Her Mucosa, Her Rules:

Regulation of Memory T Cells in the Female Genital Tract

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Abstract

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The lower female genital tract (**FGT**) is a mucosal barrier and site of exposure to various pathogens, including fungi, parasites, bacteria, and viruses. The FGT faces the unique challenge of maintaining tolerance to a semi-allogenic fetus and the optimal vaginal microbiome, in addition to preventing infection through cell-mediated surveillance for pathogens and subsequent measured immune activation without excessive damage to host tissues. The lower FGT (vagina and ectocervix) is covered in multi-layered squamous epithelial cells, whereas the upper FGT (endocervix, uterus and fallopian tubes) is covered by a single layer of columnar epithelial cells. Beneath the epithelium is a layer of stromal fibroblasts providing structure to the tissue. Dynamic populations of immune cells are distributed throughout the stroma, with T lymphocytes being the most abundant immune cell subset within the lower FGT[1–3]. Mechanisms involved in immune responses within the FGT are further complicated by changes in sex-hormones throughout an individual's life. In addition to regulating changes in epithelial and stromal structural reorganization and repair, hormonal fluctuations

throughout the menstrual cycle can also participate in modulation immune cell function to maintain tolerance to innocuous antigens and protection against pathogens [3–5].

Tissue-resident memory T cells (**Trm**) mediate protection within tissue sites of prior or persisting infection. Upon pathogen re-exposure and antigen recognition, Trm elicit robust immunity locally within the tissue and orchestrate the recruitment of other T cells and innate immune cells. To avoid excessive tissue damage associated with this robust tissue immune reaction, we predicted that tissue recall T cell responses must be subject to regulation. We previously demonstrated that regulatory T cells (**Treg**) accumulate within infected tissue and coordinate early immune responses and T cell priming during primary infection, though their role in shaping tissue memory T cell responses remains unclear. Using transient Treg depletion in mice, we show a requirement for Treg in limiting tissue pathology following vaginal HSV-2 challenge, despite normal viral clearance. While Treg-depleted mice exhibit an elevated frequency and number of vaginal CD44+Ki67+ T cells and granzyme-B+CD8+ T cells by day 3 post-challenge, there is no difference in the magnitude of the antigen-specific CD8+ T cell response. Using adoptive transfer of TCR-transgenic OT-1 CD8 T cells, we show that Treg-depleted mice have a significantly stronger vaginal bystander-activated cytotoxic T cell (**BA-CTL**) response upon challenge. In vivo antibody blocking demonstrates that Treg limit cytotoxicity of both HSV-2-specific CD8 and BA-CTL responses via an IL-2-dependant mechanism that additionally restricts IL-15 trans-presentation by antigen presenting cells. Our findings highlight Treg' role in selectively restraining cytotoxic function during a tissue recall response, while preserving the pathogen-specific tissue T cell response, to balance viral clearance with restriction of immunopathology.

In addition to regulation by Treg, immunity can also be modulated by fluctuations in sex hormones. Characterization local FGT and systemic T cell responses throughout the menstrual cycle is paramount

to better understanding mechanisms underlying susceptibility to sexually transmitted infections and correlates of protection for improved design of vaccines and therapeutics. We sought to characterize the impact of menstrual phase on local cervicovaginal and systemic T cell responses in Kenyan women. Using high-parameter, high-throughput flow cytometry and a large panel we compared follicular vs luteal phase phenotypes in T cell populations isolated from vaginal tract (**VT**) and ectocervix (**CX**) mucosal tissue biopsies and PBMC samples. Given the abundance of T cells in the lower FGT, cervicovaginal tract (**CVT**) biopsies may better capture the mucosal immune environment than other minimally invasive sampling methods such as cervicovaginal lavage, vaginal/cervical swabs, or cytobrushes. Additionally, we evaluated 71 immunomodulatory molecules in serum and cervicovaginal secretions in follicular vs luteal phase. Overall, we did not find changes in the frequencies of T cell subsets (CD4+, CD8+, Treg) or increases in HIV susceptibility markers on CD4+ T cells (CCR5, HLA-DR, CD38) between follicular vs luteal phase in any of the tissue sample types. Interestingly, all phenotypic differences were observed in CD8+ T cells within CX and VT. Specifically, CCR7+ CD45RA- central memory CD8 T cells were significantly elevated during the luteal phase in both the CX and PBMCs, whereas CCR7- CD45RA+ terminally differentiated effector memory T cells CD8 T cells were more prevalent in the CX during the follicular phase. Additionally, CD8 T cells in the CX during the luteal phase were more activated, exhibiting higher frequencies of HLA-DR, CD38, and the exhaustion marker CD39. We also found altered expression of soluble factors in follicular phase, with increased concentrations of CCL15 and IL-2 in cervicovaginal secretions and increased CXCL12 in serum samples. These findings suggest that menstrual phase modulates CD8 T cell memory subsets and intrinsic regulation of activation, providing new insights into how hormonal fluctuations influence FGT immunity to infection.

DEDICATION

No enseñes la verdad, enseña a buscarla. - Francisco Giner de los Ríos

I would like to dedicate this dissertation to my family, near and far.

To my grandmothers, Rosi y María, who were unable to finish primary school and grew up in a world so different from my own. To my grandfathers, Manolo y Antonio, who shared their love of the natural world with me. To my aunts, uncles, and cousins who cheer me on from afar and are always present in my life. To my little brother, Ethan, who is growing into a compassionate and kind man. And to my parents, Paco y Nieves, who have worked tirelessly to provide a better future for my brother and me, who always encouraged me to be myself, and who taught me that learning is a lifelong and worthwhile endeavor.

Finally, to my husband, Jack, who has witnessed every victory and failure throughout this journey, and whose unwavering love, humor, and support keep me going.

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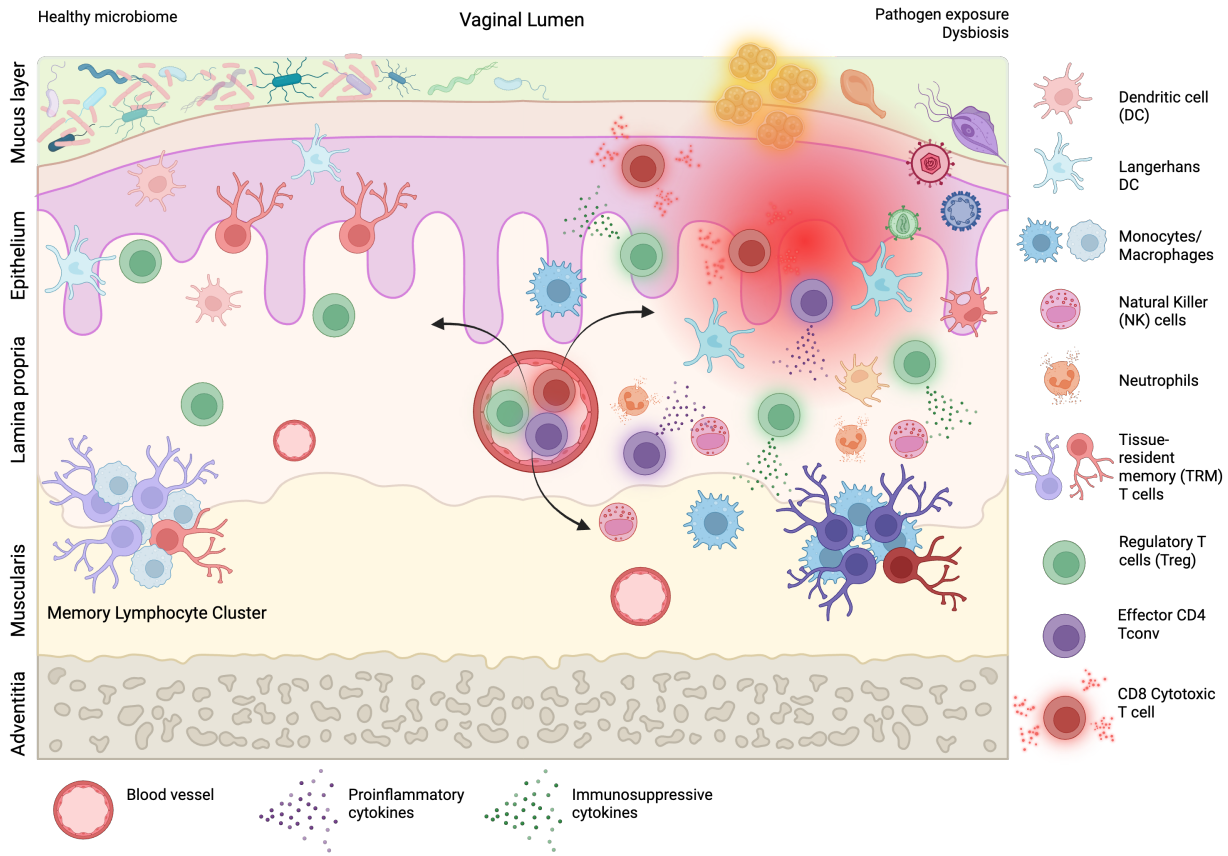

Figure 1.1: Mucosal T cell immunity in the lower female genital tract (FGT)

Mucosal barrier tissues, like the FGT, serve as entry sites for a variety of antigens and pathogens At steady state/homeostasis, the mucosal immune response must be tolerant to self and these harmless antigens while still allowing immunosurveillance for pathogens. A healthy commensal microbiome (lactobacillus dominant) interacts with epithelial cells through production of microbial metabolites and other byproducts that trigger pattern recognition receptors (**PRR**). This induces mucin production to create a protective mucus layer and recruitment of antigen-presenting dendritic cells (**DC**) and development of Langerhans cells (**LC**). In the absence of inflammatory signals, DCs produce retinoic and transforming growth factor-β (**TGFβ**), which

induces the recruitment and differentiation of regulatory T cells (**Treg**) and supports tissue-resident memory T cell (**Trm**) development and retention. Trm and antigen presenting cells (**APC**) form memory lymphocyte clusters that maintain Trm locally at the site of pathogen exposure, poised to respond in the case of infection. Treg maintain tolerance within mucosal barrier tissue sites, preventing immune activation against innocuous antigens. In the case of pathogen exposure and infection, the mucosal immune response must then promote clearance of the infection while still limiting immune-mediated damage and pathology. Microbiome dysbiosis (in the case of fungal or bacterial overgrowth) can lead to increased inflammation and epithelial barrier disruption, facilitating the probability of acquiring sexually transmitted infections [featured pathogens: yeast (*Saccharomyces* or *Candida*), *Mycoplasma genitalium*, *Trichomonas vaginalis*, human immunodeficiency virus (HIV), herpes simplex virus (HSV), human papillomavirus (HPV)]. Sensing and recognition of invading pathogens by epithelial cells, innate immune cells, and Trm triggers activation of the endothelium and recruitment of effector CD4 and CD8 T cells and proinflammatory myeloid cells (neutrophils, macrophages, DCs and natural killer cells) to prevent further spread, clear out intracellularly infected cells, and phagocytose extracellular pathogens.

1.1 Mucosal Tissue Immunity

Mucosal tissue lines almost all internal-external interfaces in the body, acting as a protective barrier to various pathogens and environmental insults. Simultaneously, it must manage and maintain a multitude of symbiotic microbial commensal communities that keep us healthy. This complexity warrants further investigation as it provides critical insight into the mechanisms that drive and balance immune protection and tolerance. The mucosal immune system is mainly distributed between the respiratory (nasal and oral cavities, airways, and lungs), gastrointestinal (intestines, stomach,) and urogenital tracts (uterus, bladder, vagina, glans penis, and urethra)[6,7]. Outside of these major organ systems, mucosal immunity is also present in the ocular conjunctiva and lacrimal glands, middle ear, and mammary glands[6,8]. Mucosal-associated lymphoid tissues (**MALT**) are also localized along mucosal surfaces and promote secretion of IgA, and antigen-specific Th1/Th2 immunity[8,9]. Lymph and blood capillaries are distributed throughout the MALT epithelium, facilitating bidirectional communication between the mucosal and central immune systems[10]. Organized mucosal MALT, known as inductive sites, are like other secondary lymphoid organs (**SLO**) in that they contain organized structures defined as B cell follicles, T cell zones, and germinal centers[9]. Diffuse mucosal MALT, known as effector sites, are composed of scattered immune cells throughout mucosal tissue lamina propria without an organized follicular structure. More recently tertiary lymphoid structures (**TLS**), identified in settings of chronic inflammation and cancer, have been defined as lymphoid aggregates within non-lymphoid tissues containing germinal centers, T cell areas, and distributed high endothelial venules in their more mature states of development[11].

The lower female genital tract (**FGT**) is a mucosal barrier tissue. At steady state, the FGT lacks organized MALT and primarily contains diffuse immune cells aggregates, making it an effector site[12]. However, there is evidence that in the context of chronic inflammation and infection inducible organized lymphoid aggregates can form[13]. Uniquely, the FGT faces the challenge of tolerance to a semi-allogenic fetus in addition to preventing infection. Despite the known negative effects of vaginal dysbiosis and sexually transmitted infections (**STI**) on female and maternal health, effective preventative measures, such as vaccines, remain elusive. Moreover, our understanding of immune responses in the FGT lags that of other mucosal tissues, such as the lung and intestine. This gap may be due in part to limited research funding for women's health, compounded by the added complexity of hormonal regulation throughout the menstrual cycle[4,5].

T lymphocytes, commonly named T cells, are main players in the adaptive immune response capable of generating immunological memory. T cells recognize and respond to peptide epitopes presented by other cells via their T cell receptor (**TCR**). There are three main subsets of effector T cells: Cytotoxic CD8+ T cells, Conventional Helper CD4+ FoxP3- T cells (**Tconv**) and CD4+ FoxP3+ Regulatory T cells (**Treg**). CD8+ T cells can directly kill cells infected with intracellular pathogens (ex. viruses) presenting pathogen peptides on major histocompatibility complex class I (**MHC-I**) molecules. Helper Tconv orchestrate immune responses by providing cytokine signals to activate and recruit other immune cells to the site of infection. Treg suppress the activation and effector function of other lymphocytes and help maintain tolerance to self, limiting excessive immune-mediated damage. CD4+ T cells (Tconv and Treg) recognize peptides presented by antigen presenting cells (**APC**) on major histocompatibility complex class II (**MHC-II**) molecules[14]. MHC molecules are specialized cell-surface glycoproteins; T cells can only

recognize pathogen peptide antigens if they are bound and presented on MHC molecules by other cells[15].

1.1.1 T cell Development and Activation

Progenitor T cells originate from multipotent hematopoietic stem cells within bone marrow that migrate via blood circulation to the thymus where they mature[14,16,17]. Developing T cells, known as thymocytes, develop into either γ:δ or α:β TCR chain lineages and undergo rigorous TCR repertoire selection in the thymus to ensure self MHC restriction and tolerance[18]. Commitment to a T cell lineage occurs after thymocytes receive signals from thymic epithelial cells in the thymic cortex through the receptor Notch1[19–21]. Notch signaling induces the expression of several T cell-specific genes and transcription factors (ie. T cell factor-1, TCF1; GATA3, CD3 complex components, *Rag1*, Bcl11b, etc.) required for TCR rearrangement and differentiation. α:β T cells eventually develop into either CD4 or CD8 T cells[22,23]. γ:δ T cells originate from the same progenitor thymocytes as α:β T cells, however they are considered part of the innate immune system and typically home to mucosal and epithelial sites throughout the body (ie. reproductive tract, lung, skin dermis)[14,24–26]. γ:δ T cells express hypervariable semi-variant TCRs and recognize non-peptide ligands (without MHC restriction), produce inflammatory cytokines (ie. IL-17, IFNγ) when stimulated, and are largely involved in tissue homeostasis, repair, and innate defense.

T cell precursors spend up to a week in the thymus differentiating before undergoing rapid proliferation (~ $5e^7$ new cells/day), most of which will not survive to maturity[14,27,28]. In total,

about 10^6 T cell thymocytes leave the thymus each day as mature T cells[14]. Early progenitors called double-negative thymocytes (**DN**) lack expression of the CD3:TCR complex and CD4 or CD8[29]. DN thymocytes develop into **DN1**s with germ-line encoded TCRs and express Kit (encoding CD117 receptor for stem cell factor; SCF) and CD44 (an adhesion molecule and activation marker)[14,29]. As they mature, DN1s begin expressing CD25 (α chain of the interleukin 2 receptor; IL-2) and become **DN2**s, in which TCR β-chain rearrangement begins and continues throughout their development into CD25+ $CD44^{low}$ Kit^{low} **DN3**s[23,30]. Cells that fail to make successful TCR β-chain rearrangements remain at the DN3 stage and soon undergo apoptosis. DN3s with productive β-chain rearrangements lose expression of CD25 and progress into $CD44^{low}$ CD25- **DN4**s. DN3/DN4 β-chains pair with a surrogate pre-T cell receptor α-chain (pTα) to form a CD3:pre-TCR[29,31,32]. The successful assembly of a pre-TCR capable of signaling in the absence of a ligand results in cell proliferation and development of double-positive (**DP**) thymocytes expressing both CD8 and CD4 co-receptors.

After proliferation and expansion of DPs, the a-chain TCR locus undergoes rearrangement to express a functional α:β TCR[33]. DPs whose TCRs can recognize self-peptide:self-MHC complexes presented by epithelial cells in the cortical stroma are positively selected for survival and go on to mature into single-positive (**SP**) CD4 or CD8 T cells[34–38]. Less abundant subsets such as invariant natural killer cells (**iNKT**) and Treg also develop in the thymus from DPs[14]. DPs also undergo negative selection during and after becoming SPs in which T cells that respond too strongly to self-antigen presented by medullary epithelial cells and dendritic cells are eliminated via apoptosis to prevent their activation and damage to self (aka. central tolerance)[39,40]. Importantly, T cells must encounter both ubiquitous and tissue-specific proteins

within the thymus before their release into circulation[14]. Stromal cells can express many tissue-specific proteins, such as pancreatic insulin, through expression of the autoimmune regulator (*AIRE*) gene[14,39,40]. Negative selection of thymocytes requires this T cell interaction with common and tissue-restricted self-antigens within the thymic cortex and medulla[39]. Though *AIRE* allows for the expression of many self-antigens in the thymus, it is unlikely that T cells encounter *all* possible self-antigens[41]. However, there are other regulatory mechanisms that operate in peripheral tissues to prevent damage caused by the expansion of self-reactive T cells that escape thymic deletion[42,43]. Studies using TCR transgenic mice expressing self-reactive TCRs have shown that autoreactive naïve T cells in the periphery can become anergic after TCR stimulation in the absence of co-stimulation and inflammatory signals or, in some cases, eliminated via activation-induced death. In the absence of infection or inflammatory signals, T cell interactions with antigen-presenting cells (**APC**) can result in a 'tolerance inducing' tolerogenic signal[44].

The specificity and strength of TCR signaling are crucial in negative and positive selection[45]. The leading hypothesis, known as the 'affinity hypothesis', is that low-affinity to self-peptide:self MHC and weak TCR signaling rescues cells from apoptosis leading to their positive selection; whereas high-affinity binding and strong TCR signaling induces apoptosis and leads to negative selection to prevent the survival of harmful self-reactive clones[46,47]. Thymically-derived Treg are a subset of CD4+ T cells arising from DP thymocytes (just as conventional CD4 and CD8 T cells) that upregulate the transcription factor forkhead box P3 (**FoxP3**)[43]. Unlike conventional T cells, Treg depend on IL-2 signaling to mature and survive[43]. Interestingly, the repertoire of Treg TCRs is composed of receptors with a high affinity for self-antigens and TCR

signaling that is slightly weaker than that of negatively selected thymocytes; this positive selection of moderately self-reactive Treg progenitors is called 'agonist selection' [27,42,46,48].

After surviving positive and negative selection, mature SP thymocytes leave the thymus and emigrate into the bloodstream. Mature thymocytes recognize lipid molecule sphingosine 1-phosphate (**S1P**) via binding by **S1PR1**; blood and lymph contain high concentrations of S1P, drawing mature thymocytes out of the thymus and into circulation[49–52]. At this stage, mature thymocytes also express L-selectin (**CD62L**), which enables their localization to peripheral lymphoid organs via interaction with P-selectin (**CD62P**) and E-selectin (**CD62E**) expressed on vascular endothelium[49,50]. T cell migration and entry into lymph nodes also requires chemokine-dependent activation of integrins (CD11a:CD18; leukocyte functional antigen-1, LFA-1) expressed on their surface[50,53]. LFA-1 on T cells binds intercellular adhesion molecules (ICAM) on expressed by the endothelium to enable migration though blood vessel walls[50]. CCL21 and CCL19 expressed by dendritic cells, vascular high endothelial cells, and stromal cells within lymphoid tissues bind CCR7 expressed on naïve T cells, increasing integrin binding affinity and aiding in extravasation and retention within lymph node T cell zones[54]. Naïve T cells then scan for activated dendritic cells presenting their cognate antigen[14,55]. APCs deliver three critical signals to activate naïve T cells: 1) activation via binding of antigen-peptide:self MHC by the TCR and co-receptor (CD4 or CD8); 2) a co-stimulatory survival signal, such as B7 interaction with CD28 expressed on T cells; 3) and a differentiation/inflammatory cytokine signal (i.e. IL-1, type IFN, IL-6, IL-12, IL-23, IL-4), which is particularly important for CD4 T cells[14,56–60].

Upon antigen stimulation, activation of the transcription factors NFAT, AP-1, and NFκB induces IL-2 production in T cells[61,62] and expression of the high affinity IL-2 a-chain receptor, CD25[63]. IL-2 signaling supports T cell activation, differentiation, and expansion[64]. Importantly, IL-2 is essential for Treg maintenance and survival; Treg do not produce IL-2 upon activation and constitutively express CD25, sinking IL-2 and competing with conventional T cells[65]. Activated T cells also express various other co-stimulatory signals that can modulate the activation and differentiation[66]. For example, inducible co-stimulator (**ICOS**) on the surface of activated T cells binds ICOSL expressed by activated APCs and regulates the expression of IL-4 and IFNγ in CD4 helper T cells necessary for B cell isotype switching[66–68]. Another important co-stimulatory molecule is cytotoxic lymphocyte antigen 4 (**CTLA-4)**, which dampens T cells by competing with CD28 for B7 molecules expressed by APCs[66,67,69]. Antigen-activated T cells in the lymph nodes upregulate expression of **CD69** and downregulate S1PR1 expression, keeping them in T cell zones where they proliferate for several days before re-expressing S1PR1 and leaving the lymph node as effector T cells[52,70]. Effector T cells no longer require co-stimulatory signals for TCR-mediated activation[71]. Differentiated effector T cells exhibit altered cell-surface receptors and signaling thresholds and can respond to peptide-MHC on target cells without needing classical co-stimulatory ligands from APCs[71]. Unactivated naïve T cells that fail to find their cognate antigen within the lymph node exit via the S1P gradient between lymphoid tissues and lymph or blood, drawing them back into circulation[52].

1.1.2 Conventional CD4 and CD8 T cells

Positive selection to develop into either CD8 or CD4 depends upon engagement of the TCR (antigen receptor) and co-receptors (CD8 or CD4) by MHC-I or MHC-II molecules[27,34,36,72]. Successful signaling from both the antigen receptor and co-receptor results in the survival of an SP T cell and commitment to one of the two lineages. TCR signaling regulates lineage commitment through downstream expression of the transcription factors, ThPOK and Runx3[14,73]. ThPOK is expressed in MHC-II restricted DPs after strong TCR signaling and represses the expression of Runx3[74]. Thus, the expression of ThPOK and absence of Runx expression within the same thymocyte results in CD4 lineage commitment and effector function[73–75]. In the case of weaker TCR signaling in MHC-I restricted thymocytes, ThPOK expression is not induced and Runx3 is expressed leading to silencing of CD4 gene expression and commitment to the CD8 lineage[73].

CD4 T cells can differentiate into several different functional effector subsets associated with the activity of certain cytokines and downstream transcription factors, each specialized to orchestrate immune responses to defend against different kinds of pathogens[76]: **Th1** (IFNγ, IL-12; via STAT1/4 and T-bet), **Th2** (IL-4, IL-5, IL-13; via STAT6 and GATA-3), **Th17** (IL-6, IL-23, TGF-β, IL-17, IL-22; via STAT3 and RORγT), and **Tfh** (IL-6, IL-21; via STAT3 and Bcl-6)[14]. Th1 cells mediate protection against intracellular pathogens, such as viruses and intracellular bacteria, by inducing intracellular killing in phagocytes, promoting B cell class-switching to produce IgG antibodies, and inducing the expression of interferon stimulated genes (ISG) in both immune and non-immune cells (ie. epithelial and endothelial cells, fibroblasts)[77]. Th2 cells mediate defense against extracellular pathogens, helminths and other multicellular parasites, by

promoting mast cells, eosinophils, and IgE antibodies. Notably, Th2 immunity is also associated with allergic reactions and other autoimmune conditions[78]. Th17 cells respond to extracellular bacterial and fungal infections by inducing neutrophil responses, IgG2 and IgG3 antibodies, and production of cytokines (IL-17 and IL-22) that activate barrier epithelial cells to produce anti-microbial peptides such as β-defensins[79] and induce tissue remodeling and repair[80]. Lastly, Tfh cells aid in high-affinity antibody production and differentiation of B cells within lymphoid follicles[81]. CD4 Tconv can also differentiate into induced Treg (induced by TGF-β, IL-2, IL-10; via STAT5 and FoxP3) in the periphery[80,82,83]. In contrast to natural thymic Treg (**nTreg**), induced CD4 Treg (**iTreg**) develop from CD4 Tconv upon antigen recognition in presence of TGF-β and absence of inflammatory signals[84]. However, like nTreg, iTreg also express the transcription factor FoxP3, as well as CD25 and CTLA-4, and can comparably dampen unwanted or excessive inflammatory responses[80,83].

CD8 T cells can differentiate into cytotoxic lymphocytes (**CTL**), which are particularly important for immune defense against intracellular pathogens, like viruses[14]. They can selectively kill infected target cells expressing their cognate antigen on MHC-I through either extrinsic or intrinsic apoptotic pathways[14]. The extrinsic pathway is mediated by the expression of TNF-related apoptosis-inducing ligand (TRAIL), (Fas-ligand) FasL, tumor necrosis factor-alpha (TNFα) or lymphotoxin-alpha (LT-α); receptors for these molecules (TRAIL-R1/2**,** Fas/CD95, TNFR1) can be expressed by both immune and non-immune cells, though their distribution is somewhat restricted[14,85,86]. TRAIL expressed on the CTL cell surface binds to TRAIL-R1/R2 expressed on target; TRAIL receptors contain a conserved death domain motif that triggers recruitment of apoptosis molecules, inducing programmed cell death[87]. Fas-FasL

mediated apoptosis involves interaction of Fas protein (receptor for cytotoxic signal) expressed on the surface of target cells and FasL expressed on CTLs to transduce cell death signals[88]. Similarly, soluble and membrane-bound TNFα produced by CTLs interacts with TNFR1 on target cells to trigger apoptosis[89]. Lastly, LT-α is the closest homolog to TNFα, also inducing cell death through interaction with TNFR1[85]. Interestingly, in particularly inflammatory settings CD4+ Th1 cells can also utilize Fas-FasL, LT-α, and production of TNFα to exert cell-mediated cytoxicity[88,90–93], whereas TRAIL expression has been primarily observed in CD4+ Th2 cells[94,95].

The intrinsic apoptotic pathway is a more universal mechanism in which CTLs can directionally deliver cytotoxic granules into the immunological synapse, often to several target cells in succession, rapidly inducing apoptosis in the receiving cells[96]. Cytotoxic granules are essentially modified lysosomes that contain perforin, granzymes, and granulysin coexisting as multimeric complexes bound together by scaffolding proteoglycan serglycin[97]. Perforin functions by "poking holes" in the target cell's cytoplasmic membrane, directly damaging cell integrity as well as providing a necessary conduit through which to deliver other cytotoxic effector molecules[96,98]. Granzymes induce apoptosis once delivered into the target cell cytoplasm by activating caspases and damaging mitochondria; 10 types have been identified in mice, and 5 in humans[99]. Granulysin is only expressed in humans[100] and is also pore-forming and induces reactive oxygen species in the receiving cell, synergizing with granzymes[101]. These cytotoxic molecules are synthesized de novo shortly after TCR-stimulation in naïve and effector CD8 T cells and are stored as inactive forms within cytotoxic granules for rapid delivery [12,94,100,101]. Interestingly, human alloantigen-specific Th1-like CD4 Tconv can also deliver cytotoxic granules to kill target cells in the context of graft-vs-host-disease[104]. CTLs also produce IFNγ[105]

and TNFα[106], which inhibit viral replication and induce increased expression of peptide-loading proteins and MHC-I molecules, increasing recognition of intracellularly infected cells[14,107]. Perhaps due to their potential for cytotoxic killing, CD8 T cells require more co-stimulatory signals to become effectors[14,108,109]. In most cases, CD4 effector T cells can further amplify CD8 T cell activation by secreting IL-2 and increasing expression of B7 on APCs via presentation of CD40 ligands that bind the CD40 receptor on APCs[110]. Co-stimulation by highly activated dendritic cells can be sufficient to induce IL-2 production required for differentiation in CD8 T cells, without help from CD4 T cells[111].

1.1.3 Memory T cells

Protective immunity is dependent upon the development and capability of long-lived pathogen-specific memory CD4+ and CD8+ T cells (**Tmem**) to rapidly respond to re-encountered pathogens. Tmem are often categorized into discrete subsets based upon their tissue homing patterns and functions[112]. Central memory T cells (**Tcm**) home to secondary lymphoid organs (**SLOs**) and recirculate via blood and lymph[112,113]. When stimulated by antigen presenting cells in SLOs, Tcm can generate new populations of effector T cells to respond to infection. Effector memory T cells (**Tem**) surveil and recirculate through both lymphoid and non-lymphoid tissues[112,113]. As their name suggests, they can provide a rapid and robust immune response via direct cytotoxic function and effector molecule expression to eliminate infection[112]. Over the past couple of decades, we have come to recognize a unique subset of stable non-circulating tissue-resident memory T cells (**Trm**) that persist, patrol, and self-renew within non-lymphoid tissues [114–116]. Upon recognition of a previously encountered pathogen, Trm can directly kill infected

cells via cytolytic function and secrete pro-inflammatory cytokines (IFNγ, TNFα, IL-2), eliciting a rapid local anti-viral response and sounding the alarm for the rest of the immune system to respond[114,117–120]. Trm-derived cytokines lead to induction of chemokines (ie. CXCL9 and CXCL10), activation of endothelial adhesion molecules (ie. VCAM-1), and recruitment of other immune cells, including Tem, from circulation into infected tissues (Fig1.1)[120,121]. Their localized *in situ* cytokine and chemokine expression also activates nearby immune cells, even those not specific to the primary pathogen such as bystander activated cytotoxic T lymphocytes (**BA-CTL**), which can kill infected cells in an innate-like TCR-independent manner[122–125].

Trm have been identified in virtually every tissue type in both mice and humans[126]. They constitutively express CD69, and/or integrin αEβ7 (CD103) in the case of CD8 Trm at mucosal barrier sites and skin[116,127–130]. TGFβ has been shown to directly induce CD69 and CD103 expression on CD8 T cells, aiding in their retention within tissues by inhibiting egress from tissues and binding to E-cadherin expressed by epithelial cells respectively[131,132]. Trm also upregulate expression of the receptors for IL-7 (IL-7Rα) and IL-15 (IL-15Rα/CD122/CD132) [133,134]. More recently, Jarjour et al. found that deletion of *IL-7Rα* in memory CD8 T cells caused a gradual decline in their numbers systemically and impaired basal proliferation of Tcm and Trm at steady state, suggesting IL-7 signaling is important for memory T cell self-renewal at homeostasis[135]. Similarly, IL-15 supports maintenance and survival of CD8 Trm in tissues such as the skin and lung[134,136], though it does not seem to be a requirement for Trm in all tissues[137,138]. Interestingly, *IL-7Rα* deletion also reduces Tmem proliferation in response to IL-15 stimulation, and conversely *IL-15* deletion increases IL-7Rα surface expression in Tmem[135]. Rather than the requirement for one cytokine of the other in Trm within different tissues, this

work suggests there is “immune crosstalk’ and collaboration between IL-7 and IL-15 signaling pathways, allowing Trm to adapt to changes in cytokine availability and inflammation.

1.1.4 Regulatory T cells

Treg are a CD4+ T cell subset characterized by their expression of the *FoxP3* transcription factor and known for their role in regulating the effector function and activation of other immune cells via various direct and indirect mechanisms (aka. peripheral tolerance) [7,139–146]. Treg are essential to maintaining tolerance to self and steady-state homeostatic immunity[7,147,148]. Their critical importance is apparent in patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, in which loss-of-function mutations in *FoxP3* results in early-life presentation of lymphoproliferation, autoimmune enteropathy, autoimmune endocrinopathy, and eczematous dermatitis[149]. The clinical presentation and immunopathology of IPEX syndrome results from a loss of Treg and unchecked T cell activation[149], and the main treatment option includes hematopoietic stem cell transplantation and immunosuppressive therapy from an early age[150]. Similarly, male scurfy mice[151] and FoxP3-deficient mutant mice[152] show signs of severe autoimmunity and allergic inflammation involving multiple tissues and organs early in life, and a significantly shortened life-span. Furthermore, an early study showed that deletion of *FoxP3* in already mature Treg leads to a loss of their immunosuppressive functions and acquired ability to produce IL-2, IFNγ, and TNFα[153]; continuous *FoxP3* expression seems to be a requirement to maintain the stability, lineage, and function of mature Treg *in vivo*[153,154].

Similar to Tconv helper types, Treg and their functions can also be characterized based on their complementary expression of transcription factors and cytokine profiles[155]. Type 1 Treg express the Th1 transcription factor Tbet, limit immunopathology and suppress Th1 cell-mediated responses, and often express the chemokine receptor CXCR3 which recognizes CXCL9/10/11[156]. Type 2 Treg express the Th2 transcription factor GATA3 and are particularly enriched in barrier epithelial sites and tissues[157]. Unlike Type 1 Treg which are involved in managing Th1 responses, Type 2 Treg are not as commonly associated with managing Th2 responses; instead, they appear to be the main subset involved in directing the rest of the Treg response, maintaining tolerance at steady state, and tissue integrity/repair[157,158]. GATA3 induces the expression of the receptor ST2, which recognizes the alarmin IL-33, a cytokine commonly associated with tissue damage[155,159,160]. IL-33 signaling induces intrinsic regulation within Treg critical to maintaining their expression of FoxP3 and immunosuppressive function, as well as preventing their differentiation into Th17 cells[159,160]. Notably, ST2 is co-expressed with CXCR3 and CCR6 in Type 2 Treg, which suppress Tconv proliferation through IL-10 and TGFβ release[155,161]. Type 3 Treg are enriched in the gut lamina propria alongside Th17 cells and characterized by their expression of the transcription factor RORγt and cooperation with type 3 innate lymphoid cells [155,162]. In cooperation with colonic Type 2 Treg, Type 3 Treg help control Th17 and Th2-mediated colonic inflammation and maintain tolerance to the gut microbiome[163–165]. Type 3 Treg are thought to be largely peripherally induced Treg that recognize short chain fatty acids, as well as commensals and other oral antigens[166].

Treg are a heterogenous cell type consisting of both natural Treg (**nTreg**) and induced Treg (**iTreg**)[82]. As previously mentioned, nTreg arise from self-reactive α:β T cells in the thymus

with a high binding affinity for self-peptide:MHC-II complexes[167], whereas iTreg develop from CD4 Tconv in the periphery or can be differentiated *in vitro* in the presence of IL-2 and TGF-β[83,84,168]. In *in vivo* mouse studies using TCR-transgenic mice lacking thymically derived nTreg[169] or through adoptive transfer of antigen-specific naïve CD4+ CD25- FoxP3- into *Rag*-deficient mice[170], iTreg develop independently in the periphery and functionally prevent autoimmune sequalae comparable to nTreg. The differentiation of iTreg is dependent on many factors, including the strength of TCR signaling, co-stimulation signals from APCs, and the local cytokine environment[139,171–173]. Though nTreg and iTreg can comparably maintain immune tolerance, they also seem to display setting-dependent biological and functional differences[172]. For example, in the context of Th17-driven autoimmunity, synovial fluid (SF)-primed iTreg appear to be more protective than nTreg, maintaining immunosuppressive function against Th17 cells and stable expression FoxP3 and CD25[174]. Furthermore, SF-primed nTreg from patients with rheumatoid arthritis lost their suppressive capacity and even transdifferentiated into Th17 cells[174,175]. Th17 cells, like Treg, require TGF-β for their development in vivo, revealing an interesting link in their development[176,177]. An earlier study from the Shevach research group utilizing a murine model of Th17 driven autoimmune gastritis similarly found that *in vitro* TGF-β-induced antigen-specific iTreg (but not polyclonal nTreg or iTreg) could prevent Th17-mediated autoimmune disease in athymic nude mice[178], demonstrating a role for shared antigen-specificity in Treg-mediated suppression of Tconv. Though it is widely accepted that nTreg tend to be self-antigen specific based on what has been observed in mice[172], laboratory mice are generally specific-pathogen-free (**SPF**) and therefore lack the variety of exposures experienced by humans[179]; it is still unclear what proportion of the whole Treg response is foreign-antigen specific and whether they tend to be naturally derived or peripherally induced[180]. More recently,

work by the Savage research group[181] and others[180,182–185] support the theory that Treg selectively restrain T cells with a shared antigen-specificity in the context of inflammatory innate immune activation during autoimmunity or infection. However, they also found that shared antigen-specificity was not a requirement for Treg suppression of self-reactive Tconv in the absence of a strong pathogenic immunological challenge, suggesting that nTreg and iTreg may work together to maintain tolerance at steady state and highlighting the importance of context and tissue microenvironment[181]. Moreover, distinguishing these subsets *in vivo* and *ex vivo* has proven difficult, as knowing which markers should be used to differentiate them has been unclear[186]. Originally nTreg, but not iTreg, were reported to express high levels of transcription factor Helios and semaphorin receptor neuropilin-1 (**Nrp-1**) and thus were used as defining markers for Treg of intrathymic origin[173]. However, in recent years their status as bonafide markers for nTreg has been contested as others have reported expression of Helios is upregulated in all Treg that are antigen-activated outside of the thymus, even by CD4 Tconv-derived iTreg[187,188]. Furthermore, another study showed that allogenic iTreg could also express Nrp-1 in response to TGFβ[189]. Taken together, though Treg have been described as a stable self-renewing cell lineage resistant to many physiological and inflammatory changes[154], they appear to be a heterogenous population that retains some developmental plasticity and versatility in their functions[80,83,177,190].

Like conventional CD4 and CD8 T cells, Treg require antigenic stimulation to induce their immunosuppressive functions, however once activated they can dampen immune response in an antigen-independent manner[191]. Treg-mediated suppression of other T cells can be direct (cell-cell contact) or indirect (ie. immunosuppressive cytokine secretion, inhibition of APC immunostimulatory function, CD25-mediated IL-2 sinking). For example, Treg can directly

inhibit TCR-induced proliferation and transcription of IL-2 in Tconv upon cell-cell contact in the absence of APC involvement[146,192]. Interestingly, our lab and others found Treg also utilize contact-dependent perforin/granzyme to selectively kill effector T cells[193–195]. Conversely, Treg can indirectly inhibit the activation of Tconv through their modulation of APC antigen-presentation or function. Human and murine Treg constitutively express the costimulatory molecule CTLA-4[196,197], which reduces immunostimulatory function in APCs through competitive binding with CD28 on T cells for CD80 (B7-1) and CD86 (B7-2)[198], inducing expression of immunosuppressive indoleamine 2,3-dioxygenase (**IDO**)[199] in APCs, and even destruction of APC-expressed CD80 and CD86 via trogocytosis[200]. Another mechanism of indirect Treg-suppression of Tconv through regulation of APCs involves the adhesion molecule, lymphocyte activation gene-3 (LAG-3). LAG-3 expressed on Treg binds MHC-II molecules and rapidly exerts contact-dependent suppression of APC maturation[201]. IDO catalyzes tryptophan degradation to kynurenine, starving effector T cells and causing metabolic dysfunction and cell cycle arrest; while concomitantly resulting in iTreg differentiation[202]. Similarly, Treg can contribute to metabolic distress and dysfunction in effector T cells via expression of the ectoenzymes **CD39** and **CD73,** which hydrolyze available extracellular ATP into AMP, and generate adenosine and cyclic-AMP[203–205]. Adenosine activates adenosine receptor 2A signaling in effector T cells, resulting in T cell anergy and generation of iTreg[206]; while cyclic-AMP can be delivered by Treg through gap junctions in a contact-dependent manner to inhibit effector T cell function and proliferation[207]. IL-2 sinking by CD25+ Treg is also an indirect form immunosuppression via metabolic disruption[192]. Additionally, Treg can produce various immunosuppressive cytokines that indirectly modulate nearby immune cells. Treg secretion of IL-10 and TGFβ not only contributes to the generation of iTreg, but also induces STAT3 and

SMAD3 signaling (respectively) in receiving cells that inhibits inflammatory gene expression, IL-2 production, and antigen-presentation[208,209]. IL-35 is a more recently described Treg cytokine that strongly inhibits effector T cell proliferation and cytotoxicity[210,211]. Altogether, Treg utilize a myriad of mechanisms to dampen innate and adaptive immune responses and to further expand the Treg response through the generation of iTreg.

1.1.5 Specialized Non-lymphoid Tissue Treg

The characterization of non-lymphoid tissue Treg and their non-canonical roles in maintaining homeostasis and restoring tissue integrity has rapidly become a growing area of research[212]. Tissue Treg with specialized functions and unique TCR specificities have been described in various tissues including the skin, gut, muscle, visceral adipose tissue (**VAT**), lungs, and FGT[7,164,193,213–222]. Specialized non-lymphoid Treg were first described by the Mathis and Benoist research groups; they found that Treg enriched in VAT from lean mice expressed a distinct transcriptional program, exhibiting high expression of peroxisome proliferator-activated receptor γ (PPAR-γ) and cytokines that influenced production of inflammatory mediators (such as serum amyloid A3) and glucose uptake (adiponectin-mediated) in adipocytes, and modulating insulin resistance. Treg found in human skin tissue are enriched at hair follicles and regulate epithelial stem cell development, promote wound-healing, and limit pro-fibrotic responses[220,223–225]. Similarly, during muscle-damage Treg respond to IL-33 secreted by fibroblast-like adipogenic progenitor (FAP) cells and produce epidermal growth amphiregulin (**Areg**) to facilitate tissue repair[214,226,227]. Treg have also been found to prevent tissue damage and promote tissue integrity in immune privileged tissues such as the central nervous system[217,228], the

maternal-fetal interface, and the eyes. Gut Treg are essential regulators of oral tolerance to microbiota and dietary antigens, and prevent the development of autoimmune inflammatory bowel diseases[229–231]

Despite their organ-specific roles and heterogeneity, tissue Treg often share a transcriptional signature and common features such as expression of the transcriptional regulator basic leucine zipper transcription factor, ATF-like (**BATF)** and interferon regulatory factor 4 (**IRF4**), IL-33 receptor (**ST2**), chemokine receptor CCR8, killer cell lectin-like receptor (**KLRG1**), IL-10, T-cell immunoreceptor with Ig and ITIM domains (**TIGIT**), and Areg[221,232]. More recently, an alternative 'Pan-tissue Treg' theory has emerged. Instead of seeding different tissues and differentiating into a tissue-specific specialized Treg with a unique phenotype and TCR, Burton et al. suggest that non-lymphoid tissue Treg (except for those in the gut) originate from progenitors expressing PPAR-γ, as well as share TCR-specificities to self-antigens and are capable of multi-tissue migration and residency, allowing for pan-tissue Treg to maintain homeostasis throughout the body[233]. Furthermore, they found that adoptively transferred Treg from 7 different tissues were tissue-agnostic upon re-entry, and that tissue-residency was short-lived (about 3 weeks) in comparison to conventional tissue resident memory T cells (years to decades)[121,127,234,235].

1.1.6 Tissue Treg in infection

Tissue Treg localized at mucosal barrier sites has also been of particular interest due to their roles in tissue-healing during or following infections. In the context of infection, gut Treg can

also promote mucosal immunoglobulin A (IgA) responses, blocking intestinal pathogens from infecting the mucosal epithelium and supporting the maintenance of beneficial commensals[236,237]. Treg can form tissue memory in the lungs and antigen-specificity to influenza virus nucleoprotein (NP) after primary infection in mice; upon secondary infection, they display rapid accumulation in the lung parenchyma and control lung inflammation and tissue damage[184]. Importantly, replacement of NP-specific memory Treg with naïve Treg failed to restore protection from CD8 T cell-mediated damage during secondary infection[184]. Another study also investigating the role of lung Treg in influenza infected mice found that IL-18 and IL-33 induced Treg production of Areg in the absence of TCR signaling that directly contributed to tissue healing during infection[213]. In conjunction with work by many others, these studies suggest that eliciting Treg responses during particularly inflammatory infections associated with immunopathology may be beneficial[238–240]. Our lab has shown that CTLA-4+ Treg are critical to preventing early death and orchestrating HSV-2-specific CD4 T cell responses in mice upon primary infection[241,242]. Additionally, we've found that Treg in healthy human and mouse vaginal mucosa are highly activated at steady state and are further activated upon primary intravaginal HSV-2 infection in mice, displaying a unique phenotype and transcriptional signature similar to that of VAT Treg[193,218,219]. Further research is required to better understand the mechanisms by which tissue Treg establish memory and tissue-residency and how they participate in immunity in the case of episodic, chronic, or life-long conditions such as sexually transmitted infections (HSV-2), chronic urinary tract infections, bacterial or fungal infections (bacterial vaginosis, candidiasis, tinea).

1.1.7 Bystander Activated CD8 T cells

The mechanisms involved in regulating Trm induction of BA-CTL responses remain unclear, particularly within mucosal barrier tissues such as the respiratory, digestive, and urogenital tracts[118,243,244]. BA-CTLs are transiently activated CD8+ Tmem of irrelevant TCR specificity that can participate in early anti-pathogen immunity[125,245,246]. They can be stimulated by pathogen associated molecular pattern (**PAMP**) signals via toll-like receptors (**TLR**), Type I interferons, and pro-inflammatory alarmins IL-18, IL-12, and IL-15[247]. Instead of recognizing cognate antigen, they sense infected cells via NKG2D-dependent recognition of stress ligands and directly kill targets cells through secretion of cytotoxic granules[123,247]. An early study using MHC-I tetramers and mice previously infected intranasally with Sendai virus found that heterologous influenza A infection elicited increased recruitment of Sendai-specific BA-CTLs from circulation into the lung airways shortly after intranasal influenza[124]. Similarly, our previous work in mice challenged intravaginally with herpes-simplex virus type 2 (**HSV-2**) after systemic immunization with an irrelevant antigen showed that BA-CTLs recruited to the vaginal tract (VT) are sufficient to significantly reduce clinical symptoms and early viral burden shortly after challenge. This prompt orchestration of local immunity by Trm is an efficient and robust way of preventing further spread of the pathogen, giving the rest of the immune system time to respond. Conversely, it can also result in exaggerated inflammatory responses and collateral tissue damage to the host. For example, in the context of sustained inflammation during acute hepatitis A, Zika virus, *Leishmania major*, or *Borrelia burgdorferi* infection, BA-CTLs can cause enough immunopathology to impact host organ function if left uncontrolled[122,123,248,249]. Thus, proper regulation of the duration and intensity of BA-CTL responses warrants further study.

Our research group previously demonstrated that Treg can restrain "virtual memory" CD8 T cells (Tvm) within SLOs in part through limiting IL-15 trans-presentation by CD11b+ dendritic cells during West Nile Virus (WNV)[250]. Tvm are unconventional naive CD122+ CD44+ CD8 T cells that display a memory phenotype and are similar to BA-CTLs in that they do not require recognition of cognate antigen for transient activation and IFNg production in the presence of inflammatory cytokines like IL-15[251]. Additionally, we have found that Treg limit APC trans-presentation of IL-15 and tissue damage, both within ZIKV infected mucosal tissue and distal tissue sites such as the brain[252]. Associated with the increased immunopathology scores and IL-15 trans-presentation in transiently Treg-depleted ZIKV infected mice, was a significant increase in cytotoxic CD8 T cells not specific for the immunodominant ZIKV$^{94\text{-}302}$ epitope. Though we hypothesized that this expanded population of tetramer negative CD8 T cells may include BA-CTLs causing increased immunopathology in the absence of Treg, we did not further confirm their precise lack of specificity to ZIKV.

1.2 Herpes Simplex Virus and Genital Herpes

Herpes simplex virus type 1(**HSV-1**) and type 2 (**HSV-2**) are globally prevalent sexually transmitted infections. Around 846 million people between 15 and 49 years of age are living with genital herpes infections worldwide, with Sub-Saharan African countries experiencing the highest incidence rates [253]. HSV-2 nearly always causes genital infection, whereas HSV-1 more commonly causes oral mucocutaneous infections[253]. Historically, HSV-2 was the primary cause of genital herpes, however in recent years HSV-1 is increasingly associated with genital infection, now accounting for more than half of all new infections as the leading cause of first-

episode genital herpes [254,255]. HSV virions are encapsidated large double-stranded DNA alphaherpesviruses within the *Herpesviridae* viral family, characterized by their short replication cycle and latent infection of sensory ganglia[256–258]. Both types can enter host mucosa via nectin-1[259] and replicate within epithelial cells in mucocutaneous tissue upon primary infection before spreading through peripheral sensory neurons and travelling retrogradely to the lumbosacral dorsal root ganglia (**DRG**)[260,261]. In the case of oral infection, HSV-1 typically establishes latency in the trigeminal dorsal root ganglia[262]. Once in the DRG, HSV establishes a latent infection and sporadically reactivates, often causing recurrent painful genital lesion at or near the primary site of exposure. Reactivation can be triggered by various immunosuppressive events, such as changes in emotional or physical stress, UV/sunlight exposure, unrelated viral infections or fevers, lack of sleep, or dietary insufficiencies[260,263–265]. Though there is no visual distinction in terms of clinical presentation, genital HSV-1 infection is generally less severe with a six-fold lower frequency of symptomatic recurrences, likely due to a lower colonization of lumbosacral DRG than HSV-2 [262,266,267]. In mice, this difference in ability to colonize DRG between has been attributed to HSV-2's ability to evade type II interferon responses, thus leading to delayed NK cell-mediated IFNγ and T cell response kinetics during primary intravaginal infection[267]. Risk of transmission is highest in the prodromal phase and upon development of herpetic lesions, though sub-clinical asymptomatic viral shedding is common, even during high-dose treatment with antiviral medications (acyclovir, valacyclovir, and famciclovir), and greatly contributes to HSV transmission[268–270]. Around 20% of individuals with positive serology for HSV-2 are asymptomatic[271]. Though antiviral medications can accelerate lesion healing, reduce the severity of lesions, and decrease viral shedding, they are not a cure. Furthermore, to date there is no effective HSV vaccine available to prevent acquisition of this life-long infection[272].

Notably, cis-gender women have a significantly increased risk of genital herpes with HSV-2 infection rates 2 to 3 times higher in heterosexual male-to-female transmission[253], though this sex-based difference in transmission has not been observed in HSV-1 infections[254]. Risk factors for recurrent genital herpes in women include hormonal contraceptive use, group B streptococcus colonization, and bacterial vaginosis[273]. Though genital HSV-2 infections are not generally life threatening in immunocompetent adults, they are associated with an altered vaginal microbiome and a significantly increased risk of acquiring and transmitting other STIs[274–276]. In regions with high HSV-2 prevalence, a large proportion of newly acquired human immunodeficiency virus (HIV) infections are attributable to existing HSV-2 infection[277,278]. HSV-2 infection increases the risk of HIV infection by about three-fold due to compromised barrier tissue integrity, increased inflammation, and an increase in responding activated CCR5+ CD4+ target T cells[279–281]. Additionally, findings from a study in women on antiretroviral treatment living with HIV-HSV-2 co-infection found that a higher percentage of co-infected women had detectable levels of HIV in cervicovaginal lavages compared to women without HSV-2, suggesting that coinfection may also contribute to HIV shedding and transmission[276]. HSV-2 infection in women also greatly impacts neonatal health. Mother-to child-transmission of HSV-2 can occur in utero, during peripartum, or postnatally[282] and risk of transmission to infants also depend on whether primary maternal infection occurs before or during pregnancy[283]. Congenital HSV infections in neonates are rare, but without prompt treatment there is a high risk for disseminated infection, which has a 60% mortality rate and can cause severe ocular disease, recurrent cutaneous lesions, and neurological damage in survivors[284]. Strategies to prevent congenital herpes include rapid diagnosis of maternal infection, antiviral treatment during late

pregnancy and labor, and cesarian section delivery instead of vaginal birth to prevent newborn contact with infected vaginal fluids[285]. Despite the disproportionate global burden of HSV-2 infection on women and impact on maternal-fetal-health, we still lack clear understanding of how to elicit protective mucosal immune responses within the FGT[286]. Therefore, it is crucial to gain an improved understanding of the mucosal tissue immune response at homeostasis and during natural infection in the FGT to determine how to best therapeutically tune optimal immune responses to limit infection and disease in this unique tissue compartment.

1.2.1 HSV-2 FGT immunity

Upon primary HSV-2 infection, the innate immune response is the first line of defense at the site of infection. Stratified squamous epithelial cells in the female genital tract kick off innate immunity through initial sensing of the virus and subsequent production of antimicrobial mucins, proinflammatory cytokines (Type I IFNs, IL-36γ, IL-18, IL-12), chemokines (CXCL1, CXCL17, CCL3, CCL5, CXCL9/10/11), antimicrobial peptides (defensins, lysozymes), and expression of toll-like receptors (TLR) to recruit innate immune cells[287–291]. The first innate responders include Type I IFN-producing plasmacytoid dendritic cells (**pDCs**)[292], natural killer cells (**NK cells**)[293] that serve as an early source of IFNγ and mediate NKG2D-dependent cytolytic killing of infected cells to contain viral speread[294,295], and DCs that collect and present viral antigens to prime HSV-2 specific T cells in the draining lymph nodes[242,296–299]. In primary intravaginal murine infection, recruited pDCs sense HSV-2 infection via TLR9-mediated recognition to produce large amounts of Type I IFNs[300,301]that can directly suppress viral replication[302,303], and contribute to recruitment of T cells into the vaginal tissue[304]. However, one study using

diphtheria toxin (**DT**) depletion of pDC in CLEC4C-DTR mice found that pDC were not protective during either local primary intravaginal HSV-2 infection or cutaneous HSV-1 infection. Conversely, pDC-derived Type I IFNs promoted protective NK cell and CD8 T cell responses during systemic HSV infection, suggesting that the role of pDC in HSV immunity may dependent on the infection route[305]. Importantly, Type I IFNs promote early NK and T cell recruitment to the vaginal mucosa in murine intravaginal infection through the induction of the CXCR3-CXCL9/10 chemotactic pathway[306,307].

Studying innate immune responses to HSV-2 infection in humans is largely within the context of naturally infected tissue donors with preexisting immunity and experiencing recurrent active lesions outbreaks. Primary HSV lesion samples are rare, making it difficult to study the human primary immune response; it is estimated that 25% of patient experiencing their first episode of genital lesions have evidence of prior antibody-immunity, suggesting prior acquisition[308]. Thus, many studies using human tissue samples from HSV-2 seropositive individuls are observational or utilize primary cell lines for *in vitro* studies. In a study using primary human foreskin epithelial cells, *in vitro* HSV-2 infection of induced expression of TLR9 in genital epithelial cells though, it did not trigger the TLR9 signaling pathway[287]. In another human study, pDCs were identified via confocal microscopy analysis of biopsies collected from patients recurrent HSV-2 lesions 4-10 days after onset, found to co-localize with NK cells and T cells within lesions, and stimulated virus-specific T cell proliferation[292]. Additionally, TLR9 expression was expressed intracellularly in autologous PBMC pDC from all donors, indicating that endocytosis of virions by human pDC is required for TLR9-mediated recognition[292]. However, in contrast to primary murine infection studies, there seem to be lower expression of Type I IFNs and lower numbers of

pDCs at HSV-2 reactivation lesion sites in humans, suggesting that recall responses do not depend as much on Type I IFN signaling[309].

Though early innate responses alert the immune system and prevent early spread of the virus, full protection requires adaptive immunity[310]. HSV-2 specific IgG and IgA antibodies are produced in the genital tract by both mice and humans[311,312], and seem to provide partial protection by lowering viral burden[313–315]. However, HSV-2-specific tissue T cells are particularly critical for proper protection of the vaginal mucosa and sensory ganglia in mice[127,316,317]. The Iwasaki group first coined the 'prime-and-pull' vaccine approach to establish a protective T cell response in the FGT utilized a murine model in which naïve mice are first given adoptive transfer transgenic HSV-2 specific CD8 T cells and subsequently primed with a subcutaneous parenteral immunization with attenuated thymidine kinase deficient HSV-2[318]. Subcutaneous immunization alone did not elicit recruitment of HSV-2 specific CD8 T cells to the vaginal tract, however topical intravaginal application of CXCL9 and CXCL10 shortly after prime induced recruitment, pulling them into the tissue and establishing a Trm response that was protective upon challenge with WT HSV-2[318]. Later, the same research group demonstrated local networks of macrophages provide chemokine signals to maintain protective tissue-resident memory T cell clusters within the vaginal tract[130,319]. Taken together, innate and adaptive immunity work together through early anti-viral immune innate responses that recruit HSV-2 specific T cells to the site of infection and support their retention as Trm within mucosal tissue.

1.2.2 Role of T cells in HSV-2 Immunity and Reactivation

While the Trm response is clearly potent and effective at controlling pathogen expansion upon re-exposure, the mechanisms involved in regulating this robust tissue memory response remain unclear (Fig1.1) [126,320]. We predicted that regulation of this potent and rapid response is critical to avoid excessive collateral damage to host tissues. Though Treg are crucial in promoting tolerance and preventing autoimmunity, whether they help or hinder protective immunity and disease outcomes during infection seems to be dependent on the tissue microenvironment and invading pathogen. Treg have been shown to limit immune-mediated pathology by restraining the magnitude and intensity of adaptive immune responses, but often at the expense of timely pathogen clearance[312]. Early studies utilizing mouse infection models of *M. tuberculosis*, *L. major*, HSV-1, Friend virus, LCMV, and *Plasmodium* demonstrate that inhibition or depletion of immunosuppressive Treg function results in robust protective antigen-specific T cell responses[183,322–329]. However, in the context of mucosal infection within barrier tissues such as the lung and vaginal tract, we and others have shown Treg are necessary for orchestrating the development of an appropriate anti-viral responses during primary infections[239,241,242,330]. During respiratory syncytial virus (RSV) infection in mice, in vivo depletion of Treg prior to infection resulted in hampered recruitment of RSV-specific CD8 T cells to the lung early in infection with consequent exacerbation in disease, morbidity, and delayed viral clearance. Similarly, in our model of intravaginal viral infection in mice, systemic depletion of CTLA-4+ Treg during primary herpes simplex virus type 2 (HSV-2) infection in mice caused early death and increased viral titers due to delayed arrival of innate immune cells, impaired priming and migration of HSV-2 specific CD4+ T cells to the site of infection, and decreased antiviral cytokine production in the vaginal tract[241,242]. In both primary viral infection studies, FoxP3+ Treg rapidly proliferate

and accumulate in draining secondary lymphoid organs (SLOs) and traffic to mucosal infection site (i.e. lung or vaginal tract) with kinetics similar to that of CD4+ conventional T cells (Tconv). Furthermore, these mucosal Treg are highly activated based on increased expression of activation and immunosuppressive markers[193].

Notably, this influx of activated Treg has also been observed in human studies focused on tissue T cell responses within biopsies of HSV skin lesions. HSV-specific Trm at sites of infection actively patrol the tissue, confer protection, and control viral reactivation and lesion outbreaks[120,318,331–334]. Mathematical modeling and spatial analysis of human genital tissue suggest that Trm density and functionality are major determinants in the severity of reactivation[335–338]. However, HSV-2 is still frequently shed in the genital tract in the absence of symptomatic outbreaks, indicating that reactivation and replication occur despite the presence of HSV-2 specific Trm[270,271]. Milman et al. found that higher Treg:CD4+ Tconv and Treg:CD8+ T cell ratios within HSV lesions correlated with higher viral loads, and Treg persist alongside HSV-2 specific T cells throughout lesion healing[339]. Similarly, our research group found that Treg were highly activated in human tissue early after lesion presentation and decreased as lesion healing progressed, mirroring conventional T cell kinetics[340]. Yet, whether these Treg are the cause of increased viral replication or a response to robust tissue responses during viral reactivation is unclear[7,130,339,340]. In mice, Treg have been implicated in the suppression of HSV-1 vaccine-generated memory CD8+ T cell responses[325] and promotion of ocular HSV-1 latency[341], leading us to speculate that they may play a similar role in restraining the activation and function of tissue recall responses. Furthermore, though it is well known that Treg limit excessive

inflammatory T cell responses in a myriad of direct and indirect mechanisms, whether Treg regulate non-specific BA-CTL responses to infection is unknown.

1.3 Thesis Goals

Treg have been shown to infiltrate and persist alongside HSV-2 specific Trm throughout lesion healing[339,340], though their role in shaping Trm recall responses upon reactivation remained unclear. Others have reported the localization and specialization of non-lymphoid tissue Treg in the context of inflammatory settings, such as viral respiratory infection, sterile muscle damage, and adipose-tissue inflammation[181,213,214,216,218,223]. However, despite the frequent exposure to innocuous antigens and the bacterial/fungal microbiome, in addition to sexually transmitted pathogens, few studies have investigated Treg in the female genital tract outside of their essential role in fertility and tolerance during pregnancy. Furthermore, though it is well known that Treg limit excessive inflammatory T cell responses, how Treg modulate recalled memory T cells and non-specific BA-CTL responses to infection is not well understood.

We and others have shown that human and murine Treg in non-lymphoid tissues have a more activated and immunosuppressive transcriptional and phenotypic signature compared to Treg in circulation and lymphoid tissue [139,193,213,214,219,220,223,233]. Given this heightened activation in addition to their presence in barrier tissues, we hypothesized that they participate in anti-pathogen immunity. Therefore, we hypothesize that Treg form memory responses to mucosal virus infection, allowing them to be poised to respond to re-infection within affected tissues. Thus, in Chapter 2 we sought to investigate the role of regulatory T cells in regulating the tissue

recall T cell response and disease progression upon secondary intravaginal HSV-2 infection using a mouse model of systemic Treg depletion.

In addition to Treg-mediated immune modulation, other non-immune factors can impact the regulation of T cell responses, such as normal fluctuations in hormones. Innate and adaptive immune cells express receptors for sex-hormones and respond to fluctuations in estrogens, androgens, and progesterone[342–345]. In Chapter 3, we explore how endogenous hormones involved in directing menstruation impact T cell phenotypes across human tissue compartments through analysis of cervicovaginal biopsies and peripheral blood mononuclear cells (PBMC) from women in follicular (progesterone low) vs luteal (progesterone high) phase using high parameter flow cytometry, as well as multiplex detection of soluble immune factors from serum and vaginal secretion samples.

Chapter 2 : Regulatory T cells restrain IL-15-mediated cytotoxicity and bystander T cell activity in mucosal tissue without compromising antigen-driven memory

2.1 Introduction

The tissue microenvironment plays an influential role in guiding phenotypic and functional aspects of lymphocytes such that mucosal tissue T cells have distinct qualities compared to their circulating counterparts[114–116]. As a notable example, tissue-resident memory T cells (Trm) have been identified as a subset of memory T cells with a unique transcriptional signature that persist, patrol, and self-renew within non-lymphoid tissues[116,118,346–348]. Upon recognition of their cognate antigen via their TCR, Trm can accelerate protection *in situ* by directly killing infected cells via cytolytic function and through secretion of pro-inflammatory cytokines such as IFNγ, TNFα, and IL-2[114,117–119]. This cytokine production not only elicits a rapid local anti-viral response, but also serves as a 'sense and alarm' function[118] that potentiates robust tissue immunity through recruitment and activation of other immune cells[117,120,349]. Thus, in addition to canonical antigen-driven memory CD8 T cell activity elicited by pathogen re-exposure in the tissue, memory CD8 T cells can also become bystander activated via cytokine signals to acquire cytotoxic functions[122–125,245,350–352]. It was recently shown that at steady state, human memory CD8 T cell cytotoxicity, including expression of granzyme B, is lower in nonlymphoid tissues compared to the circulation, and inversely correlates with CD8 Trm phenotype[353]. Nevertheless, exposure of human tissue memory CD8 T cells to IL-15 is sufficient to increase expression of Granzyme B and perforin, each of which is tightly correlated with target cell killing capacity[353]. Thus, while human tissue memory CD8 T cells maintain a state of relative quiescence at steady-state, signals within the tissue environment, such as those elicited during infection, lead to rapid acquisition of cytotoxic function.

However, a knowledge gap remains in terms of how the cytotoxic activity of tissue memory CD8 T cells is controlled, particularly in settings of active inflammation or infection. Moreover, the overall Trm response is clearly potent and effective at controlling pathogen upon re-exposure, yet the mechanisms involved in regulating this potent tissue memory response remain unclear. We predicted that regulation of this robust and rapid tissue memory T cell response is critical to avoid excessive collateral damage to host tissues. Recent work from Lee *et al* demonstrated a cell-intrinsic mechanism whereby IL-15-driven bystander activation of human memory CD8 T cells is reduced in context of TCR signaling[354]. However, it remains unclear how IL-15 is regulated to restrict the cytotoxic program that is induced without antigenic signals. We hypothesized that regulatory T cells (Treg) could provide a layer of cell-extrinsic regulation of the tissue cytotoxic program in settings of mucosal re-infection.

Treg are a CD4+ T cell subset characterized by their expression of the forkhead box protein 3 (FoxP3) transcription factor and known for their role in regulating the effector function and activation of other immune cells via various direct and indirect mechanisms[139–144]. Though Treg are crucial in promoting tolerance and preventing autoimmunity, whether they help or hinder protective immunity and disease outcomes during infection seems to be dependent on the tissue microenvironment and invading pathogen[183,241,321,323,324]. Treg have been shown to limit immune-mediated pathology by restraining the magnitude and intensity of adaptive immune responses, but often at the expense of timely pathogen clearance[183,323,329]. Early studies utilizing mouse infection models of *M. tuberculosis*, *L. major*, HSV-1, Friend virus, LCMV, and *Plasmodium* demonstrated that inhibition or depletion of immunosuppressive Treg function results in robust protective antigen-specific T cell responses[182,183,185,322–329,355]. However, in the context of primary infection within barrier tissues, such as the lung and vaginal tract, Treg are necessary for orchestrating the development of an appropriate anti-viral responses[239,241,242,330]. During respiratory syncytial virus (RSV) infection in mice, *in vivo* depletion of Treg prior to primary infection

resulted in hampered recruitment of RSV-specific CD8 T cells to the lung early in infection with consequent exacerbation in disease, morbidity, and delayed viral clearance. Similarly, in a model of intravaginal viral infection in mice, systemic depletion of activated CTLA-4+ Treg during primary herpes simplex virus type 2 (HSV-2) infection in mice caused early death and increased viral titers due to delayed arrival of innate immune cells, impaired priming and early migration of HSV-2 specific CD4+ T cells to the site of infection, and decreased antiviral cytokine production in the vaginal tract[242]. In primary viral infection studies, FoxP3+ Treg rapidly proliferate and accumulate in draining secondary lymphoid organs (SLOs) and traffic to mucosal infection sites (i.e. lung or vaginal tract) with kinetics similar to that of CD4+ conventional T cells (Tconv)[239,241,242]. Furthermore, these mucosal Treg are highly activated based on increased expression of activation and immunosuppressive markers[7,193]. Notably, this influx of activated Treg has also been observed in human studies focused on tissue T cell responses within biopsies of HSV skin lesions. Higher Treg:CD4+ Tconv and Treg:CD8+ T cell ratios within HSV-2 lesions correlated with higher viral loads, and Treg persisted alongside HSV-2 specific Trm throughout lesion healing[339]. However, whether the presence of these Treg are the cause of increased viral replication or a response to robust tissue immunity resulting from viral reactivation is unclear[7,339]. In mice, Treg have been implicated in the suppression of HSV-1 vaccine-generated memory CD8+ T cell responses[325], leading us to speculate that they may play a similar role in restraining the activation and function of tissue recall responses.

We sought to interrogate the role of Treg in regulation of the tissue memory T cell response to re-infection. We previously demonstrated that Treg limit IL-15 trans-presentation in context of flavivirus infections[250,252]. Here, using a mouse model of genital HSV-2 infection, we demonstrate that during a tissue recall response to secondary infection, Treg limit IL-15 trans-presentation by tissue innate immune cells. Furthermore, we demonstrate that Treg selectively restrict the BA-CTL response in tissues while sparing the protective memory HSV-specific tissue CD8 T cell response. This selective

Treg-mediated restriction of the cytotoxic activity of tissue memory CD8 T cells is dependent on both IL-2 and IL-15. Our findings highlight the essential role for Treg in regulating BA-CTL within barrier tissue sites, preserving an appropriate and protective pathogen-specific tissue memory T cell response while limiting cell-mediated immunopathology.

2.2 Results

2.2.1 Regulatory T cells are poised to restrain IL-15 trans-presentation by tissue antigen-presenting cells upon secondary infection.

To address whether Treg could modulate antigen-specific and bystander tissue memory T cells via control of the local immune microenvironment, we first sought to establish antigen-experienced Treg and CD8 T cells in the vaginal tract. We have previously shown that human and murine vaginal Treg are highly activated at steady state, and acquire a phenotypic and transcriptional tissue signature distinct from baseline following primary infection with HSV-2[193]. Here, we infected mice intravaginally with thymidine kinase-deficient (TK-) HSV-2 and allowed them to recover for approximately one month. To characterize and define transcriptional and phenotypic changes in vaginal Treg at a memory timepoint following primary HSV-2 infection, Treg (CD4+ FoxP3^{eGFP}+) were FACS-sorted from the VT and dLN of naïve and previously infected mice for bulk RNA sequencing (Fig S1A-B). Compared to Treg isolated from vaginal tissue of naïve mice, VT Treg from TK- HSV-2-infected mice clustered independently and had elevated transcripts for canonical markers of immunosuppressive function (*Il10, Ctla4, Icos*) and tissue-homing receptors (*Ccr8*). Though the subset of differentially expressed genes between VT Treg from naïve and TK- infected mice was relatively small, this data suggests that infection-experienced VT Treg retain heightened activation and suppression potential long after resolution of acute infection. We next assessed Treg abundance and phenotypes at day 7 and day 90 after TK- HSV-2 infection using flow cytometry. As previously

demonstrated, Treg numbers increased significantly both within the VT and dLN at day 7 post-infection (p.i.)[193,241]. However, at day 90 p.i. Treg numbers remained elevated compared to baseline in the VT but not the dLN (Fig S2A). CD4 FoxP3- Tconv and CD8 T cell numbers similarly peaked at day 7 but returned to near baseline numbers both within the VT and dLN (Fig S2B). In addition to Treg abundance, we assessed markers of activation and immunosuppressive function. VT Treg displayed significantly increased frequencies of CD25+ CTLA-4+, ICOS+, Tim-3+, and GITR+ out to day 90 post-HSV-2 infection, whereas dLN Treg only transiently expressed these markers during acute infection (Fig S2C-E), consistent with transcriptional profiling that showed more persistent changes in VT Treg compared to dLN Treg after infection (Fig S1B). Thus, highly activated Treg accumulate within the VT tissue, remain transcriptionally and phenotypically activated, and persist long after clearance of mucosal viral infection, potentially poised to participate in governance of the recall tissue T cell response.

To address the mechanism whereby Treg may modulate the tissue cytotoxic memory CD8 T cell response upon secondary infection, we utilized Foxp3^{DTR} mice along with an HSV-2 re-infection model to assess IL-15 trans-presentation. It is well-known that IL-15 is an essential cytokine that can induce cytotoxicity in memory CD8 T cells[122,245,350–354]. IL-15 must be trans-presented by IL-15Ra to be biologically active and is constitutively expressed by many different cell types, including monocytes, macrophages, and dendritic cells (DC)[356]. Thus, we infected Foxp3^{DTR} mice intravaginally with TK- HSV-2 and allowed them to recover. Unlike wildtype (WT) HSV-2, mice survive and recover from intravaginal TK- HSV-2, and are then protected from death upon subsequent infection with WT HSV-2[317,318,357]. One month after primary infection, mice were systemically depleted of Treg and subsequently challenged with WT HSV-2 (Fig 2-1A). Successful Treg depletion was confirmed in both the lymphoid tissues and vaginal tract (Fig 2-1B). Treg sufficient and depleted mice previously infected with TK- HSV-2 only (primary infection) were included as controls. We then assessed IL-15

trans-presentation by innate immune cells in the mucosa (gating shown in Fig S3) upon Treg depletion and HSV-2 challenge via flow cytometry. We found a more than two-fold increase in the number of IL-15+ cells compared to Treg-replete and challenged mice (p=0.0650; Fig 2-1C). Thus, during a memory response to secondary infection, Treg play a role in limiting the trans-presentation of IL-15 within mucosal tissue, suggesting that they may play an active role in extrinsic regulation of cytotoxic activity of tissue memory CD8 T cells.

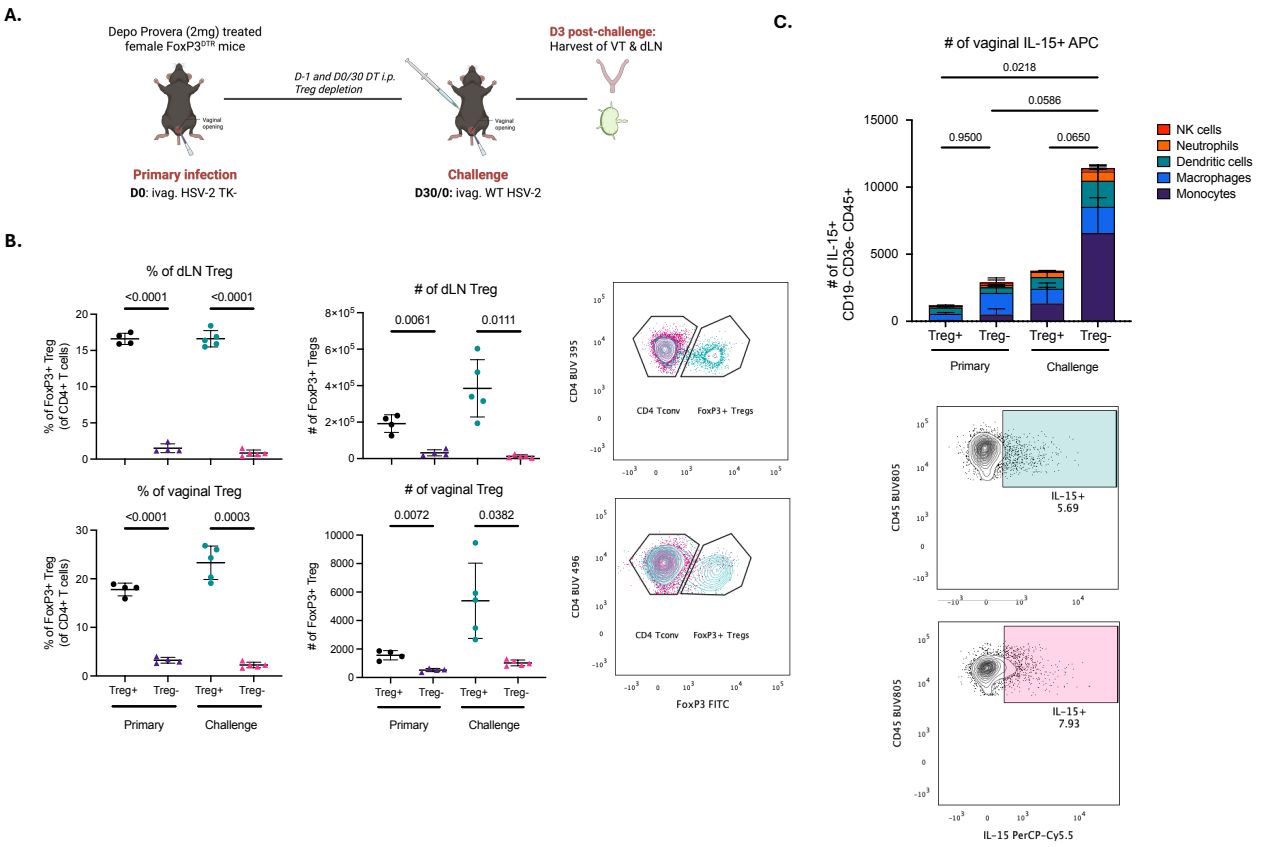

Figure 2-1: Regulatory T cells are poised to restrain IL-15 trans-presentation by tissue antigen-presenting cells upon secondary infection.
Female FoxP3^{WT} and FoxP3^{DTR} Depo Provera-treated mice were vaginally infected with 10^5 pfu of attenuated HSV-2 TK- and allowed to recover for 28-30 days. Mice were again treated with Depo Provera 5-7 days before WT HSV-2 (10^4 pfu) challenge and treated intraperitoneally with diphtheria toxin (DT) on days -1 and 0 (with respect to challenge) to ablate Treg prior to infection (FoxP3^{DTR} mice, Treg-depleted group, Treg-). A dose of 30 µg/kg was administered the day before WT challenge, followed by a dose of 10 µg/kg on d0. VT and dLNs were harvested at d3 p.c. and characterized via flow cytometry. Representative data from two experiments (n= 4-5 mice per group) are shown. The experimental scheme is shown in (**A**). (**B**) dLN frequencies and absolute numbers of FoxP3+ Treg in Treg depleted and sufficient mice after WT HSV-2 challenge and representative staining (top). VT frequencies and absolute numbers of FoxP3+ Treg in Treg depleted and sufficient mice after WT HSV-2 challenge and representative staining (bottom). (**C**) The absolute number of IL-15+ CD19- CD3e- CD45+ APC is shown. Stacked bar plot displays cell type subset as a fraction of the total number at day d3 (left). Representative flow staining of total IL-15 expression in CD19- CD3e- CD45+ APC (right). Data shown is representative of two experiments. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test for B-C.

2.2.2 Treg limit the magnitude and cytotoxic potential of the CD8 T cell recall response shortly after WT HSV-2 challenge.

Given that Treg restrict trans-presented IL-15 in the tissue, a cytokine known to be critical for activation of memory CD8 T cells, during a local recall response, we next sought to assess how Treg impact the tissue memory T cell response upon HSV-2 challenge (as in Fig 2-1A). We first evaluated the abundance and phenotypic changes of CD4+ FoxP3- Tconv and CD8+ T cells in dLN and VT shortly after challenge using flow cytometry. Treg depleted and challenged mice had a significantly higher frequency and number of CD4 Tconv and CD8 T cells in the VT at day 3 post-challenge (p.c.) compared to Treg sufficient challenged mice; these increases were not observed in the dLN (Fig 2-2A and Fig S4A-C). In alignment with their increased number, a statistically larger frequency and number of VT CD8 T cells from Treg depleted and challenged mice expressed Ki67 and CD44, indicating that Treg restrict proliferation of the total T cell recall response (Fig 2-2B and S4D). Finally, after HSV-2 challenge, vaginal tissue CD8 T cells from Treg depleted mice also expressed the cytotoxic molecule granzyme B (GrzmB) at a higher frequency and number compared to Treg sufficient mice, indicating increased cytotoxic potential (Fig 2-2C; S4D).

Next, we specifically assessed Trm to determine the extent to which Treg may restrict Trm compared to total CD8 T cells found in tissue upon rechallenge. To better identify tissue-resident cells, we injected CD45.2 mice intravenously with anti-CD45.2 APC-conjugated antibody label 3 minutes prior to euthanasia to label circulating CD45.2+ lymphocytes while sparing those within the tissue[358]. Like the total T cell response, there was a significantly higher number of CD45.2- CD103+ CD69+ CD8 T cells in the VT of Treg depleted and challenged mice at day 3 p.c. (Fig 2-2D). Furthermore, in absence of Treg, there was a significantly increased frequency of VT Trm expressing Ki67 or GrzmB upon HSV-2 challenge (Fig 2-2E). To confirm that Treg limit proliferation of Trm during the tissue recall response, we treated mice daily with intraperitoneal (i.p.) injections of FTY720 (an agonist of the S1P

receptor that blocks T cell egress from SLOs) starting just prior to and during challenge. Thus, FTY720 administration aimed to prevent T cell egress from the dLN and therefore block new recruitment to the mucosal tissues to allow for assessment of the effect of Treg on restriction of Trm proliferation in situ. We found that even upon blockade of T cell recruitment into the VT during challenge, there was a significant increase in the frequency of Ki67+CD44+ CD8 T cells in the vagina of Treg-depleted compared to Treg-sufficient mice (Fig 2-2F), indicating that Treg limit in situ proliferation of Trm in context of a tissue recall response. Altogether, these findings show that in concert with their restraint of tissue IL-15, Treg limit the total tissue recall T cell and Trm response at the site of re-infection by limiting their proliferation and cytotoxic potential.

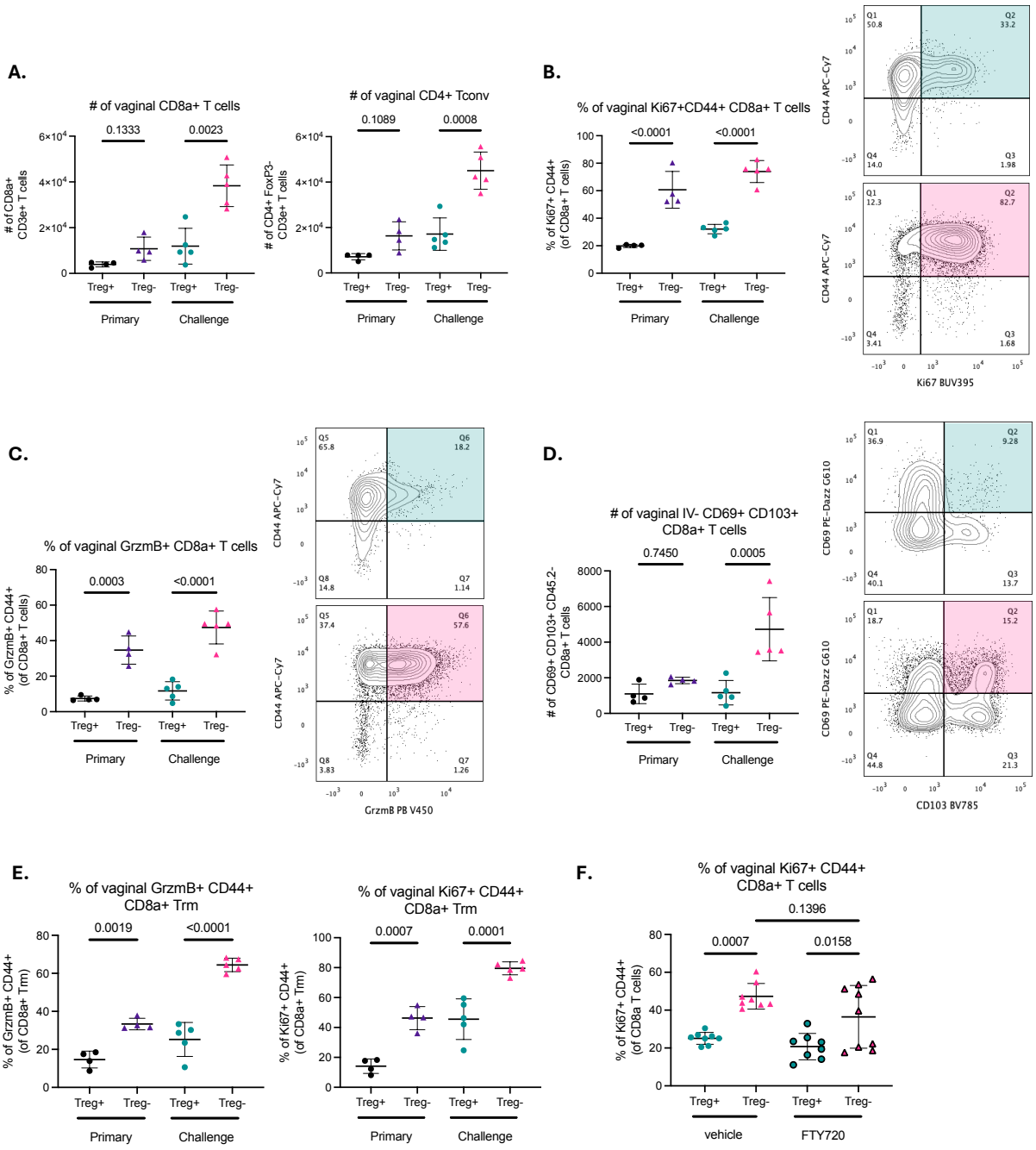

Figure 2-2: Treg limit the magnitude and cytotoxic potential of the CD8 T cell recall response shortly after WT HSV-2 challenge.

Female FoxP3^{WT} and FoxP3^{DTR} mice were infected and treated with DT as in Figure 1. (**A**) The absolute number of VT CD8+ T cells (left) and CD4+ Tconv (right) in Treg depleted vs Treg sufficient unchallenged and challenged mice. (**B**) Frequency of CD44 and Ki67 expression by VT CD8+ T cells d3 p.c. (**C**) Frequency of CD44 and GrzmB expression by VT CD8+ T cells d3 p.c. (**D**) The absolute number of VT Trm. Mice were retro-orbitally injected with anti-CD45.2-APC antibody 3 minutes before euthanasia. Trm were defined as CD69+ CD103+ CD45.2 IV- label negative. (**E**) Frequency of VT CD44+ GrzmB+ CD8+ (left) and CD44+ Ki67+ Trm (right). Data shown are representative of at least two experiments with 4-5 mice per group. Error bars represent mean and SD. (**F**) Frequency of Ki67+ CD44+ CD8 T cells in FTY720 and vehicle control treated mice. Mice were treated with 1mg/kg FTY720 via intraperitoneal injections on days -1, 0, and 2 with respect to WT HSV-2 challenge. Control mice were treated with vehicle (2% cyclodextrin in sterile PBS). Data combined from two experiments with 4-5 mice per group. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in A-F.

2.2.3 Treg do not limit the magnitude or cytokine expression of protective HSV-2 gB-specific CD8 T cells in the VT.

Having observed significant increases in the total CD8 T cell and Trm recall response in the absence of Treg, we wanted to determine the impact of Treg depletion on the HSV-specific tissue memory T cell response. Surprisingly, we did not observe significant differences in the frequency or number of CD44+ CD8 T cells in the VT that stained positive with an MHC class I tetramer specific for the HSV glycoprotein B immunodominant epitope following HSV-2 challenge, regardless of the presence of Treg (Fig 2-3A). Likewise, there was no difference in the frequency and number of CD69+ CD103+ I.V. label negative gB+ CD8 T cells between Treg depleted and sufficient groups (Fig3B). We wondered whether gB-specific CD8 T cells mostly infiltrate from circulation or expand *in situ* upon challenge. Treating mice with FTY720 just prior to and during WT challenge revealed that infiltrating gB-specific CD8 T cells comprise a large fraction of the recall response; forced retention of T cells in the secondary lymphoid organs resulted in a significant decrease in the number of HSV-specific CD8 T cells in the vagina, regardless of the presence or absence of Treg (S5A). In accordance with their comparable numbers, there was a similar frequency of HSV-specific CD8 T cells from Treg depleted and challenged mice that expressed Ki67 compared to Treg sufficient mice (Fig 2-3C; S5B). Additionally, Treg depleted and HSV-2 challenged mice displayed a significantly higher frequency of vaginal HSV-specific CD8 T cells that were GrzmB+ compared to Treg sufficient mice (Fig 2-3D), just as we observed with **the total** CD8 T cell and Trm response (Fig 2-2C and E). To more deeply assess CD8 T cell effector function beyond cytotoxic potential, we assessed cytokine expression by T cells following HSV-2 challenge. We stimulated single cell suspensions of vaginal tissues with glycoprotein B (SSIEFARL) peptide in complete media for 4 hours at 37°C before intracellular cytokine staining (ICS) for IFNγ and TNFα. In concordance with our gB tetramer data, there was no difference in the frequency or number of responding IFNγ+ and TNFα+ CD8 T cells in the VT regardless of the presence of Treg (Fig 2-3E and S6A, B). Taken together, we demonstrate that Treg

allow for the expansion and cytokine effector function of a protective antigen-specific local CD8 T cell response within infected tissues, while limiting the cytotoxic potential of the memory recall response locally, which may selectively minimize cell-mediated tissue damage while allowing for effective viral control.

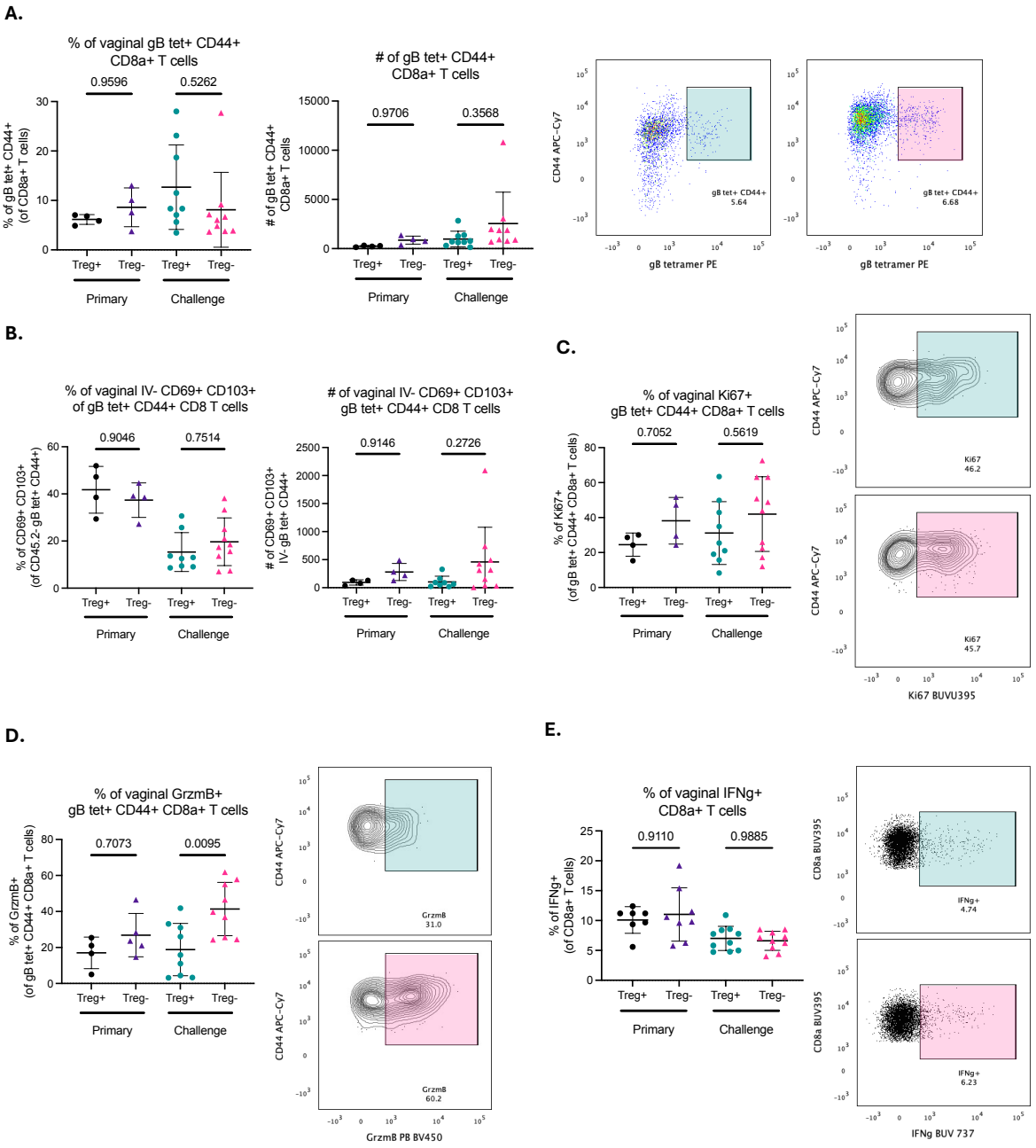

Figure 2-3: Treg do not limit the magnitude or cytokine expression of protective HSV-2 gB-specific CD8 T cells in the VT.

Female FoxP3^{WT} and FoxP3^{DTR} mice were infected and treated with DT as in Figure 1. (**A**) Frequency and absolute number of gB tetramer+ CD44+ CD8 T cells in VT (left) and representative staining (right). (**B**) Frequency and absolute number of CD69+ CD103+ gB-tetramer+ IV label negative CD8 T cells in the VT. (**C**) Frequency of Ki67+ expression in gB tetramer+ CD44+ T cells (left) and representative staining (right). (**D**) Frequency of GrzmB+ expression in gB tetramer+ CD44+ T cells (left) and representative staining (right). (**E**) VT tissue from mice euthanized at 3 days p.c. was processed into a single-cell suspension and stimulated in complete media with gB peptide (immunodominant MHC-I epitope) for 4 hours at 37°C before conducting intracellular cytokine staining for IFNγ production in CD8 T cells. Data shown are combined from two experiments with 4-5 mice per group. Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in A-E.

2.2.4 Treg restrain the bystander-activated cytotoxic T cell response in the tissue following viral challenge.

Given the comparable expansion of antigen-specific responses between challenged Treg depleted and sufficient mice, we wondered whether Treg might regulate memory T cells responding to challenge independent of TCR signaling (ie. bystander activation, or BA-CTL). BA-CTL are transiently activated CD8+ memory T cells that can participate in early anti-pathogen immunity via exposure to cytokines such as type I interferons and pro-inflammatory alarmins IL-18, IL-12, IL-15 rather than via exposure of TCR to cognate antigen[125,320,359,360]. BA-CTL sense infected cells via NKG2D-dependent recognition of stress ligands and directly kill target cells through delivery of cytotoxic granules[123,125]. Our previous work utilizing data from humans with HSV-2 reactivations to inform mathematical modeling[337] and a murine HSV-2 challenge model after systemic immunization with an irrelevant antigen suggest that BA-CTL recruited to genital tissue can significantly reduce clinical symptoms and early viral burden shortly after viral reactivation or challenge. To now examine whether Treg regulate BA-CTL in the tissue upon challenge, we adoptively transferred $5x10^5$ – $1x10^6$ naïve TCR transgenic Thy1.1+ OT-I CD8 T cells into $FoxP3^{DTR}$ mice one day before primary intravaginal infection with TK- HSV-2-OVA to establish a memory population of OVA-specific CD8 T cells in the vagina. One month later, mice were depleted of Treg and challenged vaginally with WT HSV-2 not expressing OVA (Fig 2-4A). As a control, groups of $Foxp3^{WT}$ or $Foxp3^{DTR}$ mice received the adoptive transfer prior to primary TK- HSV-2-OVA infection, and one month later, mice were treated with DT followed by collection of VT and dLN tissues at a timepoint matched to mice that received vaginal HSV-2 challenge (3 days p.c.). OT-I BA-CTL were identified as Thy1.1+ CD44+ NKG2D+ GrzmB+ CD8 T cells after WT HSV-2 challenge. Following HSV-2 challenge, we observed a significant increase in the number of Thy1.1+ CD44+ CD8 T cells in the VT of Treg-depleted compared to Treg-sufficient mice

and a significantly higher frequency and number of them were GrzmB+ NKG2D+ BA-CTL (Fig 2-4B, C). Furthermore, a significantly larger proportion of VT Thy1.1+ BA-CTL from Treg depleted and challenged mice expressed Ki67 (Fig 2-4D). Interestingly, removal of Treg at the time of challenge led to a significant increase in the fraction of Trm that were bystander activated (S7). Thus, our findings are consistent with a role for Treg in restraining the expansion of BA-CTL and cytotoxic responses in the VT upon secondary intravaginal infection, thereby coordinating an appropriate and pathogen-specific memory T cell response finely regulated to clear pathogen while limiting cytotoxic activity that could compromise host tissue integrity.

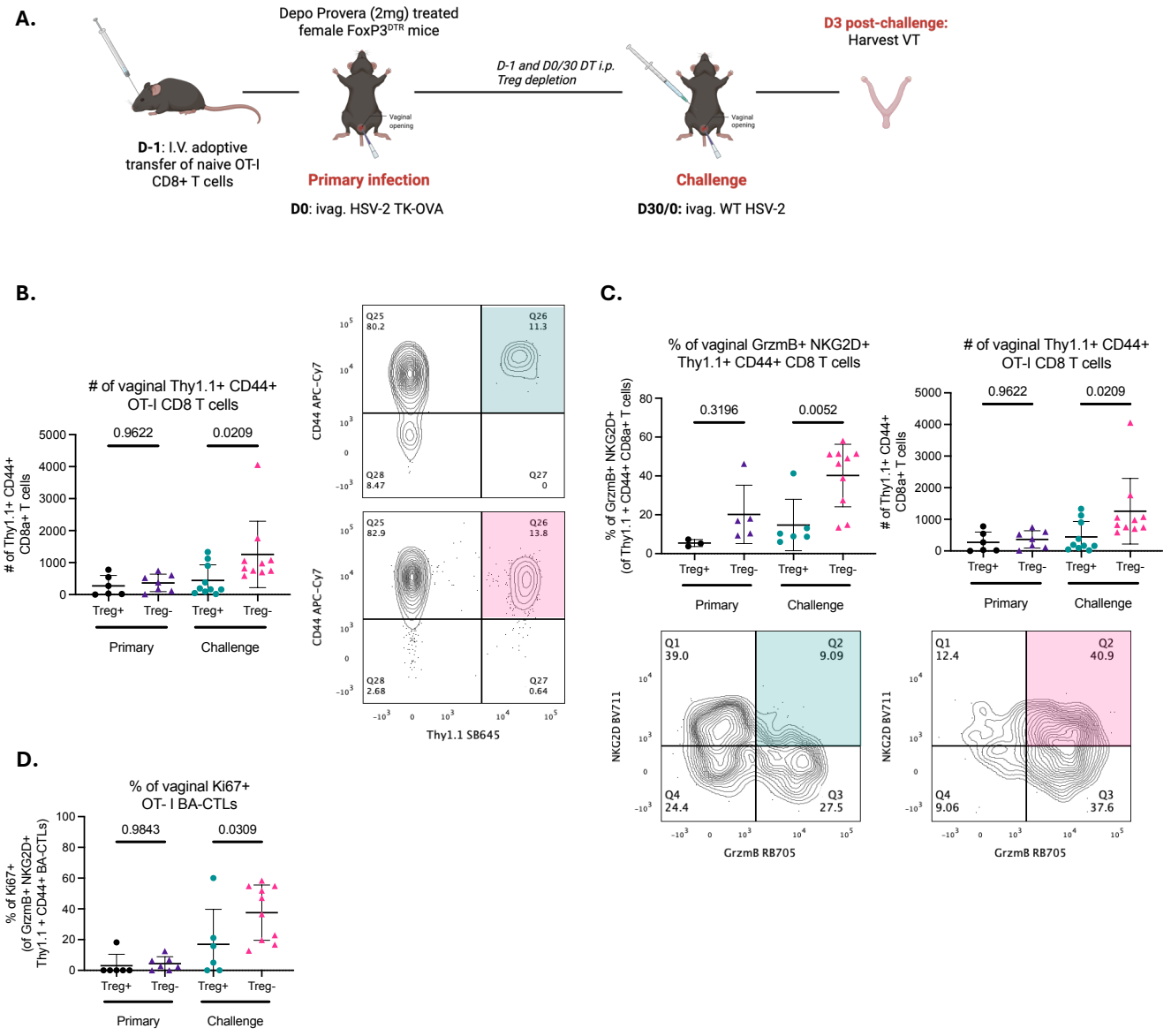

Figure 2-4: Treg restrain the bystander-activated cytotoxic T cell response in the tissue following viral challenge.

Thy1.1+ CD45.2+ TCR transgenic OT-I CD8 T cells were first isolated from healthy adult female OT-I mice using an immunomagnetic negative selection kit; 500,000-1 million OT-I cells were then adoptively transferred into female FoxP3^{WT} and FoxP3^{DTR} mice treated with Depo Provera 24hrs before vaginal infection with 10^5 pfu of HSV-2 TK-OVA and allowed to recover for 28-30 days. Mice were treated with Depo Provera again 5-7 days before WT HSV-2 (10^4 pfu) challenge and treated intraperitoneally with diphtheria toxin (DT) to ablate Treg prior to infection (FoxP3DTR mice, Treg-depleted group, Treg-). A dose of 30 µg/kg was administered the day before WT challenge, followed by a dose of 10 µg/kg on d0. VT and dLN were collected d3 p.c. and assessed via flow cytometry. Part (**A**) shows the experimental scheme. (**B**) Absolute number of VT CD44+ Thy1.1+ OT-I cells (left) and representative staining (right). (**C**) Frequency and absolute number of VT GrzmB+ NKG2D+ Thy1.1+ CD8 T cells (top) and representative staining (bottom). (**D**) Frequency of Ki67 expression in cytotoxic bystander-activated Thy1.1+ OT-I cells. Data shown are combined from two experiments with 4-5 mice per group. Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in B-D.

2.2.5 Treg limit the cytotoxicity of antigen-specific and bystander CD8 T cells via an IL-2 dependent mechanism.

Next, we wanted to further investigate mechanisms by which Treg can suppress the expansion and cytotoxic potential of memory T cells, and particularly the BA-CTL. A canonical mechanism of Treg immunosuppressive function is their ability to sink IL-2 availability in the local immune environment through expression of the high affinity IL-2 receptor subunit, CD25[141]. Their efficient binding and obligatory consumption of IL-2 sequesters it from other T cells, depriving them of the potent activating cytokine. Furthermore, others have shown that strong IL-2 signaling is necessary to induce cytolytic function in effector CD8 T cells[361], and more recently, it was shown that IL-2 was able to modestly induce expression of Granzyme B and NKG2D by human memory CD8 T cells, though to a lesser extent compared to IL-15[354]. Thus, we hypothesized that in the absence of Treg there is more IL-2 available in the local immune milieu, resulting in the activation and expansion of CD8 T cells leveraged in the memory tissue recall response.

To address our hypothesis that unrestrained availability of IL-2 in the absence of Treg was contributing to the expansion and activation of BA-CTL and other effector T cells in our HSV-2 challenge model, we blocked IL-2 signaling *in vivo* prior to WT challenge using a monoclonal anti-IL-2 antibody (clone JES6-1A12)[362] (Fig 2-5A). IL-2 blocking just prior to challenge significantly decreased the frequency of GrzmB+ CD44+ CD8 T cells within the VT of Treg depleted mice compared to isotype-treated Treg depleted mice also challenged with HSV-2 (Fig 2-5B). We also observed a subtle decrease in the frequency of HSV-specific CD8 T cells expressing GrzmB in Treg depleted mice treated with anti-IL-2 compared to isotype control-treated mice ($p=0.0803$; Fig 2-5C). However, just as in our initial experiments (Fig 2-3), there was no significant difference in the frequency of Ki67 expression by HSV-specific CD8 T cells between Treg sufficient and depleted challenged mice treated with anti-IL-2 or isotype control monoclonal antibodies (Fig 2-5D). Notably, IL-2 blocking significantly inhibited the

bystander activation of Thy1.1+ cells (as identified by co-expression of NKG2D and GrzmB) and their expression of Ki67 in Treg depleted mice (Fig 2-5E, F). Altogether, our findings suggest Treg limit IL-2 in the immune environment to restrain bystander activation and proliferation of memory CD8 T cells during a tissue recall response.

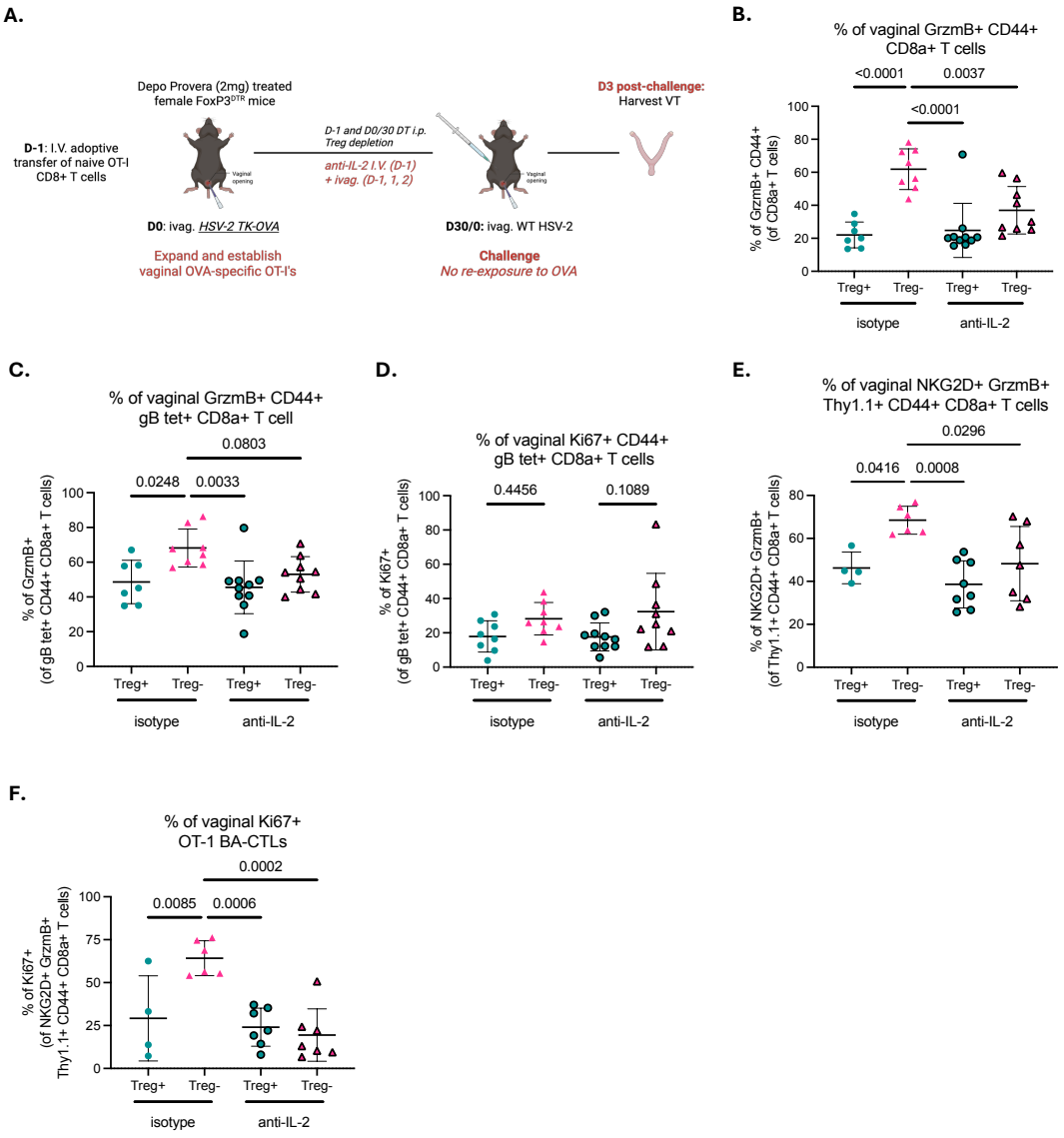

Figure 2-5: Treg limit the cytotoxicity of antigen-specific and bystander CD8 T cells via an IL-2 dependent mechanism.

500,000-1 million OT-I cells were adoptively transferred into female Depo provera treated FoxP3^{WT} and FoxP3^{DTR} mice 24hrs before vaginal infection with 10^5 pfu of HSV-2 TK- OVA and allowed to recover for 28-30 days. Mice were again treated with Depo Provera 5-7 days before WT HSV-2 (10^4 pfu) challenge and treated intraperitoneally with diphtheria toxin (DT) to ablate Treg prior to infection (FoxP3DTR mice, Treg-depleted group, Treg-). Mice were also administered both intravenous (150ug) and intravaginal (80ug) anti-IL-2 monoclonal antibody (clone JES6-1A12) or rat IgG2a isotype control on day -1 with respect to WT challenge. 40ug of anti-IL-2 or isotype control was administered intravaginally d1 and 2. VT was collected d3 p.c. and assessed via flow cytometry. (**A**) shows the experimental scheme. (**B**) Frequency of total VT CD44+ GrzmB+ CD8 T cell response in WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control. (**C**) Frequency of GrzmB+ in VT gB tetramer+ CD44+ CD8 T cells from WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control. (**D**) Frequency of Ki67+ in VT gB tetramer+ CD44+ CD8 T cells from WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control (**E**) Frequency of NKG2D+ GrzmB+ Thy1.1+ BA-CTL in VT from WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control Mab. (**F**) Frequency of Ki67 expression in VT NKG2D+ GrzmB+ Thy1.1+ BA-CTL from WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control. Data shown are combined from two experiments with 3-5 mice per group. Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in B-F.

2.2.6 Antigen presenting cells increase IL-15 trans-presentation in response to available IL-2 in the absence of Treg.

In addition to depriving memory CD8 T cells of IL-2, we wondered if an increase in available IL-2 in the absence of Treg might impact APC activation and IL-15 trans-presentation, either indirectly through CD4 Tconv or directly via APC IL-2-receptor (IL-2R) binding and signaling. For example, activated macrophages have been shown to express a functional IL-2R that upon IL-2 binding promotes anti-microbial functions such as intracellular killing of bacteria, tumoricidal activity, and IFNγ production[363–366]. To determine if there was a relationship between IL-2 levels and IL-15 trans-presentation, we evaluated IL-15+ APC in challenged Treg sufficient and depleted mice after in vivo IL-2 blocking. Indeed, we found that IL-2 blockade resulted in significantly decreased IL-15 trans-presentation by macrophages, monocytes, and dendritic cells within Treg depleted mice (Fig 2-6A; S8A). To ascertain whether this could be a direct effect of sensing of IL-2 by APC, we first evaluated expression of the IL-2R components by APC in VT of Treg sufficient unchallenged mice and found that monocytes, macrophages, and dendritic cells all express CD25 and CD132 to equal or higher levels as observed in NK cells (Fig6B; S8B). However, CD122 was expressed at much lower levels in comparison to NK cells (Fig 2-6B). Nonetheless, we observed a significant increase in the number of mucosal tissue IL-2R+ (CD25+ CD132+ CD122+) APC in Treg depleted and challenged mice at day 3 p.c. (Fig 2-6C). To further assess the relationship of IL-2 and IL-15 trans-presentation in vitro, splenocytes were isolated from healthy naïve FoxP3^{WT} mice; half of the single cell suspension was directly plated at 1 million cells/well, while the remaining half was put through a CD3e T cell magnetic microbead depletion kit before plating. The total vs CD3-depleted splenocytes were then cultured with increasing concentrations of recombinant murine IL-2 in vitro overnight at 37C. We found that macrophages directly responded to IL-2 stimulation with significant increases in the frequency of IL-15 trans-presentation, in a dose-dependent manner, in T cell depleted cultures (Fig 2-6D). Additionally, we observed a similar dose-dependent increase in the frequency of CD25

expression in IL-15+ macrophages (Fig 2-6E). To our knowledge, this is the first report of high levels of IL-2 directly increasing IL-15 trans-presentation in macrophages and adds mechanistic insight into the pathways by which Treg coordinate the tissue recall response upon secondary infection.

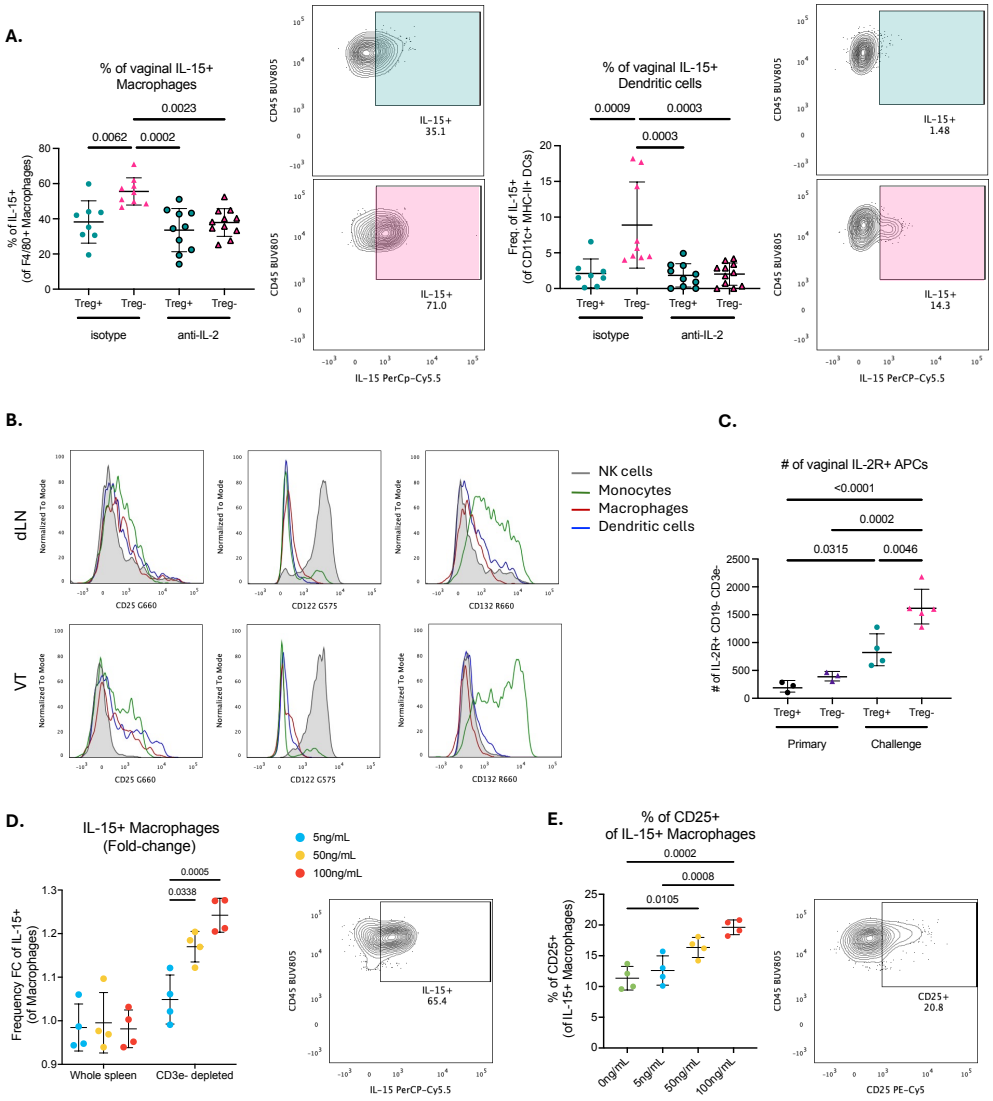

Figure 2-6: Antigen presenting cells upregulate IL-15 trans-presentation in response to available IL-2 in the absence of Treg.

Female FoxP3^{WT} and FoxP3^{DTR} mice were infected and treated with DT as in Figure 1, and with anti-IL-2 or isotype control Mab as in Figure 5. (**A**) VT was collected d3 p.c. and assessed via flow cytometry. Shown is the frequency of IL-15 trans-presentation by macrophages (CD3e-CD19-Ly6G-NK1.1-F4/80+) or DC (CD3e-CD19-Ly6G-NK1.1-F4/80-CD11c+MHC-II+) in VT from d3 WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control Mab. Data shown are combined from two experiments with 4-5 mice per group. (**B**) Representative histograms showing expression of IL-2R components (CD25, CD122, CD132) by NK cells (CD3e-CD19-Ly6G-NK1.1+), monocytes (CD3e-CD19-Ly6G-CD11b+Ly6C+), macrophages, and dendritic cells from VT tissues of unchallenged Treg sufficient mice about a month after primary TK- infection (normalized to mode). (**C**) Female FoxP3WT and FoxP3DTR mice were infected and treated with DT as in Figure 3. Absolute count of IL-2R+ (CD25, CD122, CD132) + CD19- CD3e- CD45+ lymphocytes. Data shown are representative of two experiments with 4-5 mice per group. (**D**) Single-cell suspensions of total spleen and CD3e depleted spleen from healthy FoxP3^{WT} mice were plated at 1 million cells/well and stimulated with increasing concentrations of recombinant murine IL-2 (rmIL-2) in complete media overnight at 37C. Representative flow plot of IL-15+ gating of macrophages and graph showing frequency of IL-15+ trans-presentation by macrophages after culture. Data presented as a fold-change in frequency in comparison to media only control (0ng/mL of rmIL-2). (**E**) Representative flow plot and frequency of extracellular CD25 expression in IL-15+ macrophages after overnight culture of CD3e depleted splenocytes with increasing concentrations of rmIL-2. Data shown are representative of two experiments with 4-5 mice per group. Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in A, C-E.

2.2.7 Treg limit immune-mediated tissue pathology upon HSV-2 re-infection.

Finally, we wanted to assess the impact of Treg on disease progression and pathogen clearance upon challenge with WT HSV-2. FoxP3^{DTR} mice were first infected with TK- HSV-2 and allowed to recover. One month after primary infection, mice were systemically depleted of Treg and subsequently challenged with WT HSV-2. VT was harvested from Treg sufficient and depleted mice on days 3, 7, 10, and 14 post-challenge (p.c.), and formalin-fixed, paraffin embedded tissue was H&E stained and blindly scored by a pathologist for tissue damage and signs of inflammation (Fig 2-7A). We utilized a total sum histopathology scoring system in which different regions of the VT (lumen, epithelium, lamina propria, and muscularis) were individually scored, summed, and averaged for a total score per mouse (Fig 2-7B). Daily vaginal washes were also collected from challenged mice up to the time of harvest to assess changes in viral load via RT-PCR[245]. Naïve Treg sufficient and depleted mice were included as controls for inflammation caused by the transient systemic ablation of Treg[367]. Mice challenged with WT HSV-2 did not show signs of infection or disease progression as assessed by clinical scoring[318], irrespective of Treg depletion. However, Treg-depleted and challenged mice displayed significantly higher total sum pathology scores internally in the VT at 7 days p.c. (Fig 2-7C). Despite this increase in pathology scores, we did not observe a difference in vaginal viral load or ability to clear the infection (Fig 2-7D), suggesting that the tissue damage was likely immune-mediated, and not due to an increase in viral burden in the absence of Treg as observed in primary infection[241]. Critically, this indicates that the protective tissue memory T cell response was not compromised in the absence of Treg. Overall, we demonstrate that Treg are critical to manage a protective tissue recall response that limits host tissue pathology.

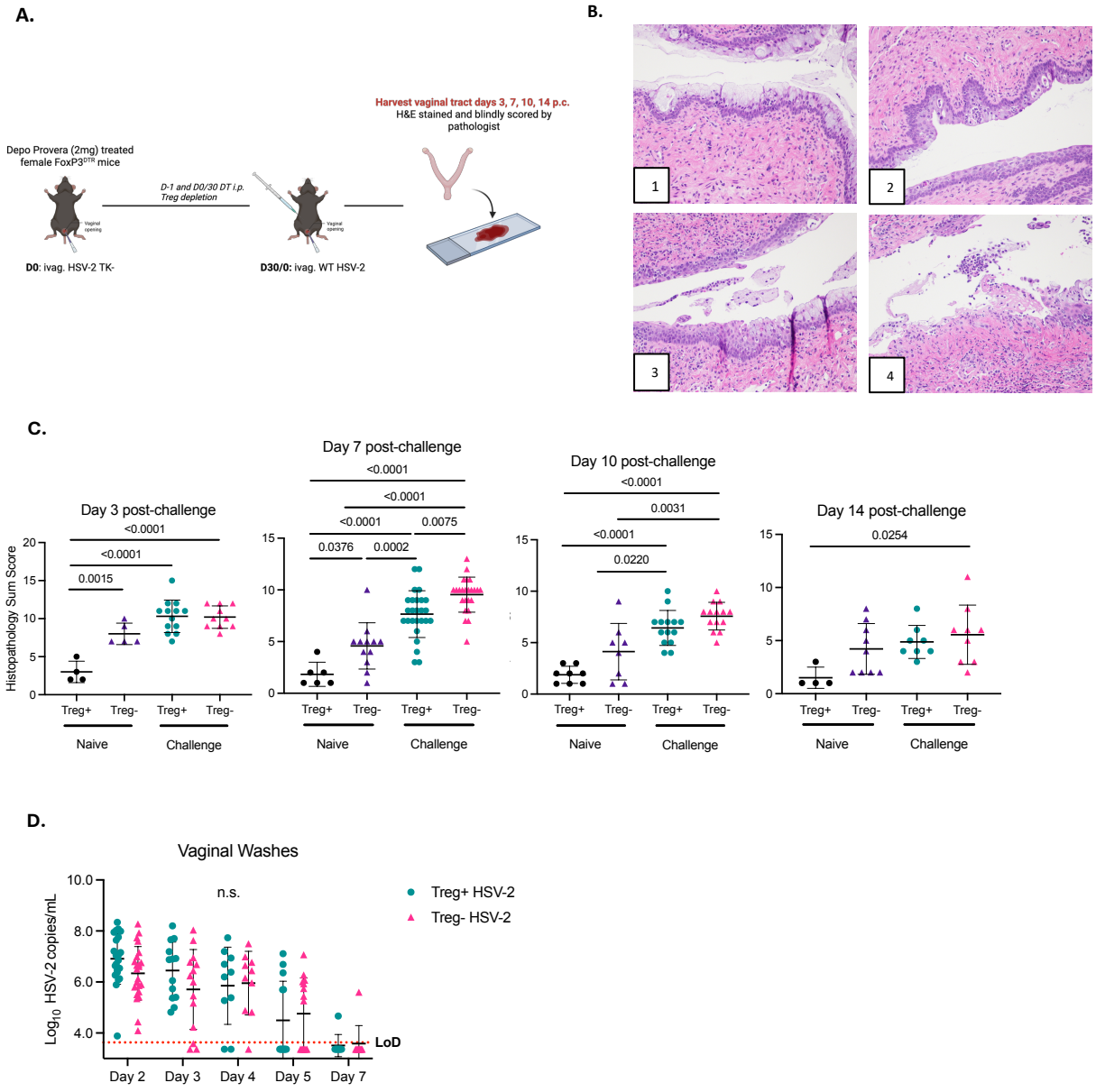

Figure 2-7: Treg limit immune-mediated tissue pathology upon HSV-2 re-infection.
FoxP3^{WT} and FoxP3^{DTR} mice were infected and treated with DT as in Figure 1. (**A**)VT tissues were carefully harvested at 3, 7, 10, and 14 days p.c., formalin fixed, paraffin-embedded and scored blindly by a pathologist using a total sum pathology rubric. (**B**) An example of pathology scoring (1-4) in H&E-stained vaginal epithelium. (**C**) Total sum vaginal pathology scores on days 3, 7, 10, and 14 p.c. Total sum pathology is composed of averaged and summed scores within the mucosal epithelium, lamina propria, and muscularis mucosa, and lumen layers of the VT. Data combined from 2-3 representative experiments per timepoint (n = 3-7 mice per group). Error bars represent mean and SD. Statistical significance determined by Two-Way ANOVA and Tukey's multiple comparisons test. (**D**) Vaginal washes were collected daily during the first week of infection and assessed for viral load via RT-PCR. Data shown are combined from 3 representative experiments per timepoint. Error bars represent mean and SD. Statistical significance determined by multiple unpaired T-tests with Welch correction and Holm-Šídák multiple hypothesis testing.

2.3 Discussion

Here, we sought to identify extrinsic signals that regulate tissue memory CD8 T cells during a recall response to mucosal infection. We reasoned that such regulation would be critical, given that an over-exuberant tissue recall response could lead to significant tissue injury, thereby contributing to disease, particularly in chronic or frequently recurrent infections such as HSV-2[270]. Previous work identified IL-15 as a major player in induction of the cytotoxic program in CD8 T cells[353,354], yet regulatory mechanisms of local IL-15 within tissues remained unclear. Mucosal tissues including the genital tract are frequently exposed to inflammatory threats and commensals that could induce IL-15, suggesting that this response must be subject to immunoregulation to therefore restrict the tissue memory CD8 T cell response and avoid immunopathology. Notably, while Lee *et al* investigated cell-intrinsic mechanisms of restraint of the IL-15-induced bystander activation of memory CD8 T cells and found that TCR signaling could quell the cytokine-induced cytotoxic program[354], this does not account for regulation of cytokine-induced cytotoxicity in absence of cognate antigen. The latter is predicted to occur frequently given the diverse range of microbial exposures encountered at a mucosal surface. Thus, our work for the first time focuses on extrinsic environmental and cellular cues in mucosal tissues that restrain tissue memory CD8 T cell cytotoxic activity and notably BA-CTL.

As the most abundant memory T cell subset, Trm are critically important to coordinating rapid and long-term immunity against infections at mucosal surfaces[130,368]. Early work in mice identified a core Trm gene expression profile[119,134,347] that was later found to be remarkably conserved in human Trm[116]. Additionally, TGF-β and IL-15 have emerged as pivotal cytokines involved in regulating Trm formation and persistence in which TGF-β supports the differentiation and sequestration of Trm within tissues, while IL-15 (along with IL-7) support their survival and maintenance[134,135]. More recently, work by Niessl et al. demonstrated that local cytokines assist in directing human CD8+ T cell function and residency within tissues. Though CD103+ Trm across tissues expressed low levels of

perforin/granzyme B expression at steady state, their work suggests that high doses of IL-15 can induce proliferation in Trm and override the effects TGF-β, which promote tissue-residency and suppress cytotoxic function[353]. Despite the sustained interest and research efforts in understanding their formation, activation, and localization since their discovery over 2 decades ago[116,127,319,346,348], we still lack a clear understanding of Trm regulation, particularly during a tissue recall response, which motivated our study of Treg.

We and others have shown that human and murine Treg in non-lymphoid tissues have a more activated and immunosuppressive transcriptional and phenotypic signature compared to Treg in circulation and lymphoid tissue[7,139,193,213,214,219,220,223]. Given this heightened activation in addition to their presence in barrier tissues, we hypothesized that they participate in anti-pathogen immunity. It is plausible that Treg also possess the ability to form immunological memory just as conventional T cells do. In support of this idea, previous reports have documented the formation of memory Treg[181,184,369–371]. A study from the Rudensky group revealed that prior exposure to an autoinflammatory environment or systemic LCMV infection imprints a stable non-lymphoid tissue localization preference for Treg, as well as a limited set of transcriptional changes that are consistent with a memory of prior inflammation[372]. However, they also observed that Treg reversed many, though not all, activation induced changes. Many studies assessing tissue Treg or memory Treg are carried out in settings of autoimmunity, steady-state, systemic infection, or sterile tissue inflammation or injury[216,371–373]. Yet, such models do not precisely mimic natural pathogen encounter or chronic exposure, in which microbial antigens and pathogen-associated molecular patterns drive distinct inflammation types and responses in addition to tissue-specific changes in the microenvironment due to localized pathogen replication. Therefore, we hypothesize that Treg form memory responses to mucosal virus infection, allowing them to be poised to respond to re-infection within affected tissues.

Here, using a murine model of localized mucosal viral infection, we demonstrate that Treg limit the expansion and cytotoxic potential of the recall memory CD8+ T cell and non-specific BA-CTL response to mucosal viral challenge by sinking local IL-2 and sequestering it from conventional T cells and APC. We have previously demonstrated that at steady state and in context of primary infection, Treg limit IL-15 trans-presentation by antigen-presenting cells (APC) including DC and monocytes[252,374]. Here, we build on that work to now focus on the tissue during a recalled memory response. Further, we propose that restricted availability of IL-2 in the local immune environment in the presence of Treg restrains T cell activation, proliferation, and survival as well as limits trans-presentation of IL-15 by APC to T cells. Though IL-2 and IL-15 signaling share the common gamma chain (CD132) and IL-2/IL-15 receptor beta chain (CD122), they seem to play different roles in immunity. Notably, IL-15 is produced by many cell subsets, including non-myeloid epithelial and stromal cells. IL-15 expression can be induced both in response to microbial pathogen-associated triggers[375] and in instances of sterile inflammation like autoimmunity[376]. IL-15 overexpression is commonly linked to various tissue-specific autoimmune disorders such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, coeliac disease and insulin-dependent diabetes[377–380]. Interestingly, in the context of cancer, metastatic patients with low expression of IL-15 due to a deletion at the IL-15 genetic locus have increased risk of relapse and lower density of proliferating intratumoral immune cells, hinting at a role for IL-15 in tissue immunity and pathology[381,382]. In contrast, IL-2 deficiency as opposed to overexpression typically contributes to autoimmunity that can be ameliorated by IL-2-based therapies that target *in vivo* expansion of Treg to restore immune homeostasis[383,384]. Our findings here support a model wherein the tissue microenvironment keeps Trm in check at least in part via Treg control of IL-2 and IL-15.

Finally, we propose that Treg, in addition to their direct modulation of both innate and adaptive responses, are also critical in preventing sustained excessive local inflammation that contributes to

pathologic BA-CTL responses. Importantly, Treg restrain the cytotoxic potential of the total memory recall T cell responses and immune-mediated tissue damage upon secondary mucosal viral infection, while still allowing for an optimal pathogen-specific protective tissue T cell response. The fact that presence or absence of Treg did not significantly impact the immunodominant HSV-2 specific CD8 T cell memory recall response in terms of size and cytokine expression is particularly interesting. Furthermore, a growing body of research on the role of Treg during infection supports the idea that they are essential in limiting excessive inflammation that can distract the immune response from focusing on the formation protective pathogen-specific T cell responses[7,184,242,374,385]. Our research group has previously shown that Treg are a critical source of TGF-β within infected tissues, shaping the generation of CD103+ CD8+ Trm and supporting their maintenance in brain tissue after primary WNV infection in mice[374]. Furthermore, recent work by Jarjour et al. shows that, once formed, CD8+ memory T cells can dynamically adapt to changes in the cytokine milieu during viral infection, making them resilient to potential loss of homeostatic signals[135]. Taken together, by modulating the cytokine environment, Treg are critically important in coordinating priming of T cells and Trm formation within tissues; yet once differentiation establishes a Trm program in T cells, their survival may be less impacted by cytokine alterations upon Treg-depletion during infection and even supported by compensatory inflammatory cytokines.

Here, we have demonstrated a role for Treg in directing the tissue memory CD8 T cell response to viral re-challenge. Importantly, Treg soak up excess IL-2 in the local tissue microenvironment, thereby depriving memory CD8 T cells of this cytokine but also restricting local IL-15 trans-presentation. Thus, we demonstrate for the first time that Treg govern the environmental cues that induce the cytotoxic program of memory CD8 T cells, including bystander activation. Importantly, Tregs do not restrict the protective, antigen-driven and antigen-specific tissue memory CD8 T cell response, as demonstrated by the lack of difference in tissue pathogen control (Fig 7D), though they are critical to

reducing tissue injury in context of a tissue recall response. Altogether, our findings reveal the importance of Treg mediation in recall innate and adaptive tissue responses and have implications for the design of a successful vaccine against mucosal infections that can elicit protective, but not pathogenic, Trm populations poised at the site of pathogen exposure.

Limitations of the study

We assessed bystander activation using an adoptive transfer model that leveraged OT-I cells to ensure that cells were not activated via TCR signals (Fig 5). We recognize that assessment of endogenous rather than TCR transgenic T cells would be preferable. We did attempt to establish an endogenous OVA-specific memory CD8 T cell response in the mucosa by priming mice intravaginally with TK-HSV-2-OVA and then assessing the frequency of endogenous GrzmB+ NKG2D+ OVA-specific T cells after depletion of Treg and WT HSV-2 challenge. Unfortunately, this strategy was unable to establish sufficient numbers of OVA-specific memory CD8 T cells in the VT to properly enumerate and assess their bystander activation phenotype after challenge (generally less than 25 endogenous OVA-specific CD8 T cells could be detected in the vagina). Thus, we instead relied on adoptive transfer to increase the number of T cells detectable in the vagina.

Additionally, although we report that increased IL-2 availability results in a direct dose-dependent increase in IL-15 trans-presentation by macrophages, we have not addressed the exact mechanism by which IL-2R signaling results in this response from APC. Future studies aimed at understanding the distinct effects of IL-2 and IL-15 on immune cells present in non-lymphoid tissue would aid in the improved design of vaccines and therapeutics to achieve improved immunity and minimize collateral tissue damage.

2.4 Methods

2.4.1 Mice

6–10-week-old female wildtype C57BL/6J (Jackson Laboratories, Bar Harbor, ME) or B6.129(Cg)-Foxp3tm3(HBEGF/GFP) Ayr "FoxP3 DTR" knock-in mice (bred at Fred Hutch) were used for in vivo experiments and in vitro stimulations. C57BL/6-Tg(Tcra/Tcrb)1100Mjb/J OT-I Thy1.1 were used for isolation of CD8 OT-I from splenocytes for adoptive transfers. All animal experiments were approved by Fred Hutch IACUC and the study was conducted in strict compliance with the PHS Policy on Humane Care and Use of Laboratory Animals. Foxp3DTR mice are a gift from Dr. Alexander Rudensky (Sloan Kettering Institute) and bred in-house. C57BL/6J mice were purchased from Jackson (Bar Harbor, Maine, USA, Strain #:000664) and used as controls and in non-depletion experiments. Due to the HSV-2 infection route used in this model (vaginal), only female mice were used.

2.4.2 Infections

Mice were injected subcutaneously in the neck ruff with 2mg of medroxyprogesterone acetate (Depo Provera) dissolved in sterile PBS, 5–7 days prior to intravaginal (ivag.) infection. Mice were infected with either 10^5 PFU TK- HSV-2 (HSV-2 TK-/kpn) or 10^5 PFU of TK- OVA HSV-2 (HSV-2 OVA TK-/kpn) for primary infection. Mice were challenged 30 days post-primary HSV-2 TK- infection with 10^4 PFU of wild-type HSV-2 (186syn+) derived from a human clinical isolate.
Unchallenged/uninfected mice were also administered Depo Provera to control for any estrus cycle effects on immunity.

2.4.3 Intravascular labeling

Mice were administered an intravenous (i.v) retro-orbital injection (ROI) of APC-CD45.2 (3ug in 200uL per mouse) three minutes prior to CO2 euthanasia to label and differentiate circulating lymphocytes from tissue-resident cells.

2.4.4 Mouse Tissue Processing

Murine vaginal tracts (VT), including the cervix, were harvested into RP10 media and minced with scissors prior to a 30 min incubation in DM10 (10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, 5 mM sodium pyruvate) containing collagenase D (2mg/mL; Sigma Cat.11088858001) and DNAse I (15ug/mL; Sigma Cat.10104159001) at 37 °C with gentle shaking. Tissue samples were then mashed through a 100um strainer and washed with 20mL of RP10 media. VT samples were spun down, and the entire cell pellet was resuspended with fluorescence-activated cell sorting (FACS) buffer into a single-cell suspension stained and used for flow cytometric analysis. Vaginal-draining lymph nodes (dLN), iliac and inguinal, were harvested into RP10, mashed through a 100um strainer and resuspended in FACS buffer to prepare a single-cell suspension. Spleens were harvested into RP10, mashed through a 100um strainer, and incubated in 5 mL ACK lysis buffer for 5min. The ACK-treated cells were suspended in RP10 medium before proceeding with flow staining. All samples were spun down at 300g for 5 min to pellet. All samples were put through 35um nylon strainer shortly before flow cytometric analysis.

2.4.5 Flow Cytometry

Cells were incubated in fixable Live/Dead amine-reactive blue viability dye (Thermo Fisher Cat. L23105) for 25 min. (FLOW PANELS INCLUDED IN A TABLE). Cytosolic and intranuclear proteins were detected after permeabilization with the FoxP3 Transcription Factor

fixation/permeabilization buffer set (eBioscience Cat. 00-5523-00). Samples were acquired on the FACSymphony instrument (BD Biosciences) using BD FACSDiva software. AccuCheck Counting Beads were used according to manufacturer instructions to calculate absolute cells in samples (Invitrogen; Cat. PCB100). UltraComp™ ebeads (ThermoFisher; Cat. 01-2222-42) and ArC™ Amine Reactive compensation beads were used for compensation. Analysis was performed using FlowJo software version 10.10.0.

2.4.6 IL-15 trans-presentation extracellular staining

After staining VT and dLN samples with viability stain (LIVE/DEAD™ Fixable Blue Dead Cell Stain; Cat. L23105) and washing with 1X PBS, samples were stained with L-15 Polyclonal Antibody, Biotin (PeproTech®; Cat. 500-P173BT-25UG) alone at 1:100 in FACS buffer for 30 min at 4C. Samples were washed twice with 200uL of FACS buffer before staining with an extracellular APC panel also containing the secondary antibody, PerCP/Cyanine5.5 Streptavidin (Biolegend; Cat.405214).

2.4.7 In vitro peptide stimulation and intracellular cytokine staining

VT and dLN single cell suspensions from experimental and control mice were incubated at 37°C for 4 hours with 10uM HSV-2 gB498-505 peptide (GeneMed Synthesis Inc. Lot# 104865) and 7uM HSV-2 gD290-302 (GeneMed Synthesis Inc. Lot# 106044) diluted in RP10 media with GolgiStop (BD Biosciences Cat. 555029). Unstimulated and PMA/Ionomycin conditions were included as negative and positive controls. After peptide stimulation, cells were washed, pelleted, and stained for flow cytometric analysis of intracellular cytokines using the FoxP3 Transcription Factor fixation/permeabilization buffer set (eBioscience Cat. 00-5523-00) to stain for intracellular IFNγ and TNFα expression.

2.4.8 OT-I Adoptive transfers

CD8+ T cells were isolated from naive Thy1.1 CD45.2 TCR-transgenic OT-I mouse splenocytes using a murine CD8+ T cell isolation kit (Stemcell Tech, Cat. 19853). Isolated cells were washed with PBS and transferred into naive recipient mice via ROI; each mouse received 5e5 – 1e6 OT-I cells intravenously. 24 hours later, mice were intravaginally infected with 105 PFU of HSV-2 TK- OVA. 7 days post- HSV-2 TK- OVA infection, mice were bled via the submandibular (facial) vein using 5mm Goldenrod lancets. Bleeds were stained using an MHC-I OVA tetramer (conjugated to SA-PE) and congenic markers (Thy1.1, CD45.2) and analyzed using flow cytometry to identify the expanded adoptively transferred population.

2.4.9 In vivo FTY720, Treg depletion, and monoclonal antibody depletion

Treg depletion: On days –1 and 0 relative to WT HSV-2 challenge, mice were administered 30ug/kg and 10ug/kg respectively of diphtheria toxin (DT) via intraperitoneal injection to ablate Treg systemically in FoxP3 DTR mice.

FTY720 treatment: Mice previously infected with either 105 PFUHSV-2 TK- or TK- OVA virus 30 days prior, received 1mg/kg FTY720 (Sigma Cat. SML0700) dissolved in 2% cyclodextrin (ApexBio; Cat.B6413; in sterile PBS) via intraperitoneal (i.p) injection on days –1, 0, 1, and 2 relative to WT HSV-2 challenge. Control mice received vehicle only injections (2% cyclodextrin in PBS).

IL-2 blockade: On day –1 relative to WT HSV-2 challenge, mice were administered 150ug of i.v. anti-IL-2 (Bio X Cell, clone JES6-1A12) or 150ug of i.v. isotype control (Bio X Cell InVivoMAb rat IgG2a isotype control, anti-trinitrophenol). Anti-IL-2 treated mice received 80ug of anti-IL-2 Mab in 10uL intravaginally on day –1, and 40ug intravaginally on days 1 and 2. Control mice received isotype control clones via identical route, concentration, and treatment schedule as experimental mice.

2.4.10 Histology

VTs (including cervix) were carefully harvested into PBS. The tissue was then laid flat in a cassette with a nytex square and submerged in 10% NBF (Sigma; Cat. HT501320) for 3-5 days. Formalin fixed, paraffin embedded samples were then sectioned and progesterone&E stained. Sections were sent to the Fred Hutch Histopathology core to be blindly scored by a pathologist (Amanda Koehne) as previously described[252]. Scores were given to reflect changes in the mucosal epithelium, inflammation within the lamina propria, inflammation within the muscularis, and cellular debris within the vaginal lumen. Inflammatory changes were scored in the mucosal epithelium, lamina propria, and muscularis mucosa, and lumen layers of the VT and then reported as a sum composite. Scoring reflected severity and extent of lesions whereby 0 = no significant change, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe.

2.4.11 HSV-2 viral load PCR

The mouse vaginal canal was carefully swabbed before washing by gently pipetting 50uL of titration buffer in and out. The 50uL of vaginal wash was then added to a screw-top 2mL tube containing 950uL of titration buffer and frozen at -80°C. Vaginal washes were thawed once prior to quantifying HSV-2 viral titers by RT-PCR at UW Virology. DNA was extracted from 200 ul specimen using QIAamp 96 DNA blood kit and eluted into 100 ul of AE buffer (Qiagen). Real-time Taqman PCR detects HSV gB gene was applied to quantify HSV in the samples[386]. Each 30 ul PCR reaction contains 10 ul of purified DNA, 833 nM primers, 100 nM probe, 15 ul of 2x QuantiTect Multiplex PCR master mix, 0.03 units of UNG. To monitor PCR inhibition, EXO internal control was spiked into all the reactions. The thermocycling conditions are as follows: 50°C for 2 minutes, 95°C for 15 minutes, followed by 45 cycles of 94°C for 1 minute and 60°C for 1 minute. The limit of detection is 3 viral copies/reaction.

2.4.12 Bulk RNA sequencing

Bulk RNA-sequencing was performed on 100-200 CD4+ FoxP3+ Treg from the dLNs and VTs of previously infected and naïve mice. Cells were not pooled between mice, 16 samples were sequenced in total (D28 post-TK- n=4, naïve depo-treated mice n=4). Treg were sorted into directly into SMART-Seq v4 Ultra Low Input lysis buffer (Takara Bio USA) using the BD Symphony S6 and Flash frozen; upon thawing samples underwent reverse transcription followed by PCR amplification to generate full length amplified cDNA. cDNA was then submitted for bulk RNA-seq performed by the Genomics Core at Benaroya Research Institute. Sequencing libraries were constructed using the NexteraXT DNA sample preparation kit with unique dual indexes (Illumina) to generate Illumina- compatible barcoded libraries. Libraries were pooled and quantified using a Qubit® Fluorometer (Life Technologies). Sequencing of pooled libraries was carried out on a NextSeq 2000 sequencer (Illumina) with paired-end 59-base reads (Illumina) with a target depth of 5 million reads per sample. Base calls were processed to FASTQs on BaseSpace (Illumina), and a base call quality-trimming step was applied to remove low- confidence base calls from the ends of reads. The FASTQs were aligned to the GRCm38 mouse reference genome, using STAR v.2.4.2a and gene counts were generated using htseq-count. QC and metrics analysis was performed using the Picard family of tools (v1.134). We received a report including QC, gene counts, and differential gene expression.

Analysis: Raw gene counts were analyzed for top differentially expressed genes by the Benaroya Research Institute sequencing core. For comparison of differentially expressed genes, genes with a False Discovery Rate (FDR) of less than 0.1 and an absolute expression fold-change of greater than 1.0 were considered differentially expressed. Volcano plots were visualized using ggplot2 (https://ggplot2.tidyverse.org) and heatmaps were generated in base R (R version 4.5.1).

2.4.13 Statistical Analyses

All statistical analyses were performed using Prism software (GraphPad Software). Statistical significance was determined using unpaired t-tests, one and two-way ANOVA with ad hoc Tukey's or Dunnet's multiple comparisons tests, or multiple unpaired t tests with Welch correction and Holm-Šídák multiple hypothesis testing. For all flow cytometry, virology, and histology studies, each measurement was taken from a distinct sample as biological replicates.

2.5 Acknowledgements

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Chapter 3 : Menstrual Phase-Dependent T Cell Phenotypic Changes in the Female Genital Tract and Circulation - Implications for Vulnerability to Sexually Transmitted Infections

3.1 Introduction

The menstrual cycle, which varies in length between individuals (21-35 days) with an average of 28 days, consists of four main phases: the menstrual phase (menstruation), the follicular (proliferative) phase characterized by rising estrogen (estrogen) and very low progesterone (progesterone) levels prior to ovulation, mid-cycle during which estrogen drops precipitously and ovulation occurs, and the luteal (secretory) phase marked by elevated progesterone produced by the corpus luteum following ovulation. In addition to their role in regulating physiological changes throughout the menstrual cycle, estrogen and progesterone can also alter immune responses[4]. The steep increase of endogenous progesterone levels to over 40 times higher during the luteal phase shortly after ovulation to allow for successful implantation of an embryo[3,387–389]. Accordingly, progesterone is thought to be a likely mediator of luteal phase immunosuppression, leading to the hypothesis first proposed by Wira and Fahey that this reduced immunity may also serve as a "window of vulnerability," increasing susceptibility to STIs[3,390].

CD4+ T cell lymphocytes in the FGT have been a major focus of investigation in the context of hormonal changes on immunity because of their role as target cells for HIV infection. CD4+ T cells treated with estrogen *in vitro* had lower susceptibility to HIV infection in a dose-dependent manner[391]. Similarly, studies in humans and non-human primates (NHP) have found that higher progesterone levels correlate with the recruitment of CD4+ T cells expressing HIV-susceptibility markers (ie. CCR5, CD38, HLA-DR, CXCR3, TNFa) into the lower FGT during luteal phase[392–394]. In the upper FGT, estrogen has been found to promote Th1 responses, whereas progesterone skews CD4+ T cells

towards Th2[395]. The lower FGT has poor Th1 immune responses to pathogens in comparison to the upper FGT due to higher expression of TGFβ and IL-10[396]. Interestingly, both progesterone and estrogen can skew CD4+ T cells away from a Th17 phenotype and towards a CD4+ FoxP3+ regulatory T cell (Treg) or Th2 expression profile respectively[395,397]. Systemically in human peripheral blood mononuclear cell (PBMC) samples, IL-10+ Tregs have been observed at a higher frequency during the luteal phase[344].

CD8+ T cells are important mediators of mucosal immunity against intracellular pathogens by clearing out infected cells via cytotoxic function[318,332,346]. The vaginal mucosa is a primary inductive site for CD8+ T cell responses upon pathogen exposure due to the presence of antigen presenting cells (APCs) that can prime and activate CD8+ T cells *in situ*[398]. CD8+ T cells make up a substantial proportion of intraepithelial lymphocytes in the lower FGT, though how hormonal fluctuation impacts their activity and function *in vivo* is not well characterized in the literature[399,400]. One study showed that uterine CD8+ T cells lose their cytolytic function during the luteal phase (progesterone high), though this difference was not observed in CD8+ T cells within the vaginal tissue. They found that cytolytic function in CD8+ T cells begins to decrease near ovulation when estrogen peaks, and that samples from post-menopausal (low estrogen and progesterone) women had the highest CD8+ cytotoxic activity[400]. However, another study by the same research group later demonstrated that cytotoxic function in human CD8+ T cells was directly suppressed in culture with addition of estrogen and indirectly suppressed by progesterone via secretion of TGFβ by epithelial cells[401]. Other groups have conversely reported activating effects of estrogen on CD8 T cells using murine and NHP models[402–404]. Though the existing literature is sparse, there is some evidence to support the expression of estrogen and progesterone binding receptors in human CD8 T cells[405,406]. However, there are contradictory

reports on the direct downstream effects of female sex hormones on CD8 T cell function and phenotypes.

Further characterization local FGT and systemic T cell responses throughout the menstrual cycle is paramount to better understanding mechanisms underlying STI susceptibility and correlates of protection for improved design of vaccines and therapeutics. Here, we sought to characterize the impact of menstrual phase on local cervicovaginal and systemic T cell responses in Kenyan women. Using high-parameter, high-throughput flow cytometry and a large panel we compared follicular vs luteal phase phenotypes in T cell populations isolated from vaginal tract (VT) and ectocervix (CX) mucosal tissue biopsies and PBMC samples. Given the abundance of tissue resident T cells in the FGT[126,407], cervicovaginal tract (CVT) biopsies may better capture the tissue immune environment than other minimally invasive sampling methods that only sample the mucosal epithelial surface or secretions such as cervicovaginal lavage, vaginal/cervical swabs, or cytobrushes[408]. Additionally, we evaluated 71 immunomodulatory molecules in serum and cervicovaginal secretions in follicular vs luteal phase. Overall, using a cross-sectional study design, we did not find differences in the frequencies of T cell subsets (CD4+, CD8+, Tregs) or increases in HIV susceptibility markers on CD4+ T cells (CCR5, HLA-DR, CD38) between follicular vs luteal phase in any of the tissue sample types. Interestingly, all phenotypic differences were observed in CD8+ T cells. Specifically, CCR7+ CD45RA- central memory CD8 T cells were significantly higher during the luteal phase in both the CX and PBMCs, whereas CCR7- CD45RA+ terminally differentiated effector memory T cells CD8 T cells were more prevalent in the CX during the follicular phase. In the VT, the frequency of CD69+ CD103- CD8 T cells was significantly higher during follicular phase, suggesting an increase in activated tissue-resident memory (Trm) CD8 T cells within the lower FGT. Additionally, CD8 T cells in the CX during the luteal phase were more activated, exhibiting higher frequencies of HLA-DR, CD38, and CD39. We also found altered expression of soluble factors in follicular phase, with

significantly higher concentrations of CCL15 and IL-2 in cervicovaginal secretions and higher CXCL12 in serum samples. These findings suggest that menstrual phase modulates CD8 T cell memory subsets and regulation of activation, providing new insights into how hormonal fluctuations may influence FGT immunity to infection.

3.2 Results

3.2.1 Study population and sample collection.

Samples for this analysis were provided by a subset of participants from the Kinga Study (Clinicaltrials.gov ID# NCT03701802). The Kinga Study enrolled a total of 406 heterosexual Kenyan couples from October 2018 through December 2019 to study how STIs and other exposures affect local FGT and systemic immune responses. Among these couples, 110 were HIV serodifferent: one partner living with HIV (PLWH) with their heterosexual partner being HIV-exposed; the remaining 298 couples involved partners who were both HIV seronegative at enrollment. HIV-serodifferent couples were excluded if the PLWH had initiated antiretroviral therapy (ART) that resulted in undetectable HIV viral load, or the HIV-exposed partner had initiated tenofovir-based pre-exposure prophylaxis (PrEP) prior to enrollment. Upon enrollment, ART was provided to PLWH and PrEP to HIV-exposed partners. This cross-sectional analysis includes samples from non-pregnant participants that reported not using hormonal contraception or experiencing amenorrhea (>35 days since last menstrual period). Five sample types were requested (in addition to diagnostic clinical samples) from each Kinga Study participant for immune characterization studies. These include a 3-mm vaginal tract (VT) tissue biopsy and a 3-mm cervical (CX) tissue biopsy, all of which were cryopreserved for subsequent flow cytometry analysis. Fractionated PBMC samples were also collected and cryopreserved for flow cytometry analysis performed in batches. CVT fluid was collected by menstrual cup (Softcup®) and analyzed along with serum samples, for the presence and concentration of soluble

immune factors. To limit the effect of confounding variables on CVT immune phenotyping analyses, we adjusted flow cytometry analysis of the CX and VT tissue samples for the following variables (selected *a priori)*: BV status diagnosed by vaginal Gram-stain and classified using the method of Nugent and Hillier (0-3 negative, 4-6 intermediate, 7-10 positive, or unknown for a small number of samples)[409], HSV-2 serology status, HIV exposure (defined as participants with heterosexual partner living with HIV and semen exposure defined by the number of unprotected sex acts in the last thirty days (continuous). For soluble immune factor analysis from CVT fluid, BV status, HSV-2 serostatus, HIV exposure, hormonal contraceptive use, and semen exposure were adjusted. Systemic immune analyses of PBMC flow cytometry analysis and serum soluble immune factor analysis were unadjusted. This was an exploratory analysis in which we used nominal p-values adjusted for confounders but not post-hoc multiple comparisons tests.

Samples collected from non-pregnant women not using hormonal contraception were classified as follicular phase specimens if collected 5-12 days after self-reported last menstrual period (LMP), or as luteal phase specimens if collected 19-24 days after LMP. To determine these phases, days after LMP were calculated as time from LMP to date of sample collection. Samples from participants living with HIV were excluded due to the known impact of HIV infection on tissue and systemic T cell immunity and phenotypes. Seventy-eight specimens fell into a classified menstrual phase either at enrollment (n= 44) or 6-month visit (n= 34) and were included for flow cytometric analysis. Fifty-eight samples were collected during the follicular phase, and twenty samples were collected during the luteal phase. Overall, the study population was younger (46% under 30 years of age) and sexually active (median of 8 days of unprotected vaginal intercourse in the past month), with 38% seropositivity for HSV-2, 14% bacterial vaginosis positive by a Nugent score of 7-10, and 18% with a sexual partner living with HIV (HIV exposed) (Table 3-1). Seventy-five ectocervical (CX) biopsies (follicular n= 55, luteal n= 20), seventy-four vaginal (VT) biopsies (follicular n= 54, luteal n= 20), and seventy-one PBMC samples

(follicular n= 52, luteal n= 19) were included for flow cytometric analysis. Additionally, sixty-eight participants provided soft cup samples (follicular n= 51, luteal n= 17) (Table 3-2), and seventy-two participants provided serum samples (follicular = 52, luteal = 20) (Table 3-3) for soluble immune factor analysis via Luminex. Sample collection and clinical testing procedures for the Kinga Study, including HIV-1/2 and HSV-2 serology diagnostic methods, were performed in the same manner as previously described[410,411].

Table 3-1 : Flow cytometry analysis study population

Characteristic	**Follicular**, N = 52[1]	**Luteal**, N = 20[1]	**p-value**2
Age			0.3
<=24	12 (23%)	3 (15%)	
25-29	12 (23%)	6 (30%)	
30-34	7 (13%)	6 (30%)	
35-39	11 (21%)	4 (20%)	
40-49	10 (19%)	1 (5.0%)	
>=50	0 (0%)	0 (0%)	
HIV Exposure			0.5
HIV exposed - Female Partner, Discordant Couple	9 (17%)	5 (25%)	
HIV unexposed - Female Partner, Concordant Couple	43 (83%)	15 (75%)	
HSV2 status			0.6
Indeterminate	5 (9.6%)	0 (0%)	
Negative, nonreactive	27 (52%)	11 (55%)	
Not done	1 (1.9%)	0 (0%)	
Positive, reactive	19 (37%)	9 (45%)	
BV Nugent score			0.6
0-3	33 (63%)	16 (80%)	
4-6	6 (12%)	2 (10%)	
7-10	9 (17%)	1 (5.0%)	
Not done	4 (7.7%)	1 (5.0%)	
Number of unprotected vaginal intercourse in 30 days	6.5 (1.0, 12.0)	6.0 (1.0, 12.0)	0.7

[1]n (%); Median (IQR)
[2]Fisher's exact test; Wilcoxon rank sum test

Table 3-2: Softcup soluble immune factor analysis study population

Characteristic	Follicular, N = 51[1]	Luteal, N = 17[1]	p-value[2]
Age			0.3
<=24	12 (24%)	2 (12%)	
25-29	11 (22%)	5 (29%)	
30-34	7 (14%)	6 (35%)	
35-39	12 (24%)	3 (18%)	
40-49	9 (18%)	1 (5.9%)	
>=50	0 (0%)	0 (0%)	
HIV Exposure			0.3
HIV exposed - Female Partner, Discordant Couple	8 (16%)	5 (29%)	
HIV unexposed - Female Partner, Concordant Couple	43 (84%)	12 (71%)	
HSV2 status			0.4
Indeterminate	5 (9.8%)	0 (0%)	
Negative, nonreactive	28 (55%)	8 (47%)	
Not done	1 (2.0%)	0 (0%)	
Positive, reactive	17 (33%)	9 (53%)	
BV Nugent score			0.8
0-3	33 (65%)	13 (76%)	
4-6	5 (9.8%)	2 (12%)	
7-10	8 (16%)	1 (5.9%)	
Not done	5 (9.8%)	1 (5.9%)	
Number of unprotected vaginal intercourse in 30 days	7.0 (1.0, 12.0)	4.0 (1.0, 10.0)	0.4

[1]n (%); Median (IQR)
[2]Fisher's exact test; Wilcoxon rank sum test

Table 3-3: Serum soluble immune factor analysis study population

Characteristic	Follicular, N = 52[1]	Luteal, N = 20[1]	p-value2
Age			0.3
<=24	12 (23%)	3 (15%)	
25-29	12 (23%)	6 (30%)	
30-34	7 (13%)	6 (30%)	
35-39	11 (21%)	4 (20%)	
40-49	10 (19%)	1 (5.0%)	
>=50	0 (0%)	0 (0%)	
HIV Exposure			0.5
HIV exposed - Female Partner, Discordant Couple	9 (17%)	5 (25%)	
HIV unexposed - Female Partner, Concordant Couple	43 (83%)	15 (75%)	
HSV2 status			0.6
Indeterminate	5 (9.6%)	0 (0%)	
Negative, nonreactive	27 (52%)	11 (55%)	
Not done	1 (1.9%)	0 (0%)	
Positive, reactive	19 (37%)	9 (45%)	
BV Nugent score			0.6
0-3	33 (63%)	16 (80%)	
4-6	6 (12%)	2 (10%)	
7-10	9 (17%)	1 (5.0%)	
Not done	4 (7.7%)	1 (5.0%)	
Number of unprotected vaginal intercourse in 30 days	6.5 (1.0, 12.0)	6.0 (1.0, 12.0)	0.7

[1]n (%); Median (IQR)
[2]Fisher's exact test; Wilcoxon rank sum test

3.2.2 Menstrual phase does not impact T cell subset frequencies across tissue sites.

We examined lymphocytes from cryopreserved CX and VT tissue biopsies, and PBMC samples in order to phenotypically characterize T cells within the mucosal tissues as well as in circulation via high-parameter flow cytometry as previously described[412]. As previously reported, the majority of CD45+ lymphocytes within the lower FGT are CD3e+ T cells, and we did not find that the concentration of CD3+ T cells differed based on menstrual phase (Fig 3-1A)[1,2]. We first assessed the

frequency of CD25- CD127-/+ CD4+ conventional T cells (Tconv) and CD8+ T cells as a fraction of CD3e+ T cells within each tissue compartment and found no significant differences between follicular versus luteal phase specimens (Fig 3-1B, C). Similarly, we did not find a significant difference in the frequency of CD25+ CD127lo FoxP3+ regulatory T cells (Treg) within any of the sample types (Fig 3-1D). Although this finding contrasts with a previous study in which authors found the frequency of Treg within PBMC samples were increased during the follicular phase[413], that study did not include the marker CD127 to subset human Treg accordingly[414].

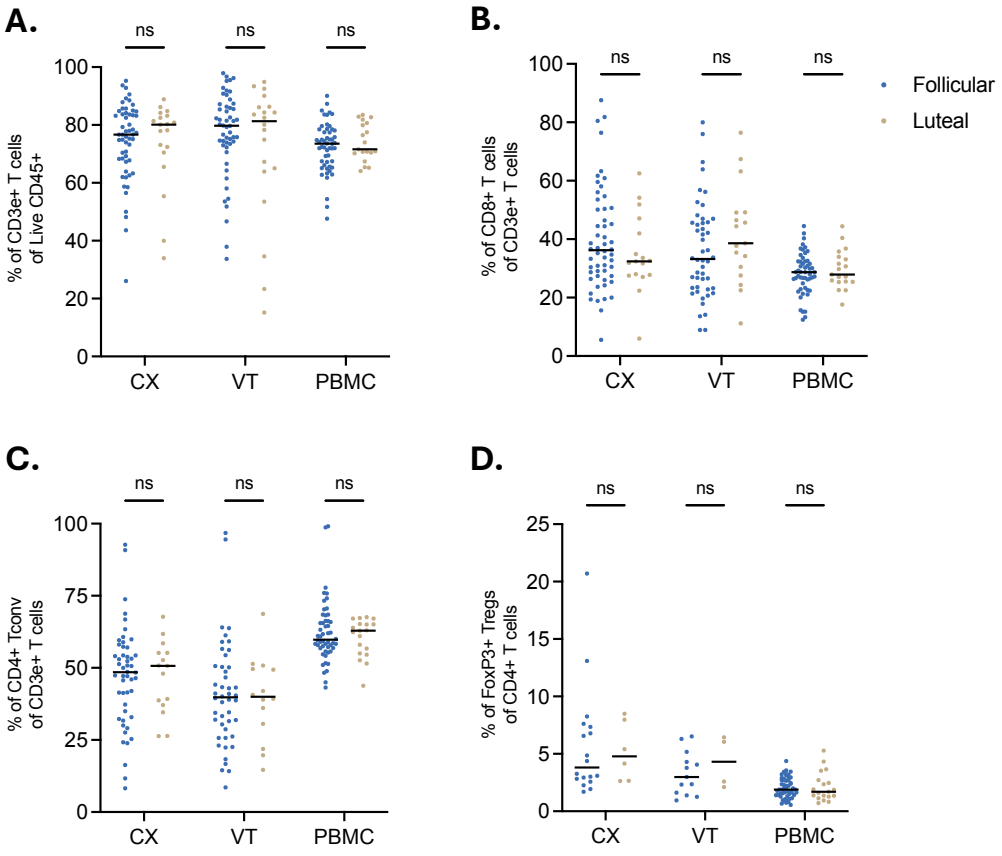

Figure 3-1: Menstrual phase does not impact T cell subset frequencies across tissue sites.
Flow cytometry was used to quantify the proportions of T cells within different tissue sites as indicated. **A**) Frequency of CD3e+ among total CD45+ cells (follicular CX n = 55, luteal CX n = 19; follicular VT n = 52, luteal VT n = 20; follicular PBMC n = 52, luteal PBMC n = 19). **B**) Frequency of CD8+ T cells among CD3e+ CD45+ cells (follicular CX n = 54, luteal CX n = 17; follicular VT n = 48, luteal VT n = 17; follicular PBMC n = 52, luteal PBMC n = 19). **C**) Frequency of CD4+ Tconv among CD3e+ CD45+ cells (follicular CX n = 50, luteal CX n = 15; follicular VT n = 46, luteal VT n = 14; follicular PBMC n = 52, luteal PBMC n = 19). **D**) Frequency of CD4+ CD25+ CD127- FoxP3+ Treg among CD3e+ CD45+ cells (follicular CX n = 18, luteal CX n = 6; follicular VT n = 13, luteal VT n = 4; follicular PBMC n = 52, luteal PBMC n = 19). Comparisons between medians were made using an adjusted rank regression model; ns indicates non-significant results, significant adjusted p-value for tissue biopsies, unadjusted p-value for PBMC greater than 0.05.

3.2.3 Menstrual phase does not impact CD4 Tconv memory subsets or phenotypes.

We next sought to assess menstrual phase associations with T cells' phenotypic expression of markers associated with activation/exhaustion state (CD38, CD39, CD101, HLA-DR, PD-1, TCF-1, CTLA-4) immunity type (Th1; T-bet and Th17; CD161+ CCR6+), chemokine migration (CCR6, CCR5, CXCR3), memory subsets (CCR7, CD45RA, CD69, CD103), and cytotoxic potential (Granzyme B) in CD8 T cells. Our flow cytometry dataset lacked sufficient Treg cell counts within CVT biopsies to assess expression of other surface markers by this tissue subset, and we observed no significant differences in phenotypic markers between follicular vs luteal phase Treg from PBMC (Fig B-S1).

Due to particular interest in CCR5+ HIV target cells in the field, we first assessed any differences in CCR5 expression between CD4 Tconv collected in follicular vs luteal phase. Previous reports by others lack a clear consensus as to whether menstrual hormones impact CCR5 expression by CD4 T cells in the FGT[393,394,415,416], nonetheless we did not observe a difference in the frequency of CCR5+ CD4 Tconv between follicular and luteal phases, though the majority of CVT Tconv were CCR5+ (Fig 3-2A-B). We similarly did not observe any differences in any other markers of activation, immunity type, or chemokine migration in CD4 Tconv between menstrual phases (Fig 3-2A). Our findings suggest that though CCR5 expression in CVT CD4 Tconv is higher than in PBMC, menstrual phase is not associated with detectable changes in CCR5 expression in CD4 Tconv, both in circulation and within lower FGT mucosal tissues.

Based on previous work by others showing that distinct alterations in the frequency of CD4 T cell memory subsets are associated with menstrual phase[392–394,416], we next sought to assess changes in CCR7 and CD45RA expression[417]. CCR7 expression directs T cells towards T cell zones in secondary lymph nodes via CCL19/21 chemotaxis and is expressed by circulating naïve (TN; CCR7+ CD45RA+) and central memory T cells (TCM; CCR7+ CD45RA-). CD45 isoforms are used to distinguish

between naïve (CD45RA+ CD45RO-) and memory T cells (CD45RA- CD45RO+)[417–420]. However, terminally differentiated effector memory T cells re-expressing CD45RA (TEMRA) are an exception to this categorization. TCM are long-lived memory T cells that continuously circulate throughout the body to SLO, serving as homeostatic surveillance with high proliferative capacity and ability to give rise to effector memory T cells (TEM; CCR7- CD45RA-) that migrate to peripheral tissues via the blood[417,421]. Conversely, TEMRA exhibit a reduced proliferative capacity but rapid effector functions, circulate peripherally via blood, are highly activated, and can be found in non-lymphoid tissues[417,421]. In contrast to previous studies that found a preponderance of CCR7+/hi CD4 Tconv using CVL samples[392–394], the majority of CD4 Tconv in CVT biopsies were CCR7- CD45RA- TEM with the highest frequency of CD4+ CCR7+ TN and TCM observed in PBMCs, and very low frequencies of CD4 TEMRA across all tissues (Fig 3-2C-D). As expected, most CD4 Tconv in CVT biopsies were CD69+ and consistent with a Trm phenotype (in comparison to only around 1% in PBMC samples), but we did not find any notable differences in CD69 or CD103 expression in CD4 Tconv between menstrual phases (Fig 3-2A). Though our statistical analysis found a significant difference in the frequency of CD103+ CD69- CD4 Tconv from PBMC samples, we doubt biological relevance due to the very low frequencies (median of 0.50% in follicular phase vs 0.37% in luteal phase).

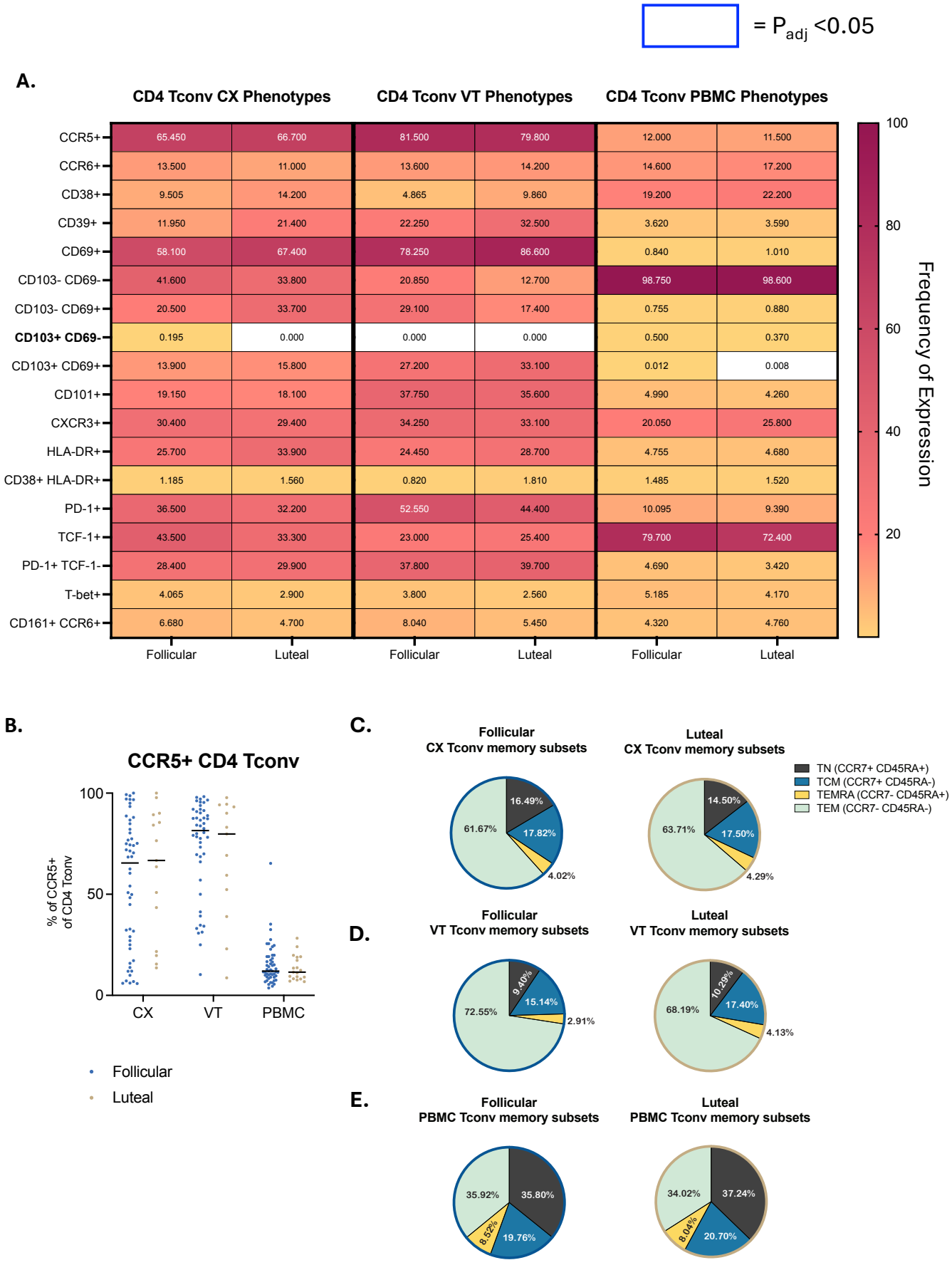
= P_{adj} <0.05
A.
CD4 Tconv CX Phenotypes
CD4 Tconv VT Phenotypes
CD4 Tconv PBMC Phenotypes
CCR5+ 65.450 66.700 81.500 79.800 12.000 11.500
CCR6+ 13.500 11.000 13.600 14.200 14.600 17.200
CD38+ 9.505 14.200 4.865 9.860 19.200 22.200
CD39+ 11.950 21.400 22.250 32.500 3.620 3.590
CD69+ 58.100 67.400 78.250 86.600 0.840 1.010
CD103- CD69- 41.600 33.800 20.850 12.700 98.750 98.600
CD103- CD69+ 20.500 33.700 29.100 17.400 0.755 0.880
CD103+ CD69- 0.195 0.000 0.000 0.000 0.500 0.370
CD103+ CD69+ 13.900 15.800 27.200 33.100 0.012 0.008
CD101+ 19.150 18.100 37.750 35.600 4.990 4.260
CXCR3+ 30.400 29.400 34.250 33.100 20.050 25.800
HLA-DR+ 25.700 33.900 24.450 28.700 4.755 4.680
CD38+ HLA-DR+ 1.185 1.560 0.820 1.810 1.485 1.520
PD-1+ 36.500 32.200 52.550 44.400 10.095 9.390
TCF-1+ 43.500 33.300 23.000 25.400 79.700 72.400
PD-1+ TCF-1- 28.400 29.900 37.800 39.700 4.690 3.420
T-bet+ 4.065 2.900 3.800 2.560 5.185 4.170
CD161+ CCR6+ 6.680 4.700 8.040 5.450 4.320 4.760
Follicular Luteal Follicular Luteal Follicular Luteal
100
80
60
40
20
Frequency of Expression
B.
CCR5+ CD4 Tconv
% of CCR5+ of CD4 Tconv
100
50
0
CX
VT
PBMC
Follicular
Luteal
C.
Follicular CX Tconv memory subsets
16.49%
17.82%
4.02%
61.67%
Luteal CX Tconv memory subsets
14.50%
17.50%
4.29%
63.71%
TN (CCR7+ CD45RA+)
TCM (CCR7+ CD45RA-)
TEMRA (CCR7- CD45RA+)
TEM (CCR7- CD45RA-)
D.
Follicular VT Tconv memory subsets
9.40%
15.14%
2.91%
72.55%
Luteal VT Tconv memory subsets
10.29%
17.40%
4.13%
68.19%
E.
Follicular PBMC Tconv memory subsets
35.80%
19.76%
8.52%
35.92%
Luteal PBMC Tconv memory subsets
37.24%
20.70%
8.04%
34.02%

Figure 3-2: Menstrual phase does not impact CD4 Tconv memory subsets or phenotypes.
A) Heatmaps show the median frequency of CD4 Tconv marker expression in CX, VT, and PBMC samples (left to right) collected in follicular vs luteal phase. Adjusted rank regression model for CVT samples, unadjusted for PBMC. Significant ($p < 0.05$) comparisons between follicular and luteal phase denoted by blue box. **B**) Frequency of CCR5 expression in CD4 Tconv in CX, VT, and PBMC between menstrual phases. Median shown, comparisons between phases within tissues using adjusted rank regression model, unadjustes rank regression for PBMC. **C-E**) Pie charts depicting average frequencies of CD4 Tconv memory subsets categorized by expression of CCR7 and CD45RA in CX (**C**), VT (**D**), and PBMC (**E**) between follicular (left, blue outline) and luteal phase (right, tan outline).

3.2.4 Menstrual phase alters CD8 T cell memory and tissue resident subsets.

As we observed in CD4 Tconv, most FGT CD8 T cells also displayed a Trm phenotype (CD69+ CD103+/-), with highest Trm frequencies in VT biopsies. Additionally, we found a significant increase in the frequency of CD69+ CD103- VT CD8 T cells in follicular phase (Fig 3-3A). These data are in agreement with reported human Trm frequencies of 50-90% in the FGT[126]. We also observed a significantly higher frequency of CD8+ CCR7+ CD45RA- TCM in CX and PBMC samples collected during luteal phase and reciprocally, a significantly higher frequency of CD8+ CCR7- CD45+ TEMRA in follicular phase CX samples (Fig 3-3B-F). CD8 TEMRA are specialized for rapid activation and pathogen killing, expressing elevated levels of cytotoxic molecules, proinflammatory cytokines, and NK-like receptors[422,423]. Altogether our data suggests that there is increased recruitment or expansion of CD8 Trm and TEMRA in the FGT during the first half of the menstrual cycle (low progesterone), supporting the theory that follicular phase is characterized by increased immune activation whereas luteal phase is less inflammatory[4,387].

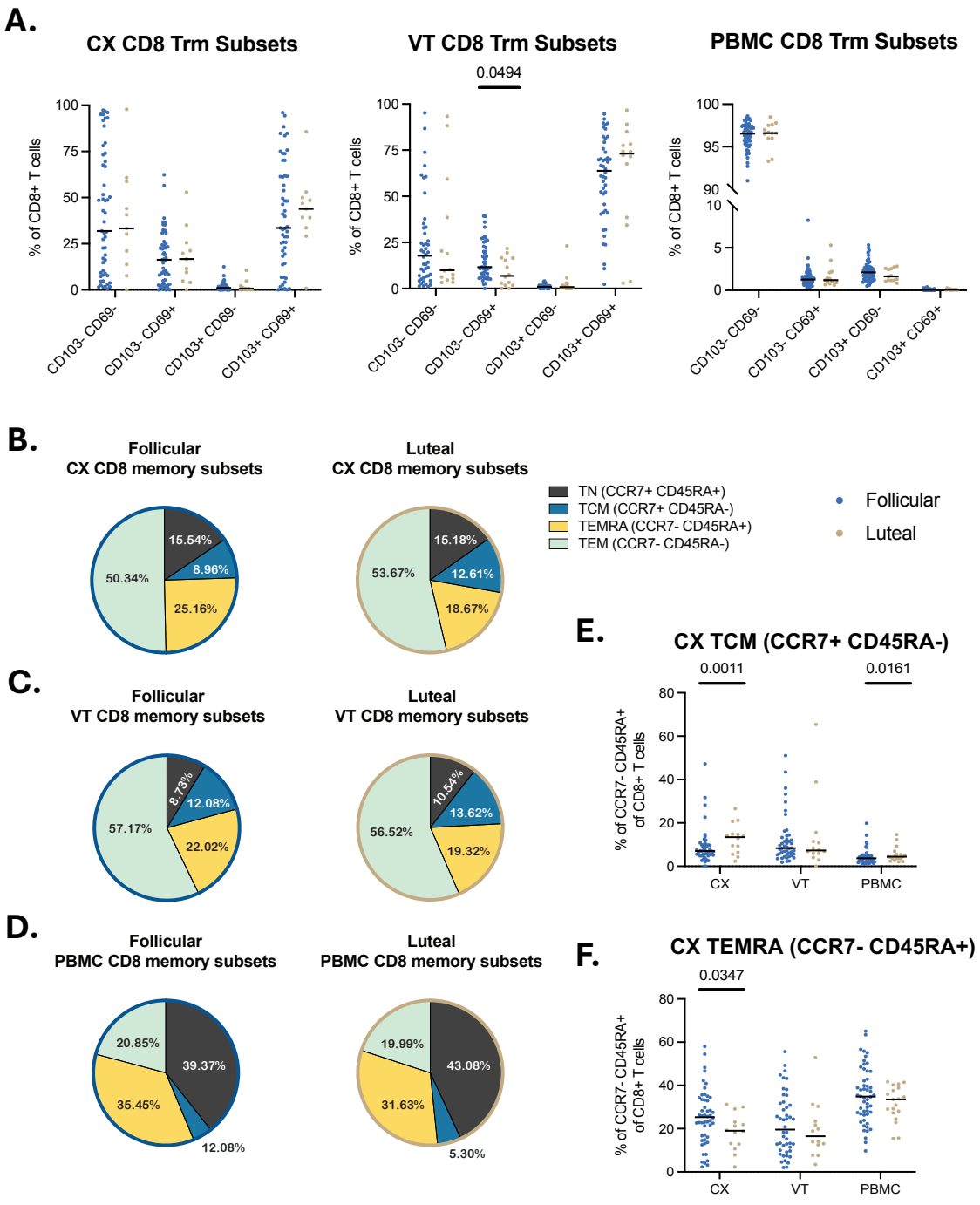

Figure 3-3: Menstrual phase alters CD8 T cell memory and tissue resident subsets.
A) Frequency of tissue memory subsets categorized by expression of CD69 and CD103 in CX, VT, and PBMC samples (left to right) between menstrual phases. **B-D**) Pie charts depicting average frequencies of CD8 T cell memory subsets categorized by expression of CCR7 and CD45RA in CX (**B**), VT (**C**), and PBMC (**D**) between follicular (left, blue outline) and luteal phase (right, tan outline). **E**) Frequency of CCR7+ CD45RA- CD8 TCM in CX tissue between menstrual phases. **F**) Frequency of CCR7- CD45RA+ CD8 TEMRA in CX tissue between menstrual phases. Median shown in **A, E, F**. Comparisons between menstrual phase medians were made using an adjusted rank regression model; ns indicates non-significant results, significant adjusted p-value for tissue biopsies, unadjusted p-value for PBMC greater than 0.05.

3.2.5 CD8 T cells express markers of activation state and metabolic regulation during luteal phase

Like CD4 Tconv, CVT CD8 T cells expressed higher frequency of CCR5 than PBMC, though frequencies were comparable between menstrual phases within each sample type (Fig 3-4A). CVT CD8 T cells also generally expressed higher frequencies of CD101, CD161, CXCR3, HLA-DR, and PD-1 than PBMC (Fig 3-4A). Notably, CX CD8 T cells displayed significantly higher frequencies of CD39, CD38 and co-expression of CD38 and HLA-DR during luteal phase (Fig 3-4 A-B). CD38 and CD39 are ectoenzymes expressed by many different cell types (immune and non-hematopoietic) that generate immunosuppressive adenosine (ADO) via cooperation with CD73[424,425]. Historically, CD38 has been widely used as a marker of T cell activation and CD4 Tconv marker of increased HIV susceptibility in the HIV/AIDS field[426–428]. Similar to CD38, CD39 expression is also associated with increased viral load in human HIV infection, as well as prolonged antigen-TCR stimulation in chronic infections and terminal exhaustion in cancers[429,430]. However, there is a lack of consensus concerning the exact contexts and roles of CD38 and CD39 surface expression in modulating human CD4 Tconv and CD8 T cell development, localization, and function[431,432].

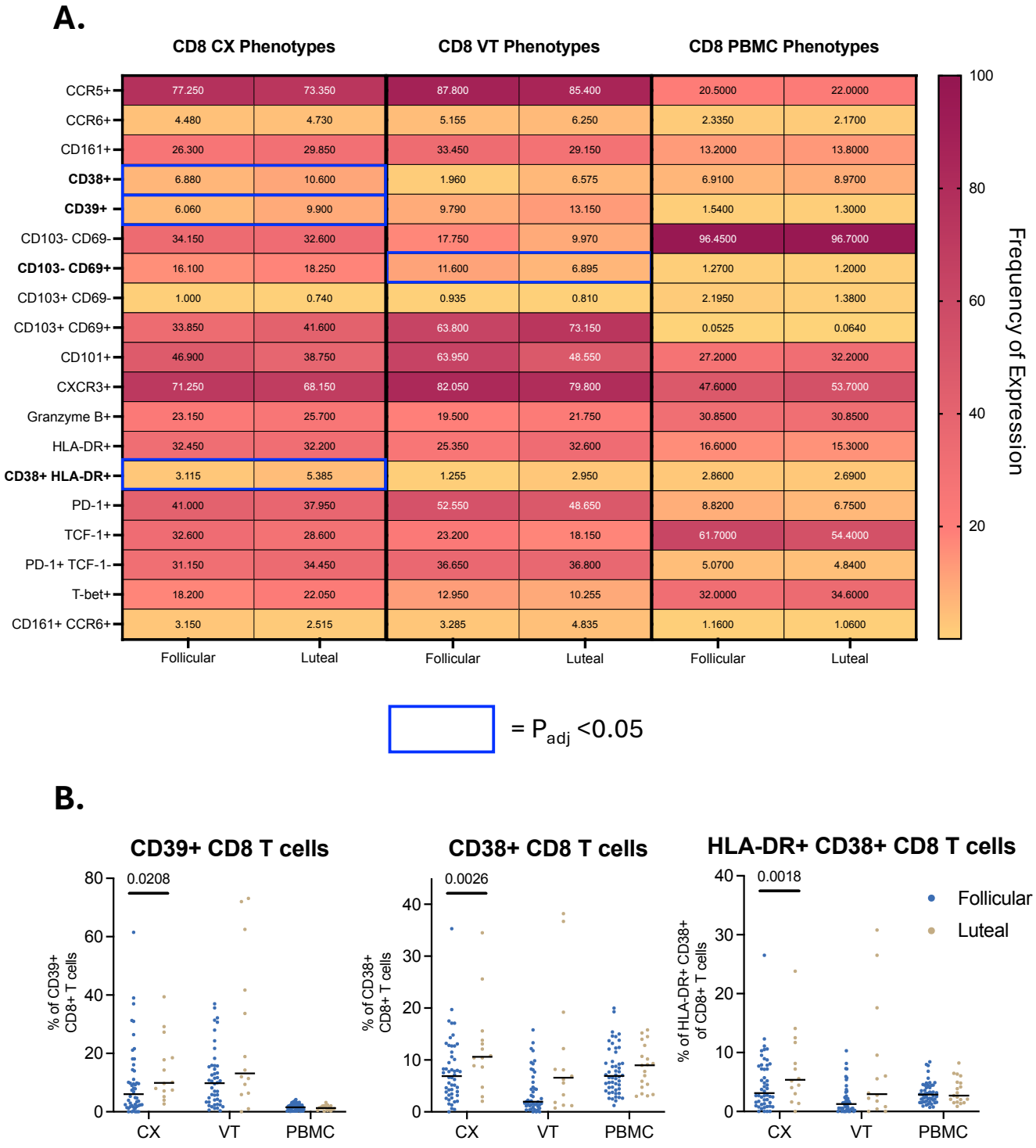

Figure 3-4: CD8 T cells express markers of activation state and metabolic regulation during luteal phase.
A) Heatmaps show the median frequency of CD8 T cell marker expression in CX, VT, and PBMC samples (left to right) collected in follicular vs luteal phase. Adjusted rank regression model for CVT samples, unadjusted for PBMC. Significant ($p < 0.05$) comparisons between follicular and luteal phase denoted by blue box. **B**) Frequency of CD39 expression, CD38expression, and HLA-DR CD38 co-expression of CD8 T cells within CX, VT, and PBMC samples (left to right) between menstrual phases. Medians shown. Comparisons between menstrual phase medians were made using an adjusted rank regression model; ns indicates non-significant results, significant adjusted p-value for tissue biopsies, unadjusted p-value for PBMC greater than 0.05.

3.2.6 Increased detection of T cell-related cytokines and tissue homing chemokines in vaginal secretions during follicular phase.

To assess changes in soluble immune factors associated with menstrual phase we analyzed 71 cytokines and chemokines in serum and softcup (vaginal secretion) samples using a multiplex Luminex platform assay. 60/71 soluble factors were detectable within range and met criteria for continuous analysis (linear regression) in softcup samples, and 53/71 in serum; out-of-range values were imputed by assigning the highest observed value (if out-of-range high). The rest in which fewer than 80% of samples were quantifiable were analyzed dichotomously (detected vs undetected) (Fig B-S2). We used logistic regression to estimate the odds ratio for the effect of menstrual phase on detection of dichotomously categorized cytokines/chemokines, and IL-3 was excluded from both softcup and serum analysis as it was not detected in any luteal phase samples and less that 80% of follicular phase samples. In softcup samples, IL-2 and MIP-1d/CCL15 log10-transformed concentrations were significantly higher in follicular phase samples, while BCA-1/CXCL13 was elevated in luteal phase samples (Fig 3-5A, C). Only SDF-1αβ/CXCL12 was significantly different between phases in serum samples, with higher serum concentrations in follicular phase (Fig 3-5B). Similarly, the unadjusted softcup dichotomous outcome analysis revealed that CXCL12 was detected significantly more frequently in follicular phase, though this was not significant in the adjusted dichotomous outcome (Fig B-S2).

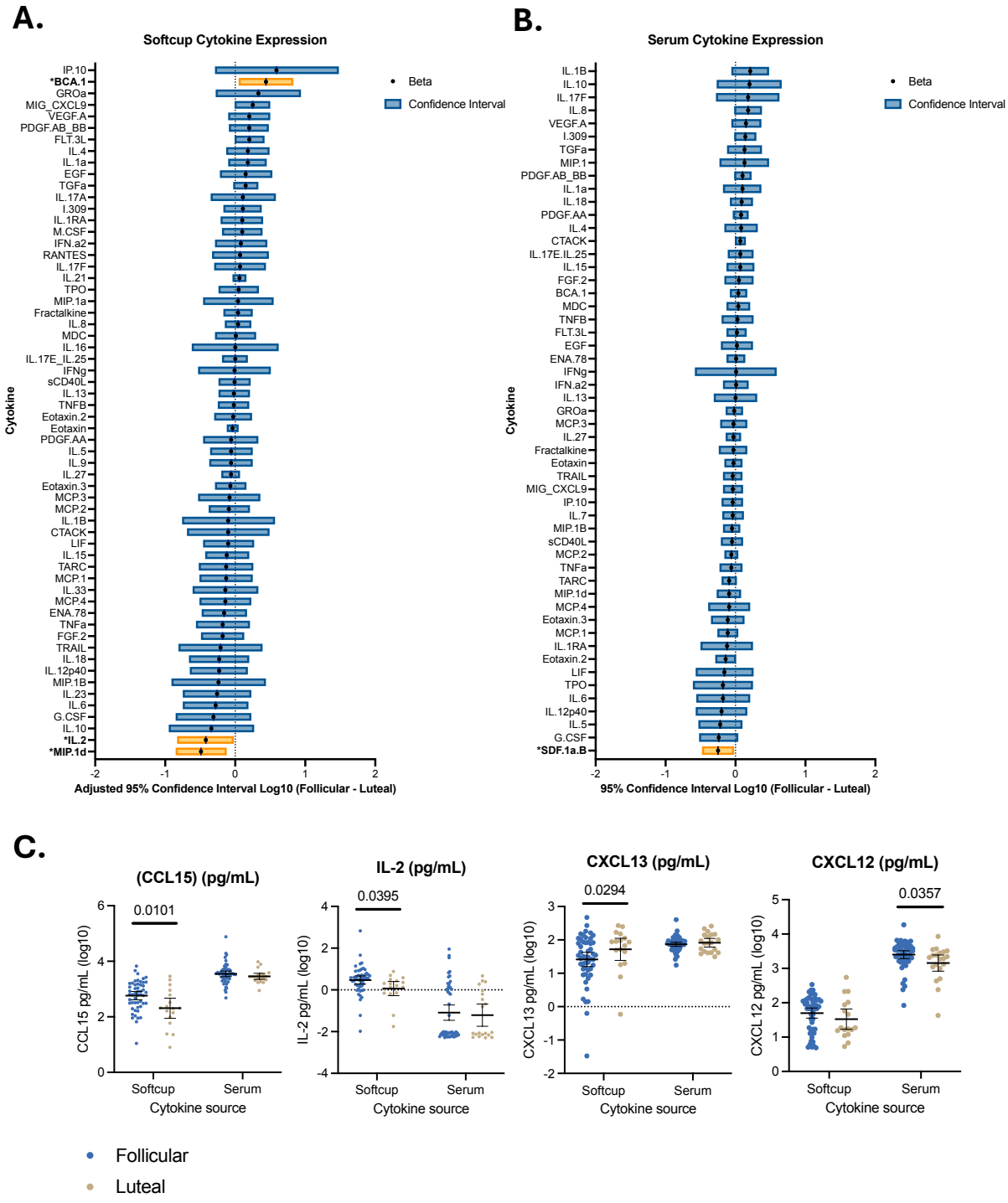

Figure 3-5: Increased detection of T cell-related cytokines and tissue homing chemokines in vaginal secretions during follicular phase.

Cytokines and chemokines were quantified using the multiplex Luminex assay from A) CVT fluid (follicular n = 51, luteal n = 17) and B) serum samples (follicular n = 52, luteal n = 20). Adjusted mean difference (follicular - luteal) for CVT and serum soluble immune factors that met quantification criteria (80% or more detectable samples). Adjusted 95% confidence interval shown for CVT comparisons, unadjusted for serum comparisons. Significant results (adj. p-value < 0.05) colored in orange and non-significant comparisons in blue. CVT fluid comparisons adjusted for *a priori* for HSV-2 status, HIV exposure, and semen exposure.

3.3 Discussion

Despite the growing interest in the role of endogenous sex hormones and how their fluctuation throughout the menstrual cycle and life (ie. from puberty to reproductive years, to menopause) impact mucosal FGT and systemic immunity, we still lack clear understanding of the underlying mechanisms contributing to STI susceptibility. Recent data show that adolescent and adult women are at greater risk of acquiring and suffering from complications caused by *Chlamydia trachomatis*, *Neisseria gonorrhea*, *Trichomonas vaginalis*, *Treponema pallidum,* human papillomavirus, HSV-2, and HIV than men worldwide[433]. Although the prevailing theory suggests that elevated progesterone levels in women (such as during pregnancy, the luteal phase, or progestin-based contraceptive use) create an immunosuppressive "window of vulnerability"[3,434], empirical evidence remains limited, the clinical implications are unclear, and consensus across studies is lacking[435]. Furthermore, perhaps due to the historically devastating impact of AIDS and an ongoing global HIV pandemic, the field has largely focused on the impact of sex-hormones on FGT CD4 T conv susceptibility to infection, despite the essential role of CD8 T cells in controlling HIV viral replication after primary infection[436] and the indispensable contributions of both types of T cells in combatting STI. Our findings here provide new insight into the effect of menstrual phase (follicular vs luteal) on CD8 T cell phenotypes and memory subsets, both in circulation and the lower FGT.

Although several studies have explored how the menstrual cycle and hormonal fluctuations affect susceptibility to STI, the mechanistic impact of menstrual phase on systemic vs local immunity in the FGT remains poorly understood. In women infected with *Neisseria gonorrhoeae*, positive detection in cultures were more frequently obtained during the early follicular phase (low progesterone) of the menstrual cycle after several previous consecutive negative cultures during the luteal phase[437]. Another study demonstrated that both hormonally driven host-factors (ie. pH, immune response) and direct effects of progesterone itself impacted *N. gonorrheae* virulence[441]. The opposite has been observed in

the case of *Chlamydia trachomatis,* with higher detection in samples from women in during luteal phase (high progesterone)[439]. In mice, progesterone treatment and/or deletion of the estrogen receptor promoted clearance of *Candida albicans* by enhancing neutrophil migration into the vaginal lumen[440]. In the case of herpes simplex virus type 2 (HSV-2), early follicular phase was associated with higher HSV-2 shedding. Micks et al. reasoned that luteal phase immunosuppression may lead to increased shedding and detection of HSV-2 in the following weeks[441]. Supporting this hypothesis, treating mice with estrogen after HSV-2 vaccination resulted in better protection against intravaginal HSV-2 challenge[391]. Interestingly, human immunodeficiency virus (HIV) genital shedding patterns are also influenced by hormonal fluctuation with highest frequency and magnitude occurring immediately after menses[442]. Studies using non-human primate (NHP) models have shown that susceptibility to vaginal simian-human immunodeficiency virus (SHIV) infection is significantly elevated during the late luteal phase, with detection in the early follicular phase[392,443,444]. In humans, use of progesterone-based hormonal contraceptive methods increases the risk of HIV-1 acquisition[445,446]. However, there is a lack of consensus in human studies limited to *ex vivo* HIV-1 infection of cervicovaginal tissue explants[447–449]. Collectively, these findings illustrate the complexity of untangling the impact of hormonal changes on the vaginal environment, local immune response, and susceptibility to STIs.

We utilized a large T cell phenotyping flow cytometry panel to cross-sectionally analyze CD3+ T cells in CVT tissue biopsies and PBMC samples from Kenyan women collected in either luteal or follicular phase of the menstrual cycle. Our data suggest that menstrual phase does not significantly impact T cell subset (CD8, CD4 Tconv, Treg) frequencies in the cervix, vagina, or PBMC. Contrary to other reports[446–448], we did not observe a significant difference in frequency of CCR5 expression in CD4 Tconv between phases either. There may be a few of reasons for these discrepancies. 1) Studies do not consistently use similar FGT sampling methods or only assess circulating PBMC T cells (CVL, cytobrush, tissue biopsy, tissue sections, cervical swabbing); these sampling methods can vary

significantly in the tissue compartment they sample, as well as the viability and types of cells recovered. One study demonstrated that CVL and single cervical cytobrush sampling recovered lower numbers of viable lymphocytes than biopsies[408]. However, tissue biopsies and cytobrushes were biased towards recovery of macrophages and neutrophilic granulocytes, and T cells respectively[387,408,412]. 2) Other studies include more cycle time points (early/mid/late follicular and luteal phase) allowing for deeper analysis of changes as hormone levels naturally fluctuate from early to late *within* each phase[451]. 3) Additional biological (ie. genetic variation in immunity, autoimmune conditions, reproductive disorders, other unknown health conditions) and environmental variables (ie. exposure to allergens, pollution or contaminants in food and air) not accounted for those we adjusted for may impact the abundance and phenotype of T cells[452]. 4) Methods for reporting and categorizing participant samples by menstrual phase vary between studies. Studies that include systemic hormonal levels in addition to reported LMP may have more accurate assessments of how menstrual phase hormones impact immunity.

Earlier work by Swaims-Kohlmeier et al. found that CD4 T cells from human CVL samples were primarily CCR7hi, and that roughly half of them expressed CD69[393]. Additionally, they observed an increase in CCR7hi CD4 T cells during luteal phase that correlated with increased serum progesterone levels[393]. More recent studies by the same group utilized CVL samples from NHP and found that memory T cell subsets in the FGT displayed cycle-associated fluctuations with changes in migratory memory (CCR7hi CD62L-, CD69+/-, CD103-) and tissue resident memory T cells (CCR7lo CD62L-, CD69+, CD103+/-)[394]; they found that migratory memory CD4 T cells were increased in the luteal phase, whereas Trm were reciprocally decreased in the FGT[392,394]. Similarly, another study found an increased frequency of CD69+ CD4 T cells in human cervical cytobrush samples during follicular phase[416]. We found significant shifts in CD8 T cell memory and tissue resident subsets in the cervix between menstrual phases. In follicular phase (progesterone low) samples we found an increase in

frequency of CX CCR7- CD45RA+ CD8+ TEMRA, whereas in luteal phase we observed a reciprocal increase in CX and PBMC CCR7+ CD45RA- TCM. TEMRA development and expansion is associated with immune senescence and chronic antigen stimulation during viral infections, such as cytomegalovirus (CMV), hepatitis C virus, and HIV[238,412,453]. Though in the past TEMRA were viewed as a senescent and potentially dysfunctional subset, they have been credited with superior antigen-specific antiviral activity in recent years[453–455]. To date, only one paper[456] has reported on CD8 TEMRA frequencies within the upper FGT in which the proportion of CD8 TEMRA were lowest in the endometrium and comparable between the endocervix and blood; there are very few reports on frequencies of CD8 TEMRA in the lower FGT. Previous work by our research group and others found a higher frequency of CD8 TEMRA in CVT samples[407,457,458] and that CD8 T cells found in healthy human CVT from multiple donors lacked TCF-1 expression, suggesting a tissue-driven short-lived memory phenotype[457].

Furthermore, in addition to our data presented here we have also previously reported that Trm-like CD69+ CD8 T cells in the CVT display a distinct tissue cytokine secretion profile[407]. In humans, CD103 expression in tissue-resident CD8 T cells differs across tissue types and may indicate differences in migration capabilities and antigen or tissue compartment-specific features[116,126,459]. Though not significant, there was a slightly higher frequency of CD69 CD103 double positive CD8 T cells within luteal phase CVT biopsies (CX adj.p = 0.1834). CD103 expression in CD8 T cells is induced by the pleiotropic cytokine TGFβ[126,347,374]; it is a potent immunosuppressor often produced by Treg and can have differing downstream effects on CD8 T cells based on timing, location, and concentration[460]. Though TGFβ was not included in our Luminex panel, others have reported progesterone-driven increases in TGFβ during luteal phase[461,462], and thus it is plausible that this could modulate CD103 expression in CD8 T cells within the FGT throughout the menstrual cycle[401,463].

Taken together with previous work by others, our data support the theory that unlike the long-lived CD8 Trm in the skin and the gut, CVT Trm may be a more migratory and dynamic pool[456], and thus could be in part regulated by in fluctuations menstrual hormones.

In luteal phase, we found significantly higher frequencies of CD39+, CD38+, and HLA-DR+CD38+ CX CD8 T cells. In the CD39/CD73 adenosinergic pathway, CD39 first degrades extracellular adenosine triphosphate (ATP) into adenosine diphosphate (ADP), and subsequently into adenosine monophosphate (AMP); CD73 then further degrades AMP into ADO[425]. In the case of CD38, it degrades extracellular nicotinamide adenine dinucleotide (NAD+) into adenosine diphosphoribose (ADPR) and AMP in cooperation with CD203a, and CD73 further degrades AMP into ADO[425]. In addition to producing ADO, these pathways impair metabolism by decreasing extracellular ATP which can act as a pro-inflammatory damage associated molecular pattern (DAMP)[431,464]. Increased expression of CD38 and CD39 by highly immunosuppressive Treg has been well characterized[203,465,466]. One early longitudinal study using peripheral lymphocytes from a cohort of persons living with HIV (PLWH) found that disease progression correlated with an increased susceptibility to apoptosis in HLA-DR+ CD38+ memory (CD45RA-) CD4 and CD8 T cells[427], supporting the theory that chronic immune stimulation and activation is associated with worse disease outcomes (such as lower CD4 counts) during HIV infection. Similarly, others reported that co-expression of CD38 and CD8 T cell exhaustion markers (PD-1, Lag-3, and Tim-3) in blood samples from PLWH correlated with disease progression[467]. Another human study found that primary HIV infection (before antiretroviral treatment) in humans not only resulted in expansion of CD38+ HIV-specific CD8 T cells in circulation, but also elevated frequencies of bystander activated CD38+ CD8 T cells specific for other viral infections (Epstein-barr virus, EBV; CMV, influenza, FLU)[468]. Others have reciprocally reported that HIV-controllers with lower viral loads and higher CD4 T cell counts display higher frequencies of cytotoxic HLA-DR+ CD38- CD8 T cells and delayed progression to

AIDS[469,470]. Furthermore, they found that HIV viral load also correlated with CD38 expression on bystander activated non-HIV specific CD8 T cells, though the authors never identified their exact role[468]. Interestingly, a more recent study using an *in vivo* murine model and *in vitro* co-culture of human PBMC found that IL-15, a cytokine known for its ability to activate bystander T cells, induced high CD38 expression in CD8 T cells. However, instead of inducing cytotoxic function or increased activation, IL-15 promoted the expansion of 'regulatory' CD38high CD8 T cells with the ability to suppress effector CD4 Tconv proliferation *in vitro*[471]. Importantly, others have also observed increased progesterone-driven IL-15 expression in the FGT during luteal phase and pregnancy[472–474]. In murine chronic LCMV infection, CD38 has a dual cell-intrinsic effect in exhausted CD8 T cells where it decreases their proliferation and function through metabolic regulation and promotes their survival *in vivo*[475]. Our findings of increased frequency of CD38+ CD8 T cells and co-expression with HLA-DR in cervical samples collected during the more putatively immunosuppressive luteal phase align with the findings from these latter studies associating CD38 expression with cell-intrinsic regulation and immunosuppression[471,475]. Though the role of CD38 expression in modulating CD8 T cell function and exact mechanism by which it may increase susceptibility to viral infection remains unclear.

Similar to CD38, CD39 expression is also associated with increased viral load in human HIV infection, chronic antigen-TCR stimulation, and terminal exhaustion[429,430]. CD39 is more widely accepted to be marker of metabolic dysregulation, senescence, and exhaustion in CD8 T cells, commonly observed and studied in the context of cancer[476,477]. A study by the Newel lab utilizing mass cytometry and a large phenotyping flow panel to assess CD39 expression in tumor infiltrating CD8 T cells (TIL) demonstrated that tumor-specific cells were commonly CD39+ and displayed a highly exhausted phenotype and transcriptional profile; whereas bystander activated pathogen-specific TIL lacked expression of CD39, providing a novel biomarker for distinguishing tumor-specific from bystander TIL[432]. Additionally, they found that an abundance of CD39- TIL was associated with poorer

responses to checkpoint blockade cancer treatment. More recent work has shown CD39 is often co-expressed with markers of tissue resident effectors (CD69 and CD103) and also induced by TGFβ in the setting of ongoing TCR signaling[478–480]. In the context of vaccination, increased CD39 expression in T cells contributed to age-dependent impaired T cell memory after immunization in older individuals[477]. However, we were unable to find previous reports of CD39 expression in lower FGT CD8 T cells or in response to fluctuations in menstrual hormones. Due to their associations with chronic antigen-stimulation, worse disease outcomes in the context of chronic viral infections, and induction by cytokines commonly upregulated in the FGT during luteal phase (progesterone high), we propose that CD38 and CD39 expression in cervical CD8 T cells suggests increased immunosuppression or impaired immune metabolism during the second half of the menstrual cycle.

Lastly, in our multiplex assay of soluble immune factors from serum and softcup samples, we found significantly increased concentrations of cytokines and chemokines (IL-2, CCL15, CXCL12) associated with T cell recruitment, immune activation, and antimicrobial effects in samples collected during follicular phase. Notably, high concentrations or prolonged IL-2 signaling in CD8 T cells within tissues promotes terminal-effector differentiation[481], aligning with our finding of increased TEMRA frequencies in follicular phase CX biopsies. IL-2 is well characterized as a vital cytokine involved in T cell survival, expansion, activation, and effector function[64,482]. A recent metanalysis paper reciprocally found that reported IL-2 concentrations in vaginal secretions were typically lower in luteal phase[483]. CCL15 is a chemokine recognized by CCR1 and CCR3 involved in tissue recruitment of T cells NK cells, and myeloid cells[484,485], and often associated with chronic allergic and autoimmune inflammation in mucosal tissues[486–488]. There are very few studies that focus on the impact of CCL15 on T cell immunity outside of autoimmunity in other mucosal tissue like the lung and gut[486–488]. However in the context of infection, one study found that gut epithelial cell-derived CCL15 displayed antimicrobial

properties in addition to chemotaxis and could directly kill *Escherichia coli in vitro*[487]. CXCL12 is commonly associated with hematopoiesis and homeostatic migration of lymphocytes to specific tissue sites (such as the bone marrow)[489]. Work using human and murine FGT tissues showed that estradiol (which is higher in follicular phase) induces CXCL12 expression in human endometrial stromal cells[490] and increased stem cell migration and hematopoietic stem cell renewal[491], suggesting a role in regulating reproduction and immunity. CXCL12 also involved in angiogenesis and tissue repair[429,430]. In agreement with our findings, previous work by others have found that circulating CXCL12 serum concentrations are highest in the follicular phase in healthy menstruating women[494]. Lastly, CXCL13 is a chemokine that is highly expressed by endometrial epithelial cells and participates in trophoblast migration and successful blastocyst implantation[495,496]. Notably, our observation of elevated CXCL13 log concentrations in vaginal secretions from luteal phase is supported by similar findings in a study using endometrial biopsies from healthy pre-menopausal humans and macaques[497]. Taken together, our cytokine and chemokine data support the idea that FGT immunity is more activated during follicular phase when progesterone is low and estrogen is comparatively higher.

In summary, our study utilized a large comprehensive human panel for high-throughput high-parameter flow cytometry T cell to cross-sectionally assess how menstrual phase impacts T cell marker expression within cervical and vaginal tissue biopsies, and blood; and the Luminex multiplex protein detection assay soluble immune factor concentrations in CVT fluid and serum samples. Our findings show that few but biologically relevant CD8 T cell memory subsets, phenotypes, and immune signaling molecules are modulated by menstrual phase. Most menstrual phase-driven differences in marker expression were observed in CD8 T cells within cervicovaginal tissue biopsies, demonstrating that fluctuations in estrogen and progesterone are associated with local shifts in tissue residency and memory subsets. Though we did not observe significant differences in CD4 Tconv marker expression, we did find increased expression of markers in cervical CD8 T cells from luteal phase that others have

shown to be associated with local metabolic regulation, immunosuppression, and chronic TCR-stimulation. Altogether, our findings contribute to the still limited body of literature and reports on the impact of menstrual phase hormones on human CD8 T cell phenotypic marker expression and phase-associated cytokine/chemokine profiles.

Limitations of the study

Our study has several limitations that should be noted. First, the CX and VT tissue biopsies we collected were generally small (about 3mm on average) but inconsistent in size and freeze-thaw cell viability was variable, failing to capture rarer T cell subsets (ie. Treg) and phenotypes with co-expression of multiple markers. The variability in tissue size and cell viability also prevented us from reporting absolute numbers of cells in tissue, instead relying on frequencies. Secondly, we focused on characterizing T cells but did not evaluate other immune cell types known to participate in mucosal immunity and reproduction such as B cells, NK cells, and antigen-presenting cells. Third, there may be biological variables impacting our dataset that we do not account for aside from those adjusted for in our statistical analyses. Fourth, we used self-reported LMP to categorize samples, thus the use of serum hormone levels may have resulted in different menstrual phase assignments and allowed for more quantitative correlative analyses. Finally, this study was an exploratory analysis in which we used nominal p-values adjusted for confounders but not post-hoc multiple comparisons tests.

3.4 Methods

3.4.1 Sex as a biological variable

As this study focuses on cervicovaginal immunity associated with the follicular and luteal menstrual phases, all samples collected are exclusively from participants who were assigned female at birth.

3.4.2 High parameter flow cytometry analysis

Kinga study site clinicians collected VT and CX biopsies using Tischler biopsies at the VT wall or cervical os. Biopsies were placed in cryovial with 4% fetal bovine serum at 4^{o}C, transported to the lab, then cryopreserved overnight at -80^{o}C in dimethyl sulfoxide (DMSO), and transferred to liquid nitrogen for long-term storage as described previously[298,411]. PBMCs were also collected, processed, and cryopreserved in DMSO. All processing for genital tissues and PBMC samples was done as previously described[298,411]. Samples were then shipped to the University of Washington for further analysis. Cryopreserved tissue biopsies and PBMC samples were quickly thawed, processed, and stained with fluorescently labeled antibodies. After isolation, cells were incubated with UV Blue Live/Dead reagent for 30 minutes at room temperature and then stained with the antibodies and protocol previously described [298,411]. Analysis was performed using FlowJo software using the gating strategy described by MacLean et al. 2025, with a 25-cell minimum required to proceed to any downstream gate. The proportion of each cell type was then calculated by dividing the count of each phenotype by the count of its parent gate.

3.4.3 Soluble immune factor analysis

Enrolled participants who were not actively menstruating inserted a menstrual cup into the vaginal canal to collect CVT fluid for analysis of cervicovaginal soluble immune factors. Serum samples were also collected to analyze circulating soluble immune factors. CVT fluid and serum samples were processed, cryopreserved, and shipped to the University of Washington and then to Eve Technologies (Calgary, Alberta, Canada). The detection and quantification of chemokines and cytokines were done using the Human Cytokine Array/Chemokine Array 71-403 Plex Panel (Eve Technologies, HD71). All collection and processing were done as previously described[411].

3.4.4 Study approval

All participants provided written informed consent using documents reviewed and approved by the University of Washington institutional review board, and the Scientific and Ethics Review Unit of the Kenya Medical Research Institute.

3.4.5 Statistics

For flow cytometry experiments, we used rank-based regression, a nonparametric method robust to outliers, to compare the percentage of specific T cell phenotypes. For soluble immune factor analysis (both serum and CVT fluid), we first determined the proportion of total samples that were at least 80% quantifiable for each cytokine/chemokine measured. For those cytokines/chemokines with at least 80% quantifiable detection, we imputed out-of-range values by assigning the highest observed value (if out-of-range high). We then estimated differences in mean log10-transformed cytokine/chemokine concentrations comparing those who reported vs did not report vaginal washing using linear regression. If fewer than 80% of samples were quantifiable, then we categorized each value as either detected or undetected. Next, we used logistic regression to estimate the odds ratio for the effect of vaginal washing on the detection of dichotomously coded cytokines/chemokines.

To limit the effect of potentially confounding variables on local cervicovaginal tract (CVT) immunology analyses, we adjusted flow cytometry analysis of the CX and VT tissue samples for the following variables, selected *a priori*: BV status diagnosed by vaginal Gram-stain and classified using the method of Nugent and Hillier (0-3 negative, 4-6 intermediate, 7-10 positive, or unknown for a small number of samples)[409], HSV-2 serology, HIV exposure (defined as heterosexual partner living with HIV), and semen exposure (defined number of unprotected sex acts in the last thirty days; continuous). For soluble immune factor analysis from CVT fluid, BV status, HSV-2 serostatus, HIV

exposure, semen exposure were adjusted. Systemic immune analyses, including PBMC flow cytometry analysis and serum soluble immune factor analysis, were unadjusted.

As this was an exploratory analysis, we did not adjust our results for multiple comparisons. We considered all nominal p-values less than or equal to 0.05 as significant. Statistical analysis was done using R version 4.3.3.

3.5 Author contributions

ICT, FM, JBG, JLS, SCV, NP, and LW conducted the experiments. ICT, ATT, JLS, AS, and KKT analyzed data. MM provided reagents. JD and LKS contributed analysis methods. BHC, KN, NM, JRL, and JML designed the research study. ICT, JRL, and JML wrote the first draft of the manuscript.

3.6 Acknowledgements

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Chapter 4 : Conclusions and Future Directions

4.1 Conclusions

This dissertation aims to firstly, investigate the roles of and mechanisms by which tissue memory T cell responses are regulated within the FGT by Treg in the context of secondary intravaginal HSV-2 exposure; and secondly, assess menstrual phase-associated modulation of soluble immune factors and T cell phenotypic marker expression within human cervicovaginal tract tissue biopsies and in circulation (PBMC). Our findings using a mouse model of recurrent intravaginal HSV-2 exposure and Treg depletion demonstrate that Treg are critical in shaping a protective, yet measured recall tissue CD8 T cell response, composed of both HSV-2-specific memory T cells and non-specific BA-CTL, upon intravaginal challenge with wild-type HSV-2 via an IL-2-dependent mechanism. In our human data assessing the impact of menstrual phase on T cell phenotypes and associated soluble immune factor profiles in the FGT reveal for the first time that hormone fluctuations shift CD8 T cell memory and tissue resident subsets, as well as cytokine (IL-2) and chemokine (CCL15, CXCL13, CXCL14) concentrations within the cervicovaginal tract.

Our work elucidates how Treg influence extrinsic signals that restrain mucosal cytotoxic potential in tissue memory CD8 T cell recall responses to viral infection, without compromising protective antigen-driven immunity. Our study challenges the prevailing theory that Treg broadly restrain protective antigen-specific immunity during localized viral infection. Furthermore, we have uncovered a mechanism by which Treg restrain pathogenic BA-CTL responses locally at the site of infection, thereby minimizing unnecessary immune-mediate tissue damage. These findings may help inform future therapeutics and vaccine-design for mucosal infections, keeping in mind the role of Treg in regulating innate and adaptive tissue recall responses to invading pathogens and how to leverage their

activity to orchestrate focused pathogen-specific memory T cell immunity, while minimizing pathogenic off-target effects and collateral tissue damage. To our knowledge, this is the first study to show that high levels of IL-2, in the absence of Treg-mediated sinking of excess IL-2, directly increase IL-15 trans-presentation in APC and thereby promote CD8 T cell cytotoxicity and bystander activation *in vivo*, adding mechanistic insight into the pathways by which Treg coordinate an appropriate tissue recall response. Thus, we propose that Treg govern the environmental cues that induce the cytotoxic program of memory CD8 T cells, including bystander activation. Importantly, Tregs do not restrict the protective, antigen-driven and antigen-specific tissue memory CD8 T cell response, as demonstrated by the lack of difference in viral load between Treg depleted vs sufficient mice, though they are critical to reducing tissue injury upon pathogen re-exposure.

Our results on the impact of endogenous hormone fluctuations during the menstrual cycle on T cell phenotypes in the FGT and circulation support the hypothesis presented by Wira et al. that higher progesterone levels during the second half of the menstrual cycle (luteal phase) may promote a more immunosuppressive environment, thus providing a 'window of vulnerability' to pathogenic infections of the FGT. Though we did not observe differences in CD4 Tconv expression of HIV risk-associated markers between follicular and luteal phase, there were several phenotypic differences in cervicovaginal tract CD8 T cells. We found a significantly higher frequency of TEMRA within ectocervical tissue biopsies collected during follicular phase. Though TEMRA have been described as a senescent and potentially dysfunctional subset, they have also shown superior protective antigen-specific antiviral effector function in recent years[453–455]. We also observed a significantly higher frequency of CD69+ CD103- CD8 T cells in vaginal tissue biopsies from follicular phase, suggest either increased activation or expansion of Trm. Furthermore, we found increased concentrations of cytokines and chemokines involved in T cell activation and recruitment to tissues in follicular phase vaginal secretion samples, suggesting increased immune activation and signaling in the FGT.

Conversely, there were significantly higher frequencies of CD8 T cells in ectocervical tissue biopsies collected during luteal phase that expressed the activation-exhaustion markers CD39 and CD38. These markers are associated with recent or prolonged TCR-signaling and worse HIV disease outcomes[429,467,468], and cell-intrinsic metabolic regulation in exhausted cells to prevent activation-induced death [475,478]. Due to their associations with chronic antigen-stimulation, worse outcomes during chronic viral infections, and induction by cytokines commonly upregulated in the FGT during luteal phase, we propose that CD38 and CD39 expression in cervical CD8 T cells suggests increased suppression of activation state and autoregulation of metabolism during the second half of the menstrual cycle. Our findings contribute to the limited body of research on the impact of endogenous menstrual phase hormone fluctuations on human CD8 T cell phenotypic marker expression and phase-associated cytokine/chemokine profiles in the FGT.

4.2 Open Questions and Future Directions

There are still several unanswered questions to be addressed in both studies. In our study on the regulation of tissue recall T cell responses by Treg, we assessed bystander activation using an adoptive transfer model that leveraged OT-I cells to ensure that cells were not activated via TCR signals during HSV-2 challenge. Proper assessment of endogenous rather than adoptively transferred TCR transgenic CD8 T cells would be preferable. We did attempt to establish an endogenous OVA-specific memory CD8 T cell response in the mucosa by priming mice intravaginally with TK- HSV-2-OVA and then assessing the frequency of endogenous OVA-specific BA-CTL after depletion of Treg and WT HSV-2 challenge. However, we were unable to establish enough OVA-specific memory CD8 T cells in the VT to properly enumerate and assess their bystander activation phenotype after challenge (generally less than 25 endogenous OVA-specific CD8 T cells could be detected in the vagina). Instead, we utilized adoptive transfer to increase the number of detectable BA-CTL in vagina tissue. Perhaps future

experiments could include the use of topical intravaginal administration of chemokines (ie. CXCL9 and CXCL10), TLR agonists (CpG, Poly I:C), or OVA peptide (SIINFEKL) to increase and maintain the established endogenous bystander population in the vagina after primary TK- OVA HSV-2 infection. Additionally, although we found that increased IL-2 availability results in a direct dose-dependent increase in IL-15 trans-presentation by macrophages *in vitro*, and the expression of IL-2R by APC, we have not addressed the exact mechanism by which IL-2R signaling results in this response from APC. We attempted to stain for pSTAT5 as an indicator of IL-2 signaling, however many myeloid populations were very auto-fluorescent, and we suspect that methanol fixation may have impacted marker integrity before antibody staining making it difficult to assess clear differences in pSTAT5 expression in APCs between media and IL-2 stimulation conditions. Future studies *in vivo* studies could leverage conditional or inducible knock out mice in which Cre-LoxP mediated gene knockout of IL-2R in different APC subsets (macrophages, myeloid cells, dendritic cells) to assess whether IL-2 levels directly impact APC IL-15 trans-presentation through IL-2/IL-2R binding and signaling. Another follow up experiment to assess whether APC are binding IL-2 might involve fluorophore conjugation of recombinant IL-2 used in *in vitro* stimulation of APC before flow cytometric analysis. Lastly, we did not fully assess direct tissue healing function in Treg (ie. via amphiregulin), or the role of other Treg mechanisms of suppression such as the role of Treg-derived IL-10 and TGFβ. Additionally, we did not define Treg TCR specificity or assess whether vaginal Treg in our murine model were induced or natural thymic Treg.

In our menstrual phase study, we only assessed differences in expression of phenotypic markers in T cells but cannot draw empirical conclusions about their impact on T cell function. Follow up studies could include functional assays in which isolated cervicovaginal tract tissue T cells and PBMC collected in follicular vs luteal phase are cultured with TCR-dependent (CD3/CD28 beads) and

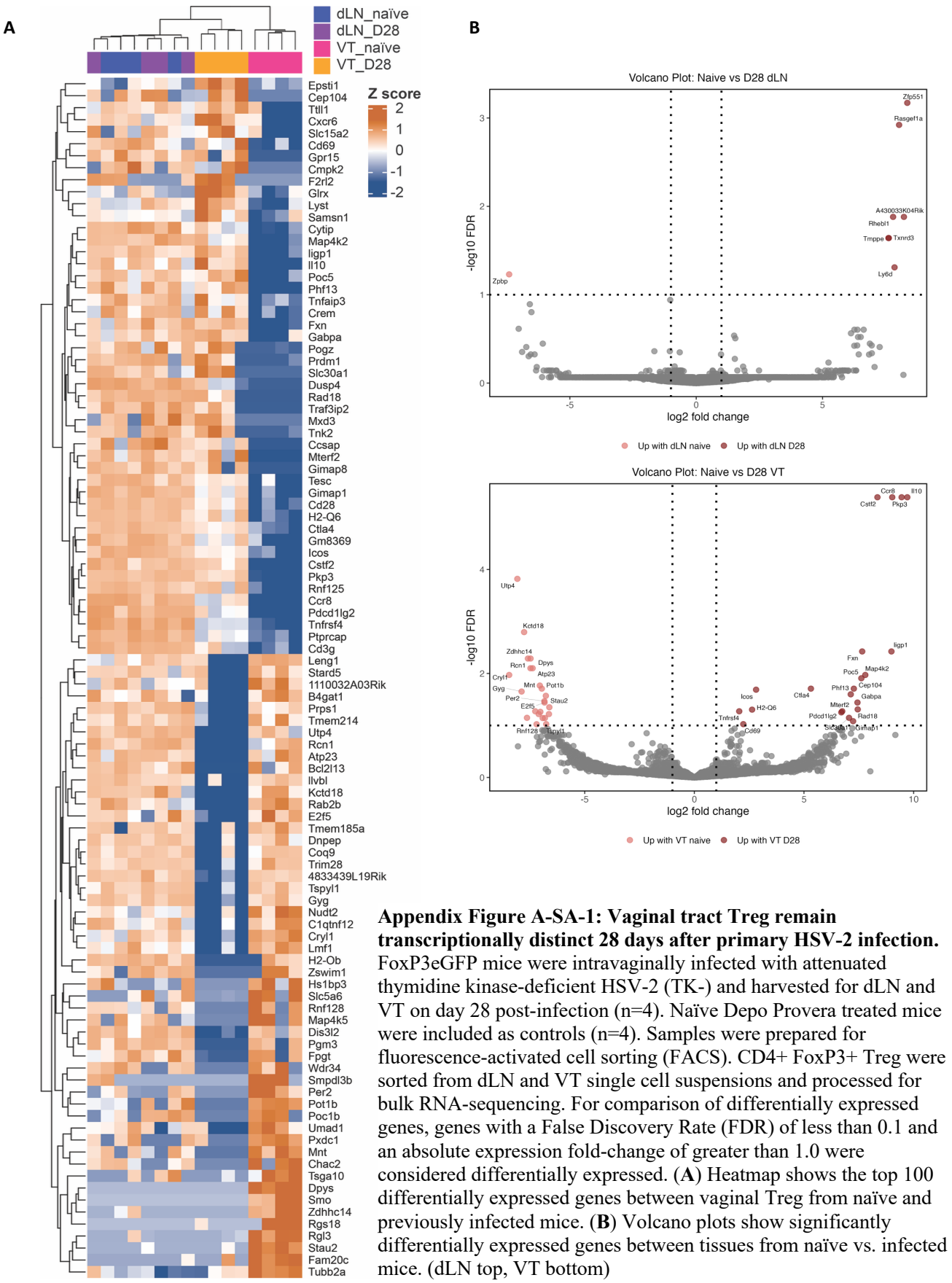
independent (PMA/ionomycin) stimulating reagents followed by intracellular cytokine staining and supernatant ELISA assays *ex vivo* to assess effector function and cytokine production. Furthermore, we did not explore the role of the vaginal microbiome in memory T cell FGT populations in this study, aside from adjusting for confounding effects from bacterial vaginosis. Future studies might also explore whether hormonal fluctuations during the menstrual cycle impact the vaginal microbiome and associated T cell responses. Another limitation of this study is the fact that participant samples were categorized into follicular or luteal phase based on self-reported last menstrual period without quantification of systemic estrogen and progesterone levels in serum. In future, serum hormone levels should be measured to confirm menstrual phase categorization of samples.

Appendices

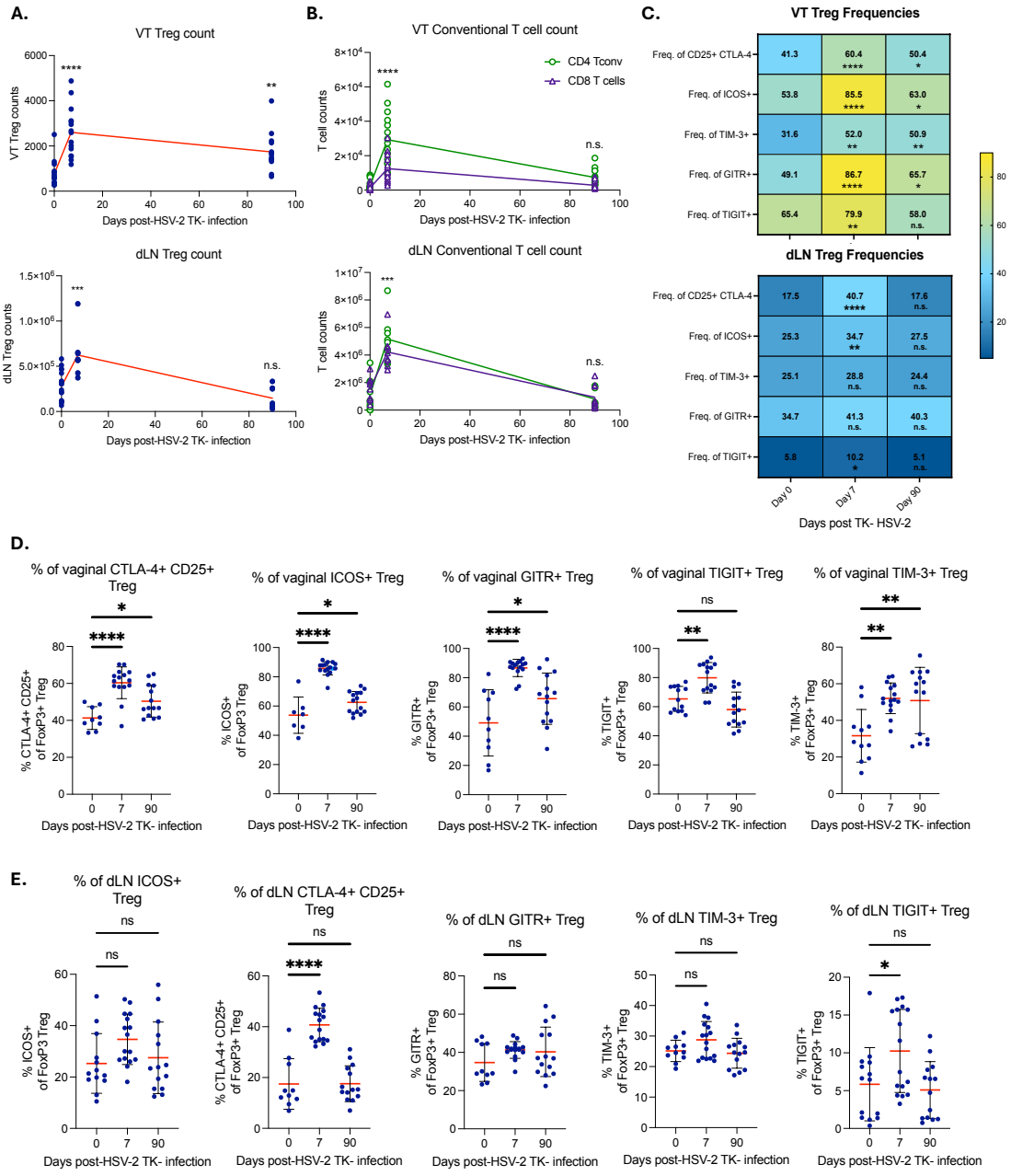
Appendix A: Chapter 2 Supplemental Figures

Table 0-1 : Flow Cytometry Panels and Antibody Details

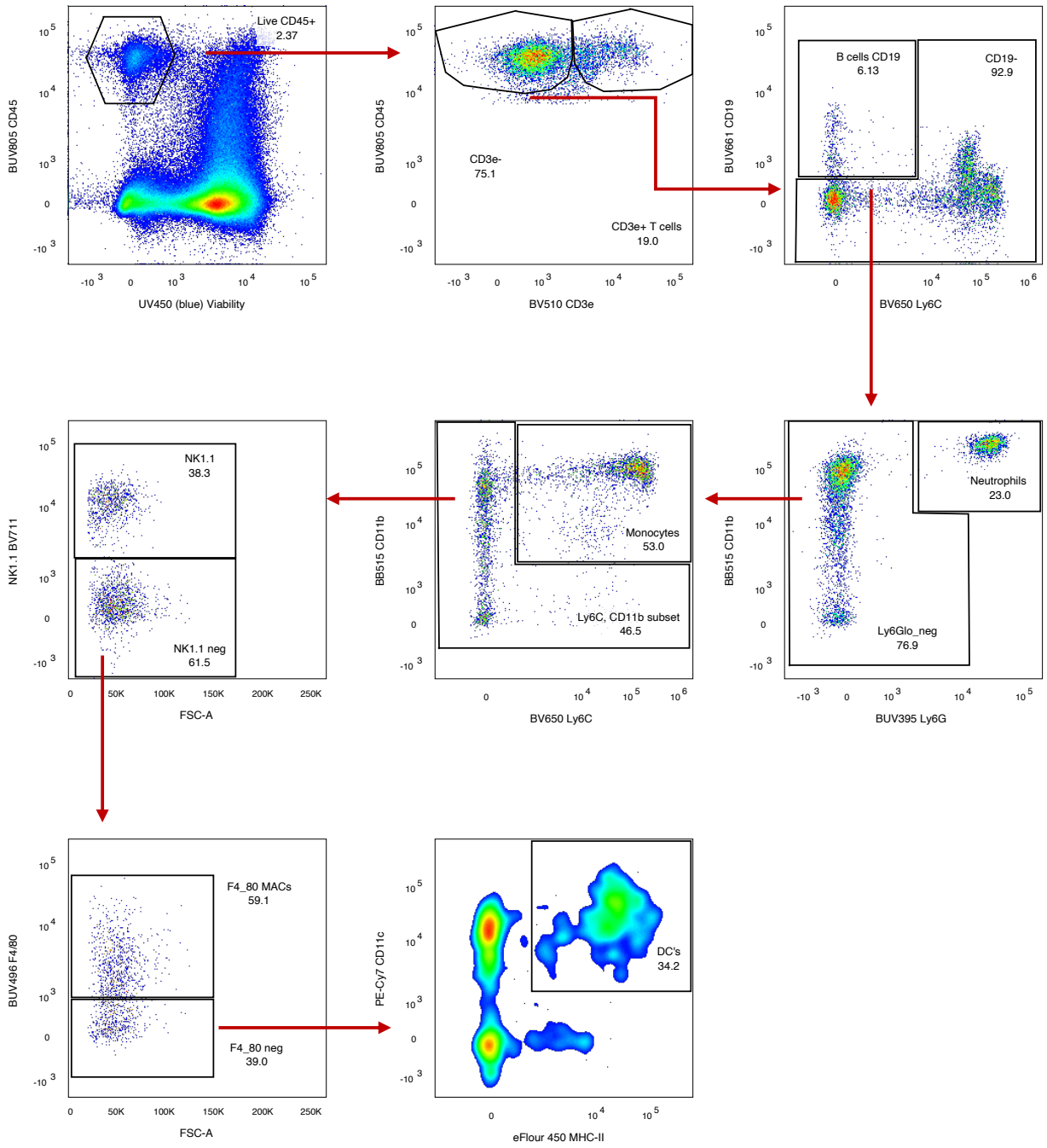
Supplementary Table 1. Flow Cytometry Antibody Details					
Antigen	**Panel**	**Clone**	**Fluorochrome**	**Manufacturer**	**Catalog Number**
CD103	T cell/Treg/BA-CTL	2.00E+07	BV785	Biolegend	121439
CD11b	APC	M1/70	BB515	BD Biosciences	564454
CD11c	APC	N418	PE-Cy7	eBioscience	25-01140-82
CD122	APC IL-2R	5H4	PE	Biolegend	105906
CD132	APC IL-2R	TUGm2	APC	Biolegend	132308
CD19	APC	1D3	BUV661	BD Biosciences	612971
CD25	T cell/Treg/APC	PC61	PE-Cy5	Biolegend	102010
CD3e	T cell/Treg/APC	500A2	BV480	BD Biosciences	746776
CD4	T cell/Treg	GK1.5	BUV496	BD Biosciences	612952
CD4	T cell ICS	GK1.5	APC-Cy7	BD Biosciences	552051
CD4	APC	RM4-5	BV605	Biolegend	100548
CD44	T cell/Treg/BA-CTL	IM7	APC-Cy7	Biolegend	103028
CD45	T cell/Treg/APC	30-F11	BUV805	BD Biosciences	568336
CD45.2	T cell/Treg/BA-CTL	104	APC	Biolegend	109814
CD69	T cell/BA-CTL	H1.2F3	PE-Dazzle 594	Biolegend	104535
CD8a	T cell/Treg/BA-CTL	53-6.7	AlexaFlour 700	Biolegend	100730
CD8a	T cell ICS	53-6.7	BUV395	BD Biosciences	563786
CD8a	APC	53-6.7	APC-Cy7	BD Biosciences	561967
CTLA-4	T cell/Treg	UC10-4B9	PE-Cy7	Biolegend	106313
F4/80	APC	BMB	PE-Dazzle 594	Biolegend	123146
FoxP3	T cell/Treg	FJK-16s	FITC	eBioscience	11-577-82
gB Tetramer	T cell/Treg	N/A	PE (Premium Grade, S21388, ThermoFisher)	Fred Hutch immune-monitoring Core	
GITR	T cell/Treg	DTA-1	BV750	BD Biosciences	747402
GranzymeB	T cell/BA-CTL	GB11	RB710	BD Biosciences	570275
GranzymeB	T cell/Treg	GB11	Pacific Blue	Biolegend	515408
ICOS	Treg	C398.4	PE-Dazzle 594	Biolegend	313532
IFNg	T cell ICS	XMG1.2	BUV737	BD Horizon	612769
IL-15 (biotinylated)	APC/APC IL-2R	N/A (polyclonal)	Streptavidin-PerC- Cy5.5	Biolegend	500-P173BT-25UG
Ki67	T cell/Treg	B55	BUV395	BD Biosciences	564071
LIVE/DEAD Aqua Fixable Viability Dye (AViD)	ALL PANELS	N/A	Aqua (405nm excitation)	Thermo Fisher	L34957
Ly6C	APC	HK1.4	BV650	Biolegend	128049
Ly6G	APC	1Ab	BUV395	BD Biosciences	
MHC-II	APC	M5/114.15.2	eFluor450	eBioscience	48-5321-82
NK1.1	APC	PK136	BV711	BD Biosciences	569723
NKG2D	T cell/Treg/BA-CTL	CX5	BV711	Biolegend	563694
OVA Tetramer	OT-I phenotyping	N/A	PE (Premium Grade, S21388, ThermoFisher)	Fred Hutch immune monitoring Core	
Thy1.1/CD90.1	BA-CTL	HIS51	SuperBright 645	eBioscience	64-0900-82
TIGIT	Treg	1G9	BV650	BD Biosciences	744213
TIM3	Treg	SA051D1	PerCP-Cy5.5	eBioscience	747619
TNFa	T cell ICS	MP-XT22	PerCP-Cy5.5	Biolegend	506322



Appendix Figure A-SA-1: Vaginal tract Treg remain transcriptionally distinct 28 days after primary HSV-2 infection. FoxP3eGFP mice were intravaginally infected with attenuated thymidine kinase-deficient HSV-2 (TK-) and harvested for dLN and VT on day 28 post-infection (n=4). Naïve Depo Provera treated mice were included as controls (n=4). Samples were prepared for fluorescence-activated cell sorting (FACS). CD4+ FoxP3+ Treg were sorted from dLN and VT single cell suspensions and processed for bulk RNA-sequencing. For comparison of differentially expressed genes, genes with a False Discovery Rate (FDR) of less than 0.1 and an absolute expression fold-change of greater than 1.0 were considered differentially expressed. (**A**) Heatmap shows the top 100 differentially expressed genes between vaginal Treg from naïve and previously infected mice. (**B**) Volcano plots show significantly differentially expressed genes between tissues from naïve vs. infected mice. (dLN top, VT bottom)

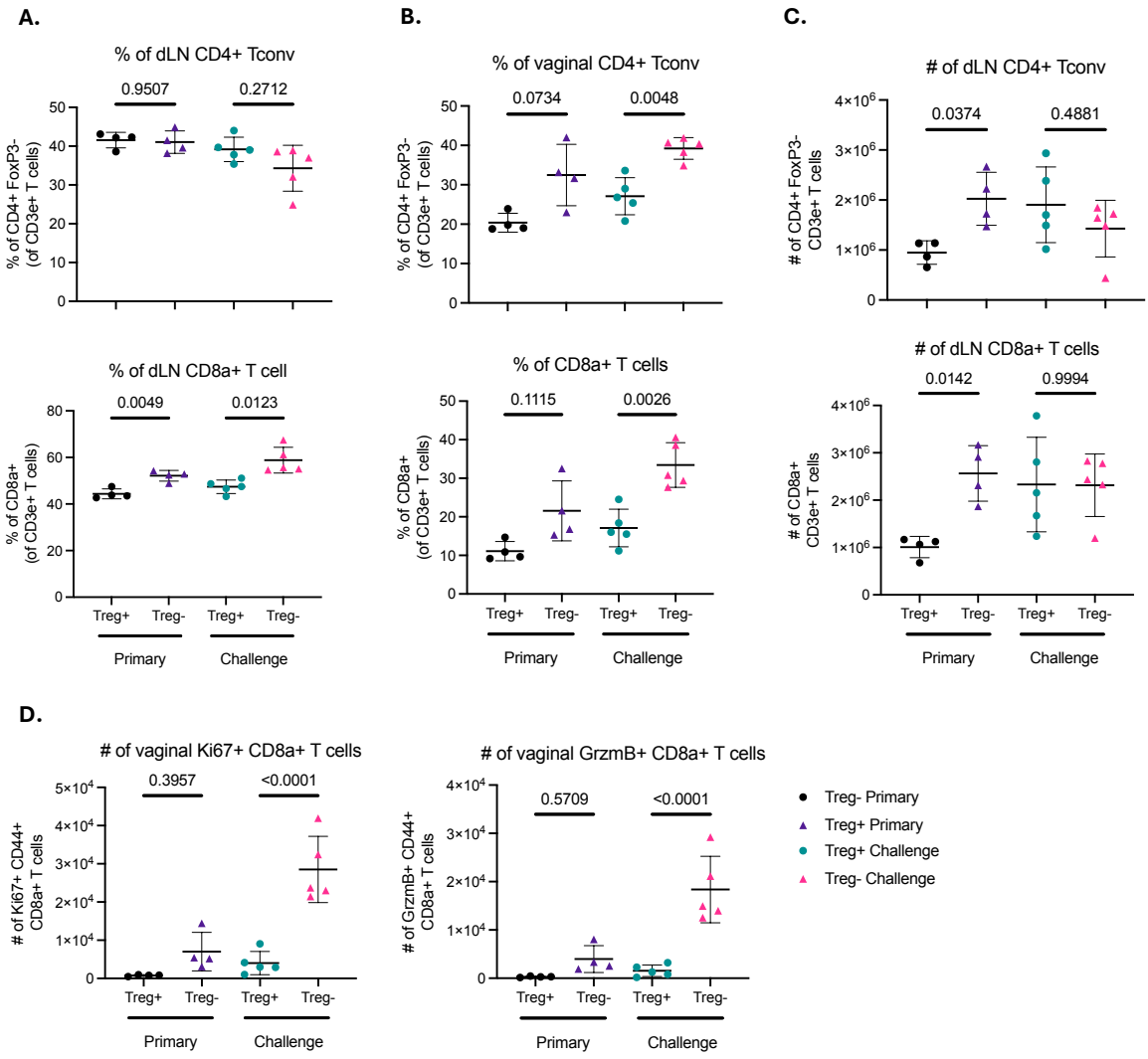


Appendix Figure A-SA-2: Treg phenotyping in the VT and dLN after primary HSV-2 infection. Female FoxP3eGFP mice were intravaginally infected with 10^5 pfu of thymidine kinase-deficient HSV-2 5-7 days after subcutaneous (neck ruff) administration of Depo Provera (2mg/kg in sterile PBS). VT and dLNs were collected at 7 and 90 days post-infection and processed for flow cytometric analysis, with naïve depo-treated mice included as day 0 uninfected controls (**A**) Absolute numbers of CD4+ FoxP3+ Treg in the VT (top) and dLN (bottom) at day 0, 7 (acute timepoint), and 90 (memory timepoint). (**B**) Absolute numbers of conventional CD4+ FoxP3- (green circle) and CD8 T cells (purple triangle) in the VT (top) and dLN (bottom) at day 0, 7, and 90. (**C**) Frequencies of Treg expressing markers of activation and immunosuppressive function in the VT (top) and dLN (bottom) at day 0, 7, and 90. (**D**) Plots show individual values of Treg frequencies for different markers of activation and immunosuppressive function in the VT at day 0, 7, and 90. (**E**) Plots show individual values of Treg frequencies for different markers of activation and immunosuppressive function in the dLNs at day 0, 7, and 90 post vaginal TK- HSV-2 infection. Data shown are combined from two experiments per timepoint (n=3-7 mice per group). Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Dunnett's multiple comparisons test in A-E.

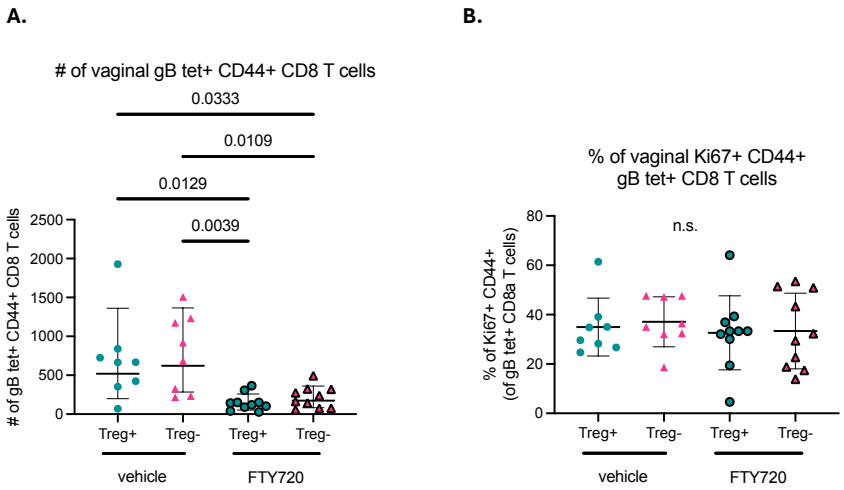


Appendix Figure A-SA-3: Murine APC flow cytometry gating.

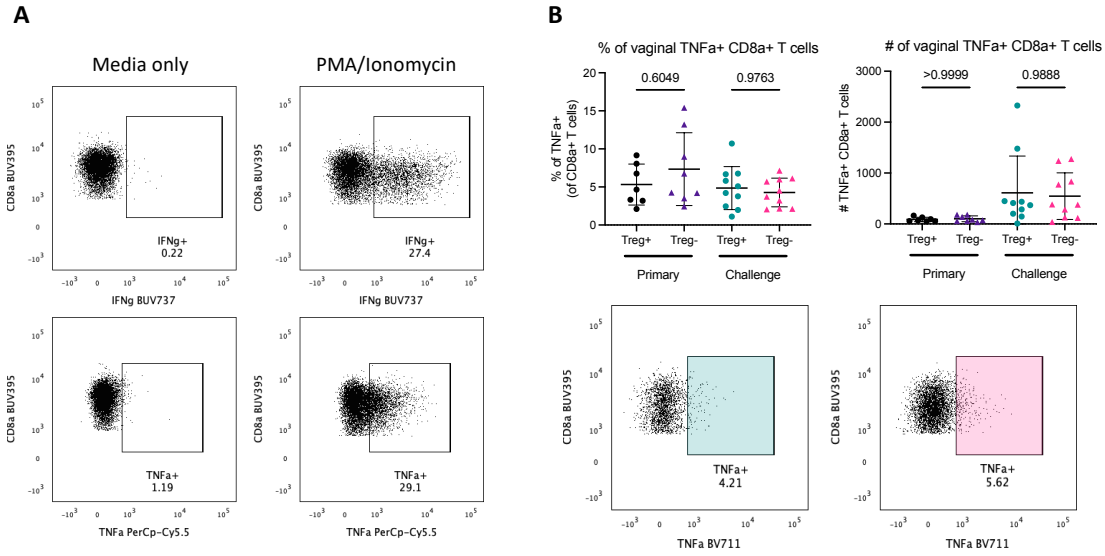
Representative gating strategy for identifying CD3e- CD19- antigen presenting cell subsets in vaginal tissue.



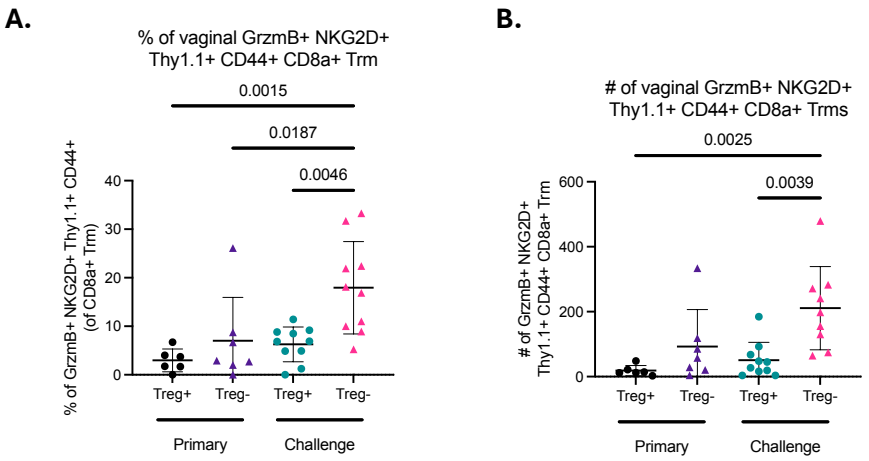
Appendix Figure A-SA-4: T cell frequencies and counts upon secondary infection.
Depo Provera treated Female FoxP3WT and FoxP3DTR mice were vaginally infected with 10^5 pfu of attenuated HSV-2 TK- and allowed to recover for 28-30 days. Mice were again treated with Depo Provera 5-7 days before WT HSV-2 (10^4 pfu) challenge and treated intraperitoneally with diphtheria toxin (DT) to ablate Treg prior to infection (FoxP3DTR mice, Treg-depleted group, Treg-). A dose of 30 µg/kg was administered the day before WT infection, followed by a dose of 10 µg/kg on d0. VT and dLNs were harvested at d3 p.c. and characterized via flow cytometry. (**A**) dLN frequencies of CD4+ Tconv (top) and CD8+ T cells (bottom) in Treg depleted and sufficient mice after WT HSV-2 challenge. (**B**) VT frequencies of CD4+ Tconv (top) and CD8+ T cells (bottom) in Treg depleted and sufficient mice after WT HSV-2 challenge. (**C**) dLN absolute numbers of CD4+ Tconv (top) and CD8+T cells (bottom) in Treg depleted and sufficient mice after WT HSV-2 challenge. (**D**) VT absolute numbers of Ki67+ (left) or GrzmB+ CD44+ (right) CD8 T cells. Representative data from two experiments (n= 4-5 mice per group) are shown. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test in A-D.



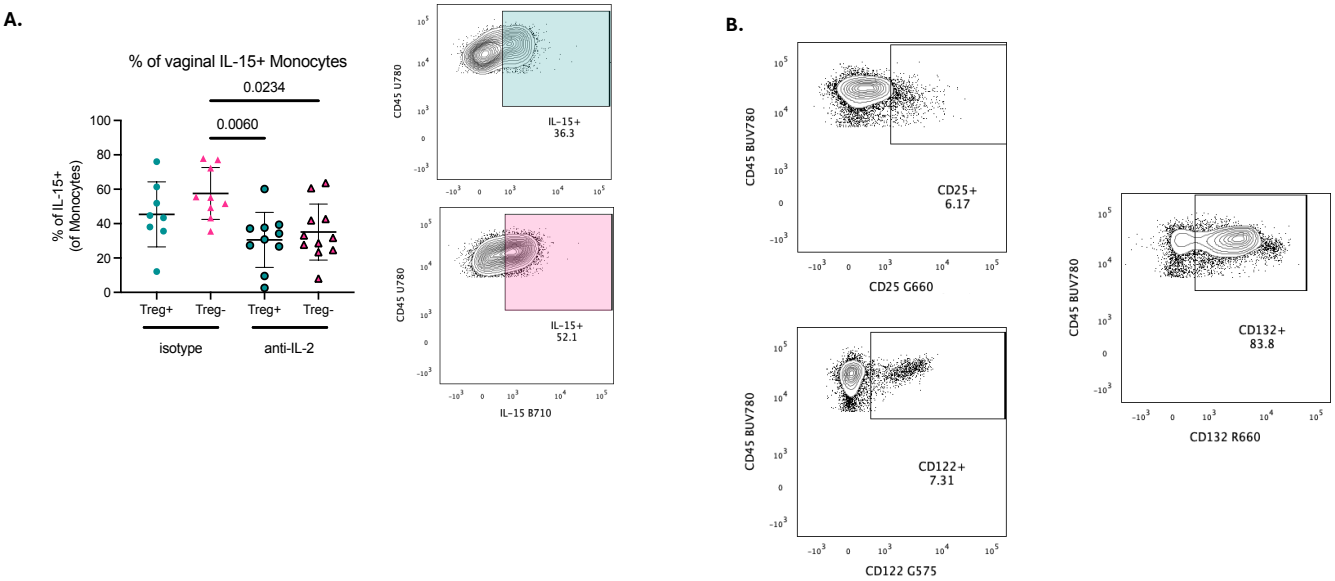
Appendix Figure A-SA-5: gB-specific CD8 T cells.
Female FoxP3WT and FoxP3DTR mice were infected and treated with DT as in Figure 1. In addition, mice were treated with 1mg/kg FTY720 via intraperitoneal injections on days -1, 0, and 2 with respect to WT HSV-2 challenge. Control mice were treated with vehicle (2% cyclodextrin in sterile PBS). (**A**)The absolute number of gB tetramer+ CD44+ CD8 T cells in VT of d3 WT HSV-2- challenged Treg depleted and sufficient mice treated with FTY720 or vehicle (2% cyclodextrin in PBS) is shown. (**B**) The frequency of Ki67+ CD44+ CD8 T cells in VT of challenged d3 Treg depleted and sufficient mice treated with FTY720 or vehicle (2% cyclodextrin in PBS) is shown. Data combined from two experiments per timepoint (n=4-5 mice per group). Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test.



Appendix Figure A-SA-6: Intracellular cytokine staining of VT and dLN CD8T cells after in vitro gB peptide stimulation.
VT and dLN from Treg depleted and sufficient mice euthanized at 3 days p.c. were processed into a single-cell suspension and stimulated in RP10 media with gB peptide (immunodominant MHC-I epitope) for 4 hours at 37C before conducting intracellular cytokine staining for IFNγ and TNFα production in CD8 T cells. (**A**) Representative flow plots of negative (RP10 complete media only condition) and positive (PMA/Ionomycin) controls for ICS. (**B**) Frequency, absolute numbers (top), and representative plots (bottom) of intracellular TNFα in vaginal CD8 T cells. Data combined from two experiments per timepoint (n=4-5 mice per group). Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test.

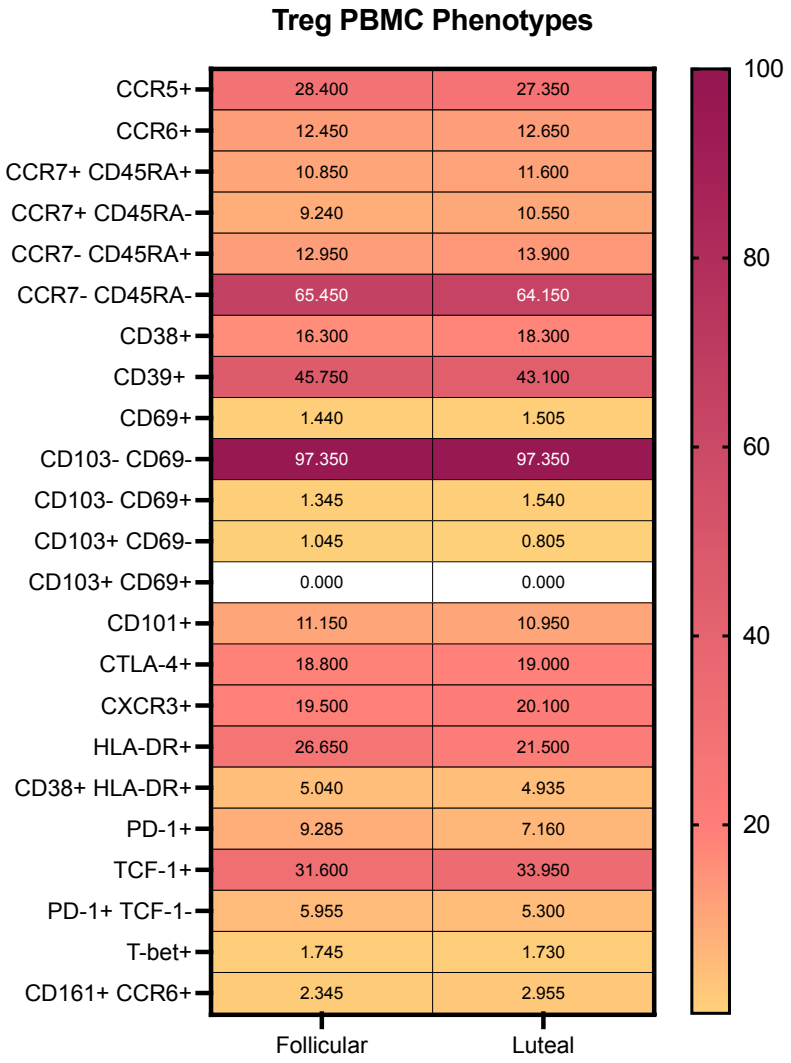


Appendix Figure A-SA-7: Cytotoxic bystander activated OT-Is.
500,000-1 million OT-Is were adoptively transferred into female FoxP3WT and FoxP3DTR mice 24hrs before vaginal infection with 105 pfu of HSV-2 TK- OVA and allowed to recover for 28-30 days. Mice were again treated with Depo Provera 5-7 days before WT HSV-2 (104 pfu) challenge and treated intraperitoneally with diphtheria toxin (DT) to ablate Treg prior to infection (FoxP3DTR mice, Treg-depleted group, Treg-). VT and dLN were collected d3 p.c. and assessed via flow cytometry. Frequency (**A**. left) and absolute number (**B**. right) of GrzmB+ NKG2D+ CD44+ Thy1.1+ BA-CTL Trm (Trm defined as IV-label negative CD69+ CD103+ CD8 T cells) from VT 3 days p.c. Data shown are combined from two experiments per timepoint (n=3-5 mice per group). Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test.

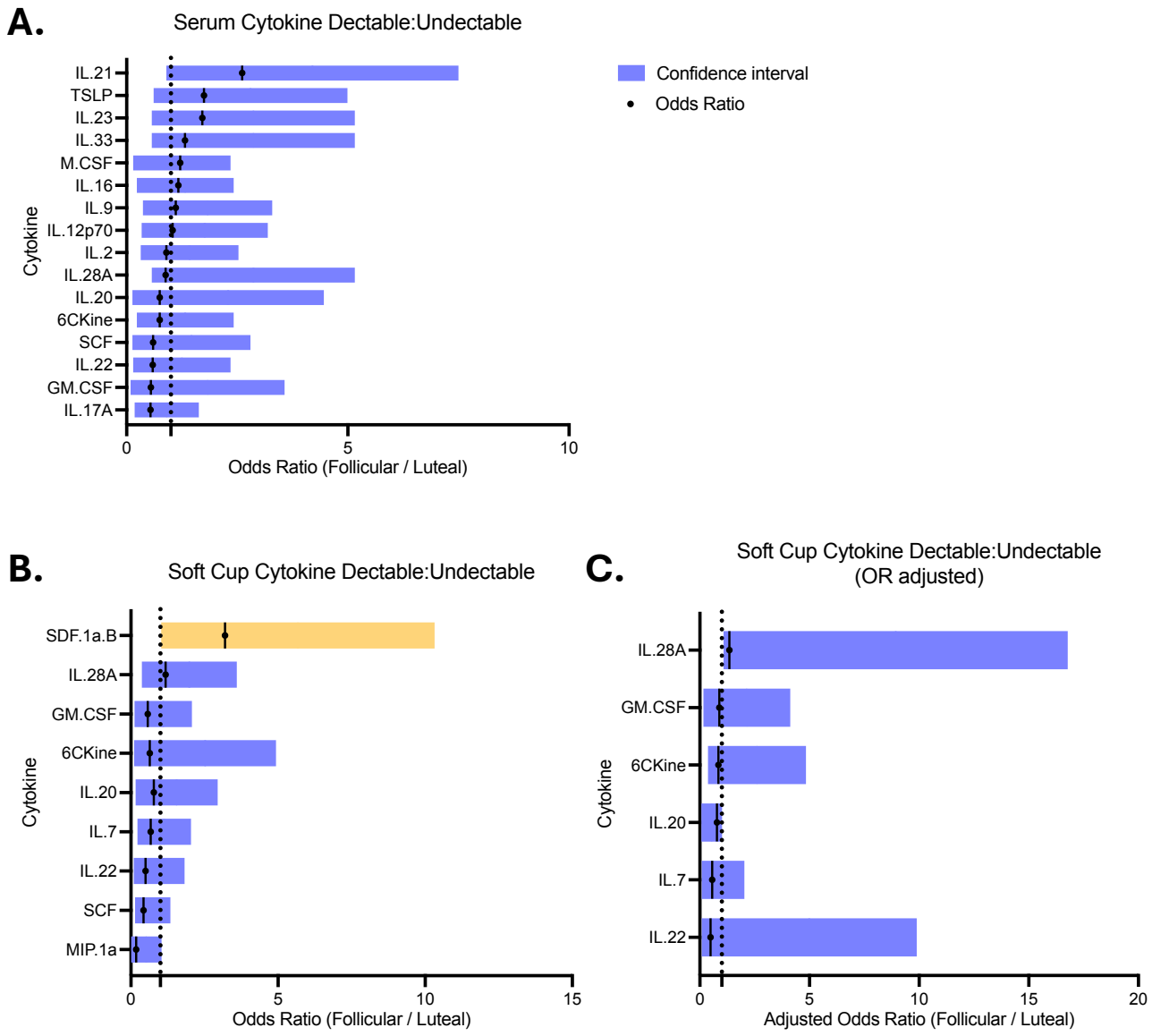


Appendix Figure A-S8: IL-15 trans-presentation in APC.
Female FoxP3WT and FoxP3DTR mice were infected and treated with DT as in Figure 1, and with anti-IL-2 or isotype control Mab as in Figure 5. VT and dLN were collected d3 p.c. and assessed via flow cytometry. (**A**) Shown is the frequency of IL-15 trans-presentation by monocytes in VT from d3 WT HSV-2 challenged Treg depleted or sufficient mice after treatment anti-IL-2 or isotype control Mab. Error bars represent mean and SD. Statistical significance determined by One-Way ANOVA and Tukey's multiple comparison test. (**B**) Representative staining of the IL-2R components in VT APC (CD25, CD122, CD132) about 33 days after primary vaginal infection with TK- HSV-2.

Appendix B: Chapter 3 Supplemental Figures



Appendix Figure B-S1: Treg PBMC Phenotypes
Heatmaps show the median frequency of CD8 T cell marker expression in CX, VT, and PBMC samples (left to right) collected in follicular vs luteal phase. Adjusted rank regression model for CVT samples, unadjusted for PBMC. Significant ($p < 0.05$) comparisons between follicular and luteal phase denoted by blue box.



Appendix Figure B-S2: Follicular phase is associated with altered detection of soluble immune factor SDF-1α/β (CXCL12) in CVT fluid.
Cytokines and chemokines were quantified using the multiplex Luminex assay from (**A**) serum samples (follicular n = 52, luteal n = 20); and (**B**) CVT fluid (follicular n = 51, luteal n = 17). Unadjusted odds ratio (follicular/luteal) for serum, unadjusted and adjusted odds ratio for CVT soluble immune factors that did not meet quantification criteria (fewer than 80% detectable samples). Unadjusted and adjusted 95% confidence interval shown for CVT comparisons, unadjusted for serum comparisons. Significant results (p-value < 0.05) colored in yellow and non-significant comparisons in purple. CVT fluid comparisons adjusted for *a priori* for HSV-2 status, HIV exposure, and semen exposure.

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