TEMs and Central Memory or TCMs. TRM only resides within the tissues. Both TEM and TCM can circulate in the system, while TCM does not circulate in the tissue itself – thus grouped as circulating memory CD8 T cells.

Memory CD8 T Cell Function & Therapeutics

The primary function of memory CD8 T cell is to preserve an immunologic memory to aid in protection against secondary, or even tertiary infection. Studies show that while TRMs recruits other circulating memory cells in response to a local reinfection, TRMs still dominates the viral immune response (Beura et al., 2018). Despite deriving from activated CD8 T cells, TRMs do not constantly secrete anti-inflammatory cytokines, and undergo homeostatic maintenance until it is presented with an antigen. TRMs are the first line of defense against viral infection in barrier organs such as vaginal mucosa, lungs, and skins (Gebhardt et al., 2018). First barrier organ in the upper respiratory tract is nasal epithelium. While both lung epithelium and nasal epithelium are part of the respiratory system, they have distinct sets of TRM subsets performing different function and maintenance. While influenza specific lung epithelium TRMs are primarily responsible for generating an immune response to influenza, nasal epithelium TRMs primarily stop viral infection spreading outside nasal epithelium to act as a protective barrier against secondary infection (Pizzolla et al., 2017).

Studies found importance of both live viral presence and location targeting to provide a long immunologic memory by TRM. In this influenza study of mouse model, TRMs in response to a live attenuated influenza virus, compared to inactivated influenza virus produced lung specific TRMs that are functionally similar to TRMs produced during a influenza infections (Zens et al., 2019). Multiple studies suggests that efforts to develop vaccination that will increase general TRMs against respiratory, reproductive and dermis infections may be highly beneficial and targeted in clearing viral infection in a faster and more efficient manner compared to using neutralizing antibodies (Rotrosen &

Kupper, 2023). Furthermore, studies with animal model shows that *in vivo* antigen challenge of exclusively respiratory dendritic cells resulted in a very strong influenza specific TRMs that were protective against a lethal dosage of influenza infection in these animals (Wakim et al., 2015). TRMs generating vaccination engineering has high potential in providing long term protection against mucosal and respiratory infections.

Memory CD8 T Cell Maintenance

TRMs are maintained localized in a niche microenvironment in different tissues (Takamura, 2018). For example, in case of influenza infection, TRMs are spatially located in specific lung compartments, rather than being ubiquitously present (Turner et al., 2014). As opposed to influenza specific TRMs, a cytomegalovirus specific TRMs were localized in both lungs and spleen, further showing viral specific location preference of TRMs, originated from the site of infection. These TRMs did not require maintenance or communication with circulating CD8 T cells. Similarly, distinct population of TRMs resides under the hair follicles, and IL-15 expression by hair follicles were required for CD8 TRM maintenance in that location (Adachi et al., 2015). In small intestine, two distinct location contains subsets of TRMs – intraepithelial lymphocyte (IEL) and lamina propria lymphocytes (LP) (Takamura, 2018). The recruitment of both of these TRMs is mediated by integrin $\alpha 4\beta 7$ and integrin CCR9, both of which are present during site specific APC activation. Both CD69 and CD103 are highly expressed in intestinal TRMs as they act as both homing molecules and residency markers. Retinoid acid homeostasis, TGF β signaling and continuous IL-15 production in the guts are essential in maintaining gut specific TRMs (Christo et al., 2021). In female reproductive tract, TRMs are maintained in two compartments, similar to lungs – upper female reproductive tract and lower reproductive tract (Beura et al., 2018). IFN γ and CCL5 are two important homing cytokines that are essential to retain CD4 TRMs in the female reproductive tract (Baruch et al., 2014).

Estrogen and Infection

Sexual dimorphism of CD8 T cell mediated adaptive immunity is important when it comes to mortality, hospitalization, and prevalence of specific infections. For example, men suffered higher Covid-19 related mortality and increased disease severity than women (Bechmann et al., 2022). Studies suggest that sex hormone, especially estrogen may play an important role in this differential outcome, highlighting estrogen's immune protective roles (Al-kuraishy et al., 2021). Similar effects are also seen in influenza infection, where women have increased hospitalization rate than men. In addition, sexually dimorphic immune response has been reported in other infections such as influenza vaccination studies, hepatitis A studies, hepatitis B studies, measles studies, etc. (Cook, 2008). Despite these speculations and statistics, specific roles of estrogen signaling in CD8 T cell intrinsic viral immunity pathways are still largely unknown.

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Chapter 1: Role of CD8 T Cell intrinsic estrogen receptor signaling in CD8 T Cell Differentiation

Introduction:

Estrogen, the major female sex hormone, is a master regulator in biological women's health. The role of estrogen signaling has been thoroughly elucidated in innate immunity in the past decades (Jaillon et al., 2019; Kovats, 2015). However, the how CD8 T cell intrinsic estrogen signaling may impact antiviral immunity is still largely unknown. The sex biases of adaptive immunity are widely visible in both public health outcomes and in animal model studies (Fink et al., 2018; Gabriel & Arck, 2014; Klein et al., 2012; Morgan & Klein, 2019). Women have lower overall mortality rate than men in the recent Covid 19 pandemic, worldwide (Bechmann et al., 2022; Klein et al., 2020; Y. Li et al., 2020). However, women suffer more robust response and are hospitalized at a higher rate in influenza infection compared to men. Recent studies in X and Y chromosomal abnormality shows some attribution of X linked chromosome (Klein 2024). Animal studies with estrogen receptor knock out mouse show estrogen mediated increased CD4 T cells activities and function in autoimmune disorder (Mohammad et al., 2018). Despite the plethora of evidence that estrogen might impact CD8 T cell mediated viral immunity, the CD8 T cell intrinsic role of estrogen signaling is still largely unknown.

During viral infection, antigen presenting cells migrate to the lymph nodes to activate their antigen specific naïve CD8 T cells. Activated CD8 T cell undergoes further differentiation and clonal expansion to better equip themselves to clear viral infection. These activated CD8 T cells, known as effector CD8 T cells, then migrates to the site of infection to initiate antigen specific apoptotic clearance of infected cells(Pennock et al., 2013). Effector CD8 T cells can be divided to two major subsets – short lived effector

cells (SLEC) and memory precursor effector cells (MPEC). SLEC is characterized as $\text{KLRG1}^{\text{hi}} \text{CD127}^{\text{low}}$ where MPEC is characterized as $\text{KLRG1}^{\text{low}} \text{CD127}^{\text{high}}$. Upon viral clearance, majority of these SLEC also gets cleared from the system, however, MPECs differentiates into different memory subtypes (Joshi et al., 2007). Three major memory CD8 T cells are central memory T cell or TCM, effector memory T cell or TEM, and resident memory T cell or TRM. Among those, TRMs are the most abundant and are positioned to clear viral infection as a first line of defense with increased cytotoxicity (Beura et al., 2018).

This study aims to understand the CD8 T cell intrinsic estrogen signaling in response to a systemic infection. During viral infection, B cells produced antibody response and CD4 T cells control and regulate overall CD8 T cell response in clearance of viral titer. Estrogens' impact in both B cell and CD4 T cell mediated viral immunity has been thoroughly studied in the past few decades (Grimaldi et al., 2002; Hill et al., 2011; Lambert et al., 2005; Maret et al., 2003; Polanczyk et al., 2004). Thus, to exclude any B cell and CD4 T cell mediated antiviral immune response in this study, we generated a T cell specific estrogen receptor knock out model models to study CD8 T cell intrinsic antiviral response pathways. In this study, we used an adoptive transfer system to further isolate CD8 T cell specific antiviral immune response to systemic infection. We monitored CD8 T cell phenotype and function during effector, memory, and maintenance timepoints in presence and lack of estrogen signaling in the same mouse cohorts. We found an overall lack of effector response in absence of estrogen signaling that arose from early differentiation and proliferation process.

Materials & Methods:

Ovariectomy:

First, we anesthetized the mice with ketamine to perform ovariectomy and sham surgeries (mock). Briefly, we removed both ovaries in the female mouse in the ovariectomy groups (OVX/no E2) and inserted estradiol pellet in these mice in ovariectomy with estrogen pellet group (OVX + E2). The 17 β estradiol pellet had a total concentration of 0.05mg/pellet with a 60-day slow release in the system. The mice in sham surgery group had intact ovaries and did not receive the estradiol pellet. All three groups of mice were then rested for two weeks, after which we infected them with LCMV Armstrong. These mice were euthanized 40-days post infection to collect CD8 T cells from different lymphoid and non-lymphoid organs.

Animal Model:

Out of two major estrogen receptors – ER α and ER β , CD8 T cells dominantly expresses ER α . The ER α is encoded by ESR1 gene. We knocked out ESR1 gene in CD4 and CD8 T cells generating ESR1 Knock Out (ESR1 KO) mouse model using a Cre-LoxP system where the 3rd exon of the Esr1 gene encoding ER α is flanked resulting in Estrogen insensitive CD8 T cells and CD4 T cells in this mouse line. In this system, all other cells except CD4 and CD8 T cells express ER α . In presence of estrogen in the system, undergoes estrogen signaling mediated changes. However, due to the lack of ESR1 gene, the T cells cannot sense estrogen and thus does not undergo estrogen signaling mediated changes by ER α . These cells were bred to be selectively carrying specific leukocyte markers - CD45.1 and CD45.2 congenic markers to differentiate between wild

type and knock out mouse during experiments. In addition, these mice were then bred into the P14 mice to ensure that they only contain clonal CD8 T cells specific for LCMV-gp33 epitope. Thus, all CD8 T Cells in these mice are P14 transgenic, carrying specific T cell receptors specifically for the gp33 peptides. No abnormalities are visible in these mice in the lack of estrogen signaling in the CD8 T cells that are usually dominant in full body ERα knock out mouse model – such as abnormal body weight, abnormal thymus, etc. The control mice under this strain have ESR1 flox but lack CD4 Cre thereby obtaining a normal estrogen sensing CD8 T cells whereas the KO contains both ESR1 flox and CD4 Cre genes thereby obtaining Estrogen unresponsive CD8 and CD4 T cells.

Infection system:

In most cases, Lymphocytic Choriomeningitis Virus (LCMV)-Armstrong was used as the primary systemic infection. LCMV is a mouse pathogen that generates a strong antiviral CD8 T cell response and many of the tools to monitor this response are readily available. CD8 T cells recognize multiple viral epitopes in LCMV, but the gp33-41 and np396-np404 specific responses are immunodominant and well characterized. We infected mice with 2×10^5 plaque forming unit (PFU) of LCMV-Armstrong stain intraperitoneally. Systemic LCMV infection with this strain is very well studied and is usually cleared by day 8 post-infection. For protection studies, we infected the mice with 2×10^6 PFU of Vaccinia Virus intraperitoneally or 2×10^5 PFU vaccinia virus trans-cervically. Systemic Vaccinia Virus infection is also well studied for CD8 T cell protection assays. We monitored viral loads and CD8 T cell characteristics over 3 days period post this Vaccinia Virus infection using flow cytometry. For CD8 T cell memory studies, mice were infected intravaginally with LCMV virus. Briefly, LCMV-immune memory mice

were sedated and placed in a supine position with hind region of body elevated. Antigen in the form of virus (2×10^5 PFU Vaccinia virus) was delivered using a blunt gel loading tip to the cervical canal. This led to tissue resident CD8 memory T cell reactivation and the mice were assessed 12 hours later for the sensing and alarm functions of the CD8 resident memory T cells (Schenkel et al., 2013). For influenza studies, 200PFU of PR-8 strain of influenza virus was used intranasally. Briefly, the mice were sedated using ketamine and infected slowly intranasally using viral droplets.

Adoptive Transfer

To isolate and enrich naïve CD8 T cells for adoptive transfer, first, the lymphocytes were isolated from spleen and lymph nodes – mesenteric lymph nodes, iliac lymph nodes, and inguinal lymph nodes. Next, the tissues were mechanically disrupted filtered using a nylon filter. To remove all red blood cell contaminations, ACK Lysis buffer was used to lyse the red blood cell membranes using osmotic pressure. The naïve CD8 T cells were isolated using Mojo Sort magnetic beads with streptavidin and negative CD8 T cell selection antibody cocktail. CD44 marker was used in the naïve CD8 T cell negative selection antibody cocktail to remove all memory cells from the mix. Once the CD8 T cells were isolated, the purity and quality of the cells were confirmed using flow cytometry. Cells were counted using hemocytometer and Trypan Blue under inverted light microscope. For all adoptive transfer, a total of 50,000 cells were transferred each mouse for the primary infection intravenously. For adoptive transfer, a 50:50 mixture of wild type and knock out enriched CD8 T cells were transferred, with 25,000 each cell origins. The ratios were confirmed using Flow cytometry. These adoptive transferred mice were infected the next day with corresponding virus.

CD8 T Cell isolation from tissues

CD8 T cells were isolated from blood, lymphoid and non-lymphoid organs 3, 7, and 30+ days post infections. Briefly, lymph nodes were mechanically disrupted to collect CD8 T cells. Red blood cells can autofluorescence, thus can cause false signals in Flow cytometry when bound with antibody. As previously mentioned, ACK lysis buffer was used to remove red blood cell contaminations from secondary lymphoid organs such as spleen and blood. For non-lymphoid organs such as small intestine, salivary glands, kidney, and female reproductive tracts were enzymatically digested to isolate CD8 T cells. The small intestines were digested in DTE. Salivary glands, kidney and female reproductive tracts were digested in collagenase IV. For kidney, liver, and small intestines, the isolated cell mixture was further enriched using percoll gradients. These cells were characterized using flow cytometry. For intracellular and transcription factor antibody staining, Tonbo, and BD intracellular staining kits were used.

In Vitro Cell Culture studies:

For *in vitro* studies, enriched CD8 T cells were activated using 2C11 (CD3) and B7.1 Fc in 96 well plate. CD3 represent TCR signaling and B7.1 represent CD27 mediated secondary activation signaling – both necessary for CD8 T cell activations *in vivo*. The plates were coated with differential concentration of CD3 and B7.1 overnight at 4C in dark. The next day, the plates were washed twice in sterile DPBS. 200,000 – 400,000 of enriched CD8 T cells were plated in each 96 well plates. Briefly, we collected CD8 T cells from spleen and lymph nodes, isolated naïve CD8 T cells and enriched them using Biotin conjugation – magnetic bead system of negative selection as previously mentioned. Purity was monitored with FACS assays. Fresh IL-2, IL-12 and BME was

added to the restimulation media every 2 days. The T cells characteristics were monitored using flow cytometry 24 hours, 48 hours and 72 hours post plating. For cell division studies, the cells were pre-stained with CTV dyes prior to staining. For cell death studies, annexin V and caspase III antibodies were added as indicated in the protocols.

Results:

Decreased population of antigen specific memory CD8 T cells in ovariectomized mice is ameliorated by supplementation of 17 β estradiol.

To investigate the role of circulating estrogen in overall antigen specific memory CD8 T cell population during a viral infection, we compared CD8 T cell population after LCMV infection in three groups of age matched, 12 weeks old *C57Bl/6j* female mice with circulating estradiol level as a variable. The first group is mock surgery mice with intact ovary and no supplemental estradiol treatment. The second group is OVX mice with reduced circulating estradiol. The third group is ovariectomized mice with 17 β estradiol supplementation that slowly releases estradiol over a period of 60 days (OVX + E2).

Circulating estradiol concentration was confirmed by estradiol ELISA assay of blood samples of these mice over time. This allowed a direct comparison of effect of estrogen signaling in CD8 T cells population. We found that antigen specific np396+ epitopes of CD8 T cell population of spleen was reduced in OVX/No E2 group compared to mock group (1A, 1B). Furthermore, supplementation of estradiol in OVX + E2 group ameliorated this estrogen specific loss of CD8 T cell by recovering the CD8 T cell population decrease (1A, 1B). This data suggests direct role of estrogen signaling in antigen specific CD8 T cell population after a viral infection. Next, we investigated whether this impairment is CD8 T cell intrinsic.

Figure 1: Reduced Antigen Specific T Cell in Ovariectomized Mice Compared to Mock and Estradiol Treated Mice

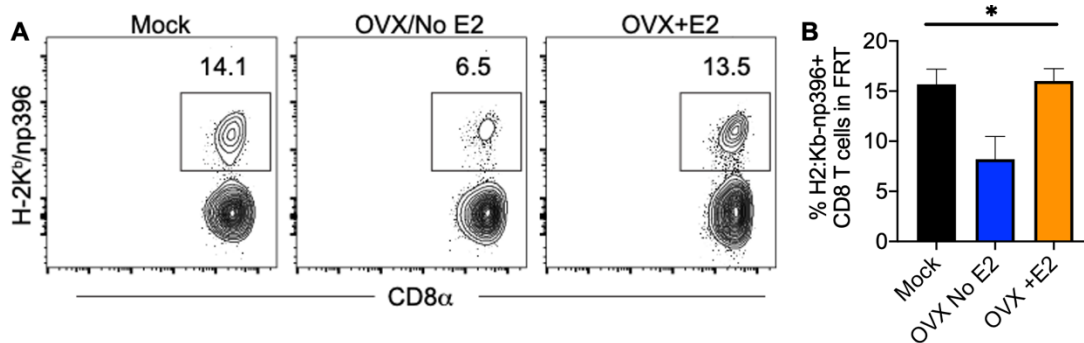


Figure 1: Reduced memory CD8 T cells in OVX mice. The 3 indicated groups of mice (mock surgery, OVX without E2, and OVX with E2) were infected with LCMV 2 weeks post ovariectomy. A. The LCMV-np396 tetramer-specific response is shown (Gated on live CD8 T cells). B. Percentage of np396⁺ CD8 T Cells shown in bar graph. This data is a representation of one of the N=3 independent trials.

Effector CD8 T cell population decreased in the lymphoid organs in the absence of ESR1 signaling in LCMV infection.

To investigate the role of estrogen signaling intrinsic to CD8 T cell mediated viral immunity, we adoptively transferred 1:1 ratio of wildtype and ESR1 KO CD8 T cells and infected them with LCMV (Fig. 2A). Both wild type and knock out mice are differentially marked with expressions of congenic lymphocyte markers CD45.1 and CD 45.2, while the host mice are marked with single expression of CD45.2, which are easily distinguishable with flow cytometry. (Fig. 2B, 2C). First, we investigated the role of estrogen signaling in effector CD8 T cell differentiation. With LCMV infection, the peak effector time is 7-8 days post infection with a P14 transfer. 8 days post infection, we harvested tissues from lymphoid organs and characterized CD8 T cells. For lymphoid organs, we collected inguinal lymph node, iliac lymph nodes and mesenteric lymph nodes because these drains in lower abdomen areas of LCMV infection route. We estimated the total population with bead counts per tissue using flow cytometry and calculated the ratio of wildtype and ESR1 KO CD8 T cells in each tissue. We observed decreased population of CD8 T cells lacking estrogen signaling compared to the CD8 T cells with intact estrogen signaling in blood, spleen, and lymph nodes (2B – 2E), while the initial transfer ratio was identical. Similar decreased population of KO effector CD8 T cells compared to their control counterpart was also observed in influenza infection in mediastinum lymph node, draining lymph node for lungs (data not shown). Our data suggests that estrogen signaling is important in CD8 T cells for effector differentiation in lymphoid organs, and that lack of estrogen signaling of the CD8 T cells results in an overall decreased antigen specific effector CD8 T cell population in lymphoid organs.

Figure 2: Reduced Effector T Cell in the Absence of ESR1 Mediated Estrogen Signaling 8 Days Post Infection in Lymphoid Organs

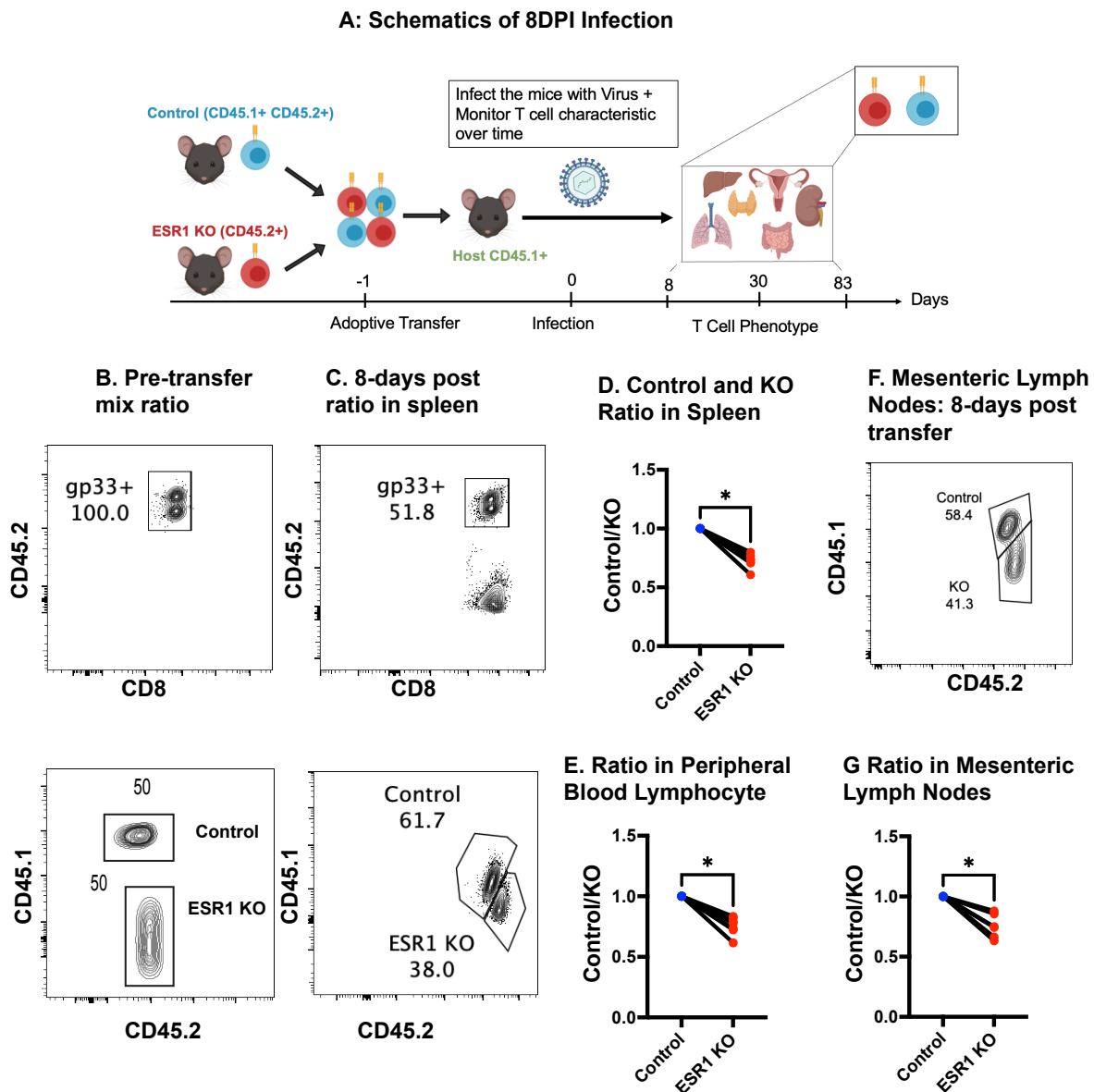


Figure 2: Reduced KO antigen specific gp33+ effector CD8 T cell population in lymphoid organs in absence of estrogen signaling. A. Schematic of infection model B. Flow plot showing ratio of Control and KO enriched CD8 T cell mix before adoptive

transfer, C. Flow plot and D. combined data of N=3 with three independent trials showing ratio of Control and KO in spleen 8-days post spleen. E. Ratio of Control and KO in peripheral blood lymphocyte 8 days post infection. F. Flow plot, and D. combined data of N=3 with three independent trials showing ratio of Control and KO in spleen 8-days post mesenteric lymph node.

Effector CD8 T cell population decreased in the non-lymphoid organs in the absence of ESR1 signaling in LCMV infection.

Next, we identified the role of estrogen signaling in effector CD8 T cell in non-lymphoid organs. After the CD8 T cell activation and clonal expansion in the lymph nodes, initiated by the antigen presenting cells, these CD8 T cells migrate to the sites of infections for viral clearance. Similarly with the lymphoid organs, we collected CD8 T cells from non-lymphoid organs – salivary gland, small intestine (intra epithelium lymphocytes only, SI - IEL), kidney, and liver. To account for the structural and anatomical differences between different part of female reproductive tract, we divided the mouse female reproductive tract into two sections while isolating CD8 T cells. The upper female reproductive tract contains ovary and uterine junction. The lower reproductive tract contains cervix and vagina. Next, we counted both wildtype and ESR1 KO CD8 T cell population in each tissue and calculated their population ratio. We stained the circulating CD8 T cells with BV711 fluorochrome prior to euthanizing the mouse to exclude circulating CD8 T cells from blood that are temporarily residing in the organs from the CD8 T cells that are migrated to the organs for viral clearance. Similar to what we observed in the lymphoid organs, we noticed that the lack of estrogen signaling also decreases CD8 T cell population in non-lymphoid organs (Fig. 3A – 3E). We concluded that the defects seen in the lack estrogen signaling in the CD8 T cells in lymphoid organs is also translated in the non-lymphoid organs, there is no visible compensatory phenomenon, and estrogen signaling is important for antigen specific effector CD8 T cell population in non-lymphoid organs. Interestingly, no difference between control and KO was observed in the SI IEL.

Figure 3: Reduced Effector Population in Absence of ESR1 mediated Estrogen Signaling in 8 Days Post Infection in Non-Lymphoid Organs

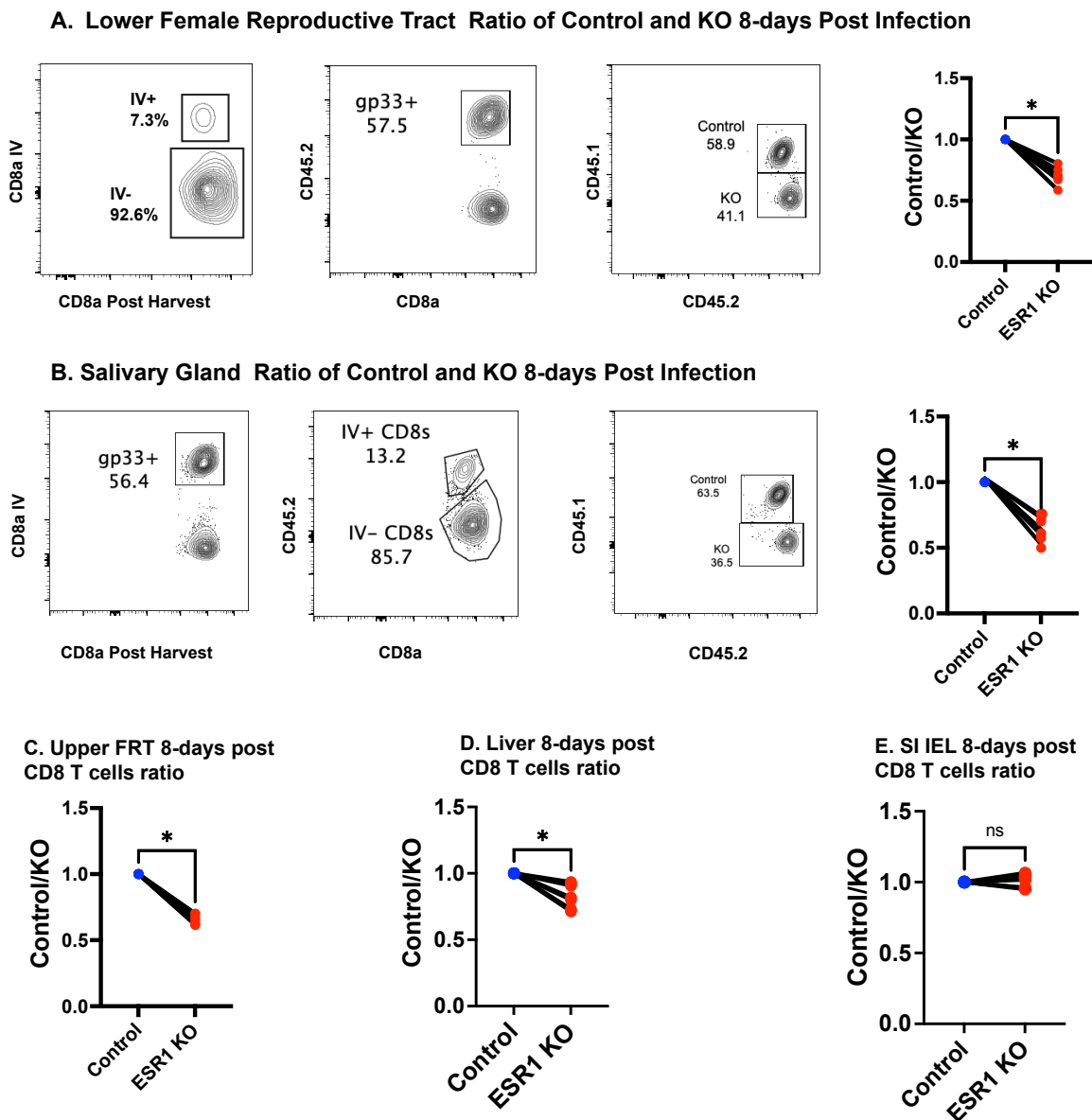


Figure 3: Reduced KO antigen specific gp33+ effector CD8 T cell population in non-lymphoid organs in absence of estrogen signaling. Flow plot and ratio of Control and KO

in A. Lower Female Reproductive Tract and Salivary Gland. Ratio of Control and KO in
C. Upper Female Reproductive Tract (Upper FRT), D. Live and E. small intestine SI IEL.
Data represented of N=4 each sets from two independent trials.

Absence of ESR1 signaling shows Reduced effector molecule expression.

Next, we identified the expressions of antiviral immunity related effector molecules and transcription factors in both control and ESR1 KO effector CD8 T cells 8 days post infection from spleen. Granzyme B is serine protease enzyme that is secreted by the cytotoxic CD8 T cell to aid in viral clearance. We observed a decreased Granzyme B expressions in cells that are lacking estrogen signaling compared to the wildtype in the same system suggesting direct impact of estrogen signaling in effector function of these CD8 T cells (Fig. 4A). We also characterized IFN γ , which acts as a pro-inflammatory cytokine during effector time, and TCF-1, a transcription factor negatively associated with the effector function of CD8 T cells during viral clearance. Interestingly, we saw a decreased IFN γ expression, and an increased in TCF-1 expression in CD8 T cells in absence of estrogen signaling (Fig. 4B, 4C.). Our data suggests that estrogen signaling may play an important role in effector differentiation and development of effector function in CD8 T cells, and lack of estrogen signaling may result in overall decreased effector function.

Figure 4: Expression of Effector molecule and a Related Transcription Factor on effector CD8 T cells in the absence of ESR1 signaling

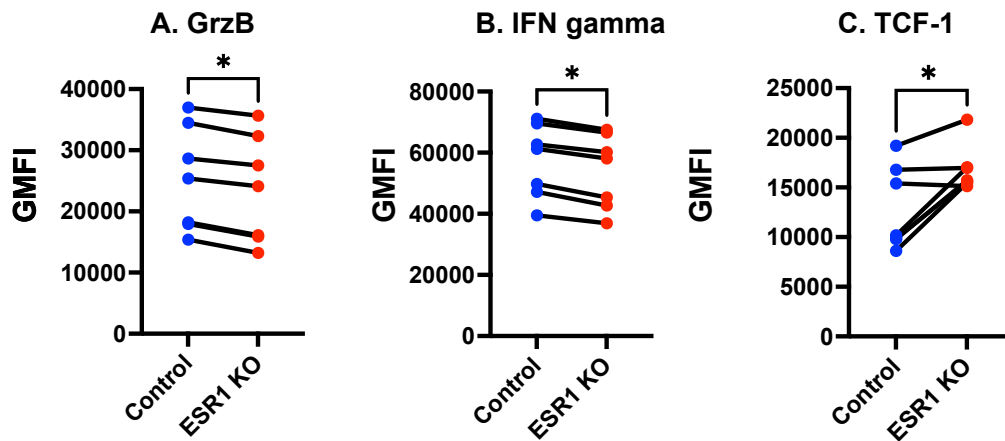


Figure 4: Transcription Factor analysis shows decreased effector function in absence of estrogen signaling. A. Granzyme B. IFN gamma and TCF-1 expression of control and KO 8 days post LCMV infection. Data represented of N=4 each sets from two independent trials.

Memory CD8 T Cell population decreased in the lymphoid and non-lymphoid organs in the lack of ESR1 signaling.

Next, we looked at the phenotype of CD8 T cells at memory timepoints. Memory T cells are poised to provide a faster adaptive immune response to a secondary infection. There are two major types of memory CD8 T cells – circulating memory cells and resident memory cells. Circulating memory cells express CD62L+ and thus continuously circulates with the blood and lymphatics. The resident memory cells express CD69+ and does not recirculate through the circulation. These resident memory cells are thus resident in the major barrier organs and are the first line of protection against secondary or tertiary viral infection. We collected CD8 T cells from both lymphoid and non-lymphoid organs 30+ days post infection when the resident memory cells are fully established after LCMV infection. Only IV- cells (cells present outside the vasculature) were screened for control and KO ratio for lymphoid organs to avoid inclusion of circulating memory cells in this dataset. Similar to the effector timepoints, we observed that the ratio of ESR1 KO is still lower in both lymphoid and non-lymphoid organs, when compared to their control CD8 T cell counterparts in the same mice (Fig 5). Similar trend was seen in 90 days post infection where the cells lacking estrogen signaling still maintained reduced population compared to their control counterpart (data not shown). SI IEL CD8 T cells also maintained similar ratio without any difference for 30 days post infection as seen at 8 days post infection. This data suggests that the lack of estrogen signaling mediated defects of effector CD8 T cells still maintained during the memory formation, and that estrogen signaling is equally important in the establishment of resident memory CD8 T cells. This data, taken

together with the effector, 30 days post, and 90 days post infection memory timepoints, suggests that whatever differentiation defects is caused by the lack of estrogen signaling in CD8 T cells are permanent and does not alter with time.

Figure 5: Reduced Memory CD8 T Cells in Absence of ESR1 Mediated Estrogen Signaling in Both Lymphoid and Non-Lymphoid Organs 30+ Days post infection

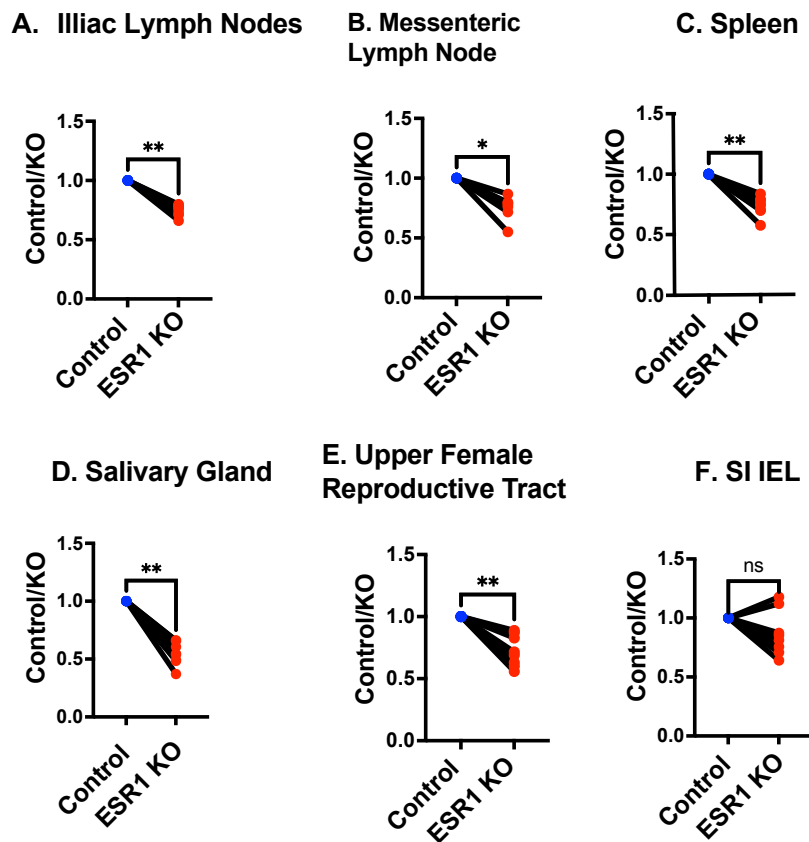


Figure 5: Reduced memory CD8 T cells in absence of estrogen signaling post infection. 30 days post LCMV infection, CD8 T cells were isolated from both lymphoid and non-lymphoid organs. Control and KO CD8 T cell ratio is presented for A. iliac lymph node, B. mesenteric lymph node, C. Spleen. Control and KO IV- CD8 T cell ratio is presented for D. Salivary Gland, E. Upper Female Reproductive Tract and F. SI IEL. Data represented of N=4 each sets from two independent trials.

TRM secondary activation was affected in the absence of ESR1 signaling, however no circulating memory CD8 T cell function remain unaltered.

To further evaluate CD8 T cell intrinsic estrogen signaling in the subsets of tissue resident CD8 memory cells and circulating memory cells, composed of both effector memory cells and central memory cells, we performed secondary viral infections. To evaluate TRM function, upon 40 days post the initial LCMV infection, we reinfected these mice locally using Vaccinia Virus. Briefly, we injected VCAM (MK2.7, 250 microgram /mouse) and VLA4 (PS2, 250 microgram/mouse) dissolved in DPBS with 200ul total volume intraperitoneally 12H before intravaginal challenge with Vaccinia Virus gp33. The VCAM and VLA4 treatment was done to prevent any trafficking of circulating T cells from central circulation to the FRT, such that we can assess the function of TRM alone. This is a local infection and thus initiates sensing and alarm functions of local TRMs. We euthanized the mice after 12 hours of secondary infections and characterized TRM sensing and alarm function. 12 hours post infection, the ratio of Control and KO was maintained similar to what was seen at memory time point (Fig 6A). However, a modest decrease in IL-2 gMFI was observed in CD8 T cells lacking estrogen signaling, suggesting intrinsic estrogen signaling may impact TRM function of CD8 T cell as it relates to sensing and alarm function (6B).

To investigate the role of estrogen signaling in circulating memory CD8 T cell function, we first adoptively transferred equal ratio of Control and KO CD8 T cells in 10 weeks old CD45.1+ transgenic female mouse. Next, we infected the mouse with LCMV virus, followed by 40 days resting period to generate circulating memory CD8 T cells. After 40 days, we isolated and sorted control and ESR1 KO circulating memory cells from spleen

and lymph nodes, and separately adoptively transferred the circulating memory CD8 T cells to *C57Bl/6j* mice. We transferred 5,000 cells per mouse, each mouse got either control CD8 T cell or KO CD8 T Cells. Next, we infected these mice with Vaccinia Virus and harvested tissues from spleen 7 days post infection. There was no significant difference in antigen specific CD8 T cell population expansion, KLRG1 expression or viral load between control and ESR1 KO mice (Fig. 6C – E). This data suggests that ESR1 signaling does not impact circulating CD8 T cell function or characteristics.

Figure 6: Lack of ESR1 affected IL-2 expression on reactivated TRM but didn't modulate circulating memory T cell function

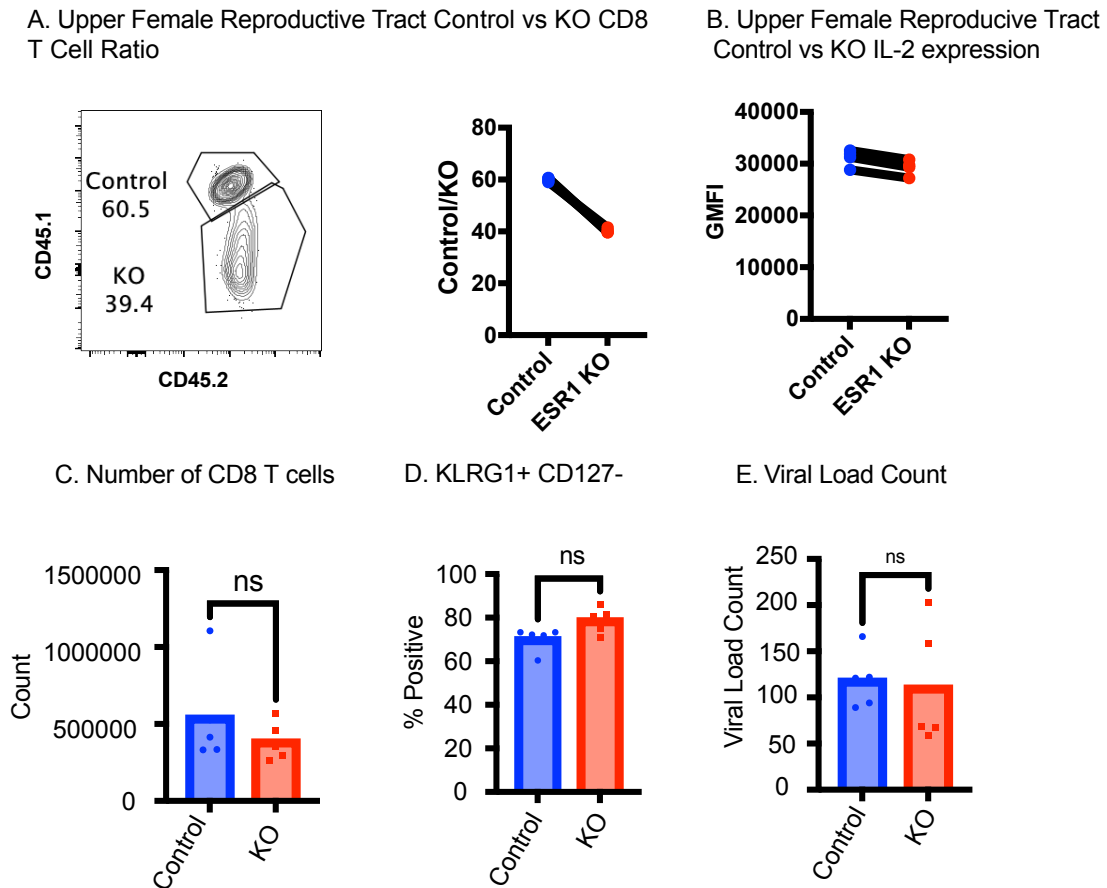


Figure 6: Slightly reduced TRM function and no difference in circulating memory CD8 T cell function in absence of estrogen signaling. A. Equal ratio of Control and ESR1 KO cells were adoptively transferred followed by LMCV infection. 40 days post infection, circulating control and KO antigen specific CD8 T cells were isolated from spleen and separately injected into naïve mice. These mice were immediately infected with Vaccinia Virus, spleen was harvested 7 days post infection. C. control and KO CD8 T cells population, D. Percentage of KLRG1+ population and E. Viral load 7 days post infection.

Lack of ESR1 signaling causes decreased cell proliferation and increased cell death *in vitro*, no difference in activation.

So far, our data suggests that the initial CD8 T cell effector differentiation defect remains constant throughout CD8 T cell mediated antiviral immune response. Since there was no difference in the ratio of control and KO CD8 T cells between lymph nodes and non-lymphoid organs, and there was no difference in the circulating TCM population in absence of estrogen signaling (6C-E), the migration is less likely affected by estrogen signaling. We suspected that the estrogen dependent defects are most likely happening earlier during the activation process. To investigate the role of estrogen signaling in CD8 T cell activation, we took an *in vitro* approach. We isolated and enriched both control and ESR1 KO CD 8 T cells as described earlier and activated them *in vitro*. CD25 and CD69 are activation markers for CD8 T cells, both expressions are increased *in vivo* during APC mediated priming in the lymph nodes post infection. We did not see any difference in CD25 and CD69 expression between activated control and KO CD8 T cells, suggesting activation is not impaired in this system (Fig. 7B-C). We next investigated clonal expansion and cell division in activated CD8 T cells using CTV assays. Our data suggested a cell division defect in absence of estrogen signaling in activated CD8 T cells where the control CD8 T cells were one cell cycle ahead of KO CD8 T cells, despite equal activation strength (Fig. 7D). We also observed increased activated Caspase 3 expression in KO cells in absence of estrogen signaling compared to their control CD8 T cell counterpart (Fig. 7E). Taken together, this data suggests that lack of estrogen signaling cause a decreased cell division and increased cell death of activated

CD8 T cells. Taken together, estrogen signaling plays an important role in activated CD8 T cell division and survival, rather than in activation of CD8 T cells.

Figure 7: Lack of ESR1 Signaling Caused by Reduced Cell Expansion and Poor Survival Post Activation

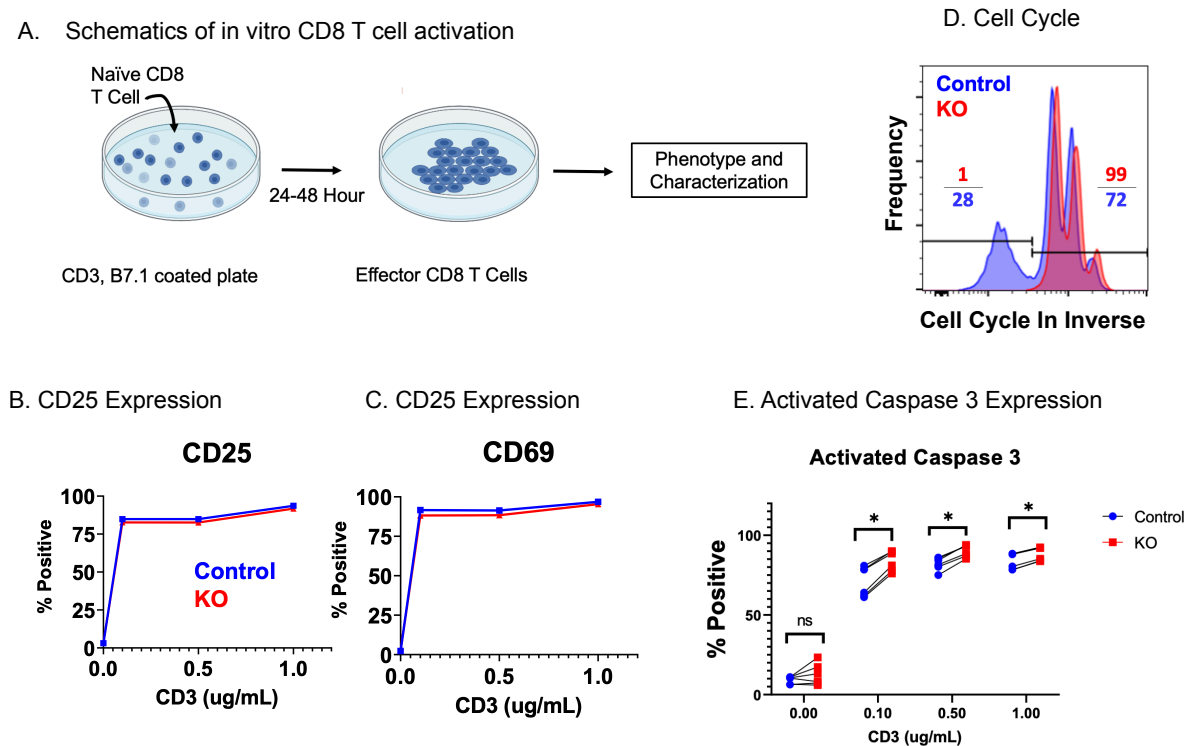


Figure 7: Impaired cell division rather than activation causes initial decreased antigen specific CD8 T cell population in absence of *Esr1* signaling. A. Schematic of in vitro CD8 T cell activation study. Expression of B. CD25 C. CD69 in activated CD8 T cells in vitro with varying activation strength 24 and 48 hours post activation. D. Cell cycle count and E. activated Caspase 3 expression between control and KO activated CD8 T cells 48 hours post activation.

Discussion:

Sexual dimorphism in antiviral immunity is well established. Mortality, disease severity and hospitalization rates can vary between men and women depending on age, type of viral infection and exposure. CD8 T cells are the epicenter of antiviral immunity, with the ability to formulate exact specific antiviral immune response. Despite these known phenomena, CD8 T cell intrinsic estrogen signaling is still poorly understood. With this study, we aim to shed some lights into how estrogen signaling might impact CD8 T cell mediated immunity. We show that, in absence of circulating estrogen, memory response is poor in women, which can be ameliorated using supplemental estrogen. In addition, absence of estrogen signaling caused defective antigen specific effector and memory cell population. We also found estrogen signaling positively contributing to overall effector function. While we did not find any correlation between circulating memory CD8 T cell and estrogen signaling, we found that TRM function may be modestly impacted in absence of estrogen signaling. Overall, we established that these defects in activated CD8 T cells are caused by estrogen's role in cell division, rather than in activation, migration, differentiation. Estrogen is a known cell cycle regulator.

While estrogen's role in innate immune cell and CD4 T cells survival is well studied, this study establishes estrogen's impact on CD8 T cell in an cell-intrinsic manner. Estrogen is an important biological modulator. The circulating estrogen concentration changes over time in one's life cycle. The level of estrogen goes down dramatically throughout post menopause. Some pathological conditions and hormone replacement therapies also alters one circulating estrogen concentration. It is well established in the scientific community that sex is a biological determinant of disease outcome in antiviral

immunity. Years of data collections for hospitalization and mortality from CDC reports shows sexual dimorphic outcome of the most common pandemic and endemics – Covid-19 and influenza. The presence and the level of sex hormones are one of the major differences between the sexes. Understanding how sex hormones may impact one's ability to fight viral infection is crucial in the current public health landscape. These findings will shed some lights on how CD8 T cell mediated immunity is impacted by ESR1 mediated estrogen signaling. These findings are also important in designing vaccination response studies in women and men through various ages for developing a more personalized vaccinations against viral infections. These findings are also useful in understanding estrogen mediated antiviral immunity in women and transgender population when estrogen level can vastly vary. As the data shows increased dependency of CD8 T cell population on ESR1 mediated estrogen signaling in response to viral infection, it will be worth understanding estrogen's role in antiviral immune response in women, assigned at birth compared to transgender population undergoing hormone replacement therapy throughout different ages.

One key aspect of our study is that we measured ESR1 mediated signaling in CD8 T cells intrinsically using Cre Lox system. Previous studies using full body estrogen receptor knock out mouse shows affected thymus weight and subsequent negative roles in T cell homeostasis defects (Staples et. al. 1999). To identify estrogen mediated defects during antiviral infections, we adoptively transferred CD8 T cells that are not able to sense estrogen signaling along with equal proportion of CD8 T cells that can sense estrogen signaling. So, both cell types have access to similar level of antigen presentation during the infection and as such is well controlled. However recent work has found different

knock out efficiency of genes using CD4 Cre, Lck Cre and Rosa25 systems (Becher et al., 2019; Kurachi et al., 2019; Reizis, 2019). Here, we didn't specifically assess the level of Esr1 knockdown. Another limitation of our study is the focus on Esr1, which is the major estrogen receptor. But immune cells can also utilize other intracellular estrogen receptor including Esr2, as well as membrane bound estrogen receptor GPER1. Moreover, all the studies are done in mice 8-16 weeks old females. Future studies using a diverse range of viral infection, sexes, and ages of mice should be conducted to address if this remains the same for different local infections, in male mice, and young vs older aged mice.

Altogether, this work demonstrated that CD8 T cell intrinsic Esr1 signaling is important for optimal CD8 T cell expansion and survival upon TCR activation. While the impact of this defect on functional protective efficacy of the CD8 memory T cells were minimal, this effect could become important where an expanded pool of T cells is important for immediate reduction in pathogen replication.

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Chapter 2: Estrogen in vaginal epithelium CD8 T

Cell characteristics in vitro

Introduction

Estrogen is the major female sex hormone produced primarily in ovary. The level of estrogen fluctuates throughout women's life cycle. Estrogen level increases from pre-pubescent towards childbearing stage and then it slowly goes down throughout the start of menopause (Berrino et al., 1996). Female reproductive tract is a complex system with multiple layers of differentiated epithelium and stem cells (Ali et al., 2020). Using single cell seeding of vaginal epithelium stem cells and multiple growth/cells differentiating factors, one can grow 3D organoids of vaginal epithelium that would contain all the differentiated and undifferentiated layer (Ali et al., 2020). Organoid models are ideal to study CD8 T cell mediated immunity because it provides with a controlled system in a short amount of time in a cost-effective way to study cell – cell interactions, maintenance, and cytokines/chemokines production.

Resident memory CD8 T cells in the female reproductive tract are a crucial component of the local antiviral defense (Rosato et al., 2017; Schenkel et al., 2013, 2014). Within the vaginal mucosa, CD8 T cells reside in close contact with vaginal epithelial cells and the CD8-epithelium crosstalk is thought to be important for maintenance and function of CD8 T cells (Beura et al., 2018). Estrogen has been shown to influence the epithelial cell proliferation and integrity (Miyagawa & Iguchi, 2015). Different tissue has different expression of ER α and ER β . Specifically, vaginal mucosal APC and CD8 T cells have differential ER expression, which are constitutive and inducible, respectively (Gu et al., 2021). Tissue microenvironment plays an important part in maintaining niche resident memory CD8 T cells in vagina and acting as the primary barrier to viral infection (Seavey & Mosmann, 2009; Takamura, 2018). However, if this estrogen mediated

modulation of epithelial biology could also affect resident CD8 T cells in the vaginal mucosa has not been investigated. In innate immunity, vaginal epithelium led changes due to estrogen signaling fluctuation was shown to alter neutrophil actions and vaginal mucosal thickness – both of which are indispensable for a strong resident memory CD8 T cell antiviral immune response (S. Li et al., 2018). This study aims to elucidate how the vaginal epithelium modulates CD8 T cell differentiation in the presence or absence of estrogen, utilizing an *in vitro* organoid model of mouse vaginal epithelium organoid model (VEO).

Materials And Methods

CD8 T Cell isolation from tissues

CD8 T cells were isolated from blood, lymphoid and non-lymphoid organs 3, 7, and 30+ days post infections. Briefly, lymph nodes were mechanically disrupted to collect CD8 T cells. As previously mentioned, ACK lysis buffer was used to remove red blood cell contaminations from secondary lymphoid organs such as spleen and blood. For non-lymphoid organs such as small intestine, salivary glands, kidney, and female reproductive tracts were enzymatically digested to isolate CD8 T cells. The small intestines were digested in DTE. Salivary glands, kidney and female reproductive tracts were digested in collagenase IV. For kidney, liver, and small intestines, the isolated cell mixture was further enriched using percoll gradients. These cells were characterized using flow cytometry. For intracellular and transcription factor antibody staining, Tonbo, and BD intracellular staining kits were used.

CD8 T Cell activation:

For *in vitro* studies, enriched CD8 T cells were activated using 2C11 (CD3) and B7.1 Fc in 96 well plate. CD3 represent TCR signaling (signal-1) and B7.1 represent CD28 mediated costimulatory signal (signal-2) – both necessary for CD8 T cell activations *in vivo* (Mohammad et al., 2018). The plates were coated with differential concentration of CD3 and B7.1 overnight at 4C in dark. The next day, the plates were washed twice in sterile DPBS. 200,000 – 400,000 of enriched CD8 T cells were plated in each 24 well plates. Briefly, we collected CD8 T cells from spleen and lymph nodes, isolated naïve CD8 T cells and enriched them using Biotin conjugation – magnetic bead system of

negative selection as previously mentioned. Purity was monitored with FACS assays. Fresh IL-2, IL-12 was added to the restimulation media every 2 days. The T cells characteristics were monitored using flow cytometry 24 hours, 48 hours and 72 hours post plating. Estradiol or ICI 182,780 was added to the media when the cells were being plated and every time forward during the media changes.

Gene Expression Studies:

For expression of ER α gene, vaginal organoid epithelial cells were lysed using Trizol to 7 days post treatment and plating. Qiagen RNA isolation micro column was used according to protocol to isolate RNA. cDNA was prepared using cDNA preparation kits (Thermo). Finally, qPCR was performed using ER α and ER β primers.

Establishment of Vaginal Epithelial Organoid (VEO):

Vaginal epithelial organoid was established using the protocol as mentioned (Ali et al., 2020). Briefly, vaginal epithelium was isolated from 8 weeks old mice using overnight pronase and DNaseI incubation. Once suspended as single cell and embedded in basement membrane extract (BME) these epithelial cells were then treated with organoid culture media containing growth factors, differentiating factors and necessary cytokines to differentiate into VEO. Grown organoids with visibly differentiated layers of epithelium can be spotted with light microscopy starting 7 days post plating. The organoid culture media for vaginal epithelium was changed every other day.

CD8 T cells and VEO co-culture:

CD8 T cells were activated in vitro as previously mentioned. 2-3 days post activation, these cells were rested for 2 more days and then co – plated with single cell suspension

of vaginal epithelium both grown in basement membrane extract. The co culture is treated with T cell organoid culture media, compared to organoid culture media, with the addition of cytokines and growth factors that helps maintenance of CD8 T cells. These co culture can be maintained up to 15 days, with every other day media changes. Co culture of CD8 T cell with vaginal epithelium induced further female reproductive tract specific resident memory CD8 T cell differentiations, characterized by CD69 CD103, markers associated with epithelial TRM. The CD8 T cell are thus embedded within VEOs in basement membrane extract.

Results

Estradiol treatment induces ER α expression in VEO, however estradiol treatment during T cell effector differentiation does not affect T cell activation.

To assess the function of estradiol on VEOs, we treated organoids with varying level of estrogen and ICI (estrogen signaling antagonist). Data shows a positive correlation with estradiol treatment with ER α expression, which goes down when estrogen receptor 1 and estrogen receptor 2 signaling inhibitor, ICI was added (Fig. 1a). ER β expression to both in response to estradiol treatment and ICI was below threshold level, suggesting ER β is not expressed in our VEO model (data not shown, below threshold). Our goal is to establish a co culture model of activated CD8 T cells with VEO to mimic vaginal resident memory CD8 T cell interactions for understanding estrogen mediated, epithelium directed, memory CD8 T cell differentiations. But before this, we wanted to test if estradiol treatment alone can modulate CD8 T cell activation in the absence of VEOs. For this CD8 T cells were exposed to estradiol or ICI during activation (Fig 1A). Similar to our previous finding, neither estradiol, nor ICI treatment affected CD8 T cell activation as seen with similar expression of CD69 and CD25 activation marker with varying level of TCR signaling strength (Fig. 1B).

Figure 1: VEOs Are responsive to estrogen and CD8 T Cell Activation Is Not Impacted by Estrogen Signaling

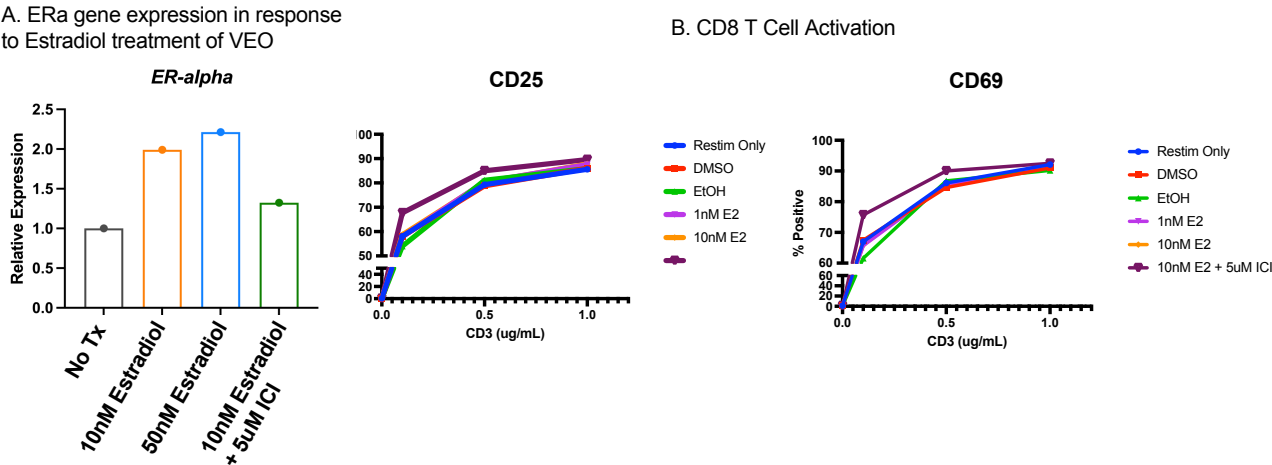


Figure 1: VEO and CD8 T cell response to estradiol treatment in vitro. A. Level of ERα gene expression in VEOs after estradiol treatment and inhibition as determined by Q-PCR. B. CD8 T cells activation marker CD25 and CD69 expression with varying level of estradiol treatment and inhibition. Data represented of one data set of N=2 independent trials.

Inhibition of estrogen signaling reduced VEO formation and size.

Next, we evaluated the role of estradiol and estradiol inhibition in VEO growth. We plated 20,000 VEO in a single cell suspension per well in a 24 well plate using previously mentioned method. These VEOs were treated with Estradiol from the start of plating up until D9 with media change performed every other day. We used 10X magnified light microscopy to visualize organoid growth over time. Data shows increase in organoid size with time (Fig. 2). There was a modest increase in size of the VEOs treated with 50nM estradiol compared to other doses as well as no estradiol treatment groups. However, ICI treatment inhibited organoid growth over time (Fig.2). No significant cell death was observed in any wells. Data also suggest that the organoid growth is slower prior to D7 (data not shown). The organoid grows exponentially 7 days post plating compared to D3 – D5 days post plating, and D9 growth is more inhibited by ICI treatment compared to D5.

Figure 2: VEO Growth Over Time with Estradiol Treatment And Antagonism

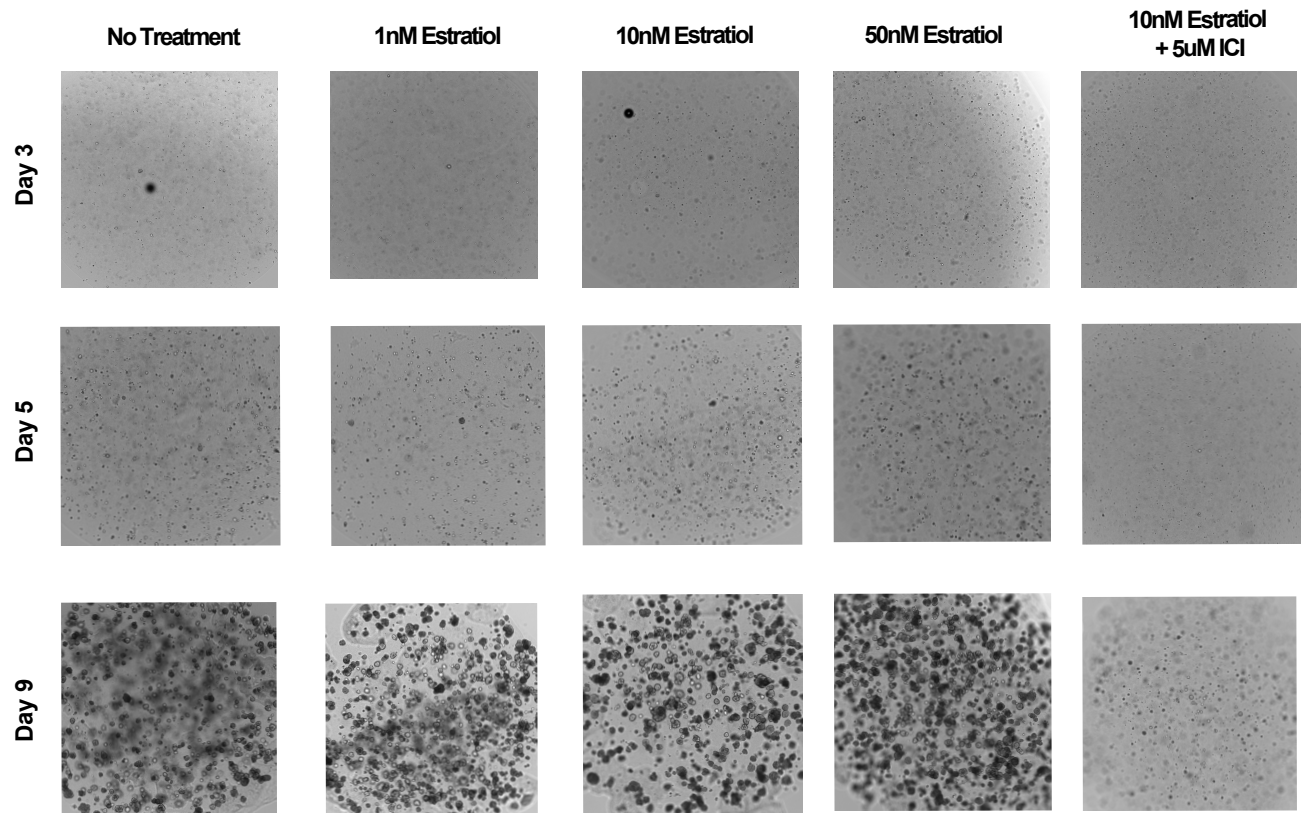


Figure 2: Estradiol treatment slightly increased organoid formation of VEOs, whereas ICI inhibits organoid growth. Organoid growth over time on 3-, 5- and 9-days post plating using 10X magnified light microscopy with various concentration of estradiol and ICI. Data represented of three independent trials.

Blocking ESR1 Mediated Estrogen Signaling Alters CD8 T Cell Residence associated marker expression.

Next, we analyzed CD8 T cell characteristics in the co culture system in response to estradiol and ICI treatment. Since VEO growth were affected by both treatments, we aimed to understand if the CD8 T cells embedded in this environment would be affected by the VEOs. Briefly, CD8 T cells were co cultured with VEOs, and similar treatment regimen was added to the T cell organoid growth media during each media changes, starting with the initial single cell suspension seeding of these cells. We waited until D9 when we established a good growth point for the VEOs and collected the CD8 T cells from each well to study CD8 T cell characteristics using flow cytometry. Data shown here are flow plots of each treatment (Fig. 3A) and overall percentage of CD69 and CD103 (Fig 3B). Presence of VEOs increased CD69+ CD103+ population which are markers for epithelial resident memory CD8 T cells in the female reproductive tract (Rosato et al., 2020; Szabo et al., 2019). Our data suggests that estradiol treatment only marginally increased double positive resident memory CD8 T cell phenotype compared to no treatment and CD8 T cell only cell culture lacking a VEO. However. Inhibiting estrogen signaling with ICI dramatically increased CD69+ population and altered the CD69 CD103 expression landscape. We did not see similar altered CD69 CD103 expression in CD8 T cell only control in absence of VEO which suggest this change is mediated by estrogen signaling in the VEO, rather than CD8 T cell intrinsic estrogen signaling (data not shown).

Figure 3: Blocking ESR1 Mediated Estrogen Signaling Alters CD8 T Cell Residency

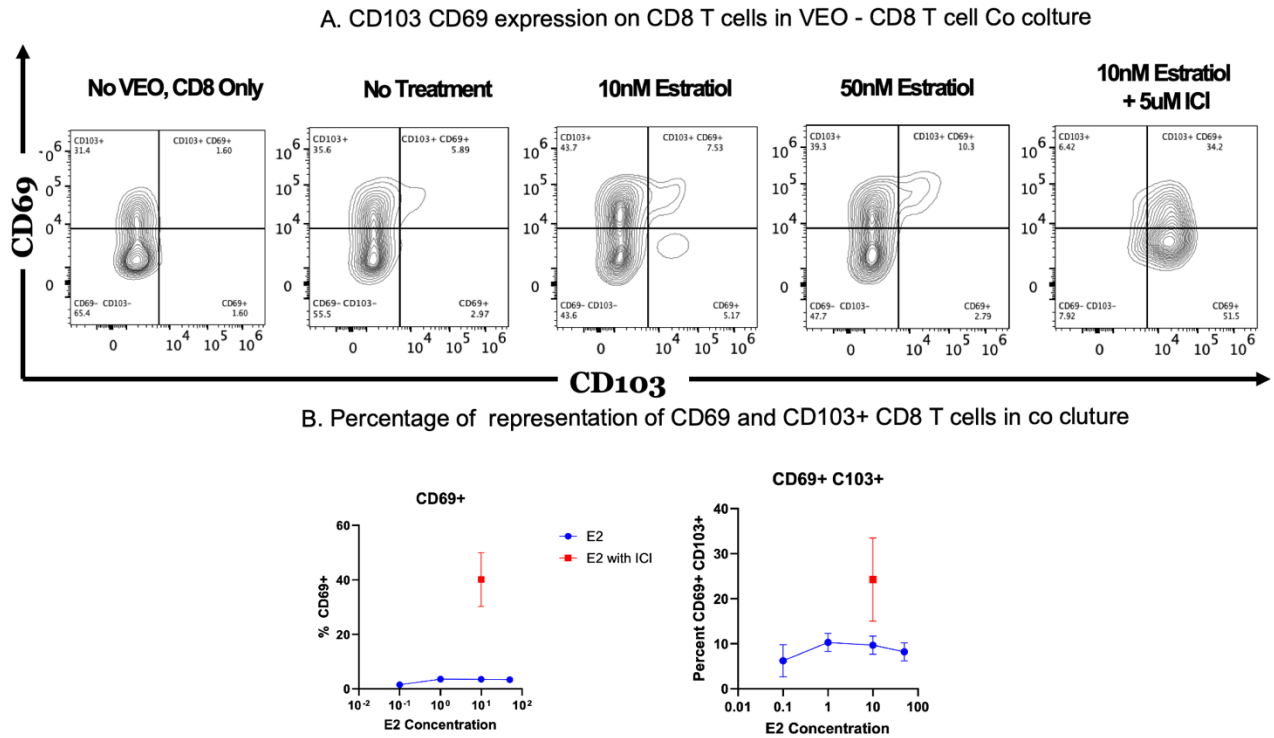


Figure 3: CD103 and CD69 expression on D9 post plating VEO – CD8 T cell co culture.

A. Flow cytometry representation and B. total percentage of CD69+ and CD69+ CD103+ CD8 T cell population 9 days post CD8 T cells and VEO co culture.

Discussion

Estrogen is a master regulator with a complex signaling network. First, we aim to understand estrogen signaling mediated changes in vaginal epithelium organoid model. Women undergoing menopause have much less circulating estrogen compared to pubescent or childbearing aged women, which is associated with multiple menopause related symptoms (Christensen & Pike, 2015; Gameiro et al., 2010; OlaOlorun & Shen, 2020; Rosano et al., 2007; Sammaritano, 2012). Resident memory CD8 T cells that are resident in local tissues and are equipped to respond quickly in a secondary immune response, rely on signaling from tissue microenvironment to maintain their population and differentiation. Understanding estrogen mediated changes to epithelium that can impact resident CD8 T cells is crucial in maintaining one's ability to fight local infection with age. We show that estrogen signaling can increase CD69⁺ CD103⁺ CD8 T cell population, defined as stable resident memory CD8 T cell population. Inhibiting them with ICI causes a transient, altered CD69 CD103 expression in these CD8 T cells, with increasing CD69 expression. It is important to notice that ICI treatment is causing a almost complete halt in VEO growth, which is not visible in the no treatment group. While ICI inhibits ER α and ER β signaling, it can increase the membrane bound GPER signaling (Nathan et. al. 2017). Further studies are required to understand this complex ICI dependent characteristics of CD8 T cells in VEO.

In broader perspective, resident memory CD8 T cells in mucosa are the primary protection against recurring viral infections. A healthy mucosal immunity is crucial in deterring various reproductive tract viruses or RTIs along with sexually transmitted infections or STIs. While being of high importance in antiviral immunity against RTIs

and STIs, resident memory T cells are difficult to study due to its short life span in any *in vitro* system. Estrogen being one of the major modulators of biological functions, scientists are still unsure its effect in vaginal mucosa. We already know the importance of tissue microenvironment in retaining long term immune memory. CD8 T cells live in niche tissue microenvironment and require both physical structures to be embedded in the tissue and signaling molecules for senescence type maintenances. To combat this issue of characterizing vaginal resident memory CD8 T cells in vaginal mucosa, our laboratory has developed organoid model that can effectively study local epitheliums, along with the CD8 T cells embedded in these epitheliums *in vitro* using organoid model in a manipulatable environment. VEO model is efficient in isolating only vaginal epithelium specific regulation of CD8 T cells in a variety of physiological and pathological conditions that can be obtained within a relatively short period of time compared to mouse models. Findings that vaginal epithelium growth is highly impacted by estrogen inhibitors and estrogen concentration is important in understanding resident memory CD8 T cell maintenance landscape in a variety of conditions and treatment, such as oral contraceptive and estrogen intervaginal cream. These products are widely used and available commercially, more understanding on how these are impacting one's antiviral immunity needs further evaluation that will open door for more therapeutics and precautions against infections.

One of the limitations of this studies that requires further inquiry is the use of ICI inhibitor to block ESR1 mediated estrogen signaling. While we did not find any ESR2 spike after estradiol treatment, we have not measured GPER expression of these cells. ICI is a known modulator of GPER, and the level of GPER in vaginal epithelium is

currently unknown. In addition, a thorough bulk RNA sequencing needs to be performed in both estradiol treated and inhibited VEOs to understand which pathways are specifically being impacted by estrogen signaling. Studies done in older adults found that the numbers of overall resident memory cells and their cytotoxicity increases as women, as assigned at birth, age. In addition, the co-culture studies were plated as a single cell layer of epithelium. Resident memory CD8 T cells in female reproductive tract exist in complex multilayer of vaginal epithelium. These studies should be repeated in matured organoid and long term co culture studies to mimic this. My preliminary data (not shown) in co culture of mature VEO with CD8 T cells found an increasing role of estrogen signaling in residency where the CD103 and CD69 double positive population increased significantly in the system with increasing estradiol treatment. Further studies are also needed to understand the function of these resident memory CD8 T cells in presence of infection during the co culture to investigate the functions of these altered characteristics of CD8 T cells with both estrogen treatment and ICI inhibition.

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Impact of the Findings

Importance of Studying Estrogen Signaling

Estrogen is the major female sex hormone that regulates a complex network of biological activities in women, assigned at birth. The level of estrogen fluctuates throughout women's life cycle. The level of circulating estradiol increases during pre-pubescent ages up to childbearing age and decreases at the same rate towards menopause to (Berrino et al., 1996). This sudden drop in circulating estrogen level in menopause causes multiple physiological and pathological changes in post-menopausal women, increasing vulnerability to autoimmune disease and infections (Sammartitano, 2012). The sex bias of immune system is well established (Cook, 2008; Fischinger et al., 2019; Gabriel & Arck, 2014; Jaillon et al., 2019; Klein & Pekosz, 2014; Márquez et al., 2020; Morgan & Klein, 2019; Moulton, 2018; Taneja, 2018). Essential role of estrogen has been suspected to play a role in Covid-19 increased mortality in men compared to women (Al-kuraishy et al., 2021). However, there is a gap in knowledge in understanding how CD8 T cells are using estrogen signaling during antiviral immunity. This study aims to bridge that gap in knowledge by investigating how estrogen impacts CD8 T cell mediated antiviral immunity using a CD8 T cell specific genetic knock out of estrogen receptor.

Estrogen mediated hormonal signaling is an intricate system and altered estrogen signaling has been linked to a plethora of disease states in women and transgender population (Deroo & Korach, 2006; Khan & Ansar Ahmed, 2016). Estrogen is a master regulator in women for various biological system. Estrogen is important in developing naïve CD8 T cells from thymus. Full body estrogen receptor knock-out mouse has smaller thymus, develop increasing amount of fats, and shows signs of diseased state

(Staples et al., 1999). Thus, it is difficult to isolate estrogen mediated CD8 T cell pathways from estrogen's effect on other cell types and naïve CD8 T cells. Using cell specific genetic knock out mouse model and adoptive transfer system of congenially marked T cells, this study is able to isolate CD8 T cell intrinsic estrogen signaling. In addition, using organoid model of vaginal epithelium, this study is able to shed some lights into understanding the impact of estrogen signaling in vaginal epithelium and subsequent effects in resident memory T cells embedded in vaginal epithelium.

Connecting The Dots Between Resident Memory CD8 T Cells and Vaginal Mucosa

Lack of ESR1 mediated estrogen signaling decreases effector CD8 T cell population, effector function and memory function in adult mice. This defect was seen in both lymphoid and non-lymphoid tissues for both systemic viral infection such as LCMV Armstrong and local viral infection such as Influenza. In addition, chemical inhibition of estrogen signaling alters CD8 T cell residency signature into an interesting effector memory- like CD8 T cell signature in our organoid system. Similar to finding in CD8 T cell intrinsic estrogen signaling, VEO growth is also impacted by the lack of estrogen signaling when induced chemically, compared to genetically. In both cases, the activation of CD8 T cell remained unimpacted under similar conditions. For effector CD8 T cells, the survival was also impacted by ESR1 mediated estrogen signaling. Altogether, this suggests a multifaceted role of ESR1 mediated estrogen signaling in CD8 T cell mediated antiviral response when data from both *in vitro*, *ex vivo* and *in vivo* studies are combined with current findings in immunology.

First, estrogen signaling impacts both VEO and CD8 T cells divisions. For epithelium, both the homeostatic division and differentiation is impacted by estrogen signaling as seen by the reduced cell number and reduced organoid growth in VEO system over time. While activated CD8 T cell clonal expansion was modulated by ESR1 mediated estrogen signaling suggested by *in vitro* cell tag it assay, the ratio of memory CD8 T cells remained similar to the ratio of original effector memory CD8 T cell for both Control and KO antigen specific CD8s. This data suggests the role of estrogen signaling in early differentiation, but not in later homeostatic maintenance over time in a CD8 T cell

intrinsic manner. The *in vivo* infection studies and CD8 T cell *in vitro* cell culture is complemented with VEO system. In VEO co culture with CD8 T cells, CD8 T cell numbers and characteristics are altered in absence of estrogen signaling when mediated by chemical inhibitors, suggesting that any decrease in CD8 T cells population in low estrogen environment might come from CD8 T cell extrinsic effect rather than CD8 T cell intrinsic effect, in this case from vaginal epithelium. Furthermore, resident memory CD8 T cell population heterogeneity may vary depending on which stage of life one has suffered the original infections, for example, pre- vs post-menopausal. Further studies in should be conducted to weed out potential age biases of estrogen sensitivity of CD8 T cells as one ages.

Second, both effector and memory function reduced in CD8 T cells in absence of ESR1 mediated estrogen signaling *in vivo*. Ex vivo peptide simulation (data not shown) showed similar defect in IL-2 mediated effector function immediately post infection, establishing that lack of estrogen signaling may slightly impact overall resident memory function post menopause. However, chemical inhibition of ESR1 mediated estrogen signaling in VEO co culture model with CD8 T cells show altered CD8 T cell residency signature with no distinct CD103 and CD69 population. This particular flow cytometric signature of CD8 T cells is typically a sign of low senescent resident memory T cells population that rapidly prepares to differentiate to fight ongoing viral infection through reentering circulatory system. It is important to mention that no CD8 T cell intrinsic ESR1 mediated estrogen signaling defect was found when investigating circulatory memory function of Control and KO. In post-menopausal women, as assigned at birth, the cytotoxicity, or function of resident memory T cell is increased when compared to

pre-menopausal women, as first assigned at birth. Together, this suggests that even though resident memory function is impaired in the absence of CD8 T cell intrinsic estrogen signaling, CD8 T cell extrinsic estrogen signaling through vaginal epithelium can alter resident memory CD8 T cell functions in these cells in low estrogen environment to combat estrogen depletion. Further studies are needed in this area to understand this multifaceted role of estrogen signaling through different tissues and pathways in overall CD8 T cells ability to mount a healthy antiviral immune response.

Advancements on Hormone Therapy, Contraceptives and Treatments During Infections

Hormone replacement therapy is a common treatment for people undergoing menopause. Studies show increasing efficacy of perimenopausal hormone therapy compared to post-menopausal hormone therapy in treatment of menopausal treatment. During menopause, there is a sudden drop in circulating estradiol concentration causing cells that are used to estrogen signaling in various biological functions are now in a low estrogen environment. Multiple studies and reviews elucidate the impact of menopause mediated low estrogen in aging related health concerns. Elderly population are more at risk of infection compared to younger population. Hormone may be an important contributing factor in this outcome, as it relates to healthy aging. Understanding estrogen signaling in antiviral immune response, both intrinsic and extrinsic to CD8 T cells are essential in bringing unique therapeutic approaches to fight antiviral infection for different populations.

Two of the major biological differences between sexes are genetics and hormonal. Isolating sex specific defects of pathological conditions is important for structuring an inclusive public health system. If estrogen level is a major risk factor for infections, as it is for many other pathological diseases such a range of cancers and reproductive disorders, it needs to be thoroughly investigated. This study is one of the first to link ESR1 mediated CD8 T cell intrinsic estrogen signaling with reduced antiviral immunity in a mouse model. In addition, I have shown one CD8 T cell extrinsic signaling through vaginal epithelium that has a high impact on CD8 T cell antiviral properties. Future studies are needed to understand the relationship between CD8 T cells and other

immune cells, muscular system, and nervous system, as well as understanding the intricate networking of epithelium and CD8s. I hope this study will serve as a model to untangle the complex relationships between multiple different cells and tissue mediated CD8 T cell antiviral immunity to our scientific and medical community for better therapeutic innovations.

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