T cell immunity in the female genital tract

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Abstract

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Many pathogens of global health significance are sexually transmitted, highlighting the importance of studying immune cells in genital barrier tissues. T cells are a critical component of the immune response to viruses, yet their role in protecting the female genital tract against viral infection is incompletely understood. Here, we combined studies of the mouse and human female genital tract to characterize T cells isolated from female genital barrier tissues. In human cervicovaginal tissue, CD8 T cells resembled effector T cells, raising the question of whether they were recently activated. By employing mouse models, we found that systemic immunization resulted in a cervicovaginal CD8 T cell compartment that was poorly maintained and underwent

progressive differentiation that was not observed in spleen, lymph nodes, or small intestine lamina propria. This progressive differentiation occurred over five months, after which CD8 T cells gained an effector-like phenotype, ultimately resembling human cervicovaginal CD8 T cells. Vaginal inflammation combined with vaginal antigen exposure accelerated this differentiation process. The CD8 T cell compartment induced by systemic immunization mediated partial protection against vaginal infection with herpes simplex virus type 2 (HSV-2). This protection waned over time as the loss and differentiation of the CD8 T cell compartment progressed. Together, these results demonstrate that tissue-intrinsic factors interact with environmental cues to dictate the final longevity and phenotype of the cervicovaginal CD8 T cell compartment.

Women living with HSV-2 often experience recurring genital herpes lesions. We explored the T cell compartment in human genital skin during these localized HSV-2 reactivation events. Upon HSV-2 lesion formation, we found that CD4 and CD8 T cells expanded in the infected skin site and upregulated markers of proliferation and activation. Some of the expanded T cells resembled circulating memory T cells, suggesting influx of immune cells from the blood. Cell-intrinsic and cell-extrinsic regulatory mechanisms were also upregulated in HSV-2 lesions, indicating that the immune system acted to control the virus as well as limit immunopathology. Meanwhile, T cells isolated from a nearby unaffected region distinct from the HSV-2 lesion were quiescent, instead resembling the T cell isolate from genital biopsies from HSV seronegative participants.

Despite the frequency of HSV-2 reactivation in people with chronic genital herpes, we did not observe evidence of T cell exhaustion or loss of functionality. After lesion resolution, the T cell infiltrate in HSV-2 lesions returned to quiescence, again resembling the T cell compartment

in contralateral and seronegative control biopsies. Given these results, we conclude that T cell responses to HSV-2 reactivation events in genital skin are highly localized, include both resident T cells and circulating memory T cells, and are rapidly constrained by regulatory T cells and intrinsic regulatory mechanisms. Taken together, our data highlight unique features of T cells in the female genital tract and their roles in vaccination and viral immunity.

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ABBREVIATIONS

ANOVA = analysis of variance

AIC = Akaike information criterion

AIDS = acquired immunodeficiency syndrome

CVT = cervicovaginal tissue

d = days

DMEM = Dulbecco's modified Eagle medium

EDTA = ethylenediaminetetraacetic acid

Eomes = Eomesodermin

FBS = fetal bovine serum

FHCRC = Fred Hutchinson Cancer Research Center

gB = HSV glycoprotein B

gBT-I = transgenic CD8 T cell specific to HSV glycoprotein B epitope SSIEFARL

gD = HSV glycoprotein D

gDT-II = transgenic CD4 T cell specific to HSV glycoprotein D epitope IPPNWHIPSIQDA

HBSS = Hank's buffered salt solution

HIV = human immunodeficiency virus

HSV = herpes simplex virus

HSV-2 TK- = thymidine kinase-deficient herpes simplex virus type 2

IFNg = interferon gamma

LM-gB = *Listeria monocytogenes* expressing HSV glycoprotein B epitope SSIEFARL

MHC = major histocompatibility complex

mo = months

PBS = phosphate-buffered saline

PCR = polymerase chain reaction

RPMI = Roswell Park Memorial Institute 1640 medium

S1PR1 = sphingosine-1-phosphate receptor 1

scRNAseq = single cell RNA sequencing

SI LP = small intestine lamina propria

TCF-1 = T cell factor 1

Tcircm = circulating memory T cell

Tex = exhausted T cell

TGFB = tumor growth factor beta

Th1 = type 1 helper T cell

Treg = regulatory T cell

Trm = resident-memory T cell

t-SNE = *t*-distributed stochastic neighbor embedding

UMAP = uniform manifold approximation and projection

YLD = years lived with disability

wk = weeks

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DEDICATION

I dedicate this work to my biological and chosen families, who have loved me, encouraged me, and kept me on a forward path. I hope to do the same for future scientists, especially they whose voices are currently too rare in the scientific community.

Chapter 1. Introduction

Many diseases of global health importance, including acquired immunodeficiency syndrome, human papillomavirus-associated genital cancer, chlamydia, syphilis, and genital herpes, are caused by sexually transmitted infectious agents. In many people, these agents first infect the female genital skin and mucosa. Several of these infections currently lack an efficacious vaccine. Vaccine development for sexually transmitted infections will benefit from a greater understanding of immune responses within the female genital tissue.

1.1 HSV-2 Virology and Pathogenesis

The herpesviruses are a class of large enveloped double-stranded DNA viruses that are generally neurotropic and possess the ability to establish latent infection in susceptible hosts (Gilden et al., 2007). Herpes simplex virus type 2 (HSV-2), one of eight herpesviruses that infect humans, is a highly prevalent sexually-transmitted infection that produces significant morbidity in symptomatic adults, causes severe neonatal infections, and predisposes for human immunodeficiency virus (HIV) acquisition (Looker et al., 2015). A preventative vaccine that protects against HSV-2 infection would be a valuable public health intervention. However, there are currently no approved vaccines for HSV-2 or the closely related virus HSV-1, and clinical trials of HSV vaccine candidates have demonstrated very limited efficacy in preventing infection or reducing the severity of symptoms (Johnston et al., 2016).

In a susceptible individual, HSV-2 rapidly replicates in permissive epithelial cells before spreading to sensory neurons, where it establishes permanent latent infection in lumbrosacral ganglia and persists for the lifetime of the host (Gilden et al., 2007; Schiffer and Corey, 2013). Frequent clinical or sub-clinical reactivation events in the ganglia lead to the shedding of virus

towards genital skin and mucosa, which can result in symptomatic disease or transmission to a new host (Schiffer and Corey, 2013). HSV-2 infection can result in a variety of clinical manifestations, many of which can also be caused by HSV-1. Although most infections are asymptomatic, the most common clinical outcomes associated with HSV are recurrent painful orolabial or anogenital lesions (World Health Organization, 2016). In adults, especially those suffering immune deficiencies, rare outcomes of HSV infection include blinding eye keratitis (Pepose et al., 2006), aseptic meningitis, recurrent radiculopathy, encephalitis, hepatitis, pneumonia, esophagitis, and recurrent zosteriform eruptions with possible associated neuropathy (Gilden et al., 2007; Koelle and Corey, 2008). In neonates that acquire HSV-1 or HSV-2 from an infected mother during childbirth, the disease is considerably more severe. Neonatal HSV infection can be limited to skin, eye, and mucosal tissues, may involve the central nervous system or can result in disseminated infection, of which the latter two are associated with high morbidity and mortality (Corey and Wald, 2009; R. Gupta et al., 2007). The main treatment for HSV infection is the antiviral nucleoside analog acyclovir, which can reduce viral shedding, lower the probability of transmission to a partner or neonate, and reduce the severity of recurrences and neonatal disease (Corey and Wald, 2009; Corey et al., 2004b; Schiffer and Corey, 2013). However, no curative therapy is currently available for HSV, and acyclovir treatment does not completely prevent viral shedding or clinical symptoms (Schiffer and Corey, 2013). Similarly, preventative methods such as condom use and acyclovir treatment of infected partners do not completely prevent transmission (Corey et al., 2004b; R. Gupta et al., 2007).

1.2 HSV-2 Epidemiology and Global Burden of Disease

HSV-2 is a highly prevalent infection worldwide. Mathematical modeling of global prevalence in the year 2012 estimated that 417 million people aged 15-49 years were living with

HSV-2 (11.3% global prevalence), of whom 64% were women (Looker et al., 2015). Additionally, there were an estimated 19.2 million incident infections. The highest burden was in Africa, but Southeast Asia and Western Pacific regions also contributed a substantial number of infections. The Global Burden of Disease attributed 311,600 and 236,400 years lived with disability (YLD) to genital herpes in 2013 and 2015, respectively (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; "Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013," 2015). Importantly, these estimates do not include YLD due to neonatal herpes or other herpes-associated morbidities.

In the U.S., where high-quality seroprevalence data is available, HSV-2 seroprevalence has decreased overall in recent years, falling from 21.2% in 1988-1994 to 15.5% in 2007-2010 (Centers for Disease Control and Prevention (CDC), 2010). However, seroprevalence differs greatly between ethnic groups. For example, in non-Hispanic Black females in the U.S., seroprevalence is approximately 50% and has remained stable since 1988 (Centers for Disease Control and Prevention (CDC), 2010). Among HSV-2 seropositive individuals, the rate of past genital herpes diagnosis is 10-20%, indicating that there are a large number of asymptomatic infections which are likely to be contagious (World Health Organization, 2016). Individuals with genital herpes may also suffer psychosocial morbidity due to anxiety associated with recurrence or fear of transmitting the infection to partners or infants (Melville et al., 2003; Swanson and Chenitz, 1990). Due to its high prevalence, genital herpes infection is also associated with a significant financial burden, with an estimated total direct cost of $541 million in the U.S in 2010 (Owusu-Edusei et al., 2013).

Global incidence estimates for neonatal herpes are not available, but the incidence of neonatal infection in the U.S. is estimated to be about 1 in 3,200 deliveries (Pinninti and Kimberlin, 2013), and is likely to be considerably higher in low- and middle-income countries. Examination of inpatient records in the U.S. suggested that neonatal herpes incidence in 2006 was between 9.6-16.6 per 100,000 and resulted in hospitalization costs of $35 million (Flagg and Weinstock, 2011). Risk factors for neonatal transmission include primary HSV infection (57% risk of neonatal transmission during primary infection, 25% during first-episode non-primary infection, and 2% during recurrent episode), low maternal antibody titer, vaginal delivery, type of HSV, and compromised cutaneous barrier function in the newborn (Pinninti and Kimberlin, 2013). It is estimated that 45% of neonatal herpes infections are limited to the skin, eyes, and mucosa, 30% involve the central nervous system, and 25% become disseminated infections that are clinically indistinguishable from bacterial sepsis (Corey and Wald, 2009; Pinninti and Kimberlin, 2013). Without antiviral treatment, 85% of neonates with disseminated herpes infection and 50% of neonates with central nervous system disease die by 1 year of age (Pinninti and Kimberlin, 2013), and many children who survive infection experience long-term neurologic sequelae (Corey and Wald, 2009). Case-fatality rates are improved substantially by antiviral treatment (Pinninti and Kimberlin, 2013); for example, analysis of 2006 inpatient records in the U.S. showed a case-fatality rate of as low as 4.1% (Flagg and Weinstock, 2011). In future years, the changing epidemiology of HSV-1 and HSV-2 infection may affect neonatal herpes incidence. HSV-1 has become a more common cause of anogenital lesions in the U.S., a shift which is commonly attributed to decreasing prevalence of childhood orolabial HSV-1 infection and increasing engagement in oral sex behavior (Centers for Disease Control and Prevention (CDC), 2016). Overall, this shift may predispose women to acquire primary HSV-1 or HSV-2 genital infection at

sexual debut and during childbearing years, thus increasing the risk of neonatal transmission (Centers for Disease Control and Prevention (CDC), 2016).

Despite the high prevalence of HSV-2 and the morbidity and mortality of neonatal herpes infection, the most detrimental effect of HSV-2 infection may be its role in the HIV epidemic (Corey et al., 2004a). In infected individuals, HSV-2 induces an inflammatory environment in the anogenital tract, compromises the mucocutaneous barrier, and recruits HIV target cells (CD4+ T cells) to the site of HIV infection (Corey et al., 2004a; R. Gupta et al., 2007; Johnston et al., 2016). By these and perhaps other mechanisms, HSV-2 infection confers an estimated 3-fold increased risk of HIV acquisition (Freeman et al., 2006). In Europe and Africa respectively, 30-70% and 50-90% of HIV-infected individuals are also infected with HSV-2 (Corey, 2007), and in areas with an especially high prevalence of HSV-2, 25-50% of HIV infections are attributable to HSV-2 (Johnston et al., 2016). In a clinical trial in Africa, acyclovir therapy in HSV-2-infected, HIV discordant couples was not effective in reducing the incidence of HIV transmission (Celum et al., 2010). A preventative vaccine against HSV-2 could have a significant effect on slowing the HIV epidemic in high-incidence areas. For example, modeling estimates suggest that 70% population coverage with a 75% effective vaccine could reduce HIV incidence by 30-40% over 20 years (Freeman et al., 2009).

1.3 Vaccines and Correlates of Protection for HSV

Both preventative and therapeutic vaccines for HSV-2 are potentially feasible, and both types of vaccine are in development (Johnston et al., 2016; 2011). The success of prophylactic and therapeutic vaccines against varicella zoster, another human herpesvirus, illustrates that both vaccination paradigms can successfully prevent herpesvirus morbidity (Johnston et al., 2016). A therapeutic vaccine for HSV-2 could reduce the frequency of viral shedding and reactivation

events, which might prevent lesions and reduce transmission of HSV-2. However, this strategy may be ineffective in disrupting HIV transmission because HSV-elicited CD4+ T cells would already have established residence at the site of HIV infection (Turner and Farber, 2014). Therefore, an ideal vaccine would act prophylactically to prevent HSV-2 infection in sensory neurons, which would protect against both asymptomatic viral shedding and symptomatic disease. A prophylactic vaccine could target adolescents prior to sexual debut but might be best delivered during childhood when HSV seroprevalence is lowest. Given that HSV-1 and HSV-2 share 80% sequence identity (R. Gupta et al., 2007), it is possible that a vaccine against HSV-2 could also confer cross-protection against HSV-1. Overall, a cross-protective prophylactic herpes simplex vaccine would hold great promise as a public health intervention.

Thus far, HSV-2 vaccines that have been tested in humans have been minimally efficacious (Johnston et al., 2016). There are many challenges to developing an effective HSV-2 vaccine. Prophylactic vaccines are particularly hard to test in clinical trials given that seronegative adults who are past the initiation of sexual activity may possess behavioral or immune factors that decrease their risk of primary HSV infection relative to the general population. Attack rates may therefore be very low, as was observed in the Herpevac Phase III trial, in which attack rates were <1% in both the vaccine and placebo arms (Belshe et al., 2012). In addition, immune correlates of protection for HSV are poorly understood (Koelle and Corey, 2008), and tissue-specific immune correlates in the mucosa or within dorsal root ganglia cannot be readily assessed in trials (Schiffer and Corey, 2013). Data from the Herpevac Phase III trial suggested that circulating antibodies to the vaccine antigen were a correlate of protection for HSV-1 (Belshe et al., 2014), but results from the Phase I/IIa trial of the therapeutic vaccine GEN-003 did not show any correlation between circulating antibody titer and reduction in viral shedding (Flechtner et al., 2016). Herpes simplex

viruses have coevolved with primates for millions of years, and a preventative vaccine must overcome the sophisticated immune evasion mechanisms of herpes viruses that allow them to rapidly infect sensory neurons and establish immunologically silent latency (Johnston et al., 2011; Schiffer and Corey, 2013). Pre-clinical vaccine investigations are hindered by the lack of an animal model that completely recapitulates the characteristics of HSV disease in humans (Dasgupta and Benmohamed, 2011). Finally, herpes vaccines will face economic and implementation roadblocks similar to those encountered by other new vaccines, such as the low financial incentive for pharmaceutical companies to pursue development (Plotkin et al., 2015), the potential for low uptake of vaccines that target sexually-transmitted infections (Henry et al., 2016; Reagan-Steiner et al., 2016), and the difficulties associated with delivery to high-incidence regions in low- and middle-income countries.

Despite these challenges, several lines of evidence suggest that an HSV-2 vaccine is feasible. The Herpevac Phase III trial of an HSV-2 vaccine showed moderate efficacy against HSV-1 infection among seronegative individuals (Belshe et al., 2012), indicating that cross-protection may be possible, and the GEN-003 Phase I/IIa trial suggests that a therapeutic vaccine can reduce viral shedding (Flechtner et al., 2016). The success of the human papillomavirus vaccine proves that an intramuscular vaccine can provide robust, antibody-mediated protection against both infection and disease caused by a sexually-transmitted viral pathogen (Johnston et al., 2016). In addition, women who are infected with HSV-1 have a lower risk of acquiring HSV-2 (Pinninti and Kimberlin, 2013), most cases of HSV-2 are asymptomatic (Schiffer and Corey, 2013; World Health Organization, 2016), and reactivation events wane somewhat over time (Phipps et al., 2011), suggesting that the immune system is capable of preventing infection and controlling reactivation in certain scenarios.

1.4 Antigen Recognition by T Cells

The low efficacy of antibody-based HSV-2 vaccines in human trials combined with the finding that T cells play an active role in controlling viral reactivation has led to the notion that T cell-based vaccination may be a successful strategy for HSV-2. T cells and B cells are the major cell types that contribute to the adaptive immune response and acquired immune memory. Unlike B cells, which produce antibodies that can bind to diverse antigens including whole virus particles, intact foreign proteins, and non-protein molecules, T cells bind to antigens presented by major histocompatibility complex (MHC) proteins on the surface of other host cells. Antigens presented on MHC molecules are short linear fragments of proteins derived from the cytosol or intracellular organelles. Conventional CD4 T cells recognize antigens that derive from endocytic pathway vesicles and are presented by MHC Class II molecules on the cell surface of antigen presenting cells, such as dendritic cells, macrophages, and B cells. Conventional CD8 T cells recognize antigens that derive from the cytosol of infected host cells, or from endocytic vesicles of antigen-presenting cells by a process known as cross-presentation. These antigens may be host-derived or infectious agent-derived and are presented by MHC Class I molecules, which are expressed on the surface of almost all cell types of the human body.

1.5 Functions of Effector T Cells

After recognition of cognate antigen presented in the appropriate inflammatory context, CD4 and CD8 T cells can differentiate into effector and memory T cell subsets and perform functions that are critical for clearance of infectious agents. In addition, effector and memory CD4 and CD8 T cells can perform some of these functions in response to inflammation alone, even in the absence of cognate antigen recognition, in a process termed bystander activation.

Upon activation by cognate antigen or exposure to bystander-activating cytokines, CD4 and CD8 T cells play key roles in antiviral immunity and control viral infection by a variety of mechanisms. Naïve CD4 T cells can differentiate into a variety of effector T cell subsets, each with its own toolbox of effector mechanisms that can act to limit viral infection and expansion. For example, type 1 helper T cells (Th1 cells) produce interferon gamma (IFNg), a cytokine of key importance in HSV-2 infection. Among other functions, IFNg drives macrophage lysosome enhancement and inducible nitric oxide synthase production, enhances MHC Class I and II expression, activates the mucosal vascular endothelium to upregulate T cell entry molecules, activates epithelial cells to produce chemokines that recruit immune cells, and can synergize with other cytokines to result directly in cell death (reviewed in (Schroder et al., 2004)).

CD4 T cells can also differentiate into other effector T cell subsets, including type 2 helper T cells, IL-17-producing T cells, follicular helper T cells, and regulatory T cells (Treg). Treg function to inhibit overzealous immune responses and enforce the correct intensity, localization, and resolution of the immune response. Treg limit the effector functions of other immune cells by producing immunosuppressive cytokines, outcompeting other cell subsets for access to IL-2, and preventing other T cells from accessing costimulatory molecules. In some infections, such as ocular HSV-1 infection and leishmaniasis, Treg limit immune-mediated tissue damage but may also suppress effective immune responses and detract from the overall efficacy of the response (Mendez et al., 2004; Sarangi et al., 2008). Conversely, in the context of vaginal HSV-2 infection, Treg improve survival outcomes after challenge (Lund et al., 2008) and are necessary for the appropriate trafficking of effector T cells into the infected tissue (Soerens et al., 2016).

CD8 T cells most famously carry out their effector function by directly killing infected host cells, thus limiting the expansion of intracellular bacterial and viral infections. CD8 T cells that

recognize cognate antigen presented by an infected cell can kill that infected cell via exposing it to cytotoxic granules that contain perforin and granzymes. CD8 T cells can also mediate host cell death by expressing molecules such as Fas ligand and tumor necrosis factor alpha. Finally, like Th1 CD4 T cells, CD8 T cells can limit viral infection by producing IFNg to activate nearby immune cells, endothelial cells, and epithelial cells, creating an antiviral inflammatory milieu and resulting in the recruitment of additional effector immune cells into the infected tissue site.

1.6 Resident-memory T cells

Upon first encounter with cognate antigen and other activating signals, naïve T cells clonally expand and give rise to a large number of effector T cells that recognize the same antigen. A minority of these effector T cells differentiates into T cells with an altered phenotype that can persist long after the effector T cell population dies. This persisting population of T cells is termed the memory T cell compartment. Upon subsequent encounters with the same antigen, memory T cells are capable of rapid re-expansion and re-generation of effector T cells, potentially mediating rapid clearance of the infectious agent before symptomatic disease occurs.

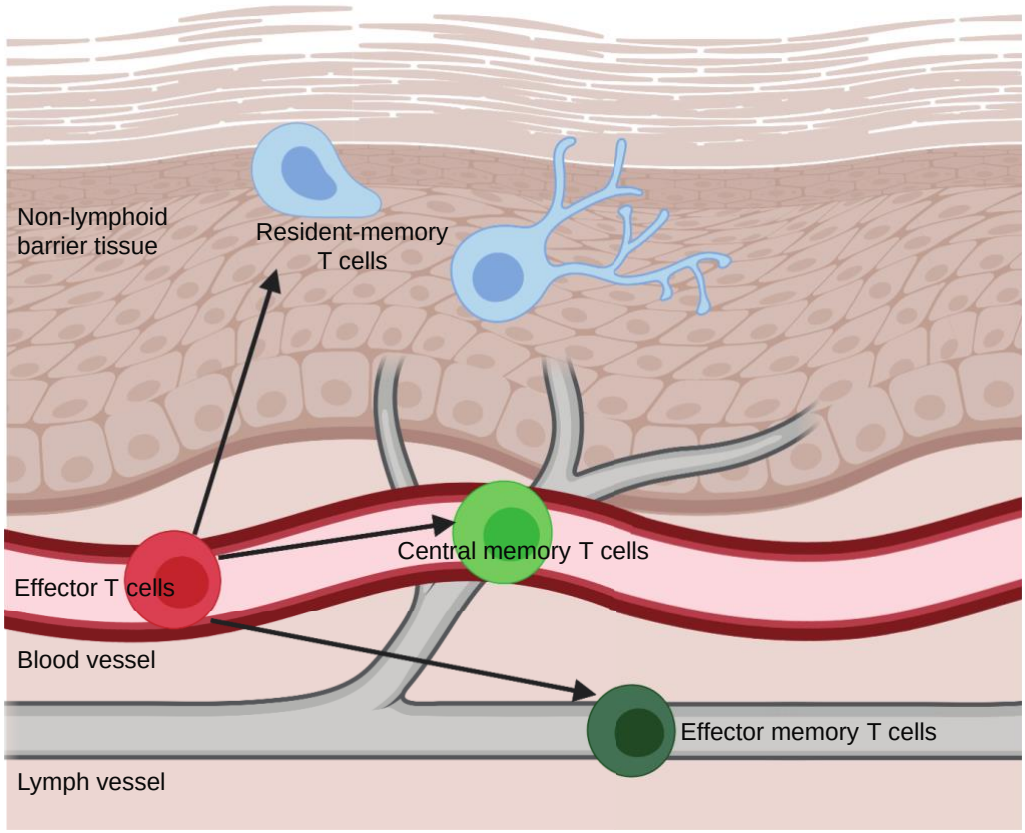

Figure 1.1. Effector T cells give rise to multiple subsets of memory T cells.

A subset of effector T cells exit the lymphoid circulatory system and enter into non-lymphoid tissues, where they largely stop recirculating and instead upregulate a gene expression program optimized for residence outside of the lymphoid system (Figure 1.1). Over the last two decades, these tissue-resident memory T cells (Trm) have been extensively characterized in many organs using mouse models and *ex vivo* studies of human tissue (reviewed in (Masopust and Soerens, 2019; Mueller and Mackay, 2016; Szabo et al., 2019b)). Before the formal naming of this subset of memory T cells, CD4 and CD8 T cells were known to exist in large numbers outside of the blood, lymph nodes, and spleen (Reinhardt et al., 2003; 2001) and possess unique phenotypes within tissues such as the intestine (Masopust et al., 2004; 2006). Parabiosis studies indicated that T cell compartments in the brain, intestine, and peritoneal cavity did not completely equilibrate

between congenic parabiotic partner mice, suggesting the existence of non-recirculating resident populations (Klonowski et al., 2004). Organ transplantation studies in mice, in which T cells in the dorsal root ganglion (Wakim et al., 2008) and small intestine (Masopust et al., 2010) stayed within the transplanted tissue after engraftment, further cemented the finding that Trm were non-recirculating. This capacity for prolonged maintenance within a single tissue without recirculation is a defining feature of Trm. Despite this, recent evidence shows that seemingly non-recirculating T cell compartments that appear to be bonified Trm can re-seed the periphery after activation in both mice and humans (Fonseca et al., 2020; Klicznik et al., 2019), suggesting that Trm cannot be stringently defined as completely lacking the ability to recirculate.

Several functions have been described for Trm. They can maintain motility within non-lymphoid tissues (Ariotti et al., 2012; Gebhardt et al., 2011; McNamara et al., 2017), sense infections and recruit effector cells from the circulating immune compartment (Schenkel et al., 2014; 2013), and proliferate *in situ* after antigen encounter (Beura et al., 2018a; Park et al., 2018). Mouse and human studies have demonstrated that heightened Trm responses are associated with enhanced pathogen control of a wide variety of pathogens (reviewed in (Masopust and Soerens, 2019)), including HSV-1 (Gebhardt et al., 2009; Mackay et al., 2012) and HSV-2 (Shin and Iwasaki, 2012; Zhu et al., 2007; 2013). Together, this body of evidence has led to an interest in leveraging Trm as a component of vaccine-induced immune responses (Iwasaki, 2016; Neutra and Kozlowski, 2006; Shin and Iwasaki, 2013).

Resident-memory T cells are characterized by their expression of a variety of transcription factors and surface-expressed proteins. Many populations of T cells that appear to be tissue-resident based on their lack of recirculation express the surface markers CD69 and CD103. CD103, also called integrin alpha-e, binds to the epithelial cell adhesion molecule E-cadherin and is

thought to be important for T cell attachment to epithelial tissue sites (Schön et al., 1999). The function of CD69 is not completely understood, but its expression suppresses the function of the sphingosine-1-phosphate receptor (S1PR1), a receptor that is important for T cell egress from non-lymphoid tissues (Bankovich et al., 2010). In addition to these two surface-expressed proteins, many other phenotypes have also been linked to Trm identity, including the loss of KLF2 and S1PR1 (Skon et al., 2013), expression of granzyme B (Casey et al., 2012), high expression of CD90 and lack of Ly6c (Stolley et al., 2020), expression of Tbet and Eomesodermin (Mackay et al., 2015), expression of Hobit and Blimp1 (Mackay et al., 2016), and expression of Runx3 (Milner et al., 2017), among others. While there is evidence that a core transcriptional signature is shared by Trm across tissues (Kumar et al., 2017), many of these phenotypes appear to differ according to tissue of residence (Steinert et al., 2015), CD4 vs. CD8 identity (Beura et al., 2019), mouse vs. human (Kumar et al., 2017), and location and method of priming (T. N. Khan et al., 2016).

1.7 Resident-memory T cells and Genital HSV-2 infection

An effective vaccine against HSV-2 must protect against primary infection of the genital skin and mucosa, and therefore might benefit from generating Trm populations at these anatomical locations. Strong evidence already exists that T cell responses are likely to be an essential component of a successful HSV-2 vaccine. For example, studies in which mice were vaginally challenged with HSV-2 after depletion of specific lymphocyte subsets concluded that protection induced by vaginal immunization was critically dependent on T cells, particularly CD4 T cells, and their production of IFNg (Dobbs et al., 2005; Milligan et al., 1998; Morrison, 2008). More recent studies show that recruiting additional CD8 T cells into the vaginal tissue before infection can enhance protection against HSV-2 (Mackay et al., 2012; Shin and Iwasaki, 2012; Shin et al., 2016).

It remains unknown whether vaccine-induced CD8 T cell responses in the cervicovaginal tissue can mediate sterilizing protective immunity against vaginal HSV-2 infection without the contributions of HSV-specific CD4 T cell responses, and past studies of this question have focused on HSV-1 infection of the skin (Orr et al., 2007) or found only modest levels of protection (Gierynska et al., 2002; Mackay et al., 2012; Muller et al., 2008). However, human studies and mathematical modeling of human data provide strong evidence for the role of CD8 T cells in controlling genital HSV-2 reactivations in the context of chronic infection. HSV-specific CD4 and CD8 Trm infiltrate and persist at sites of past HSV-2 infection (Peng et al., 2012; Zhu et al., 2007; 2009), where they exhibit antiviral and cytotoxic signatures (Zhu et al., 2013) and may be associated with protection against lesion development and future reactivation (Koelle et al., 1998). Indeed, most reactivation events are controlled prior to the development of symptoms (Schiffer et al., 2009), demonstrating that rapid and effective immune responses commonly suppress viral replication before ulcer formation. Mathematical modeling of these and other data has suggested that CD8 T cells in these sites may represent a correlate of protection (Schiffer et al., 2010), and most likely mediate this protection through the recruitment of immune effector cells from the circulation (Roychoudhury et al., 2020).

1.8 Thesis Goals

This thesis seeks to explore the unique features and functions of T cells in the female genital skin and mucosa. Through the use of mouse models and human studies, this thesis will add to the body of knowledge about the genital T cell compartment's role in immunologic protection against HSV-2. The results presented here will have implications for HSV-2 vaccine development. In addition, these findings will improve our overall understanding of T cell immunity in the female genital tract, a site of initial infection for many pathogens of global and public health significance.

Using multiple mouse models as well as direct *ex vivo* studies of human vaginal mucosa, Chapter II will explore the mechanism by which immunization impacts vaginal Trm phenotype, and link this phenotype to Trm longevity and protective function. Chapter III will describe a human study in which genital skin biopsies were used to examine the size and phenotype of the genital Trm compartment in HSV-2-negative and HSV-2-positive women, and characterize how this cellular population reacts during an HSV-2 lesion event. Chapter IV will describe the limitations of these data, suggest potential future directions of research, and explore the broader implications of these findings.

Chapter 2. Cervicovaginal tissue residence imparts a distinct differentiation program upon memory CD8 T cells

Tissue-resident memory CD8 T cells (CD8 Trm) are critical for maintaining barrier immunity. CD8 Trm have been mainly studied in the skin and gut with recent studies suggesting that the signals that control tissue-residence and phenotype are highly tissue-dependent. We examined the T cell compartment in healthy human cervicovaginal tissue (CVT) and found that most CD8 T cells were granzyme B+ and T cell factor 1- (TCF-1-). To address if this phenotype is driven by CVT tissue-residence, we used a mouse model to control for environmental factors. Using localized and systemic infection models, we found that CD8 Trm in the mouse CVT gradually acquired a granzyme B+, TCF-1- phenotype as seen in human CVT. In contrast to CD8 Trm in the gut, these CD8 Trm were not stably maintained regardless of the initial infection route, which led to reductions in local immunity. Our data show that residence in the CVT is sufficient to progressively shape the size and function of its CD8 Trm compartment.

2.1 Introduction

After infection or immunization, naive CD8 T cells differentiate into several major populations of memory T cells with distinct functions and trafficking patterns. Tissue-resident memory CD8 T cells (CD8 Trm) are a major subset defined by the fact that they do not recirculate via blood and lymph, instead remaining in the tissue sites where they were initially seeded by effector T cells during the primary response (Gebhardt et al., 2009; Masopust et al., 2010; Masopust and Soerens, 2019; Szabo et al., 2019b; Wakim et al., 2008). Because of their privileged location, CD8 Trm are uniquely poised to respond to subsequent encounters with their cognate antigen by directly killing infected cells (Casey et al., 2012; Steinbach et al., 2016), proliferating

to boost the local CD8 T cell pool (Beura et al., 2018a; Park et al., 2018), and activating and recruiting additional immune cells from both resident and circulating populations (Ariotti et al., 2014; Schenkel et al., 2013).

Many factors influence the formation of Trm, including the route of priming, exposure to antigen and inflammation, and the type of tissue in which residency is established. For example, the presence of local antigen boosts the size and function of Trm populations within the matched tissue site (Davies et al., 2017; Gebhardt et al., 2009; T. N. Khan et al., 2016; Schenkel et al., 2013). Similarly, local inflammation enhances the number and function of Trm in an antigen-independent manner (Mackay et al., 2012; Shin and Iwasaki, 2012) and cytokine cues such as tumor growth factor beta (TGFb), IL-15, and CCL5 appear to directly regulate Trm formation and maintenance (Bergsbaken et al., 2017; Iijima and Iwasaki, 2014; Mackay et al., 2015; N. Zhang and Bevan, 2013).

Despite the importance of local exposure to antigen and inflammation in Trm development, strong evidence also exists that Trm can broadly seed distal tissues after both systemic and local immunization or infection. Systemic immunization and infection models are routinely used in mice to generate Trm populations across many body sites (Masopust et al., 2001; Milner et al., 2017; Pope et al., 2001; Reinhardt et al., 2001; Steinert et al., 2015). Likewise, localized infections such as herpes simplex virus (HSV) and vaccinia virus seed CD8 Trm at distal tissue sites, albeit to a much lower extent than at the primary site of infection (Davies et al., 2017; Jiang et al., 2012; T. N. Khan et al., 2016; Masopust et al., 2004). Together, these studies suggest that Trm can develop within diverse tissue sites across a range of levels of inflammation and antigen availability, and it is currently unclear how the combination of these variables may ultimately tune the characteristics of the resulting Trm population.

The tissue of residence itself also impacts the phenotype of CD8 Trm populations. CD8 Trm have been extensively studied in skin of both humans and mice, and it is now well-known that skin-resident CD8 T cells express the putative residency markers CD69 and integrin αE (CD103), respond to local HSV infection, and rely on tumor growth factor beta (TGF-β) and IL-15 signals for their development (Gebhardt et al., 2009; Mackay et al., 2013; Watanabe et al., 2015; Zhu et al., 2013). Many reports suggest that CD8 Trm in other organs may differ in their phenotype or developmental requirements. For example, it is well-documented that CD69 and CD103 are not necessarily expressed by Trm in the uterus or pancreas (Steinert et al., 2015) but may be required by Trm in other organs such as the salivary gland, lung, or kidney (Hofmann and Pircher, 2011; Takamura et al., 2016; Walsh et al., 2019). Likewise, some organs such as the small intestine maintain Trm that constitutively express granzyme B in the absence of antigen or re-challenge (Casey et al., 2012; Kim et al., 1997), a phenotype which has not been observed as robustly in other tissue sites, including the lung and uterus (Beura et al., 2019; Piet et al., 2011). It remains an open question whether CD8 Trm phenotype is inextricably linked to tissue of residence, or whether alterations to the priming method or the inflammatory milieu within a tissue could elicit features such as constitutive granzyme B expression in tissue sites where they are not normally observed.

Mucosal barrier tissues contain large populations of CD8 Trm, and pathogens that infect mucosal barrier surfaces represent a significant global health burden. Specifically, the study of CD8 T cell memory within the cervicovaginal tissue (CVT) has broad importance in understanding the pathogenesis and vaccinology of sexually-transmitted infections. Compared to memory CD8 T cells in the upper female reproductive tract and other mucosal and lymphoid tissues, the phenotypic and functional characteristics of memory CD8 T cells in the CVT remain relatively understudied. We recently reported that the CD8 T cell compartment in human CVT includes a

subset of CD8 T cells that robustly express granzyme B (Pattacini et al., 2019). It is unclear to what extent the distinctive features we observed in human CVT may be driven by recurring local infections, exposure to inflammatory cues, or a result of signals intrinsically associated with the cervicovaginal microenvironment.

Here, we report that CD8 T cells isolated from healthy human CVT lacked expression of the memory-associated transcription factor TCF-1 and resembled effector or terminally-differentiated cells rather than a self-renewing memory T cell population. To determine if this characteristic was driven by residence in the tissue itself or was a consequence of local exposure to antigenic insults or inflammation in human CVT, we utilized multiple immunization strategies to assess memory CD8 T cell differentiation and maintenance in the mouse CVT. We found that memory CD8 T cells in the mouse CVT at later memory timepoints closely resembled the CD8 T cells found in human CVT regardless of the initial immunization route and early phenotypic differences. Memory CD8 T cell numbers were stably maintained in the periphery and gut, but gradually declined in the CVT over five months post-immunization, which was associated with a delay in viral control upon HSV vaginal challenge. We conclude that residence in the CVT is sufficient to alter the canonical differentiation and maintenance program of memory CD8 T cells.

2.2 Results

2.2.1 *Healthy human cervicovaginal tissue contains CD8 T cells that express granzyme B and lack TCF-1*

In order to study the phenotypic profile of CD8 T cells within human CVT from healthy women without any genital infections, we isolated lymphocytes from human vaginal biopsies via enzymatic digestion (Figure 2.1A) and observed that the majority of memory T cells in this tissue site expressed the residency markers CD69 and CD103 (Figure 2.2A-B). A large proportion of

these CD8 T cells expressed granzyme B ex vivo (Figure 2.2C) and the majority of granzyme B+ cells co-expressed CD103 (Figure 2.2D), suggesting that they were potentially resident within the tissue rather than transient inflammatory cells. We observed that the memory CD8 T cell compartment in human CVT lacked expression of TCF-1 (Figure 2.2E), a transcription factor associated with self-renewal potential in CD8 T cells (Zhou et al., 2010). These data indicated that human memory CD8 T cells within the CVT may possess functional and proliferative characteristics distinct from such cells in other tissue locations.

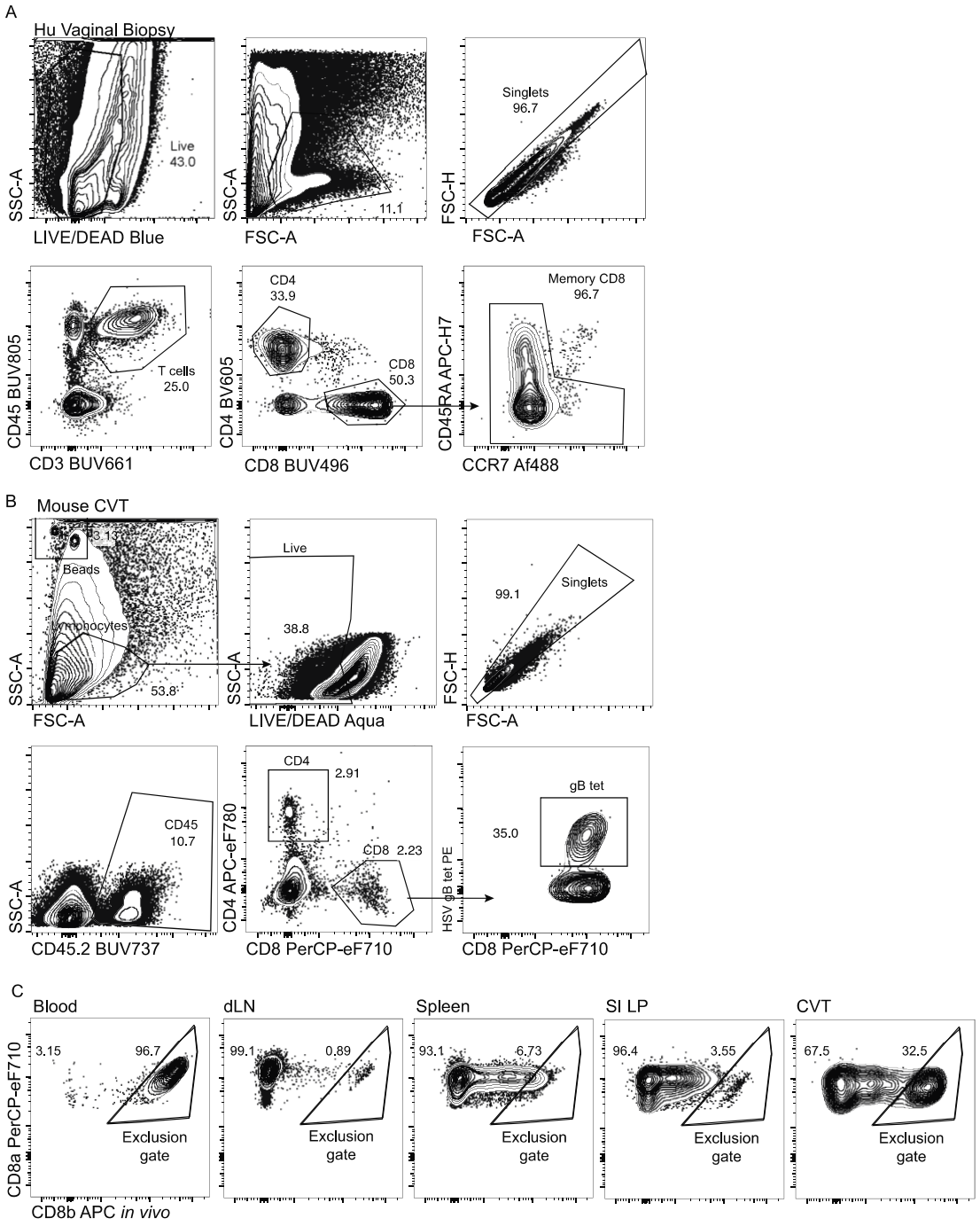

Figure 2.1. Example of gating strategies used to identify CD8 T cells in human and mouse CVT

(A) Representative example of T cell gating strategy from vaginal biopsies from one study participant. (B) Representative example from CVT of mouse sacrificed 1mo after LM-gB immunization. (C) Comparison of intravascular staining using CD8b-APC in blood, dLN, spleen, SI LP, and CVT. Representative staining examples from one mouse sacrificed 5mo after LM-gB immunization.

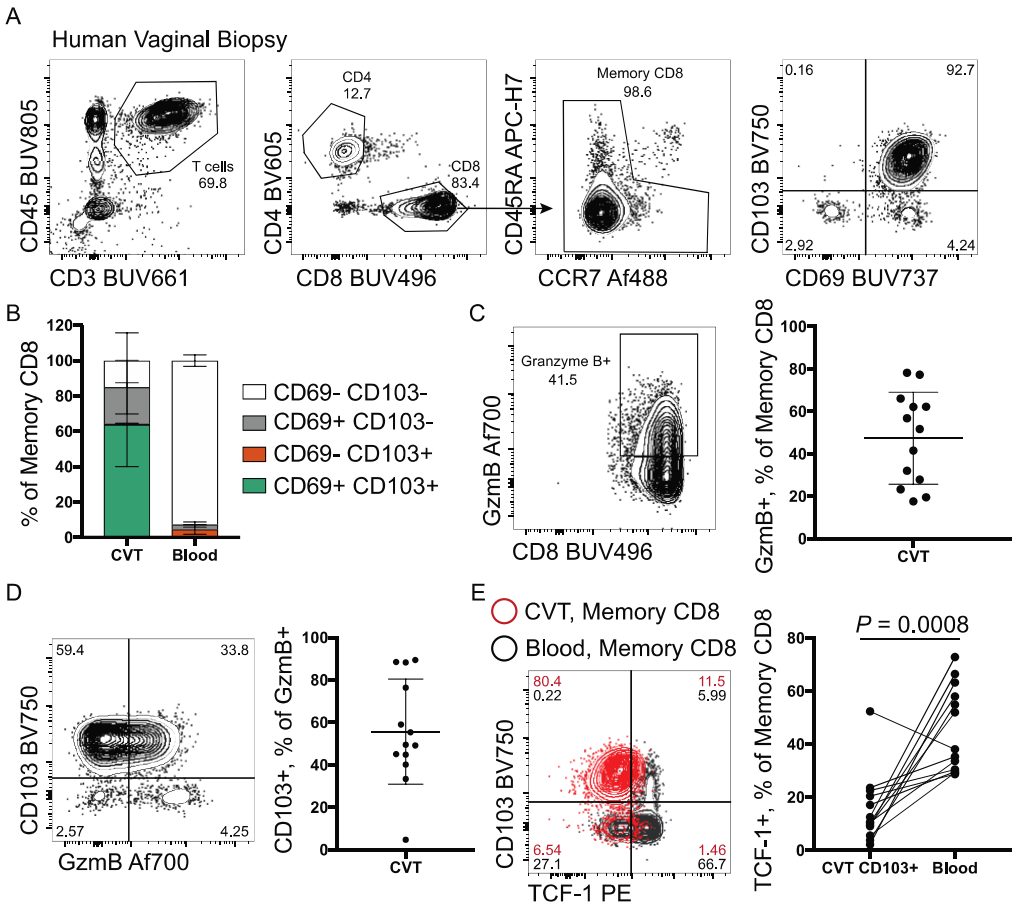

Figure 2.2. Human cervicovaginal-resident CD8 T cells lack expression of TCF-1

(A) Gating strategy to identify memory CD8 T cells in blood and CVT from healthy STI and HSV-2-negative women. (B) Relative abundance of CD69 and CD103 subsets in blood and CVT. (C) Representative flow plot and abundance of granzyme B+ cells within the memory CD8 population in blood and CVT. (D) Representative flow plot and abundance of CD103 and granzyme B co-expression in blood and CVT. (E) Representative TCF-1 staining and TCF-1+ frequency from CD103+ subset of CD8 T cells from the CVT compared to memory CD8 T cells in matched blood. Flow plots from A and C-E are from one representative participant. Data from B-E represent pooled results from study participants (n=13). Each dot in C-D represents an individual participant. Each line in E represents a participant and each dot represents an individual sample. Error bars represent mean ± SD. P-value in E was calculated via paired t-test. Exact P-values are given for all comparisons.

2.2.2 *Vaginal immunization in mice results in a CD8 T cell compartment that resembles that of human CVT*

We next wanted to address if this human CVT CD8 T cell phenotype was attributable to ongoing basal levels of antigenic insult or inflammation in the tissue, or might represent more generalized characteristics of CD8 T cells in CVT. To stringently control for external environmental factors, we sought to generate a comparable population of memory CD8 T cells in mouse CVT. We adoptively transferred naïve transgenic CD8 T cells specific to the glycoprotein B SSIEFARL epitope from HSV (gBT-I cells) and primed these cells by vaginal infection with non-lethal thymidine-kinase deficient HSV-2 (HSV-2 TK-) (Figure 2.3A). One month following infection, HSV gB tetramer+ CD8 T cells in the CVT expressed CD69, CD103, and granzyme B in similar proportions to those observed in human CVT (Figure 2.3B-C). In addition, we found that these CD8 T cells largely lacked expression of TCF-1, especially compared to gB tetramer+ cells in the vaginal-draining lymph nodes (dLN) (Figure 2.3D). Given that these mice had received a vaginal infection, we wondered if this granzyme B+ TCF-1lo phenotype might be a result of ongoing inflammatory responses in the CVT. Indeed, analysis of hematoxylin and eosin-stained sections of CVT from infected mice revealed that clusters of inflammatory cells remained in the CVT lamina propria as long as 22d after infection (Figure 2.3E), suggesting that inflammation was not resolved by this timepoint. Thus, our data suggested that prolonged local tissue inflammation could be driving this distinct memory CD8 T cell phenotype within the CVT.

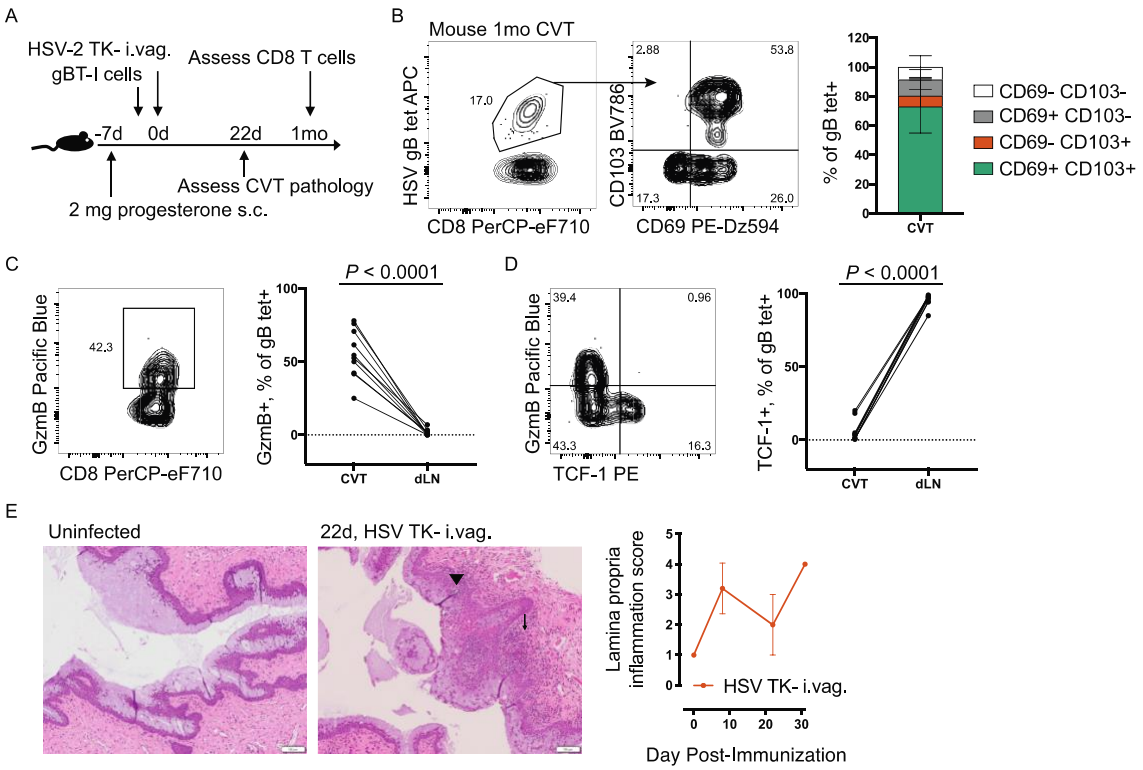

Figure 2.3. Mouse cervicovaginal-resident CD8 T cells induced by vaginal immunization mirror the phenotype observed in human samples and lack TCF-1

(A) Experiment schematic to induce CVT-resident CD8 T cells in mice via vaginal infection with 1.88 × 105 PFU of thymidine kinase-negative HSV-2 186 kpn. (B) HSV-specific cells were identified in CVT via staining with an H-2Kb SSIEFARL tetramer conjugated to APC. Left, representative flow plots gated on CD8 T cells. Right, relative abundance of CD69 and CD103 subsets within gB tetramer+ CD8 T cells in CVT. (C) Representative flow plot and abundance of granzyme B+ cells within gB tetramer+ CD8 T cells in CVT and CVT-draining lymph nodes (dLN). (D) Representative TCF-1 staining and TCF-1+ frequency within gB tetramer+ CD8 T cells in CVT and dLN. (E) Representative images of hematoxylin and eosin-stained sections of CVT at 10X objective and average lamina propria inflammation scores. Arrow indicates coalescing clusters of inflammatory cells with follicular organization. Arrowhead indicates clusters of inflammatory cells within the mucosal epithelium. Flow plots and images from B-E are from one representative mouse. Data from B-D represent pooled results from 2 independent experiments. Each dot in E represents inflammation score averaged from 1-3 mice per timepoint. Error bars represent mean ± SD. P-value in C was calculated via paired t-test. Exact P-values are given for all comparisons.

2.2.3 *Systemic immunization with LM-gB elicits stable HSV-specific CD8 T cell memory in secondary lymphoid organs and the small intestine, but not the CVT*

To determine if prolonged tissue inflammation drives this CVT memory T cell phenotype, we used a systemic immunization approach to prime gBT-I cells in the absence of significant vaginal inflammation. We generated a recombinant strain of Listeria monocytogenes that expresses the HSV-derived SSIEFARL peptide (LM-gB) and immunized mice intravenously (Figure 2.4A). We confirmed that this immunization strategy did not result in detectable levels of vaginal inflammation compared to uninfected mice, and caused significantly less inflammation than observed in mice vaginally immunized with HSV-2 TK- (Figure 2.4B). In addition, immunized mice mounted a robust systemic HSV-specific CD8 T cell response, with gB-specific T cells making up an average of 36.4% of CD8 T cells in the blood by 1wk after immunization (Figure 2.4C). At 1mo after immunization, we could identify these cells in multiple body sites including the CVT and vaginal-draining lymph nodes (Figure 2.1B and Figure 2.4D). Again, CD103+ gB-specific CD8 T cells in the CVT lacked expression of TCF-1, replicating the phenotype observed in both human vaginal tissue and CVT of vaginally-immunized mice (Figure 2.4E), despite the lack of tissue inflammation.

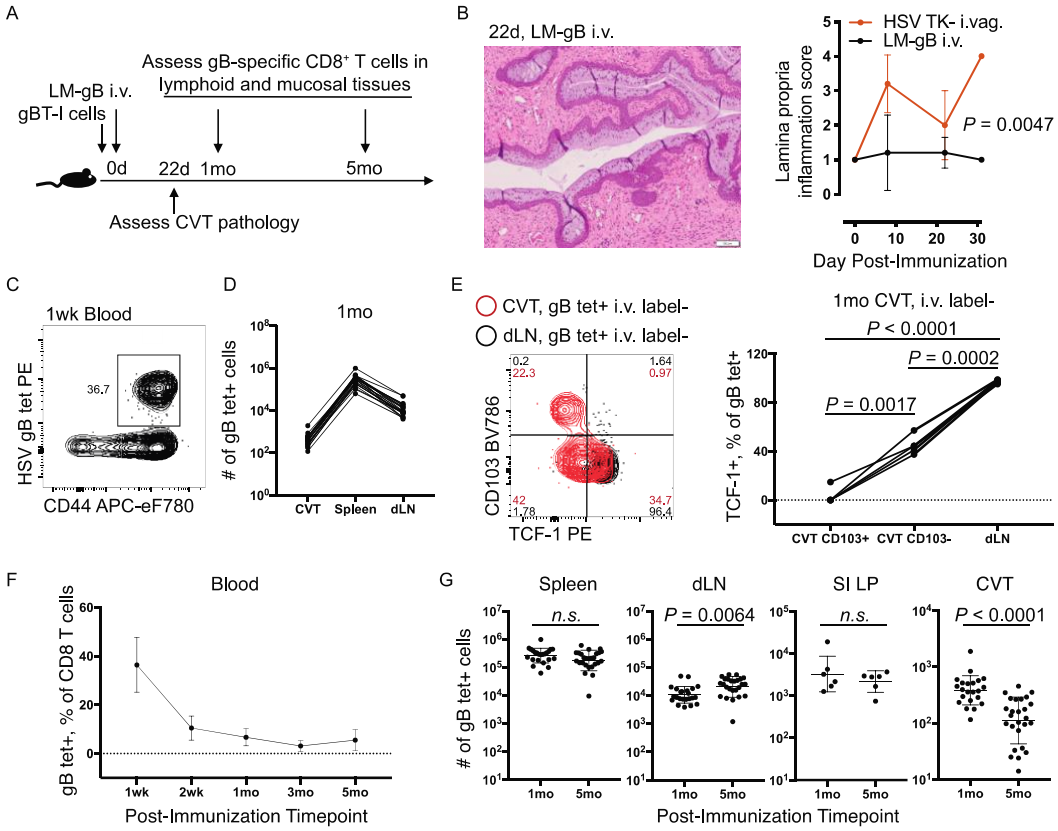

Figure 2.4. The cervicovaginal CD8 T cell memory compartment of LM-gB-immunized mice is poorly maintained after immunization

(A) Schematic of experiment to assess phenotype and maintenance of HSV-specific T cell compartment of mice immunized with 4000 CFU LM-gB i.v. (B) Representative image of hematoxylin and eosin-stained sections of CVT at 10X objective and average lamina propria inflammation scores. (C) Representative flow plot gated on CD8 T cells from blood collected 1wk after LM-gB immunization. (D) gB tetramer+ CD8 T cell abundance in spleen, dLN, and CVT 1mo after immunization. (E) Representative TCF-1 staining and quantification of TCF-1 expression within CD103+ and CD103− subsets of gB tetramer+ CD8 T cells in CVT and dLN. (F-G) Relative abundance or number of HSV-specific CD8 T cells in blood, spleen, dLN, small intestine lamina propria (SI LP), and CVT 1mo and 5mo after LM-gB immunization. Images and flow plots from B, C, and E are from one representative mouse. Data in D-G are pooled from 2-5 independent experiments. Each dot in B represents inflammation score averaged from 2-3 mice per timepoint. Each dot in F represents the mean value of 20 mice from a representative experiment. Each dot in D, E, and G represents an individual sample, with lines connecting samples from the same mice. Error bars represent mean ± SD. P-value in B was calculated via t-test comparing the two areas under the curves. P-values in E were calculated with repeated measures ANOVA with Greenhouse-Geisser correction. P-values in G were calculated via t-test using log10-transformed values. Exact P-values are given for all values < 0.05.

Given this evidence that memory CD8 T cells in CVT lack significant expression of TCF-1 regardless of immunization route, we next asked whether this memory compartment was deficient in self-renewal and would undergo gradual decay. While the frequency of memory gB-specific CD8 T cells remained relatively stable in the blood following contraction (Figure 2.4F), and the number of gB-specific CD8 T cells in the spleen, CVT-draining lymph nodes, and small intestine lamina propria (SI LP) beyond 1mo after immunization was stable, this population underwent a threefold loss in number within the CVT, with some mice having very few gB-specific CD8 T cells remaining in the CVT by 5 months post-immunization (Figure 2.4G). Interestingly, gB-specific T cells in the SI LP also lacked expression of TCF-1 (Figure 2.5A), suggesting that memory T cells induced by the same immunization may have different requirements for self-renewal.

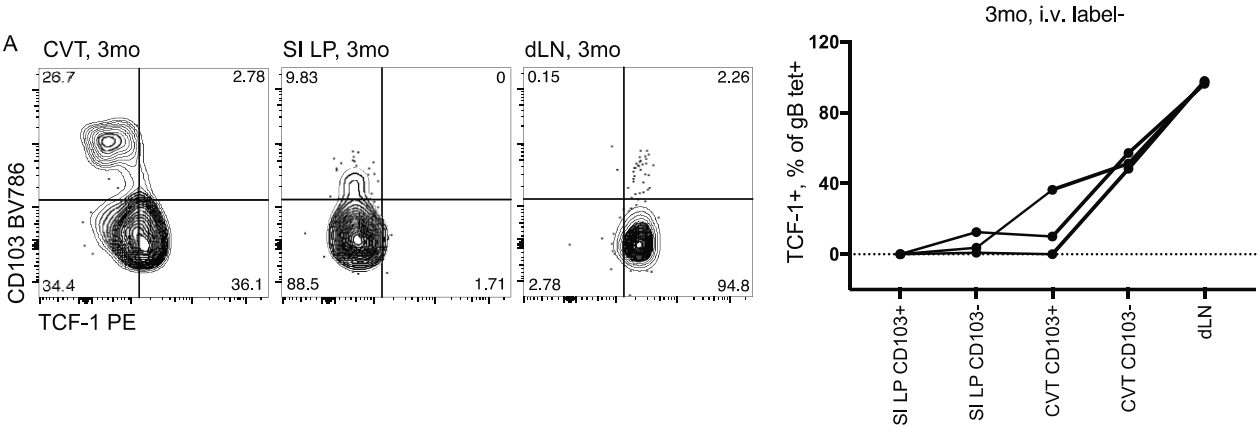

Figure 2.5. Comparison of TCF-1 protein expression in HSV-specific CD8 T cells from mouse CVT and SI LP

(A) Protein expression of CD103 and TCF-1 among HSV-specific i.v. label- CD8 T cells in CVT, SI LP, and dLN 3mo after LM-gB immunization. Flow plots represent concatenated data from 3 mice. (B) TCF-1+ frequency among CD103+ or CD103- subsets of HSV-specific CD8 T cells from CVT and SI LP compared to LN 3mo after immunization.

2.2.4 *Loss of memory CD8 T cells in the CVT occurs mainly among the CD69- CD103- subset*

Given that gB-specific CD8 T cells in the CVT continued to decline in number after T cell contraction had concluded in other tissues, we next examined the expression of canonical markers of tissue residency and effector function. To reliably determine what proportion of the gB-specific CD8 T cells in each organ were located within the tissue, we performed intravascular (i.v.) antibody labeling (Galkina et al., 2005) (Figure 2.1C). We found that an average of 85.6% of the gB-specific CD8 T cells in the CVT were protected from i.v. labeling by 1mo after immunization, similar to the fraction within the small intestine lamina propria (Figure 2.6A). The proportion of CVT gB-specific CD8 T cells that were protected from i.v. labeling remained stable across timepoints, suggesting that the smaller population of T cells remaining at late timepoints continued to be composed of cells that were mostly located within the tissue parenchyma (Figure 2.6A).

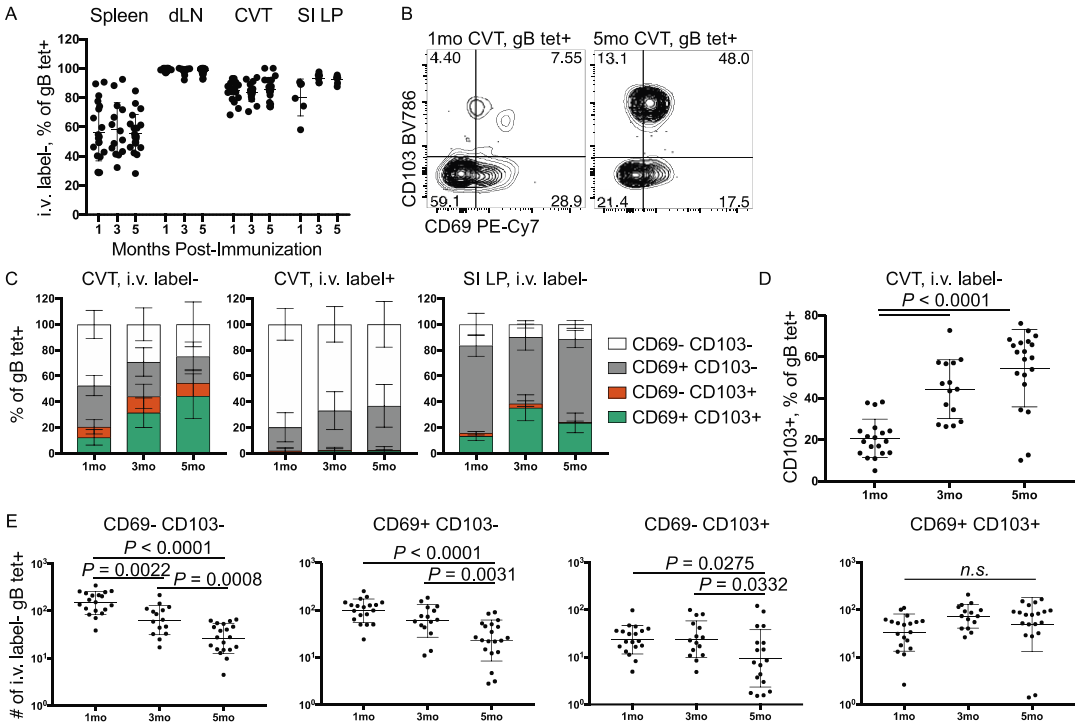

Figure 2.6. Most HSV-specific CD8 T cells remaining in the CVT by five months after immunization express CD69 and CD103

(A) Percentage of i.v. label− HSV-specific CD8 T cells in different lymphoid and non-lymphoid tissues at 1mo, 3mo, and 5mo after immunization based on gating shown in Fig. S1C. (B) Representative staining showing CD69 and CD103 expression on HSV-specific CD8 T cells in the CVT 1mo and 5mo after immunization. (C) Stacked bar plots showing relative abundance of each of four subsets of HSV-specific CD8 T cells based on CD69 and CD103 expression in the i.v. label+ and i.v. label− fractions of the CVT and SI LP. (D) CD103 expression on i.v. label− gB tetramer+ CD8 T cells in the CVT 1mo, 3mo, and 5mo after immunization. (E) Total i.v. label− count of each of four subsets of HSV-specific CD8 T cells based on CD69 and CD103 expression 1mo, 3mo, and 5mo after immunization. Data in A, C, D, and E are pooled from at least 2 independent experiments per timepoint. Data in B are from one representative experiment. Each dot in A, D, and E represents an individual mouse. Error bars represent mean ± SD. P-values in D and E were calculated via ordinary one-way ANOVA with Tukey's post-hoc test using log10-transformed values for numbers and untransformed values for percentages. Exact P-values are given for all values <0.05.

We next assessed whether gB-specific CD8 T cells expressed CD69 and CD103. We found that these markers were mostly non-expressed on gB-specific CD8 T cells at the earliest memory timepoint. However, the average frequency of CD103+ cells within the gB-specific CD8 T cell subset increased from 20.6% to 54.4% between 1mo and 5mo after LM-gB immunization (Figure

2.6B-D). While the i.v.-label+ fraction of gB-specific CD8 T cells in the CVT remained almost completely CD103- for the entire duration of the experiment, the shift from a predominantly CD103- population to a predominantly CD103+ population was especially clear within the i.v.-label- fraction of CVT gB-specific CD8 T cells (Figure 2.6C-D) and was mainly attributable to a relative loss of CD103- cells from the CVT over time (Figure 2.6E). Conversely, the equivalent i.v. label- fraction of gB-specific CD8 T cells in another mucosal tissue, the small intestine lamina propria (SI LP), did not undergo a population-level shift towards becoming CD103+ (Figure 2.6C). Finally, the total CD69+ CD103+ population in the CVT was stably maintained over time (Figure 2.6E), which could be due to conversion of other subsets to a CD69+ CD103+ phenotype or the ability to self-renew at a rate sufficient for stable maintenance.

2.2.5 *CVT memory CD8 T cells induced by systemic immunization gradually acquire expression of granzyme B*

We next wanted to determine if systemic immunization resulted in constitutive granzyme B expression by CD8 T cells in the CVT, as we observed after vaginal immunization (Figure 2.3) and as described for tissue-resident CD8 T cells in the SI LP, which express granzyme B in the absence of antigen re-exposure (Casey et al., 2012; Masopust et al., 2006). Few gB-specific CD8 T cells in the CVT expressed granzyme B at 1mo after LM-gB immunization, but the granzyme B+ population substantially increased in both number and frequency at later timepoints (Figure 2.7A-B). In contrast, a subset of SI LP gB-specific CD8 T cells expressed granzyme B by 1mo after immunization, and expression remained stable thereafter (Figure 2.7A). As expected, granzyme B expression was rarely observed among gB-specific CD8 T cells in the spleen or dLN, regardless of the memory timepoint (Figure 2.7A).

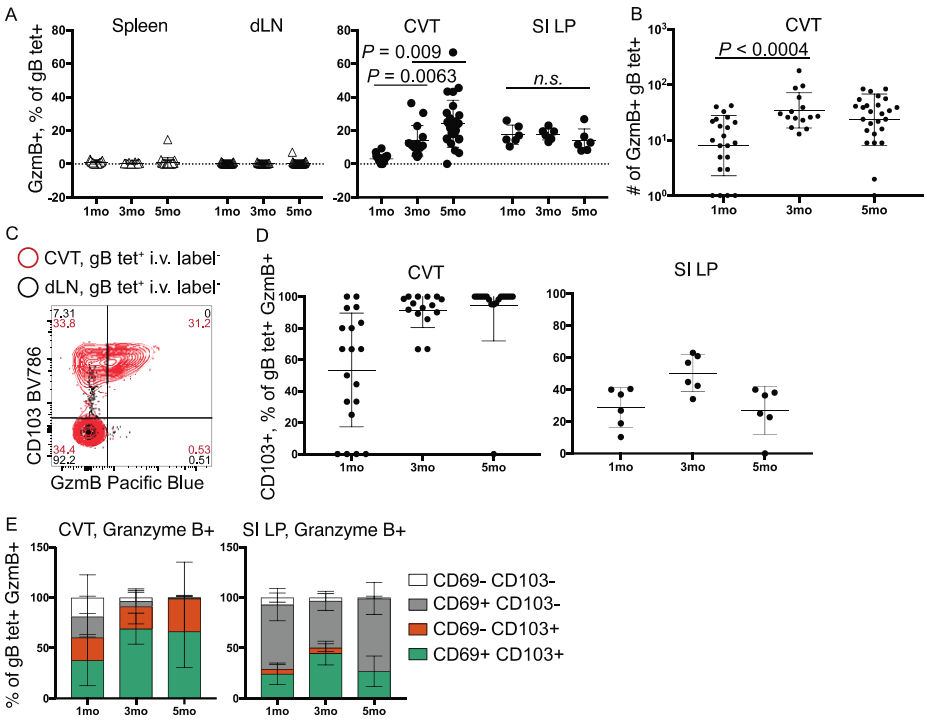

Figure 2.7. After systemic immunization, cervicovaginal-resident CD8 T cells progressively upregulate granzyme B

(A) Frequency of granzyme B+ cells among HSV-specific CD8 T cells in spleen, dLN, CVT, and SI LP at 1mo, 3mo, and 5mo post-immunization. (B) Number of granzyme B-expressing cells among HSV-specific CD8 T cells at timepoints after LM-gB immunization. (C) Concatenated flow files from 10 mice showing CD103 and granzyme B co-expression among the HSV-specific CD8b i.v. label− populations in the CVT and dLN 5mo after LM-gB immunization. (D-E) Frequency of CD69 and CD103 expression within granzyme B+ HSV-specific CD8 T cells in the CVT and SI LP 1mo, 3mo, and 5mo after immunization. Data in A-E are pooled from at least 2 independent experiments. Each dot or triangle in A, B, and D represents an individual mouse. Error bars represent mean ± SD. P-values in A and B were calculated via ordinary one-way ANOVA with Tukey's post-hoc test using log10-transformed values for numbers and untransformed values for percentages. Exact P-values are given for all values <0.05.

We next examined the phenotypic identity of these granzyme B-expressing cells. By 3mo post-immunization, granzyme B+ gB-specific CD8 T cells in the CVT were almost exclusively CD103+ (Figure 2.7C-E), resembling our results in human samples (Figure 2.2D) and suggesting that these two dynamically-expressed markers were demarcating the same cellular population. By contrast, the granzyme B+ population in the SI LP comprised both CD103+ and CD103- cells

(Figure 2.7D-E). These data suggest that immunization-induced gB-specific memory CD8 T cells differentiate asynchronously in the CVT relative to the gut mucosa or peripheral lymphoid organs.

2.2.6 *Gene expression analysis of single HSV-specific CD8 T cells from the blood or CVT reveals transcriptional differences between early and late memory timepoints*

To assess differences in the CVT gB-specific CD8 T cell compartment at early and late memory timepoints in a more unbiased manner, we FACS-sorted gB tetramer+ and gB tetramer- cells from the blood and CVT at each timepoint for single-cell RNA sequencing (scRNAseq) using the 10x Genomics platform (Figure 2.8A).

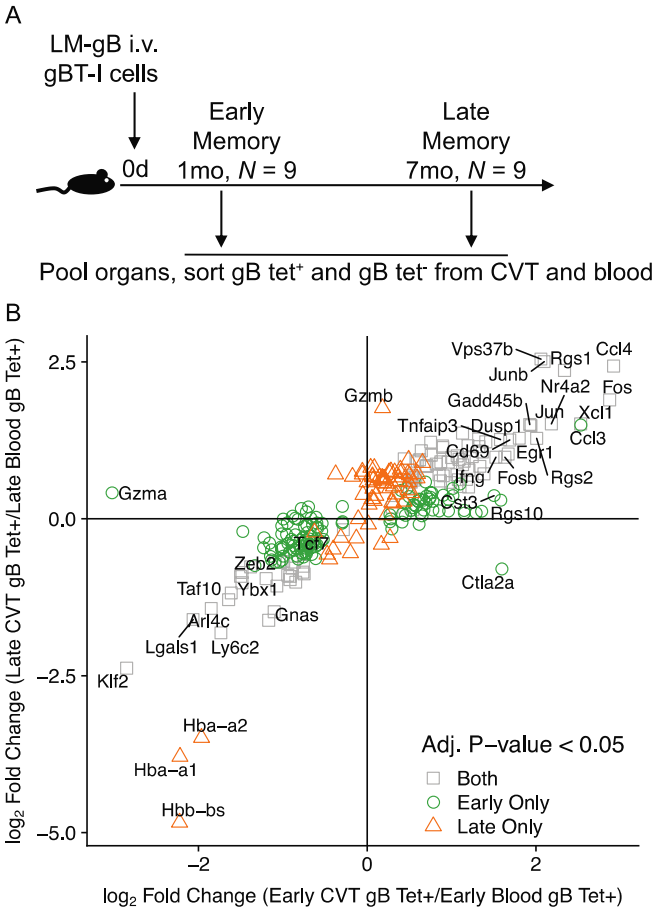

Figure 2.8. LM-gB induced memory CD8 T cells in blood and cervicovaginal tissue undergo transcriptional changes between 1mo and 5mo after immunization.

(A) Schematic of experimental design to evaluate single cell transcriptomes of HSV-specific and non-specific CD8 T cells via 10x methodology. (B) Scatter plot indicating log_2-fold changes for all genes that were significantly up- or down-regulated by gB tetramer+ CD8 T cells in CVT relative to blood at Early and Late Memory timepoints. All genes that were detected in at least 20% of cells from any one population and had adjusted P-values <0.05 for any comparison were included. Genes with a log_2 fold change >1.5 and additional genes of interest (*Tcf7*) are labeled.

Among blood-derived CD8 T cells, we observed an increase in CD62L (SELL) and IL7Ra expression in Late Memory gB tetramer+ cells compared to the Early Memory timepoint, while the early timepoint had higher expression of KLRG1 in line with the shift from effector to central memory that occurs over time (data not shown). These changes were also reflected in the CVT. In fact, when we generated a scatter plot of genes that were differentially expressed in the CVT vs.

the blood at each timepoint, we found that differences between the blood and the CVT were highly consistent over time (Figure 2.8B), with granzyme B emerging as one of the only genes uniquely upregulated in Late Memory CVT vs. blood.

We also looked directly for transcriptional subpopulations among all CD8 T cells isolated from the CVT. Uniform manifold approximation and projection (UMAP) and graph-based clustering showed that gB tetramer+ cells mostly clustered together regardless of memory timepoint (Cluster 2). This cluster was transcriptionally distinct from gB tetramer- cells (Cluster 1), which mainly derived from the Late Memory timepoint. We examined the genes that identified each of these clusters (Figure 2.9D). In line with our flow cytometry results, we found that Cluster 2 expressed *Gzmb* and lacked *Tcf7*, the genes encoding granzyme B and TCF-1. Cluster 2 was also defined by expression of *Ifng*, *Ccl4*, *Ccl5*, *Cxcr6*, and others. Unfortunately, none of these genes were obvious candidates to explain why this compartment declined numerically and changed phenotypically. Overall, this experiment did not yield an obvious answer to the question of why CVT memory T cells underwent progressive differentiation, possibly due to the limitations of scRNAseq, which are both biological (including the disconnect between transcript abundance and protein expression) and technical (detection is limited to the top ~10% of expressed genes).

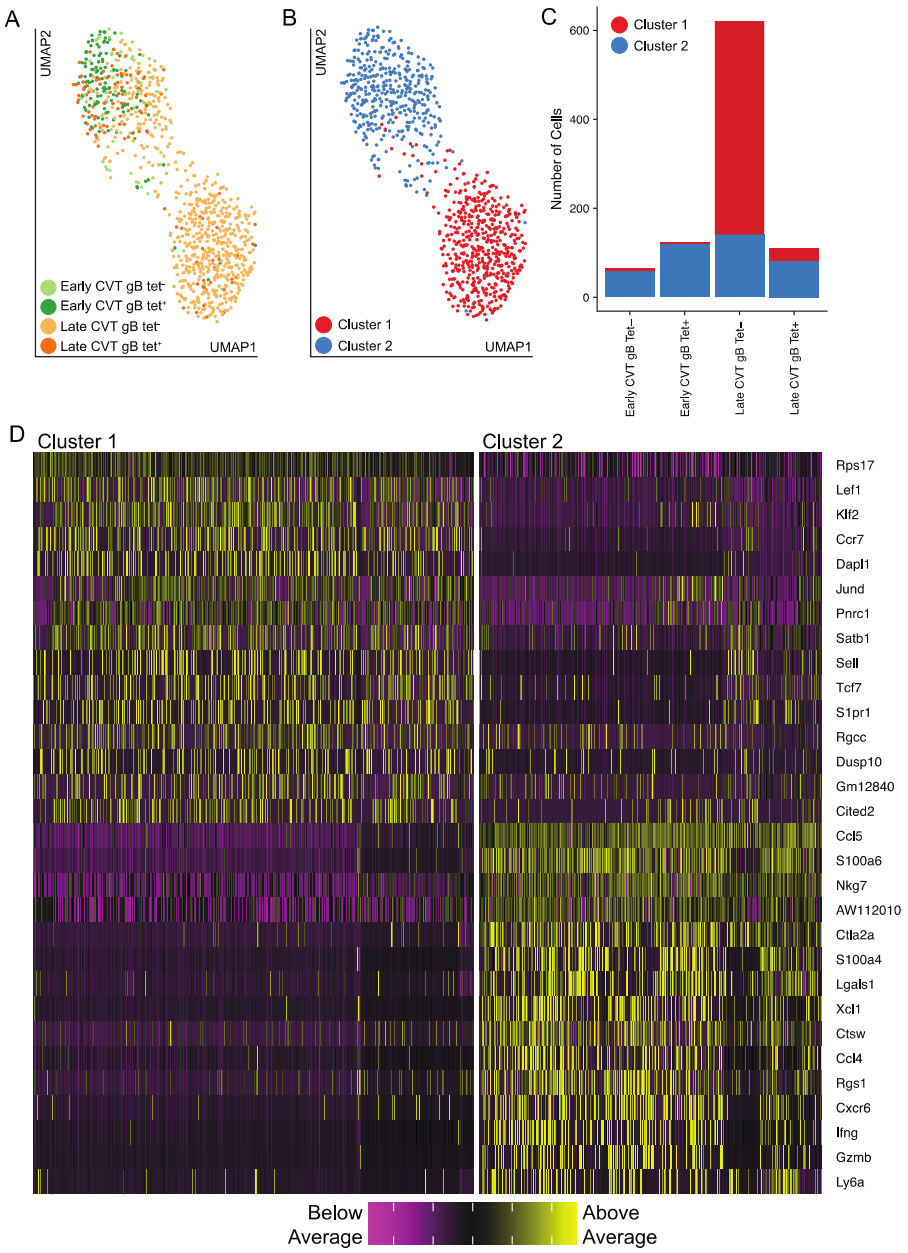

Figure 2.9. Transcriptionally distinct subsets of CD8 T cells populate the mouse CVT.

(A) Uniform manifold approximation and projection (UMAP) plots of transcriptional data from all CVT-derived HSV-specific and non-specific CD8 T cells colored by sample of origin. Graph-based clustering was used to separate CVT-derived CD8 T cells into three clusters based on gene expression patterns. (B) UMAP plot with cells colored by cluster membership. (C) Bar plots showing relative abundance of cells from each cluster within each sample. (C) Heatmap of scaled expression level for top 15 genes from each cluster that were most uniquely expressed by that cluster relative to non-cluster cells (defined as genes expressed by at least 25% of cluster cells with positive loge-transformed fold changes >0.25).

2.2.7 *Numeric and phenotypic changes of HSV-specific memory T cells in the CVT lead to delayed protection against viral challenge*

Given the decline in the number of gB-specific CD8 T cells in the CVT combined with their shift towards CD103 and granzyme B expression, we next evaluated how these changes affected the immunoprotective response to vaginal HSV-2 challenge. Because LM-gB does not prime HSV-specific CD4 T or B cells responses, this immunization system allowed us to specifically assess the protective effect of CD8 T cells in the absence of other antigen-specific responses. We presumed that the most rapid antiviral responses would likely be mediated by tissue-resident gB-specific CD8 T cells, while circulating gB-specific CD8 T cells would contribute to later responses. We challenged immunized mice at either 1mo ("Early Memory") or 4mo ("Late Memory") after immunization (Figure 2.10A). A similar proportion of mice in each group survived lethal challenge (Figure 2.10B), demonstrating that gB-specific memory CD8 T cells were sufficient to confer protection against HSV-2 lethality in a subset of immunized mice. However, mice challenged earlier after immunization began to show evidence of faster viral clearance starting as early as 3.5d after challenge (Figure 2.10C) and underwent greater T cell expansion in the first 2.5d after challenge (Figure 2.10D). We hypothesized that this was due to quantitative and qualitative differences between the early and late CD8 T cell compartment in the CVT.

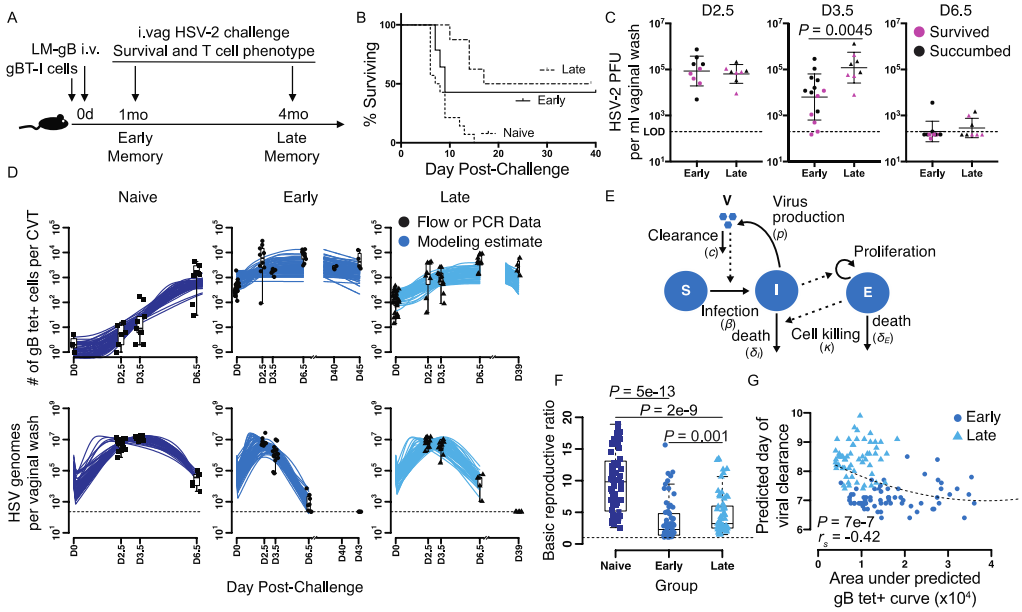

Figure 2.10. LM-gB immunized mice are protected against severe disease after lethal intravaginal challenge with HSV-2, but the efficacy of the immune response wanes over time

(A) Experiment schematic to compare protective efficacy of LM-gB immunization 1mo and 4mo after administration. (B) Survival after vaginal HSV-2 challenge of naive and LM-gB immunized mice. (C) Vaginal washes were collected after HSV-2 challenge and HSV-2 titer was determined by plaque assay. Pink dots represent mice that survived challenge, as shown in B. (D) Number of HSV-specific CD8 T cells (top) and HSV genomes (bottom) in the CVT with modeling estimates overlaid in blue. (E) Schematic of mathematical model relating CVT-infiltrating CD8 T cell immune response to HSV-2 viral expansion kinetics. S and I represent the number of susceptible and infected cells per CVT, respectively; E, the number of gB tet+ CD8 T cells per CVT; and V the number of HSV genomes per vaginal wash. (F) Model estimates of basic reproductive ratios (number of new cells infected by one infected cell when introduced into a pool of susceptible cells) for each group of mice. (G) Scatter plot of areas under curves (AUC) from D and corresponding predicted day of viral clearance. Dotted line represents a fitted line from quadratic regression. Data in B, C, and D are pooled from at least 2 independent experiments (n=8-14 per group in B). Each dot, square, or triangle in B, C, and D represents data from an individual mouse. Each dot, square, or triangle in F and G represents one model estimate. Error bars in C represent mean ± SD. P-values in C were calculated via unpaired t-test using log10-transformed values. P-values in F were calculated via pairwise-corrected Mann-Whitney's test using Bonferroni's correction. P- and rs-value in G were calculated via Spearman's rank correlation. Exact P-values are given for all values <0.05.

To better understand the relationship between CVT gB-specific CD8 T cells and viral control, we built upon our previously described approaches to model the acute T cell response to HSV-2 infection (Schiffer, 2013; Schiffer et al., 2010) and used the mathematical model in (2.1)

to characterize the different gB-specific T cell response kinetics between the early and late memory groups. In this model, susceptible cells in the CVT are infected by free HSV-2, allowing them to produce new virus or be killed by HSV-specific CD8 T cells (Figure 2.10E). We performed 100 model-fit rounds to all observations simultaneously for each group for experimentally-determined CVT gB-specific CD8 T cell counts and viral load and overlaid these predictions onto our experimental data (Figure 2.10D). In naive mice, gB-specific CD8 T cells were absent from or rare in the CVT at 0-3.5d after challenge but were abundant by 6.5d after challenge (Figure 2.10D, top left), corresponding with the entry of effector T cells into the CVT following primary infection. In mice challenged at either early or late timepoints, gB-specific CD8 T cells increased dramatically in number between 0d and 2.5d after HSV-2 infection, and HSV viral titer fell by 6.5 post-challenge (Figure 2.10D). The best model fits predicted that naive mice had the highest basic reproductive number, meaning that they had the highest number of new cellular infections predicted to derive from a single HSV-infected cell in a susceptible pool (Figure 2.10F). In addition, the greater areas under fitted gB tet+ T cell curves (shown in Figure 2.10D, top row) were associated with earlier viral clearance (Figure 2.10G). These data suggest that the numeric and phenotypic changes we observed within the CVT memory T cell compartment led to a decrease in the efficacy of the anti-viral response in the first days after challenge.

2.2.8 *Progressive differentiation by the memory CD8 T cell compartment in the CVT does not require new input of cells from circulation*

To determine if the CVT-specific changes in the memory T cell compartment occurred in a tissue-autonomous manner, we depleted Thy1.1+ gBT-I cells from the blood and lymphoid tissues of LM-gB immunized mice 1mo after immunization (Figure 2.11A), as previously described (Schenkel et al., 2013). This method depleted splenic and circulating gBT-I cells but left

the CVT gBT-I compartment intact (Figure 2.11B-C) and did not have a major impact on protection against vaginal challenge with HSV-2 (Figure 2.12A-B). By 3mo after depletion, we observed that a similar level of decay had occurred among the CVT gB-specific CD8 T cell population to that seen in undepleted mice between 1mo and 5mo after immunization (Figure 2.4G and Figure 2.11C). Similar to our previous results, we observed that this decay occurred mostly among the CD103- subset of T cells (Figure 2.11D-E) and that the persisting cells had upregulated granzyme B by 3mo after immunization (Figure 2.11F). Low TCF-1 expression remained stable from 1mo to 3mo after immunization but was reduced by gBT-I depletion, suggesting that the small TCF-1+ subset in the CVT of immunized mice was partially but not completely attributable to recirculating gBT-I cells (Figure 2.11G). These data indicate that the granzyme B+ subset of gB-specific cells stems from granzyme B- cells that were present in the tissue 1mo after immunization.

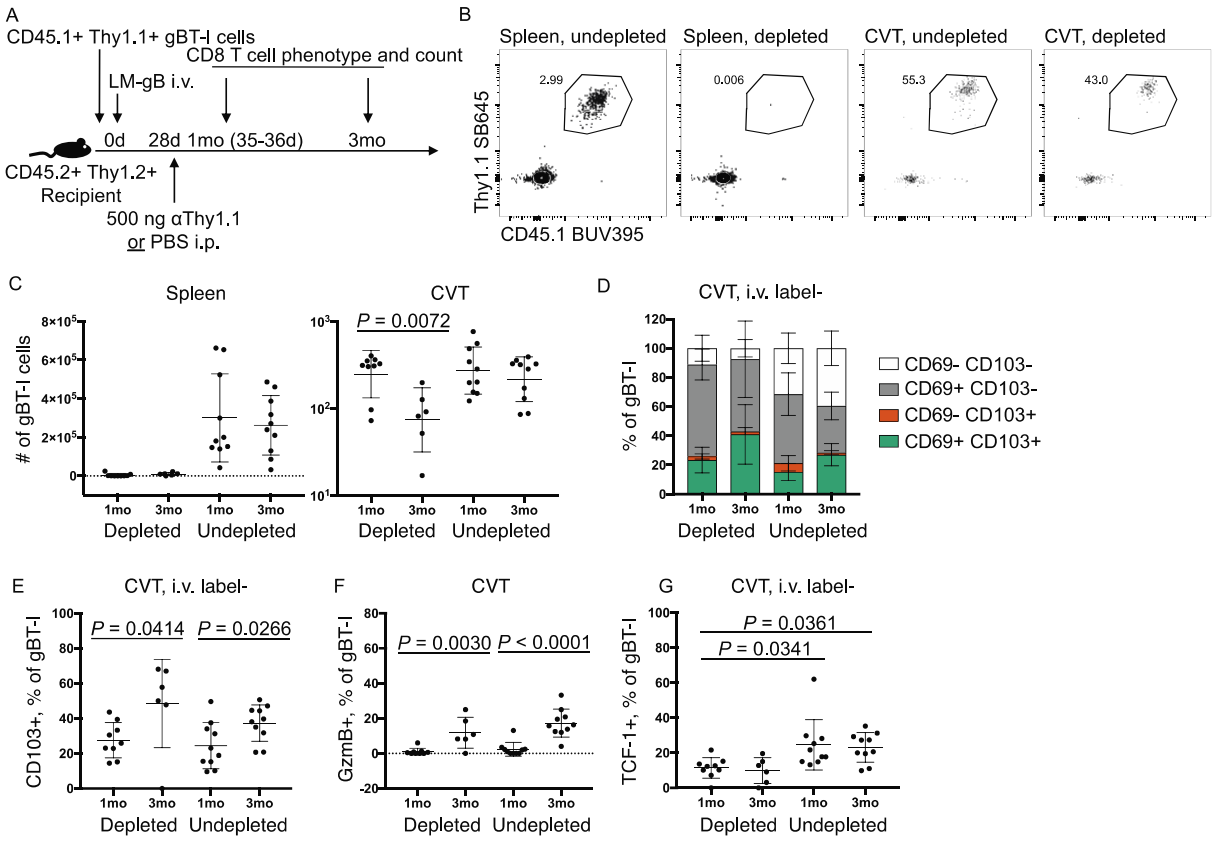

Figure 2.11. Differentiation and decay of the CVT-resident CD8 T cell compartment is tissue-intrinsic

(A) Schematic of experiment to test effect of depleting circulating gBT-I cells. (B) Example flow staining to identify gBT-I population within CD8 T cells from spleen and CVT of depleted or undepleted mice 1mo after immunization. (C) Number of gBT-I cells recovered from spleen or CVT of mice at indicated timepoints after immunization. (D-E) Relative abundance of CD69 and CD103 subsets within i.v. label− gBT-I cells in CVT at indicated timepoints. (F) Frequency of granzyme B expression among gBT-I cells. (G) Frequency of TCF-1 expression among i.v. label− gBT-I cells. Data in C-G are pooled from 2 independent experiments. Flow plots in B are derived from two representative mice. Each dot in C, E, F, and G represents an individual mouse. Error bars represent mean ± SD. P-values in C, E, and F were calculated via unpaired t-test comparing 1mo to 3mo within each depletion group. P-values in G were calculated via ordinary one-way ANOVA with Tukey's post-hoc test. Exact P-values are given for all values <0.05.

We also employed Thy1.1-based depletion of circulating gBT-I cells to test whether the increase of gB tetramer+ cells in the CVT after challenge (Figure 2.10) was primarily due to local proliferation or recruitment of circulating CD8 T cells. To address this question, we depleted circulating gBT-I cells 28d after LM-gB immunization, and challenged mice with wildtype HSV-

2 i.vag. 35d after immunization. We sacrificed groups of mice at 2.5d, 3.5d, and 6.5d after challenge to assess the number of gBT-I cells and non-gBT-I gB tetramer+ cells in the CVT (Figure 2.12C). We found that Thy1.1 depletion significantly reduced the number of gBT-I cells recovered from the CVT (ANOVA P-value < 0.0001), particularly at the 6.5d timepoint (P = 0.0097). These results indicate that the increase of CVT gBT-I cells observed in mice with intact circulating gBT-I cells is most likely due to a combination of recruitment and local proliferation. Interestingly, we also observed an increase in the number of endogenous gB tetramer+ cells in challenged gBT-I depleted animals, suggesting compensation by the endogenous memory population. The existence of this compensatory response suggests that the survival results among depleted mice in Figure 2.12B cannot be completely attributed to the CVT Trm compartment, since mice with depleted gBT-I retain some endogenous gB-specific circulating cells that are likely assisting in the immune response.

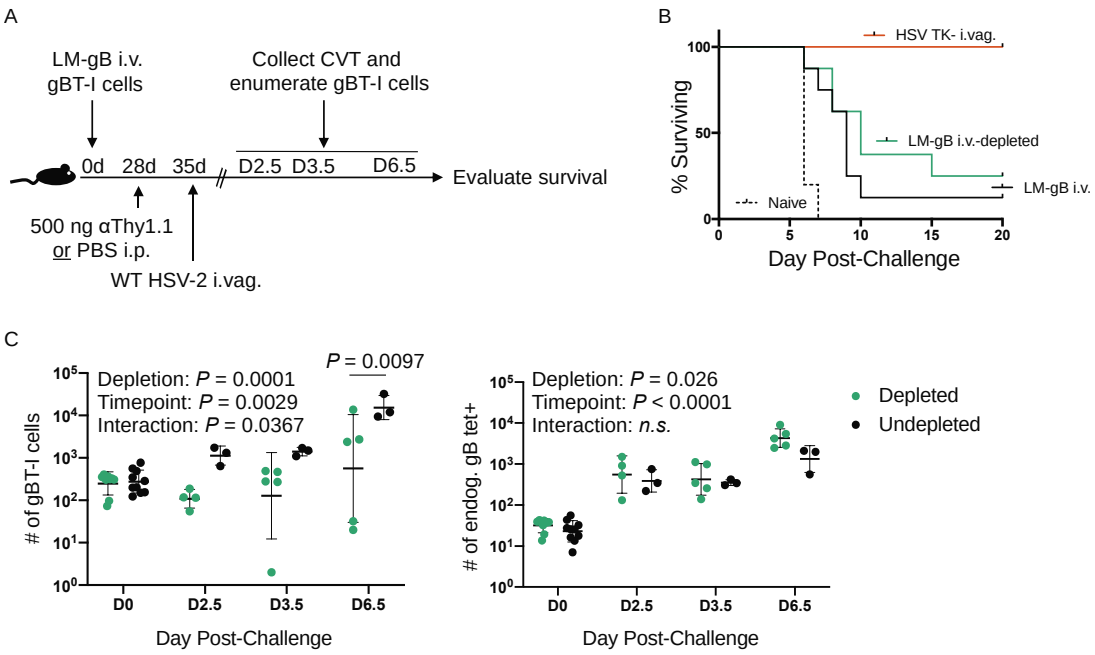

Figure 2.12. Protection against HSV-2 challenge is not affected by circulating gBT-I depletion

(A) Schematic of experiment to address role of circulating gBT-I cells in protection against HSV-2 challenge. (B) Survival after vaginal HSV-2 challenge of naive and immunized mice. Mice immunized with HSV TK- i.vag. (orange line) were challenged 4mo after immunization to test whether protection was maintained after CVT Trm decay. (C) Expansion of gBT-I cells (left) and endogenous (non-gBT-I) gB tetramer+ cells (right) within the CVT after HSV-2 challenge. Each dot in C represents an individual mouse. Error bars represent mean ± SD. P-values in C were calculated via two-way ANOVA with Sidak's post-test. Exact P-values are given for all values <0.05.

2.2.9 *The memory CD8 T cell compartment in the CVT undergoes numeric decay regardless of priming infection and immunization route*

Finally, we wished to determine whether site-matched immunization would result in improved maintenance of the CVT memory CD8 T cell compartment. We compared the maintenance and phenotype of HSV-specific CD8 T cells in the CVT of mice immunized vaginally with HSV-2 TK- or intravenously with LM-gB (Figure 2.13A).

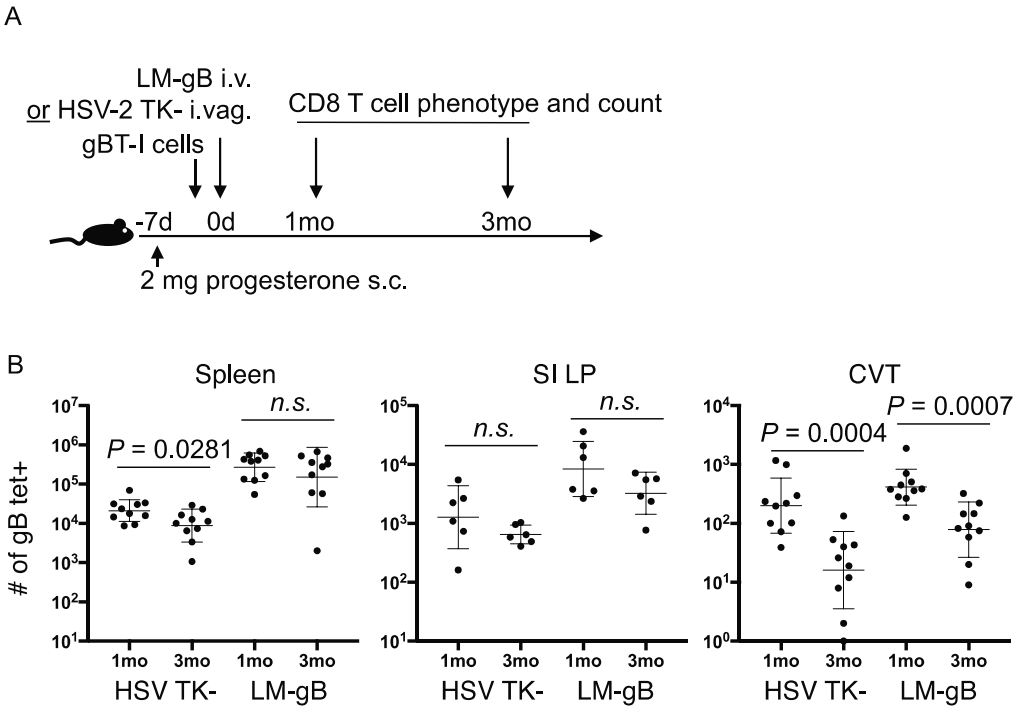

Figure 2.13. Decay of the CVT-resident CD8 T cell compartment occurs regardless of priming infection and route

(A) Schematic of experiment to assess effect of vaginal immunization on longevity of memory CD8 T cells in CVT. (B) Number of gB tetramer+ cells recovered from spleen, SI LP, or CVT of mice at indicated timepoints after immunization. Each dot in B represents an individual mouse. Error bars represent mean ± SD. P-values in B were calculated via unpaired t-test comparing 1mo to 3mo within each immunization group. Exact P-values are given for all values <0.05.

We observed that vaginal immunization with HSV-2 TK- did not result in a larger memory population of HSV-specific CD8 T cells in the CVT than systemic immunization with LM-gB (Figure 2.13B). In addition, the gB-specific population induced by vaginal immunization was relatively stably maintained in the spleen and SI LP, but underwent rapid decay in the CVT between 1mo and 3mo after immunization (Figure 2.13B). Despite this decay in the CD8 T cell compartment, HSV-2 TK- immunized mice remained completely protected against lethal vaginal challenge with wildtype HSV-2 (Figure 2.12A-B), presumably due to CD4 T cells providing substantial protection in this model (Milligan et al., 1998). These data demonstrate that this site-

matched immunization strategy did not boost the size or longevity of the HSV-specific CD8 T cell compartment in the CVT.

2.2.10 *Cognate antigen exposure within the CVT is necessary for acquisition of granzyme B+, TCF-1-, CD103+ phenotype at Early Memory timepoint*

Our data suggested that vaginal infection with HSV-2 TK- resulted in a CVT T cell compartment that expressed granzyme B and CD103 and lacked TCF-1 as early as 1mo after immunization (Figure 2.3), whereas this population took 3-5mo to emerge after intravenous LM-gB immunization (Figure 2.6, Figure 2.7). We wanted to test whether the vaginal inflammation caused by HSV-2 TK- infection (Figure 2.3E) was sufficient to induce the CD103+ granzyme B+ TCF-1lo phenotype, or whether *in situ* exposure to cognate antigen in the CVT was also required. To address this question, we primed OVA-specific CD8 T cells (OT-I cells) with intravenous ActA-deficient LM-OVA in the context of an ongoing intravaginal HSV-2 TK- infection and compared the resulting CVT memory T cell compartment to mice infected with ActA-deficient LM-OVA alone (Figure 2.14A). We found that OT-I cells isolated from the CVT were virtually identical in phenotype regardless of whether mice had been co-infected with intravaginal HSV-2 TK- (Figure 2.14B-F). In both groups, relatively few OT-I cells had adopted the Granzyme B+ CD103+ TCF-1lo phenotype, instead resembling gBT-I cells primed by LM-gB 1mo after immunization. By contrast, HSV-2 gB-specific CD8 T cells isolated from the CVT of co-infected mice expressed CD103 and granzyme B and lacked TCF-1, resembling gBT-I cells primed by HSV-2 TK-. These data indicate that vaginal inflammation alone is not sufficient to induce robust and early development of the CD103+ granzyme B+ TCF-1lo subpopulation of CVT Trm; *in situ* antigen exposure appears to be required for this phenotype to emerge by 1mo after immunization.

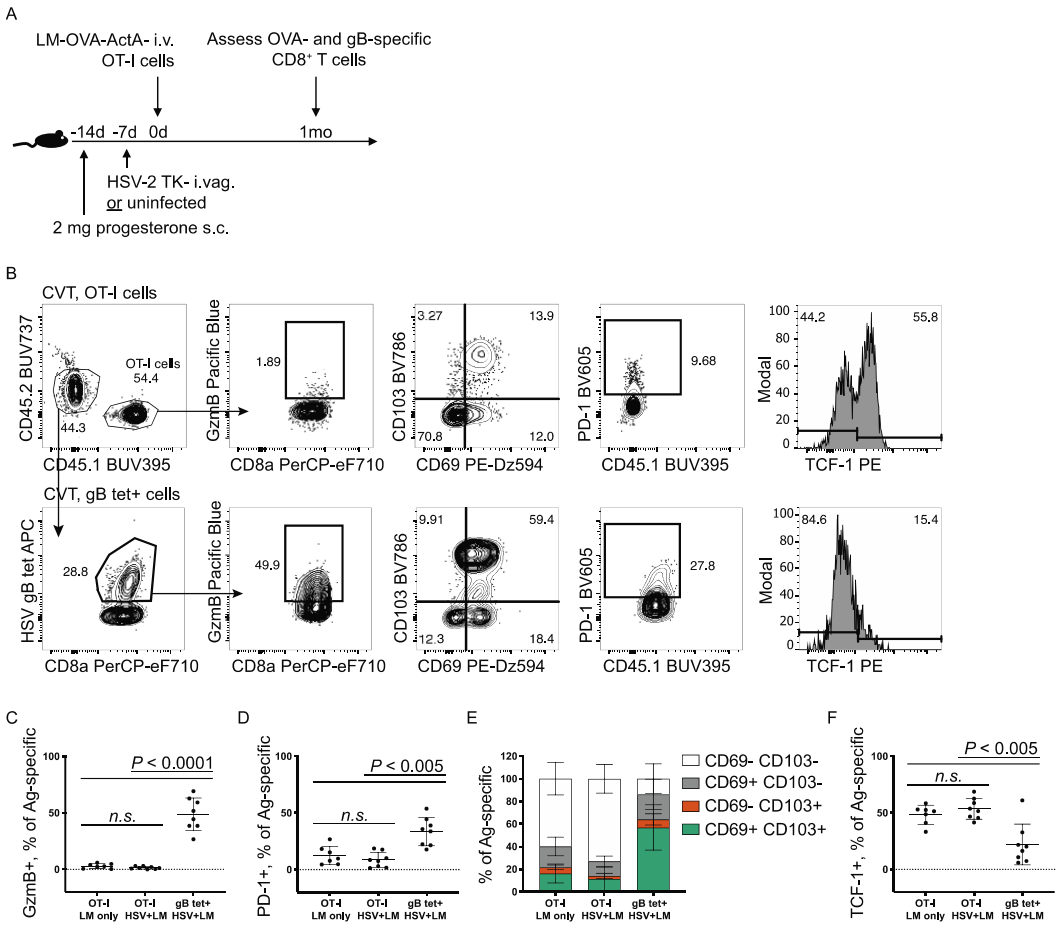

Figure 2.14. Cognate antigen exposure is required *in situ* for Early Memory loss of TCF-1 and acquisition of granzyme B, PD-1, and CD103.

(A) Schematic of experiment. (B) Concatenated flow plots from 8 mice illustrating gating strategy and phenotype of OT-I cells and endogenous gB tetramer+ cells from CVT. (C-F) Frequency of granzyme B, PD-1, CD69, CD103, and TCF-1 expression by OT-I cells and gB tetramer+ cells with and without HSV-2 co-infection. Error bars represent mean ± SD. P-values in C, D, and F were calculated via one-way ANOVA with Tukey's post-test. Exact P-values are given for all values <0.05.

2.3 Discussion

Here, we report that CVT microenvironment plays a dynamic role in shaping the numeric, phenotypic, and functional characteristics of the tissue memory CD8 T cells, which thereby impacts their immunoprotective potential. The cervicovaginal mucosa is distinct from other parts of the female reproductive tract and other mucosal tissues in terms of epithelium type and

physiology. Specifically, the vagina and ectocervix are composed of type II mucosa and the endocervix and uterus are composed of type I mucosa, with the cervical transformation zone marking the area where the simple columnar epithelial cells of the endocervix meet the stratified squamous epithelial cells of the ectocervix and vagina. In contrast, the gut and the lung, other mucosal tissues commonly studied in the case of CD8 T cell memory, are type I mucosal surfaces (Iwasaki, 2010). In addition to these differences in the structure of the epithelium, there are differences in the complexity and makeup of the microbiota at different mucosal tissue sites (Anahtar et al., 2018), which may also contribute to tissue-specific environmental differences that could affect memory CD8 T cell differentiation and function. Vaginal-resident CD8 T cells in healthy women did not express TCF-1 (Figure 2.2), which could either simply indicate recent activation of these T cells (recent infection) or a memory population with an impaired ability to self-renew. Given that the lack of TCF-1 expression was uniform across donors, we felt that a tissue-driven phenotype was more likely than recent infection in a cohort of healthy donors with no current genital infections. Using a mouse model, we similarly found that both vaginal (Figure 2.3) and systemic (Figure 2.4) immunization eventually led to a TCF-1low phenotype among CD8 T cells in the mouse CVT. These data demonstrate that mouse and human memory CD8 T cell phenotypes are remarkably well conserved in the CVT.

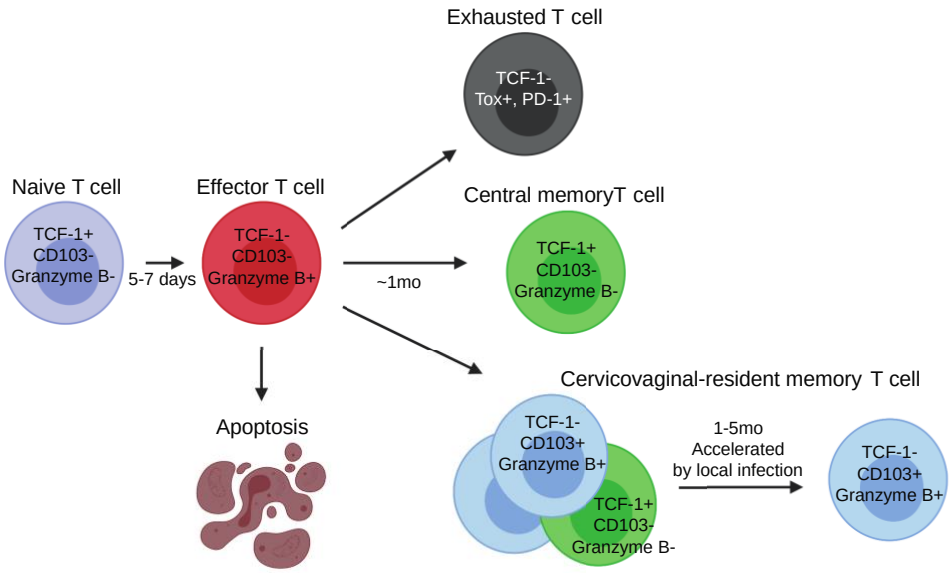

Figure 2.15. Cervicovaginal-resident memory T cells gradually decline in number and acquire expression of CD103 and granzyme B.

Over the course of 5 months following systemic immunization, antigen-specific memory CD8 T cells in the CVT numerically declined and gradually acquired expression of CD103 and granzyme B (Figure 2.15). Conversely, HSV-specific CD8 T cells that were circulating (spleen, LN) or resident in the small intestine were numerically and phenotypically stable over the same time course. This decline in CVT Trm memory occurred at a similar rate even when the circulating gBT-I T cells were selectively depleted, suggesting that peripheral T cells do not reseed the CVT under homeostatic conditions (Figure 2.11). In addition, the CVT CD8 T cell compartment upregulated granzyme B in the absence of new input from the periphery, supporting the occurrence of tissue-intrinsic memory differentiation. Interestingly, CD8 T cells primed by vaginal HSV-2 infection also underwent this gradual differentiation, but from a different starting point: by 1mo after immunization, a large fraction of CD8 T cells primed by vaginal infection with attenuated HSV-2 already exhibited a CD103+ granzyme B+ TCF-1- phenotype. We found that early

adoption of this phenotype required antigen exposure within the CVT and could not be induced by vaginal inflammation alone (Figure 2.14).

Overall, our data show that memory CD8 T cell maintenance and differentiation in the CVT is ultimately directly and uniquely regulated by the tissue environment. We were not able to conclusively identify the signals (or lack thereof) that drive the gradual decay and differentiation of CD8 T cells in the CVT. A possible explanation for these differences between the vagina and other mucosal tissues such as the gut is that the CVT tissue microenvironment is lacking signals that are important for resident-memory formation and maintenance (Liu et al., 2018). It is noteworthy that we did not observe strong TCF-1 expression in the gut Trm cells either, but these cells were stably maintained over time. This could either suggest that TCF-1 is not required for self-renewal of Trm, or the existence of other compensatory mechanisms to maintain the Trm compartment in certain mucosal tissues. TCF-1 activity is thought to be regulated by β-catenin and the Wnt signaling pathway. However, β-catenin does not regulate the phenotype of memory T cells (Driessens et al., 2010) and β-catenin-deficient T cells mount normal recall responses (Prlic and Bevan, 2011). Since β-catenin is sufficient, but not necessary to activate TCF-1 (Tiemessen et al., 2014), a β-catenin-independent upstream pathway may control TCF-1 expression in tertiary tissues with distinct cell fate outcomes compared to β-catenin-dependent activation. A recent study provides compelling genetic evidence that TCF-1 negatively regulates CD103 expression in the lung tissue (Wu et al., 2020), indicating the need to further study and dissect the role of TCF-1 in controlling differentiation and maintenance of Trm cells.

Understanding the relationship of Trm cells in mucosal tissues and the periphery is highly relevant, because memory T cell characteristics and frequencies in the blood are used as a benchmark in clinical studies to assess immune responses to vaccines and to establish correlates

of protection (Koup et al., 2011; Lewinsohn et al., 2017; Stephenson et al., 2016; Streeck, 2016). Our data indicate that there is a disconnect between memory CD8 T cell frequency, phenotype, and stability in the periphery and the CVT. The numerical and phenotypical changes of the memory CD8 T cell compartment one month vs. 5 months post-immunization ultimately influenced the rapidity of the immune response to vaginal viral challenge (Fig. 6). This may be of particular relevance to the success of a vaccine-induced memory T cell response within the CVT, as the host-pathogen events occurring within this early window likely result in the ultimate success or failure of the protective response (Haase, 2010).

Barrier immunity in the CVT remains poorly understood, but is of particular interest, because sexually-transmitted infections (STI) have a significant impact on global health. We currently lack effective vaccines to prevent the majority of these infections, including HIV, HSV, and bacterial STIs. Our data demonstrate that following systemic immunization, animals were partially protected against HSV-2 lethality by gB-specific memory CD8 T cells (Fig. 6). Importantly, this protective effect was observed despite the lack of HSV-specific CD4 T or B cell memory, elements of the immune response that are often considered crucial for protection against HSV-2 (Dudley et al., 2000; Milligan et al., 1998; Morrison et al., 1998; Petro et al., 2016). In line with this observation, results from participants with recurrent genital HSV-2 infection indeed suggest that CD8 T cell activity within lesions is associated with viral control (Peng et al., 2012; Schiffer et al., 2010; Zhu et al., 2013; 2007). It is possible that a new focus on vaccine strategies that elicit memory CD8 T cells within the genital mucosa, the site of first exposure to STIs, will increase vaccine efficacy. However, successful design of these approaches, not just in regard to adjuvant and immunization route, but also boosting intervals to optimize protection, relies on understanding how memory CD8 T cells are maintained within these genital tract mucosal tissues.

In summary, we demonstrate that the CVT tissue environment controls memory CD8 T cell differentiation and maintenance, which occurs in a tissue autonomous manner and differs across mucosal tissue compartments. The CD8 Trm compartment in the CVT declines over time with a concomitant decrease in the ability to mediate rapid antigen-specific protection.

2.4 Materials and Methods

2.4.1 *Mice*

C57BL/6J and B6.PL-Thy1a/CyJ mice were purchased from The Jackson Laboratory and maintained in specific pathogen-free conditions at the Fred Hutchinson Cancer Research Center (FHCRC). All mice used in experiments were female and were 8–12 weeks of age. Mice that rejected transferred Thy1.1+ gBT-I cells or had an undetectable HSV-2 titer in vaginal wash at 2.5d post-challenge were excluded from experiments. Experiments were approved by the FHCRC Institutional Animal Care and Use Committee.

2.4.2 *Study population*

Six women were recruited for this study and consented to vaginal biopsy and blood sampling. Eligibility criteria included aged > 18 to < 45, assigned female sex at birth, in good general health, normal PAP smear within the past 3-5 years, not menopausal, Hepatitis C negative and no report of active genital tract irritation or infection. Additionally, participants were screened to be negative for HIV, HSV-2, bacterial vaginosis, chlamydia, gonorrhea, and trichomonas vaginalis. Subjects were not using any type of steroid or medication for treatment of autoimmune disorders. Samples were not collected during menses. Informed written consent was obtained from all participants. The study and procedures were approved by the FHCRC Institutional Review Board.

2.4.3 *Adoptive CD8 T cell transfers*

CD44low CD8 T cells were purified via negative selection from spleen and lymph nodes using mouse CD8a+ T Cell Isolation kits (Miltenyi) as previously described (Prlic et al., 2001). 5 × 10^4 cells were injected into recipient B6 mice via i.v. injection into the tail vein.

2.4.4 *HSV-2 infections and quantification*

Mice were injected subcutaneously with 2 mg of medroxyprogesterone acetate. Seven days later, mice were infected intravaginally with 2 × 10^4 pfu wildtype HSV-2 (strain 186 syn+) or 1.88 × 10^5 PFU of thymidine kinase-negative HSV-2 186 kpn to the vaginal canal in a 10 ul volume (Parr et al., 1994). After infection with wildtype HSV-2, mice were monitored daily and clinical disease progression was scored as follows: 0, No sign; 1, slight genital erythema; 2, moderate genital erythema and edema; 3, significant genital inflammation with visible lesion; 4, hind leg paralysis or other severe condition requiring euthanasia; 5, moribund or dead. Mice were euthanized if they were moribund or showed signs of severe disease, including hind leg weakness, hind leg paralysis, or hunched posture. Viral titer was determined by plaque assay on Vero cells (ATCC) or by polymerase chain reaction (PCR) as previously described (Jerome et al., 2002).

2.4.5 *Listeria monocytogenes immunization*

A strain of Listeria monocytogenes (LM) was generated to recombinantly express and secrete ovalbumin containing the HSV-2 glycoprotein B-derived peptide SSIEFARL according to previously described methods (Lauer et al., 2002; Zehn et al., 2009). Stocks were diluted to 2 × 10^4 CFU per ml in sterile phosphate-buffer saline (PBS) before immunization. Naive B6 mice

received 4,000 CFU i.v. via tail vein injection. Infectious dose was confirmed by plating on brain-heart infusion media plates and enumerating colonies.

2.4.6 *Labeling of T cells in the vasculature*

To discriminate CD8 T cells in the vasculature from those located within tissues, we utilized an intravascular labeling technique as previously described (Galkina et al., 2005). Briefly, mice were injected i.v. with 3 ug anti-CD8b antibody conjugated to either FITC or APC (Clone: H35-17.2, eBioscience) in PBS via the tail vein 2-5 min before euthanasia by CO_2 inhalation.

2.4.7 *Depletion of Thy1.1-expressing T cells*

Mice were injected intraperitoneally with 500 ng anti-Thy1.1 antibody (Clone 19E12, Bio X Cell) to deplete Thy1.1-expressing cells from the circulation and peripheral lymphoid tissues.

2.4.8 *Mouse tissue collection and processing*

Mice were treated with 2 mg medroxyprogesterone s.c. 7 days before experiment endpoint for all memory experiments in order to standardize estrus cycle phase at the time of sacrifice.

For pathologic assessment, murine cervicovaginal tracts (consisting of the vagina, ectocervix, and endocervix) were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

To prepare single cell suspensions, cervicovaginal tracts were minced with scissors and then incubated in 2 ml Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 2 mg/ml collagenase D (Roche), and 1.5 mg/ml DNase I (Roche) for 30 minutes at 37C with gentle rocking. Single cell suspensions were prepared from small intestines by removing the Peyer's patches and mesentery, incubated in Hank's buffered salt solution

(HBSS) with 2% FBS, 5 mM EDTA, and 1 mM dithiothreitol for 15 min at 37C with gentle rocking, followed by collagenase treatment as outlined for the CVT.

2.4.9 *Human tissue collection and processing*

Blood was collected into acid citrate dextrose tubes. Tissues were transported from the clinic in ice cold PBS and immediately processed upon arrival. Biopsy samples were trimmed to 2mm2 pieces and digested with collagenase II (700 units/ml, Sigma-Aldrich) and DNAse I (1unit/ml, Sigma-Aldrich) for two subsequent 30 minute-digestions at 37°C as previously described (McKinnon et al., 2014). Single cell suspensions were then washed and filtered before use.

2.4.10 *Pathologic assessment of mouse vaginal tissue*

Hematoxylin and eosin-stained sections of mouse CVT were scored based on signs of inflammation within the vaginal lamina propria as follows: 1, Rare neutrophils and other leukocytes present in the lamina propria; 2, Small clusters of neutrophils and other leukocytes present in the lamina propria; 3, Multifocal clusters of leukocytes beginning to coalesce and/or form follicles within the lamina propria; 4, Leukocytes arranged in sheets within the lamina propria. Scoring was performed by a veterinary pathologist who was blinded to group identities.

2.4.11 *Cell staining for flow cytometry*

Cells were incubated in LIVE/DEAD fixable amine-reactive viability dye (Invitrogen), blocked for Fc binding (Clone 2.4G2 for mice or TruStain (BioLegend) for human), and then stained with tetramers and antibodies.

The following fluorochrome-conjugated antibodies were used to stain mouse cells for flow cytometry: anti-CD8 (Clone 53-6.7, PerCP-eFluor710), anti-CD4 (Clone GK1.5, APC-

eFluor780), anti-CD45.1 (Clone A20, Brilliant Ultraviolet 395), anti-CD45.2 (Clone 104, Brilliant Ultraviolet 737), anti-Thy1.1 (Clone HIS51, FITC or SuperBright645), anti-CD44 (Clone IM7, APC or AlexaFluor700), anti-CD69 (Clone H1.2F3, PE-Dazzle594 or PE-Cy7), anti-CD103 (Clone M290, Brilliant Violet 786), anti-granzyme B (Clone GB11, Pacific Blue), and anti-TCF-1 (Clone C63D9, PE). Anti-CD8, anti-CD4, and anti-Thy1.1 were purchased from eBioscience. Anti-CD45.1, anti-CD45.2, anti-CD44, and anti-CD103 were purchased from BD. Anti-CD69 and anti-granzyme B antibodies were purchased from Biolegend. Anti-TCF-1 antibody was purchased from Cell Signaling Technologies.

The following fluorochrome-conjugated antibodies were used to stain human cells for flow cytometry: anti-CD3 (Clone UCHT1, Brilliant Ultraviolet 661), anti-CD45 (Clone HI30, Brilliant Ultraviolet 805), anti-CD8 (Clone RPA-T8, Brilliant Ultraviolet 496), anti-CD4 (Clone RPA-T4, Brilliant Violet 605), anti-CD45RA (Clone HI100, APC-H7), anti-CCR7 (Clone G043H7, AlexaFluor488), anti-granzyme B (Clone GB11, AlexaFluor700), anti-CD103 (Clone Ber-ACT8, Brilliant Violet 750), and anti-TCF-1 (Clone C63D9, PE). Anti-CD3, anti-CD45, anti-CD4, anti-CD8, anti-CD45RA, anti-CD103, and anti-granzyme B were purchased from BD. Anti-CCR7 antibody was purchased from Biolegend. Anti-TCF-1 antibody was purchased from Cell Signaling Technologies.

Intracellular staining was conducted after fixation and membrane permeabilization using Cytofix/Cytoperm Fixation/Permeabilization Kits (BD). If TCF-1 was included in the staining panel, intracellular staining was conducted after nuclear permeabilization using Foxp3/Transcription Factor Staining Kits (eBioscience) according to the manufacturer's protocol. Samples were acquired on a FACSymphony instrument (BD) or sorted on a FACSAria (BD). To

estimate cell counts, 2 × 105 AccuCheck Counting Beads (ThermoFisher) were added to each sample immediately before acquisition.

2.4.12 *Single cell RNA sequencing and analysis*

In order to generate single cell transcriptomic data, CD8 T cell populations of interest were sorted using a FACSAria II fitted with a 70 um nozzle (BD). Live CD45.2+ CD8a+ CD8b i.v.-label+ cells from the blood and live CD45.2+ CD8a+ CD8b i.v.-label- cells from the CVT were sorted and loaded onto a Chromium Controller (10x Genomics) to generate gel beads in emulsion. After cDNA synthesis, samples were further processed using the Chromium Single Cell 3' single cell RNA sequencing kit version 2 (10x Genomics) and the resulting libraries were sequenced on a HiSeq 2500 Next Generation Sequencer (Illumina) at the FHCRC Genomics Shared Resource Facility. Cell Ranger software (10x Genomics) was used to generate FASTQ files, demultiplex samples, and count transcripts per cell. The processed data were analyzed using R (R Development Core Team) and the package Seurat version 2.3.4 (Butler et al., 2018). Quality filtering steps included removal of genes detected in fewer than three cells and removal of cells in which fewer than 200 genes were detected or in which greater than 5% of detected genes were mitochondrial. Count data were normalized for each gene by dividing the per-cell expression count by the total counts of all genes summed for that cell, multiplying the resulting value by 10,000, adding 1, and taking the natural log. Variable genes were identified on the basis of having a mean of non-zero expression values between 0.0125 and 3 and a standard deviation of all expression values greater than 0.5. Data were scaled and centered using a linear model that adjusted for the number of unique molecular identifiers per cell and the percent mitochondrial genes detected per cell. Principal components analysis was performed using the scaled and normalized data for all variable genes, and the first 20 principal components were used to compute and display uniform manifold

approximation and projection (UMAP) (McInnes et al., n.d.) plots and to cluster the data using a graph-based clustering algorithm (Waltman and van Eck, 2013) implemented in Seurat. Contaminating non-T cell subsets were identified by graph-based clustering and removed based on high expression of Krt4, Krt19, Cd74, Cd79a, and Cd79b and no expression of Cd3e. Genes that were differentially expressed between specific populations or clusters of cells were identified by model-based analysis of single cell transcriptomics (Finak et al., 2015) using criteria described in figure legends.

2.4.13 *Statistical analyses*

Sample means between two unpaired groups were compared by t-test. Sample means between three or more unpaired groups were compared by ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Sets of longitudinal measurements were compared by computing the area under each curve and the associated standard error, and then performing a t-test. Sample means between three paired groups were compared by repeated measures ANOVA with Greenhouse-Geisser correction. Paired mean fluorescence intensity or relative frequency data were analyzed via paired t-test. All cell count and viral titer data were log10-transformed before graphing and analysis. Error bars represent standard deviations. Exact P-values are given for all results where $P < 0.05$. Analysis was performed using Prism 7 (GraphPad Software).

2.4.14 *Mathematical modeling of HSV-2 and CD8 T cell dynamics*

To understand the virus and gB-specific CD8 T cell dynamic differences between the Naive, Early Memory, and Late Memory groups, we used an acute viral infection model that included a cytotoxic effect of the CD8 T cell compartment. In this model, susceptible cells (S) are

infected at rate βVS by free HSV-2 virus (V). Productively HSV-2-infected cells (I) have a clearance that is intrinsic or mediated by a humoral response with rate δII or a clearance mediated by HSV-specific CD8 T cells (E) with killing rate kEI. Free virus is produced at a rate pI and cleared at rate cV. HSV-specific CD8 T cells are cleared at rate δEE and proliferate in the presence of infected cells at a maximum rate ωEI. We also assumed the proliferation of HSV-specific CD8 T cells is constrained when infected and effector cells grow over saturation levels I50 and E50, respectively. Under these assumptions the model has the form (see schematics in Fig. 6D):

$$\begin{aligned}
\frac{dS}{dt} &= -\beta VS \\
\frac{dI}{dt} &= \beta VS - \delta_I I - kEI \\
\frac{dV}{dt} &= pI - cV \\
\frac{dE}{dt} &= \frac{\omega EI}{\left(1+\frac{I}{I_{50}}\right)\left(1+\frac{E}{E_{50}}\right)} - \delta_E E.
\end{aligned} \tag{2.1}$$

The basic reproductive ratio, R_0, which represents the number of secondary infections produced by an HSV-infected cell when introduced into a susceptible population, was calculated as $R_0 = \frac{\beta p T_0}{c(\delta_I + kE_0)}$. When $R_0 < 1$, viremia is controlled.

We performed 100 rounds of model fits to observations from all individual animals for each group with different initial parameter guesses in the optimization algorithm. We assumed that all outcomes from the 100 fit rounds were plausible predictions of the data and represented the variability in the infection dynamics in each group. For each round, we re-parameterized the model as a function of R_0 with a nonlinear least-squares approach using the differential evolution and the L-BFGS-B algorithms in R (R Development Core Team) to fit the model and to estimate parameters: V_0, pT_0, E_0, R_0, δ, c, k, ω, δ_E, I_{50} and E_{50}. Since mice were inoculated with ~2.3×106 HSV genomes, we set that number as the upper limit for V_0. We also constrained the value estimate

E_0 to the maximum and minimum HSV-specific CD8 T cell observations at the moment of virus challenge. Finally, we ensured that $R_0 > 1$ in all fitting rounds. In all simulations $t = 0$ represented the time of the HSV-2 vaginal challenge. From the best fits, we computed the predicted time of viral clearance as the time when viral load crossed the detection limit for the PCR assay of 4615.4 genomes per vaginal wash. We also computed the predicted area under the curve of HSV-specific CD8 T cells using best fits of variable E from challenge until the time of viral clearance.

We repeated the fits by exploring different possibilities for constraining HSV-specific CD8 T cell growth. Specifically, we explored a model were CD8 growth is constrained only by a saturation level in HSV-infected cells, i.e. $\frac{dE}{dt} = \frac{\omega EI}{\left(1+\frac{I}{I_{50}}\right)} - \delta_E E$, or only by a saturation for the number of effector cells, i.e. $\frac{dE}{dt} = \frac{\omega EI}{\left(1+\frac{E}{E_{50}}\right)} - \delta_E E$. To determine the best and most parsimonious model, we computed the Akaike Information Criteria, $AIC = n \ln \overline{SSE} + 2m$, where m is the number of parameters estimated, n the number of data points in each group and $\overline{SSE}$ is the average sum of squares error of all the 100 model-fit rounds in each group. We assumed a model had similar support from the data if the difference between its AIC and the best model (lowest) AIC was less than two. We selected and showed results from the model with the lowest AIC.

2.5 Acknowledgments

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Candice Teague, Nicole Potchen, Susanne Oberle, Dietmar Zehn, Joshua T. Schiffer, Jennifer M. Lund, and Martin Prlic.

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Chapter 3. Recurrent infection of human skin transiently expands the T cell compartment without driving T cell exhaustion

The immune response to chronic infections has predominantly been studied in mouse model systems, which greatly facilitates the concomitant and longitudinal assessment of how pathogen persistence affects host immunity. These studies were critical in revealing how chronic exposure to antigen leads to a change in T cell responsiveness that is referred to as a state of exhaustion or dysfunction. Comparable human data are still lacking, and it remains unclear if episodes of periodic viral reactivation and antigen exposure are sufficient to induce T cell dysfunction. Here, we use a stringent clinical surveillance protocol to track both viral load and the *in situ* immune response for 24 weeks during HSV reactivation events. Comparing lesion to contralateral control biopsies, we found that the immune compartment expands across subsets immediately after reactivation followed by a contraction phase that eventually results in a return to a phenotypical and numerical immune cell steady state. T cell expansion following reactivation appears driven by a combination of proliferation and migration to the infected area. Of note, these T cells did not express biomarkers that are associated with a state of dysfunction. Thus, our data indicate that the *in situ* immune response is functional in response to periodic HSV reactivation. We discuss the clinical implications for generating feasible therapeutic vaccines as well as the relevance for understanding T cell responses across a spectrum of chronic infections.

3.1 Introduction

HSV-2 is a globally burdensome virus that establishes a lifelong infection characterized by the presence of episodic viral shedding and recrudescent genital ulcers. The frequency of clinical HSV-2 symptom presentation including lesion formation varies widely, with the majority of HSV-

2 seropositive individuals remaining asymptomatic despite frequent detection of viral DNA from genital swabs, and in the absence of lesions 67% of the time (Mark et al., 2008; Schiffer, 2013; Schiffer et al., 2011; Wald et al., 1995; 2000). These characteristics imply a role for constant local immunosurveillance and host:pathogen engagement, either resulting in the rapid extinguishment of viral replication, or alternatively, lack of containment and clinical presentation (Roychoudhury et al., 2020; Schiffer, 2013; Schiffer et al., 2011; 2010; Schiffer and Corey, 2013; Schiffer et al., 2013; 2018a; 2018b; Wald et al., 1995). Resident memory T cells (Trm) dominate the immune response to both HSV type 1 and 2 infection (Ariotti et al., 2014; 2012; Collins et al., 2017; Gebhardt et al., 2011; 2009; 2018; Iijima and Iwasaki, 2014; Mackay et al., 2013; 2012; Park et al., 2018; Schiffer et al., 2018a; Shin and Iwasaki, 2012; Wang et al., 2016; Zhu et al., 2013; 2009; 2007). In mice, primary HSV-2 infection results in the recruitment of innate and adaptive immune cells to the effector site where T cells control infection (Harandi et al., 2001; Lund et al., 2008; 2006; Nakanishi et al., 2009; Shin and Iwasaki, 2013; Zhao et al., 2003), a fraction of which are retained as Trm (Gebhardt et al., 2009; Iijima and Iwasaki, 2014; Masopust and Soerens, 2019). Although latent HSV infection of mice does not result in episodic recurrences, as is characteristic of human disease, mice immunized with attenuated HSV-2 robustly develop CD4 Trm within memory lymphocyte clusters, which produce IFN-γ, TNF-α, and IL-2 and provide protection against lethal challenge of wild-type HSV-2 with minimal aid from circulating memory T cells (Tcircm) (Gebhardt et al., 2009; Iijima and Iwasaki, 2014; McDermott et al., 1984; Parr et al., 1994). Similarly, after HSV-1 challenge, CD8 T cells become sequestered specifically at the site of previous challenge and can provide secondary protection in the absence of Tcircm (Gebhardt et al., 2011; Mackay et al., 2015; Park et al., 2018). Protective CD8 Trm can also be elicited via antigen independent mechanisms including to the female genital tract through a prime and pull

vaccination strategy, or to the skin via contact sensitization (Mackay et al., 2012; Shin et al., 2016; Shin and Iwasaki, 2012).

Studies of human genital skin and mucosa have also identified local clusters of antigen-specific CD8 and CD4 Trm at sites of acute HSV-2 lesions and ascending mucosa (Koelle et al., 2000; Peng et al., 2012; Posavad et al., 2017; Zhu et al., 2013; 2009; 2007). T cells isolated from lesion sites express transcripts of antiviral cytokines including perforins and granzymes A and B, produce cytolytic granules, and secrete IFN-γ when restimulated with HSV-2 antigen (Peng et al., 2012; Zhu et al., 2013; 2009). Together, data acquired from human and mouse studies demonstrate that Trm abundantly congregate in response to HSV infection, and further, that they are necessary to achieve viral control (Gebhardt et al., 2009; Iijima and Iwasaki, 2014). However, little is known regarding their ability to retain functionality in the face of chronic antigen exposure, as occurs in repetitive human HSV-2 reactivation episodes wherein HSV-2 is thought to be rarely quiescent (Schiffer and Corey, 2013).

Constant antigen exposure, such as occurs during systemic chronic infection and cancer, has been shown to divert the development of functional, self-renewing memory T cells into an exhausted phenotype (Tex) (Wherry and Kurachi, 2015). Tex cells notoriously express inhibitory molecules such as PD-1, CTLA-4, Tim-3, CD244, LAG-3, CD101, and CD39, and exhibit impaired proliferative and survival protentional, as well as impaired production of IL-2, TNF and IFNg (P. K. Gupta et al., 2015; Hudson et al., 2019; Wherry and Kurachi, 2015). Moreover, recent evidence has revealed a distinct transcriptional regulation profile of Tex including expression of Tox, Tbet, and Eomesodermin (Eomes), concomitant loss of TCF-1, altered metabolic function, and epigenetic programming (Bengsch et al., 2018; Doering et al., 2012; Kaech and Wherry, 2007; O. Khan et al., 2019; Paley et al., 2012; Pauken et al., 2016; Philip et al., 2017; Scott-Browne et

al., 2016; Sen et al., 2016; Yao et al., 2019; N.-N. Zhang et al., 2020). The development of Tex may be necessary to limit long-term immunopathology, but the loss of T cell effector functions also allows pathogen or tumor persistence and/or progression (Barber et al., 2006; Frebel et al., 2012). Studies of chronic systemic mouse viruses and human infections such as HIV and hepatitis C virus have contributed greatly to defining Tex phenotypes and identifying their loss of functionality (Trautmann et al., 2006; Urbani et al., 2006; Wherry et al., 2003), but it remains unknown how chronic pathogen persistence in the periphery, for example in the context of human HSV-2, may affect T cell resilience. Moreover, potential regulatory mechanisms encouraging the retention of T cell effector functions versus transition to exhaustion remain undescribed. Here, we robustly examined T cell responses within HSV-2 lesion sites over 24 weeks, spanning early clinical presentation through healing to determine the fate, resilience, and regulatory mechanisms governing resident memory T cell populations facing chronic antigen exposure.

3.2 Results

3.2.1 *Viral exposure history does not permanently alter the frequency of bulk tissue T cells*

Given the high frequency and repetitive nature of HSV-2 viral exposure of genital skin in HSV-2 seropositive women, we sought to characterize the local T cell compartment of healthy genital skin from HSV-2 seropositive women as compared to HSV-2 naïve women. Biopsies of unaffected contralateral skin were collected from HSV-2 seropositive women one week after they had experienced an HSV-2 lesion at a disparate site. Site-matched genital skin was collected from a seronegative participant (Figure 3.1A). Using high-parameter flow cytometry (Table 3.2) we determined the frequency and phenotype of T cells present in HSV-2 seropositive versus seronegative healthy skin (Figure 3.1B). PBMCs were run in parallel as an internal control and to aid in setting analysis gates. Interestingly, we found no quantitative differences in the total number

of CD8, CD4, or regulatory T cells (Treg) present in HSV-2 seropositive women compared to seronegative women (Figure 3.1C); and moreover, the frequencies of T cells expressing the residency markers CD69 and CD103 were similar among donors (Figure 3.1D). These results suggested that although Trm are known to be instrumental in coordinating local immune responses during viral exposure events (Davies et al., 2017; Schenkel et al., 2013), upon resolution of acute HSV-2 infection, human genital skin returns to a level of homeostasis comparable to STI-unexposed skin.

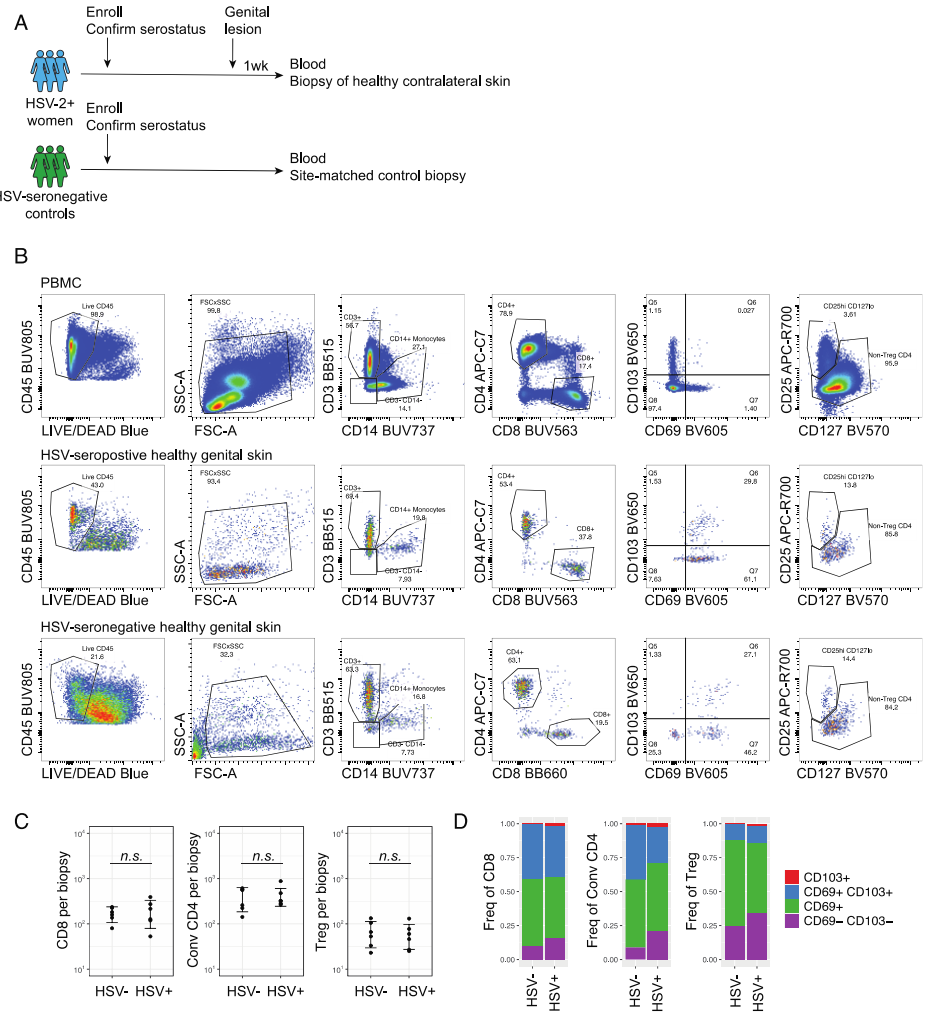

Figure 3.1. Healthy genital skin biopsies from HSV-seronegative and HSV-2+ women with a history of genital ulcers contain a comparable population of CD4 and CD8 Trm.

(A) Study schematic. (B) Gating strategy to identify CD4 Trm, CD8 Trm, and Treg populations. (C) Total number of CD8, conventional CD4, and Treg isolated from biopsy samples. (D) Frequency of expression of CD69 and CD103 within indicated population. Each dot in (C) represents an individual subject. *P*-values in (C) were calculated by t-test using log-transformed values.

3.2.2 *HSV-2 lesion formation elicits T cell and monocyte expansion but does not change the composition of the immune cell compartment in local tissue*

We next sought to characterize the size and composition of the immune cell compartment at the site of an active HSV-2 lesion. Once participants reported the appearance of a lesion, we

collected a first biopsy as soon as the active ulcer had sufficiently healed to allow safe sampling, about one week after initial lesion appearance (Table 3.1). We continued to collect biopsies from the healed lesion through 24 weeks of follow-up. We collected repeat biopsies from the lesion site in a daisy chain pattern (Figure 3.2A) and determined the frequency and total number of T cells and monocytes present over time (Figure 3.2B-C). Compared to healthy skin collected from a site contralateral to the acute HSV-2 lesion, we witnessed a significant expansion of monocytes, CD8 and CD4 T cells, and Tregs in HSV-2 lesion sites (Figure 3.2B-C). The magnitude of the expansion was especially evident at early timepoints such as 1-4 weeks, and was followed by an apparent contraction phase of tissue healing and a return to homeostasis (Figure 3.2C-D). Strikingly, the composition of the immune cell compartment remained relatively unaltered, with all analyzed subsets exhibiting expansion and contraction of proportionate magnitude. Thus, despite demonstrable expansion of tissue T cells and monocytes in HSV-2 lesions, no single cell type was responsible for the increased cellularity, leaving the overall composition of the tissue compartment similar to contralateral healthy genital skin.

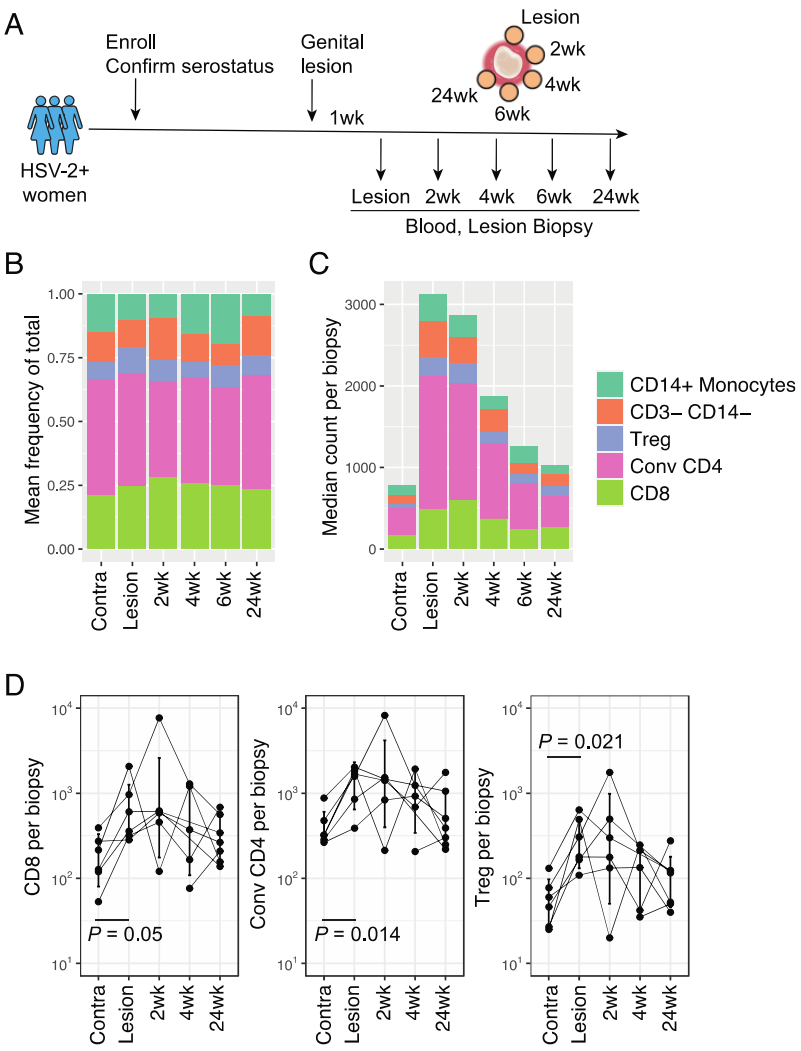

Figure 3.2. Small-scale immune cell expansion is evident during genital ulcer formation and healing, but expansion and contraction occur uniformly across major immune cells.

(A) Study schematic for assessment of longitudinal immune cell infiltrate after HSV-2+ genital ulcer formation. (B) Average frequency and (C) median count of major immune cell populations in genital biopsies at indicated timepoints. (D) Number of CD8, conventional CD4, and Treg isolated from genital biopsies at indicated timepoints. Each line in (D) represents an individual subject, and each dot represents an individual biopsy. *P*-values in (D) were calculated by mixed-effects analysis with Greenhouse-Geisser correction and Tukey's multiple comparisons test.

3.2.3 *Proliferating local Trm and recruited Trm precursors contribute to lesion healing and return to tissue homeostasis*

We next determined the role and magnitude of local T cell proliferation in HSV-2 lesion tissues as compared to recruitment of T cells from the blood. Moreover, we assessed the tissue T cell proliferation potential versus recruitment of T cells in genital tissues derived from HSV-2 seronegative women as compared to healthy contralateral skin and HSV-2 healing lesion sites of HSV-2 seropositive women. Using Ki-67 expression as a marker of recent local proliferation, we found heterogeneity in proliferation frequency among CD8 and CD4 T cells (Figure 3.3A-C). Both subsets of T cells from specific participants underwent robust early proliferation, returning to baseline frequencies by 2-4 weeks after lesion appearance (Figure 3.3B-C). Other participants demonstrated similar results but with a relative delay in the kinetics of expansion and contraction. The remaining participants exhibited little Ki67 expression regardless of the timepoint.

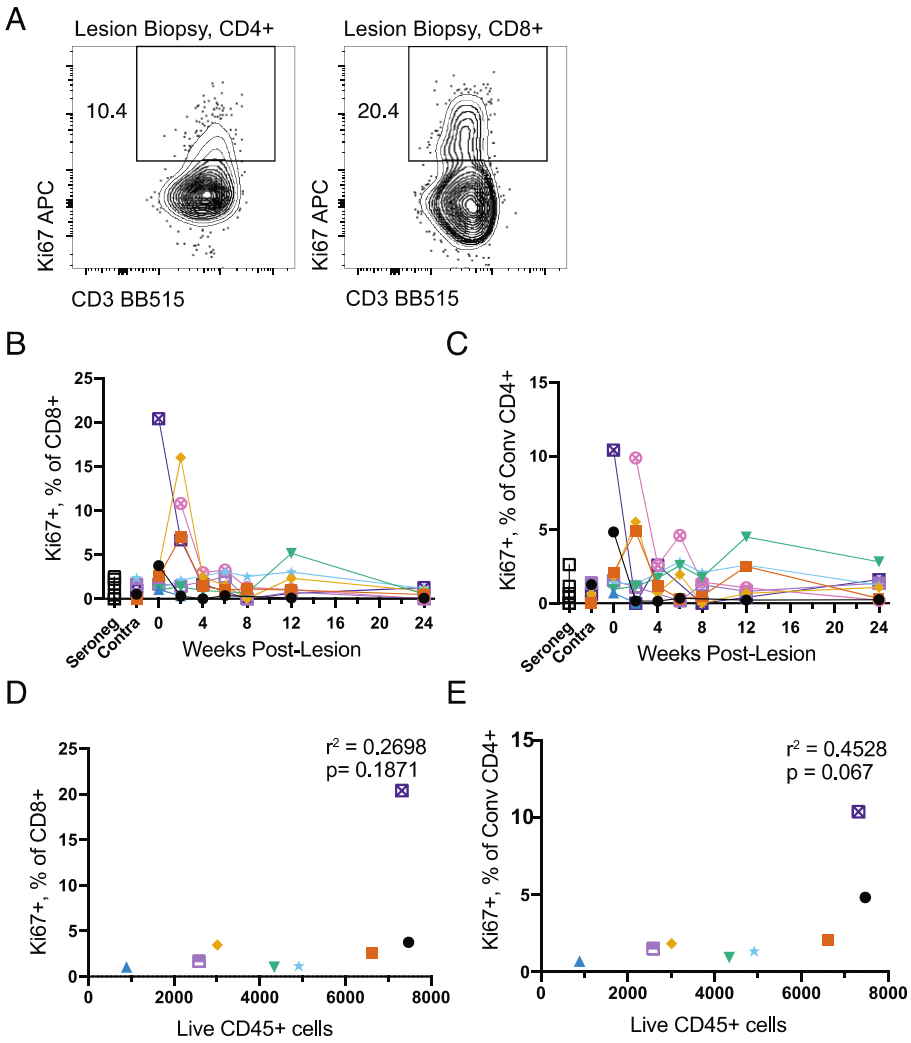

Figure 3.3. Low Ki67 expression suggests that infiltrating T cells from the circulation are contributing to the local immune response.

(A) Examples of Ki67 staining among total CD8 and non-Treg CD4 T cells from one participant at the lesion biopsy timepoint. (B-C) Ki67 expression among total CD8 and non-Treg conventional CD4 T cells. (D-E) Correlation between lesion (Week 0) total CD45+ cell count and Ki67 expression level. Each color and line in (B-C) represents an individual subject, and each shape represents an individual biopsy sample. Each dot in (D-E) represents an individual subject. *P*-values and r^2-values in (D-E) were calculated by Pearson correlation

Surprisingly, we also observed that the total number of immune cells isolated from the lesion was not correlated with Ki67 expression among CD4 or CD8 T cells (Figure 3.3D-E). Given the notable expansion of bulk T cells at the lesion site in all participants examined (Figure 3.2),

juxtaposed with underwhelming Ki-67 expression in many individuals we suspected that T cell recruitment from the blood must at least transiently contribute to the expanded tissue compartment.

3.2.4 *Circulating memory T cells likely contribute to in situ T cell expansion by influxing into HSV-2 lesions*

We next looked for evidence of potential CD8 Trm precursor cells influxing into the tissue. We expected these cells to progressively downregulate the transcription factors TCF-1 and Eomes as they more stably acquired a Trm phenotype (Mackay et al., 2015; Mackay and Kallies, 2017). To evaluate this phenotype as well as phenotypes associated with T cell exhaustion and regulation, we recruited a second cohort of participants to undergo serial biopsies that we analyzed with a flow cytometry panel designed to measure T cell activation, regulation, and exhaustion (Table 3.3). Sample collection for this second cohort was halted due to the pandemic outbreak of severe acute respiratory syndrome coronavirus 2 in early 2020, resulting in a final cohort size of 3 people, one of which had also participated in the first cohort (Table 3.1). Of these three participants, one completed the full 24 weeks of follow-up, one completed follow-up through Week 6, and one completed follow-up through Week 2. Th small size of this cohort and differences in the length of follow-up are a limitation of this study, and made it difficult for us to perform robust statistical analyses on these data. However, we found that certain patterns emerged among the three participants, and describe those patterns in the remaining figures.

We found that CD8 T cells present in homeostatic tissue (contralateral biopsies) were mainly Eomes- TCF-1- (Figure 3.4A-B). Upon lesion formation and early healing timepoints, we observed a substantial influx of CD8 T cells expressing Eomes and/or TCF-1 (Figure 3.4A-B). While the majority of these cells were negative for Trm markers (Figure 3.4A), we also observed a brief uptick in Eomes expression among CD69+ CD103+ CD8 and CD4 T cells (Figure 3.4C-

D). Consistent with a return to homeostasis, we found that beyond 2-4 weeks of healing, CD8 and CD4 Trm cells were almost exclusively Eomes- TCF-1-, suggesting the possibility that Eomes+ Trm precursors seeded the tissue at early timepoints and subsequently downregulated Eomes, consistent with transcriptional profiles associated with stable Trm populations in mouse models (Behr et al., 2019; Mackay et al., 2016).

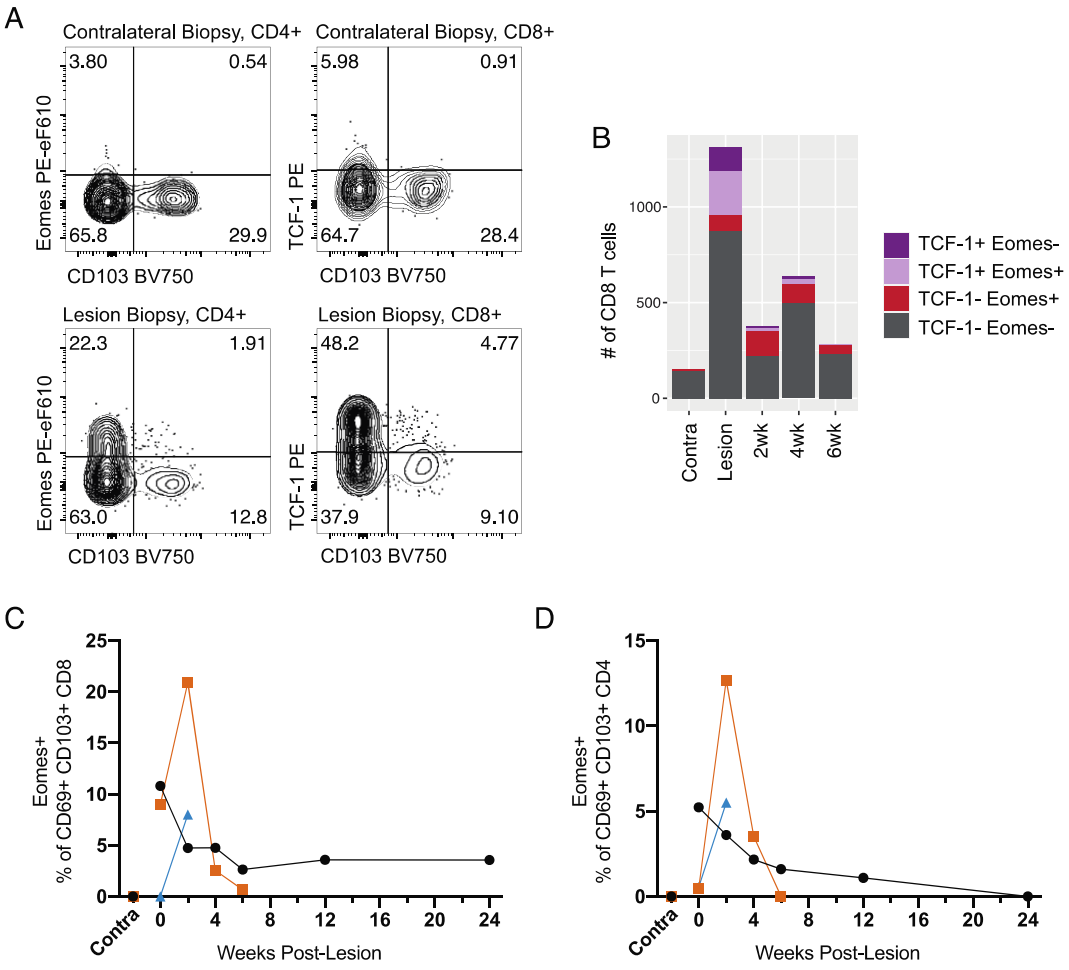

Figure 3.4. Brief uptick in TCF-1 and Eomes expression suggests influx of circulating memory T cells.

(A) Example flow plots showing Eomes and TCF-1 expression on CD103+ and CD103- CD8 T cells. (B) Median count of CD8 T cells isolated from biopsy at indicated timepoints, with bars shaded according to TCF-1 and Eomes expression. (C-D) Frequency of Eomes expression among CD69+ CD103+ CD8 and CD4 T cells in participants in which transcription factor staining was conducted (n=1-3 per timepoint). Each color and line in (C-D) corresponds to an individual participant, and each dot corresponds to an individual biopsy sample.

3.2.5 *CD103 demarcates a distinct subset of CD8 and CD4 T cells*

We next endeavored to fully characterize influxing T cells present in lesion versus healthy tissue using a broad panel of markers to identify phenotypes of activation, exhaustion, and lineage, again using matched PBMC samples to set gates (Figure 3.5A and Table 3.3). To visualize the overall composition of cells present we utilized t-distributed stochastic neighbor embedding (*t*-SNE) dimensional reduction analysis. Comparing biopsies from week 0 or week 2 lesions versus healthy tissue, we found that cells from the three sample collection contexts intermingled in most regions of the *t*-SNE plot (Figure 3.5B). We could clearly observe segregation between CD8 T cells, CD4 T cells, and Tregs (Figure 3.5C). Interestingly, we observed that CD69 was expressed promiscuously, albeit to varying degrees, by cells located in all regions of the dimensionally-reduced space (Figure 3.5D). Given that CD69 is not solely a Trm marker but also a marker of a T cell's recent exposure to antigen or inflammation (Castellanos et al., 1997), this widespread expression may be a direct result of recent HSV-2 infection. On the other hand, CD103 clearly demarcated subsets of both CD8 and CD4 T cells, and was co-expressed with the subpopulation of cells that expressed the highest level of CD69 (Figure 3.5D). This finding, combined with our hypothesis that Eomes+ and TCF-1+ cells were mostly CD103- and likely derived from the circulating memory T cell pool, implied to us that CD103 expression could be used as a surrogate marker for tissue-derived vs. circulation-derived T cells. Indeed, the number of CD103- cells was increased in lesion samples relative to contralateral samples for both CD8 and CD4 T cells (Figure 3.5E).

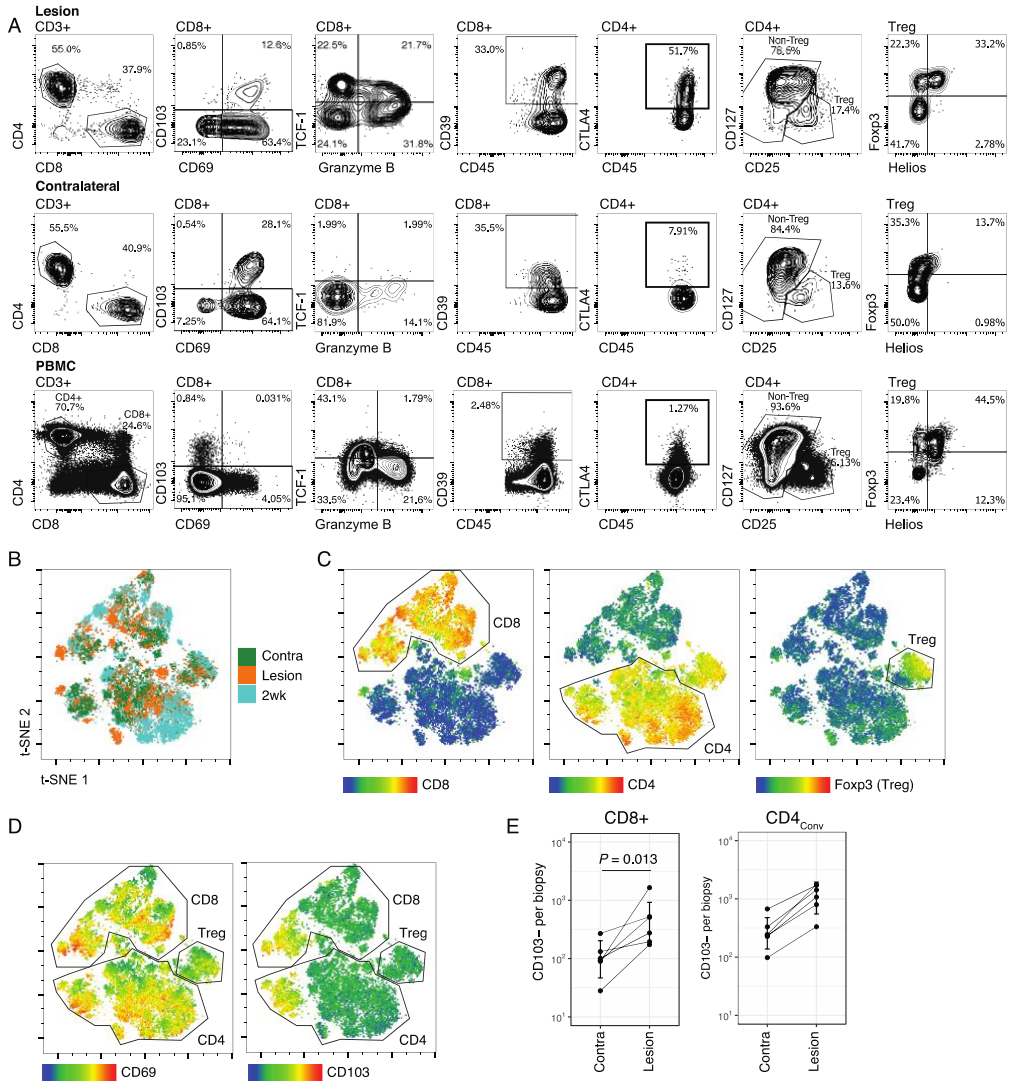

Figure 3.5. High-parameter T cell phenotyping reveals that CD103 demarcates unique clusters of CD4 and CD8 T cells.

(A) Flow cytometry staining examples of key markers in high-parameter T cell panel. (B) All viable, CD45+, CD3+ singlet events were concatenated from contralateral, lesion, and 2wk biopsy samples. These data were used to generate a *t*-SNE plot in B. (C-D) *t*-SNE plot from B colored according to expression of identity or phenotypic markers. Gates were drawn manually to highlight subpopulations. (E) Count of CD103- cells in contralateral and lesion biopsies. Each dot in (B-D) represents an individual viable CD3+ event. Each line in (E) represents an individual participant and each dot represents an individual biopsy sample. *P*-values in (E) were calculated by paired *t*-test using log-transformed values.

3.2.6 *CD103+ and CD103- T cells appear activated and functional after HSV-2 lesion formation, and then return to quiescence*

Interpreting CD103 expression as a possible proxy for tissue-derived vs. circulation-derived identity, we next assessed the activation profiles of T cells according to their CD103 status to explore their functional capacity. Comparing participant-matched healthy contralateral skin to healing lesion sites, we found that lesion-resident and infiltrating CD8 T cells expressed multiple activation markers, indicative of their antiviral capacities (Figure 3.6A). For example, the percentage of granzyme B+ and CD39+ CD8 T cells was significantly increased within the first 1-2 weeks after lesion presentation among both CD103+ and CD103- subsets. We observed a similar spike in CTLA-4 expression among non-Treg CD4 T cells (Figure 3.6A). After Week 2, the spike in the frequency of cells responding to viral insult tapered off in an apparent return to homeostasis. Consistent with expectations for homeostasis, Trm isolated from contralateral healthy skin maintained quiescence with minimal expression of activation or proliferation markers.

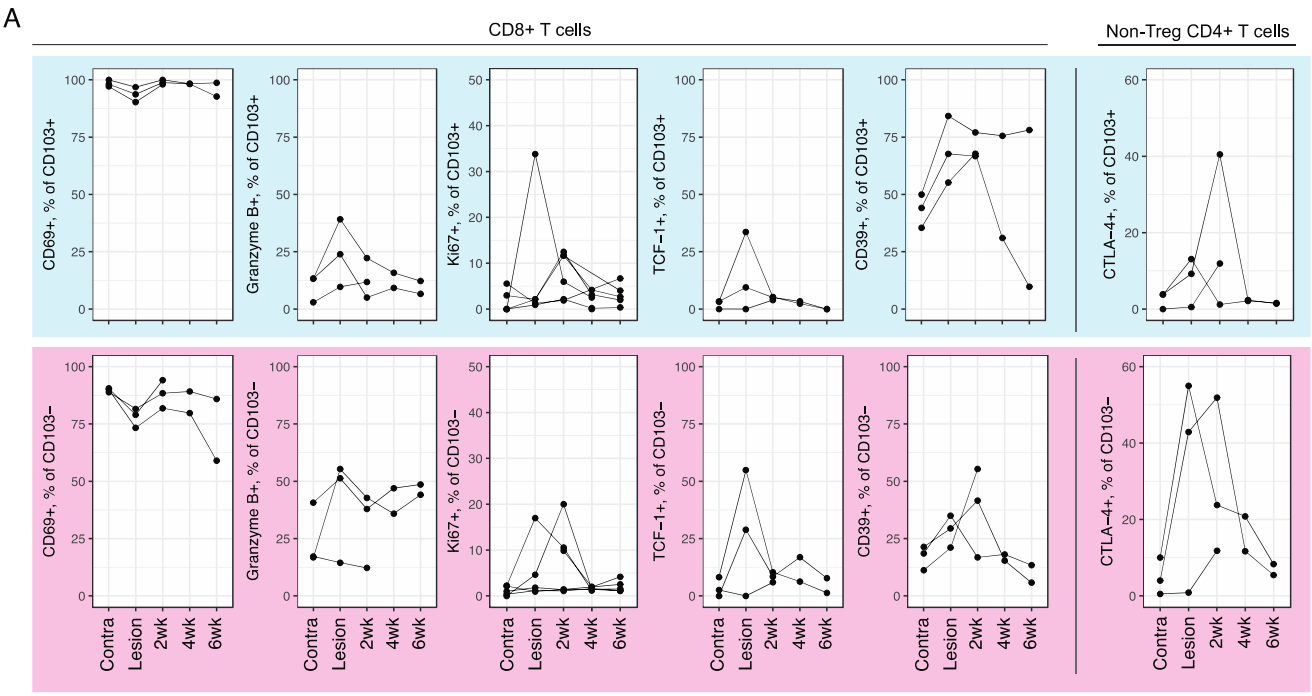

Figure 3.6. CD8 and CD4 T cells upregulate activation markers upon HSV-2 lesion formation.

(A) Frequency of CD69, granzyme B, Ki67, TCF-1, CD39, and CTLA-4 by CD8 and CD4 T cells at timepoints after lesion formation. Each line represents an individual participant and each dot represents an individual biopsy sample.

3.2.7 *No evidence for sustained acquisition of T cell exhaustion phenotypes despite the frequency of HSV-2 reactivation*

Given our observation that T cells present in HSV-2 lesion sites express multiple activation markers during the early weeks following lesion presentation, we sought to determine the fate of these cells over time. Specifically, we assessed the possibility that recurrent and frequent exposure to viral antigens and the associated heightened state of activation may lead to T cell exhaustion. We assessed staining patterns for several T cell phenotypes associated with exhaustion, including TCF-1-, Tox+, IRF4+, PD-1+, Tox+, and Tim3+ (Figure 3.7A-C and data not shown). Interestingly, we observed similar kinetics across the various parameters, characterized by early and brief peaks associated with lesion formation, followed by a decline to homeostasis as defined

by the phenotype of the contralateral biopsy. For most of these markers, little to no expression was observed at homeostasis. Some PD-1 expression was sustained during immune quiescence, a phenotype commonly associated with Trm in both mice and humans (Szabo et al., 2019b). Therefore, although the genital skin of HSV-2 seropositive women experiences chronic exposure to viral antigen, we observed no evidence for the acquisition of lasting phenotypes associated with T cell exhaustion and loss of function.

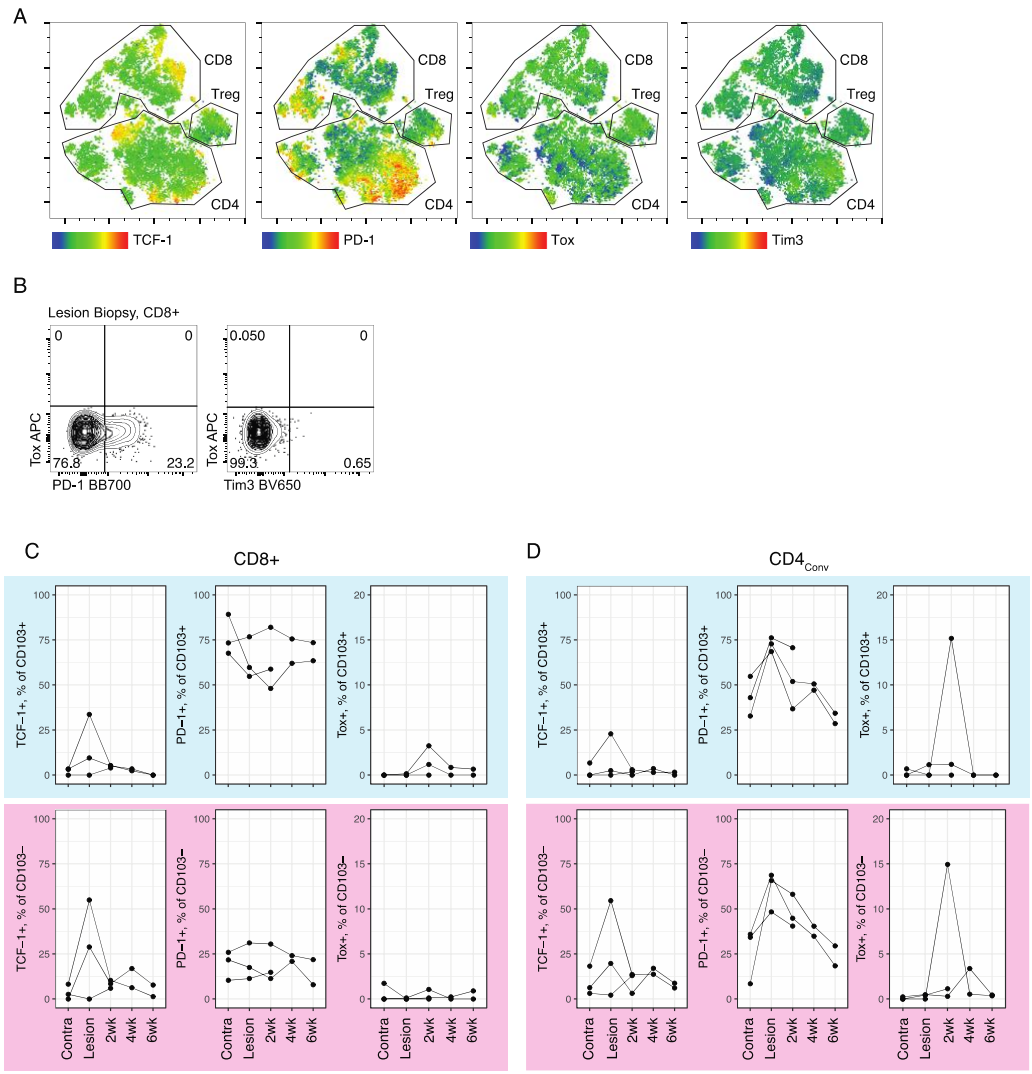

Figure 3.7. Despite frequency of HSV-2 reactivation, biopsy-derived T cells do not exhibit sustained expression of exhaustion markers.

(A) *t*-SNE plots demonstrating expression intensities of TCF-1, PD-1, Tox, and Tim3 among concatenated CD3+ events from contralateral, lesion, and 2wk biopsy samples. (B) Example flow staining for transcriptional factors and surface markers associated with CD8 T cells functional potential or exhaustion. (C-D) Frequency of expression of TCF-1, PD-1, and Tox among CD103+ (blue) and CD103- (pink) CD8 T cells and non-Treg conventional CD4 T cells. Each dot in (A) represents an individual live CD3+ event. Each line in (C-D) represents an individual participant and each dot represents an individual biopsy sample.

3.2.8 *Cell-extrinsic regulatory mechanisms are upregulated upon lesion formation*

We next sought to identify potential regulatory mechanisms governing the observed T cell resilience as opposed to exhaustion in lesion tissues. To this end, we assessed the frequency and phenotype of Tregs present in healing lesion tissue over time as compared to tissue Tregs present in healthy genital skin. Tellingly, bulk Tregs were significantly more abundant in lesion tissues during the first 2 weeks after lesion presentation, followed by an apparent return to homeostatic frequencies after week 2 (Figure 3.8A and Figure 3.2D). The majority of lesion-derived Treg were CD69+ and displayed patterns of activation including high expression of CD39, CTLA-4+, and PD-1, but not CD137 (Figure 3.8B-C). Unlike CD8 and CD4 T cells, the vast majority of tissue Tregs in lesion sites did not express CD103. We therefore used CD69 to delineate Treg that were possible tissue-resident vs. Treg that had influxed into the tissue from the blood.

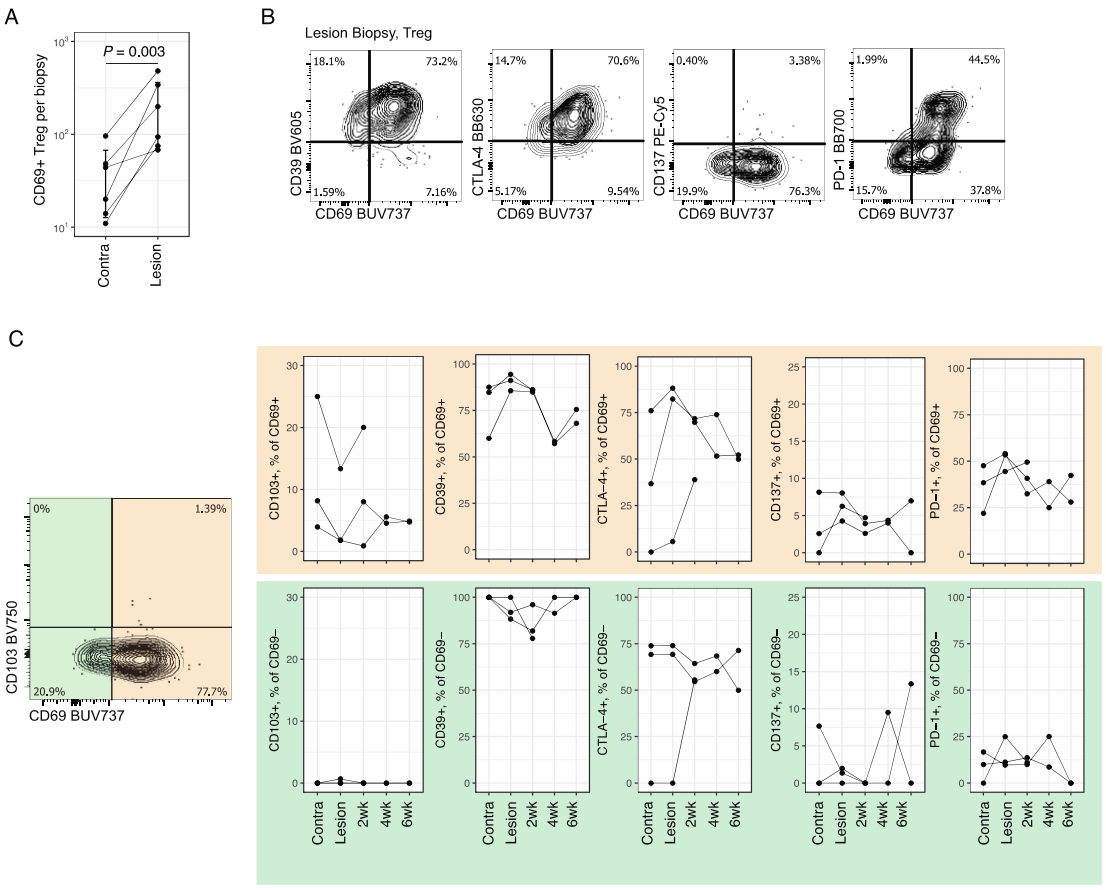

Figure 3.8. CD69+ Treg transiently upregulate markers of suppressive function upon lesion formation.

Number of CD69+ Treg in lesion and contralateral samples. (B) Examples of Treg marker staining by flow cytometery. (C) Frequency of expression of CD103, CD39, CTLA-4, CD137, and PD-1 among CD69+ (yellow) and CD69- (green) Treg. Each line in (A) and (C) represents an individual participant and each dot represents an individual biopsy sample.

Regulatory T cells modulate immune responses through a variety of mechanisms. For example, the ectoenzyme, CD39, coordinates with CD73 (also expressed on Tregs) to hydrolyze ATP to immunosuppressive adenosine (Deaglio et al., 2007). In contrast, CTLA-4 requires cell surface contact, competitively binding B7 molecules to inhibit co-stimulatory activity (Schmidt et al., 2012). We found that CD39 expression on CD69+ Tregs followed similar kinetics as we had observed for other T cell populations, namely an early upregulation followed by the return to baseline upon lesion healing (Figure 3.8C). Interestingly, CD69- Tregs appeared to constitutively

express CD39, possibly indicating their recruitment from the blood in response to viral insult, as opposed to commitment to tissue residency. CTLA-4-expression in CD69+ tissue Tregs similarly peaked in frequency early during infection and contracted upon lesion healing; whereas recruited CD69- Tregs appeared to maintain stably high CTLA-4 in 2 of the 3 individuals. Finally, we assessed the expression of the co-stimulatory molecule, CD137, which marks recent Treg:antigen encounter (Bacher et al., 2016; 2014) and PD-1, which promotes the survival and suppressive capabilities of Tregs (Asano et al., 2017) to further evaluate Treg activation and potential for suppressive function. Consistent with this expectation, we observed an expansion of PD-1+CD69+ tissue Tregs early after lesion presentation, followed by a return to baseline. CD137 was moderately upregulated in CD69+ tissue Tregs early after lesion presentation and only intermittently found in CD69- recruited Tregs. Together, our data indicate that both CD69+ resident memory Tregs, as well as recruited CD69- Tregs become activated in response to lesion presentation; and whereas tissue Tregs temporally adjust their activation patterns concordant with tissue healing, recruited CD69- Tregs appear to be less sensitive to tissue cues.

3.3 Discussion

Key to the success of the human adaptive immune system is the ability of memory T cells to respond more rapidly to antigen-matched infections upon secondary exposure than primary exposure. Our current understanding of memory T cell recall was established in mouse studies, which have the advantage of allowing researchers to fine-tune parameters such as the number of antigen-specific T cells, the timing of secondary exposure, the presence of cognate antigen, and the amount and type of inflammation (reviewed in (Kaech and Wherry, 2007; Woodland and Kohlmeier, 2009)). These studies are most commonly conducted in specific pathogen-free animals exposed to a single priming event followed by a single challenge event. In addition, infections

used to characterize canonical T cell priming and memory formation in mice are often systemic infections that are administered parenterally, such as vesicular stomatitis virus and lymphocytic choriomeningitis mammarenavirus. By contrast, at the time of primary exposure to any specific pathogen, adult humans already have a diverse history of infections and associated memory T cell populations. In addition, most infections faced by human immune cells are encountered and controlled within a specific non-lymphoid tissue location before systemic infection is established. Given these key differences, it is critical to determine whether observations about memory T cell recall in mice can be extrapolated to understanding human T cell responses.

In this study, we sought to characterize human memory T cell recall within a virus-infected non-lymphoid tissue site. To accomplish this, we performed serial biopsies of HSV-2 genital lesions with timepoints spanning initial ulcer resolution through ~24 weeks after lesion healing. We compared the T cell infiltrate from these biopsies to two types of control samples: (1) contralateral genital skin biopsies collected from the same participants who had histories of frequent genital herpes episodes, and (2) site-matched genital skin biopsies from participants who were seronegative for both HSV-1 and HSV-2 and did not have other ongoing vaginal or genital infections. Surprisingly, we found that these two types of control samples were similar in terms of the size and composition of the T cell compartment (Figure 3.1), perhaps in line with the fact that HSV-seronegative women still have a lifelong history of infection exposure. In lesion biopsies, on the other hand, we observed a uniformly expanded immune cell compartment relative to contralateral control biopsies, with higher numbers of CD8 T cells, CD4 T cells, Tregs, and monocytes. After 24 weeks of follow-up, T cells had contracted in the lesion site and the number of infiltrating T cells was similar to contralateral biopsies (Figure 3.2), as previously reported (Zhu et al., 2013; 2009).

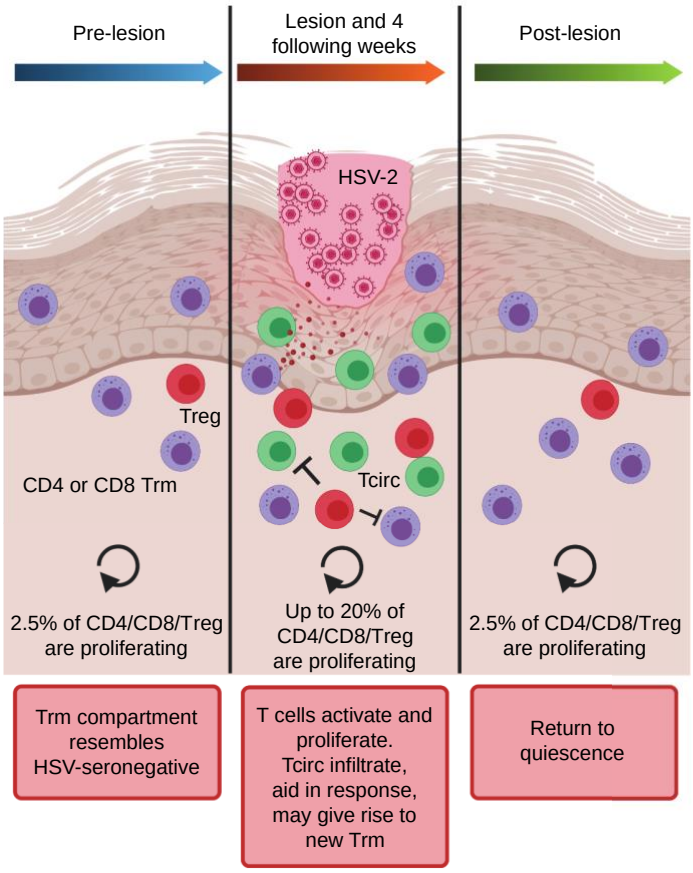

Figure 3.9. Upon HSV-2 lesion formation, genital skin T cells and infiltrating circulating T cells mount a transient response and then return to quiescence.

We found that proliferation frequency, as measured by Ki67 staining, was too low to explain the observed T cell expansion (Figure 3.3). This finding, combined with an uptick in the expression of transcription factors associated with circulating T cells and Trm precursors (Figure 3.4), suggested that we were capturing T cells infiltrating from the blood into the infected tissue. We observed that these CD8 T cells, CD4 T cells, and Tregs upregulated markers of activation (Figure 3.6) and immune regulation (Figure 3.8). Overall, our data indicate that localized viral insult in human non-lymphoid tissue results in similar memory recall features to those reported in mouse models (Figure 3.9). For example, the ability to sense cognate antigen and then recruit additional effector cells from the circulation is a well-known feature of Trm in mouse models

(Davies et al., 2017; T. N. Khan et al., 2016; Reinhardt et al., 2003; Schenkel et al., 2013). Likewise, mathematical modeling indicates that this recruitment is a key method of control in human HSV-2 lesions (Roychoudhury et al., 2020).

We observed that the *in situ* T cell response to HSV-2 occurred without the acquisition of an exhaustion-associated phenotype (Figure 3.7), resembling the immune response observed in non-lethal acute viral infections in mice, in which virus is cleared and T cells retain functionality (Bachmann et al., 2005; Khanna et al., 2003; Masopust et al., 2001). HSV-2 infection in humans recurs frequently and intermittently, with periods of viral quiescence in between periods of intense viral replication (Schiffer and Corey, 2013). HSV-2 in humans therefore lies in a space between acute infections, which quickly resolve after antigen clearance, and classically defined chronic infections, which involve ongoing inflammation and antigen persistence. Our data suggest that these periods of viral quiescence may be long enough to allow normal contraction and resolution of the immune response. This conclusion is supported by the fact that we observed transient Eomes expression associated with lesion formation, rather than the prolonged expression of Eomes that marks a pathogenic subset of T cells observed in inflammatory bowel disease (Boland et al., 2020). Therefore, we found that the T cell response to HSV-2 infection more closely resembles a series of acute recall responses, despite HSV-2's chronicity.

Conversely, previous reports indicate that blood-derived HSV-1-specific CD8 T cells from people with symptomatic ocular herpes express PD-1 and Lag-3, and that T cell responses are improved by checkpoint blockade in hamster models of ocular herpes (Coulon et al., 2020; Roy et al., 2019). While we did not stain for Lag-3, we assessed CD8 T cell exhaustion in a subset of genital biopsy samples by staining for a variety of transcription factors and surface markers, including PD-1, Tim3, Tox, TCF-1, and Eomes. Given that certain phenotypes of Tex are also

phenotypes of Trm and/or T cell activation (such as loss of TCF-1 and expression of PD-1+), we chose to stringently define T cell exhaustion in our study as sustained longitudinal expression of Tox or co-expression of multiple other Tex markers. While some of these markers were transiently expressed during early timepoints, we did not observe sustained maintenance of exhausted T cell phenotypes as have been reported in other chronic infection settings in mice and humans (reviewed in (McLane et al., 2019)). Rather, these T cells seem to retain functionality: a past report has shown that T cells expanded from genital herpes lesions can produce IFNg (Zhu et al., 2009), and we observed upregulation of granzyme B upon lesion formation. Given that our participants have a history of frequent genital herpes episodes, this suggests that their genital skin Trm compartment is either non-exhausted, or that exhaustion associated with past lesions occurs only in very small anatomical regions that are highly unlikely to overlap with future lesion occurrences. Our results may differ from those observed in ocular herpes due to the site of infection (eye vs. genital skin), source of CD8 T cells (blood antigen-specific vs. genital skin bulk), type of infection (HSV-1 vs. HSV-2), or choice of markers used to define exhaustion. We also acknowledge that this portion of our study was conducted in a very small sample cohort, and our results may not be generalizable to other people with recurrent genital herpes.

We observed that immune expansion and activation occurred at the same time as an increase in the number of Tregs and Treg suppressive marker expression. Tregs isolated from lesion biopsies expressed CTLA-4 and CD39. In addition, we found that CD8 T cells expressed high PD-1 early after lesion development and non-Treg CD4 T cells upregulated CTLA-4. Together, these data indicate that both cell-extrinsic and cell-intrinsic immune regulation mechanisms were at play after HSV-2 lesion resolution. While the function of skin-infiltrating Tregs remains incompletely understood, Tregs are present in mouse and human skin, express IL-

10 and CTLA-4, limit autoimmunity. and promote tissue repair (reviewed in (Boothby et al., 2020)). We hypothesize that these responses may be critical to limiting tissue damage associated with immune responses to HSV-2.

People living with recurrent genital herpes fail to develop sterilizing immunity against further viral reactivation. Based on this study, we believe this is unlikely to be attributable to T cell exhaustion among the genital skin Trm compartment. We suspect that the Trm compartment aids in control of viral reactivation by sensing viral antigen and recruiting additional effector cells from the circulation. Along with this response, regulatory T cells also expand in genital lesions, likely assisting in the return to quiescence and limiting tissue damage. Our data and past studies show that these responses happen on microscopic scales, with contralateral genital biopsies showing no signs of immune activation even during perineal or labial lesion events. A successful preventative or therapeutic vaccine needs to overcome the balance between site-specific immune activation and immune regulation, promoting a stronger T cell response while still avoiding T cell exhaustion and/or tissue damage. This might be achieved by designing a vaccine that incorporates other elements of the adaptive response (such as antibodies) or finding a way to boost the number of HSV-specific T cells uniformly throughout the genital skin and mucosa as well as the blood, to enable more rapid sensing and a more efficacious infiltrating immune response.

3.4 Methods

3.4.1 *Study population*

The protocol, biopsy procedures, and informed consent were approved by the University of Washington Institutional Review Board. HSV-2+ subjects had a history of clinically symptomatic genital herpes. Biopsies were collected with a 3 mm punch biopsy tool and blood was collected by peripheral blood draw. Biopsies were collected approximately 1 week after initial

ulcer appearance so as to avoid collecting a biopsy from an actively ulcerative site. Each 3 mm punch biopsy of an acute lesion included 50% of the vesicle area and 50% of the immediately adjacent erythematous skin area. Biopsies collected after lesion healing were collected in a daisy chain fashion from the predominant area of the lesion, usually contiguous to the prior biopsy. Control skin biopsies were collected from the opposite (contralateral) anatomic site of the HSV reactivation. We also enrolled HSV-1 and HSV-2 seronegative participants who underwent a single labia majorum or perineum biopsy (Table 3.1). All participants were negative for other common sexually-transmitted infections.

Table 3.1. Summary of participants

Participant	Biopsy location	Weeks Between Ulcer Appearance and First Biopsy	HSV Status	Flow Cytometry Panel
1	Labia majorum	0.71	HSV-2+	Immune Cell Subset Panel
2	Labia majorum	1.57	HSV-2+	Immune Cell Subset Panel
3	Labia majorum	1.71	HSV-2+	Immune Cell Subset Panel
4	Perineum	2.57	HSV-2+	Immune Cell Subset Panel
5	Perineum	1.71	HSV-2+	Immune Cell Subset Panel
5	Labia majorum	1	HSV-2+	T Cell Activation and Exhaustion Panel
6	Mons	1.86	HSV-2+	Immune Cell Subset Panel
7	Perineum	1.14	HSV-2+	Immune Cell Subset Panel
8	Perineum	1	HSV-2+	T Cell Activation and Exhaustion Panel
9	Buttock	1	HSV-2+	T Cell Activation and Exhaustion Panel
10	Labia majorum	NA	HSV-seronegative control	Immune Cell Subset Panel
11	Labia majorum	NA	HSV-seronegative control	Immune Cell Subset Panel
12	Perineum	NA	HSV-seronegative control	Immune Cell Subset Panel
13	Labia majorum	NA	HSV-seronegative control	Immune Cell Subset Panel
14	Perineum	NA	HSV-seronegative control	Immune Cell Subset Panel
15	Perineum	NA	HSV-seronegative control	Immune Cell Subset Panel

3.4.2 *Tissue collection and processing*

Blood was collected into heparin tubes. Biopsies were transported from the clinic in ice cold unsupplemented Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco) and immediately processed upon arrival. Biopsies were minced into pieces and subjected to two rounds of digestion in 10 ml RPMI supplemented with 2% FBS, 2 mg/ml collagenase D (Roche), and 1.5 mg/ml DNase I (Roche) for 30 minutes at 37C with gentle rocking. Single cell suspensions were washed and filtered before staining for flow cytometry.

3.4.3 *Cell staining for flow cytometry*

Cells were first incubated in LIVE/DEAD Blue fixable amine-reactive viability dye (ThermoFisher) for 10-20 min at room temperature. Two different flow panels were used to characterize immune cells. For the Immune Cell Subset Panel (Table 3.2), CCR7 staining was performed at 37C and all other staining was performed on ice. For the T Cell Activation and Exhaustion Panel (Table 3.3), samples were blocked for Fc binding using Human TruStain (BioLegend), and then stained with antibodies on ice. Cytosolic and nuclear proteins were detected using Foxp3/Transcription Factor Fixation/Permeabilization reagents (eBioscience). Samples were acquired on a FACSymphony instrument (BD). Cell counts were determined by acquiring the full sample and determining the number of cellular events recovered.

Table 3.2. Immune cell subset panel

Detector	Target	Antibody Clone	Fluorophore	Company
UV 395	CD38	HB7	BUV395	BD
UV 450	Viability	LIVE/DEAD Blue		ThermoFisher
UV 563	CD8	RPA-T8	BUV563	BD
UV 661	CD11c	B-ly6	BUV661	BD
UV 737	CD14	M5E2	BUV737	BD
UV 805	CD45	HI30	BUV805	BD
Violet 450	Helios	22F6	eFluor450	eBioscience
Violet 510	CCR7	G043H7	BV510	Biolegend
Violet 570	CD127	A01905	BV570	Biolegend
Violet 610	CD69	FN50	BV605	Biolegend
Violet 655	CD103	Ber-ACT8	BV650	BD
Violet 710	HLA-DR	L243	BV711	Biolegend
Violet 780	CD141	M80	BV785	Biolegend
Blue 515	CD3	HIT3a	BB515	BD
Blue 700	CD1c	F10/21A3	BB700	BD
Green 575	TCF-1	C63D9	PE	Cell Signaling
Green 610	Foxp3	206D	PE-Dazzle594	Biolegend
Green 660	CD123	9F5	PE-Cy5	BD
Green 780	DC-SIGN	9E9A8	PE-Cy7	Biolegend
Red 660	Ki67	Ki67	APC	BD
Red 710	CD25	2A3	APC-R700	BD
Red 780	CD4	OKT4	APC-Cy7	Biolegend

Table 3.3. T cell activation and exhaustion panel

Detector	Target	Antibody Clone	Fluorophore	Company
UV 395	CD8	RPA-T8	BUV395	BD
UV 450	Viability	LIVE/DEAD Blue		ThermoFisher
UV 496	CD3	UCHT1	BUV496	BD
UV 563	CD25	2A3	BUV563	BD
UV 737	CD69	FN50	BUV737	BD
UV 805	CD45	HI30	BUV805	BD
Violet 450	Helios	22F6	eFluor450	eBioscience
Violet 510	CCR7	G043H7	BV510	Biolegend
Violet 570	CD45RA	HI100	BV570	Biolegend
Violet 610	CD39	A1	BV605	Biolegend
Violet 655	Tim3	7D3	BV650	BD
Violet 710	T-bet	4B10	BV711	Biolegend
Violet 750	CD103	Ber-ACT8	BV750	BD
Violet 780	Ki67	B56	BV785	BD
Blue 515	Foxp3	259D/C7	AlexaFluor488	BD
Blue 630	CTLA-4	BNI3	BB630	BD
Blue 660	CD127	A019D5	Biotin-Streptavidin BB660	Biolegend
Blue 700	PD-1	EH12.1	BB700	BD
Green 575	TCF-1	C63D9	PE	Cell Signaling
Green 610	Eomes	WD1928	PE-eFluor610	eBioscience
Green 660	CD137	4B4-1	PE-Cy5	BD
Green 710	CD19	MHCD1918	PE-Cy5.5	Invitrogen
Green 780	IRF4	IRF4.3E4	PE-Cy7	Biolegend
Red 660	Tox	REA473	APC	Miltenyi
Red 710	Granzyme B	GB11	AlexaFluor700	BD
Red 780	CD4	RPA-T4	APH-H7	BD

3.4.4 *Statistical analyses*

Comparisons between different groups of subjects were evaluated using Student's *t*-tests, and comparisons between paired biopsies from the same subjects were evaluated using paired *t*-tests. Longitudinal repeated measures data was analyzed using mixed effects models with Greenhouse-Geisser correction and Tukey's post-test. These analyses were conducted in Prism for

Mac v8.4.3 (GraphPad). Flow cytometry data was visualized using dot plots and *t*-SNE plots generated by FlowJo v10.7 (BD). To calculate *t*-SNE projections, live, CD45+, CD3+, singlet events were concatenated from contralateral, Week 0, and Week 2 biopsies from participants in the T cell activation and exhaustion cohort.

3.5 Acknowledgments

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Chapter 4. Concluding Remarks

4.1 Conclusions

A major theme of these projects is the distinction between memory T cell populations that reside in non-lymphoid tissues and those that circulate through the blood, lymph nodes, and spleen. We and many others have described numerous examples of unique characteristics that make T cells in one body site unique from those in another body site. Collectively, these findings have demonstrated the importance of studying T cells in a specific time and place; characteristics of T cells in the blood often do not accurately predict characteristics of T cells in barrier tissues, where many infections first occur.

The observation that barrier tissue T cells are capable of *in situ* immune responses has led to an interest in eliciting these cellular populations with site-targeted vaccines (Iwasaki, 2016; Neutra and Kozlowski, 2006; Shin and Iwasaki, 2013). There are many reports that resident-memory T cell populations can be boosted by local inflammation and local antigen exposure (Davies et al., 2017; T. N. Khan et al., 2016; Mackay et al., 2012; Shin and Iwasaki, 2012). However, parenterally delivered systemic immunizations also generate resident memory T cell compartments across the whole body. While this seems counterintuitive, it is widely reported (Reinhardt et al., 2001; Steinert et al., 2015) and we took advantage of this facet of the immune system to generate a vaginal T cell population using intravenous *Listeria monocytogenes* infection.

These reports demonstrate that local insults are not completely necessary for large Trm populations to form in a non-lymphoid tissue. In line with this, data presented here show that local infection with attenuated HSV-2 does not promote a larger Trm compartment in the CVT than systemic infection with LM-gB (Figure 2.13). In fact, our results suggest that local infection may actually drive site-matched Trm populations to a more short-lived, terminally differentiated state

than that induced by systemic infection, with higher expression of granzyme B, PD-1, and CD103 and lower expression of self-renewal factors such as TCF-1 (Figure 2.13 and Figure 2.14). This differentiation could ultimately hinder protective immune responses by reducing the lifespan of the local Trm compartment and its ability to undergo a proliferative burst upon antigen challenge. We found that after progressive loss and differentiation had occurred in LM-gB-immunized animals, they controlled HSV-2 infection more slowly (Figure 2.10). Likewise, CD8 T cells primed by local infection with HSV-2 were more differentiated (Figure 2.3) and were lost from the CVT even earlier after immunization (Figure 2.13).

Given this, perhaps an effective Trm-stimulating vaccine does not need to be given locally at the site of most likely future exposure. Some findings in the literature have similar implications: intranasal vaccination was more successful in protecting the uterus from infection with *Chlamydia trachomatis* than intrauterine vaccination in mice (Stary et al., 2015), and intravenous vaccination with Bacillus Calmette–Guérin vaccine was more successful in protecting the lung from infection with *Mycobacterium tuberculosis* than aerosol vaccination in macaques (Darrah et al., 2020). Systemic or distal vaccination could have several advantages: (1) systemic vaccination would generate a large population of circulating T cells ready to aid in local responses, (2) Trm would not encounter cognate antigen *in situ*, meaning that they would remain in a less differentiated, TCF-1+ state, and (3) T cells would not be recruited into the high-density Trm clusters observed near HSV-2 lesions in our and others' reports (Figure 3.2) (Schiffer et al., 2013; Zhu et al., 2013; 2007), instead seeding all regions of a barrier tissue more evenly. Overall, these features could lead to a more proliferative secondary Trm response and more effective Trm patrol. Of course, these advantages would have to be balanced against safety and implementation considerations of systemic vaccination.

4.2 Limitations and Future Directions

There are several outstanding questions that we did not address in this study. Most critically, we did not elucidate the exact reasons that T cells within the CVT undergo a unique developmental timeline that is not observed in the spleen, lymph nodes, or small intestine lamina propria. We observed that CD103+ granzyme B+ CD8 T cells in the CVT completely lacked *Tcf7* transcript and TCF-1 protein expression, suggesting that these cells might be deficient in self-renewal. To evaluate this, we attempted to perform a bromodeoxyuridine (BrdU) incorporation assay to assess basal rates of proliferation among CD8 T cells in the CVT one month after immunization (data not shown). While we observed a similar level of BrdU incorporation among CD8 T cells in the CVT and the SI LP, interpretation of these data was hindered by the lack of a longitudinal experiment design in which the circulating memory T cell compartment was depleted. A future study that depleted gBT-I cells and then compared BrdU incorporation between Early Memory and Late Memory CVT CD8 Trm induced by either HSV TK- or LM-gB would reveal whether particular timepoints or immunization methods correlate with a defect in steady-state proliferation.

In addition, we did not determine the factors intrinsic to the CVT that mediate the failure to self-renew. For example, the CVT may be deficient in signals that are important for Trm survival, such as TGF-β or exogenous free fatty acids (Pan et al., 2017; N. Zhang and Bevan, 2013). Alternatively, the CVT could be physically unable to sustain Trm populations due to epithelial shedding associated with hormonal fluctuations. Future studies must further elucidate the relationship between the CVT microenvironment and Trm longevity in mice and determine whether a similar deficit in Trm longevity also exists in humans. Finally, we must also consider

whether the deficit in the longevity of CVT Trm may be an adaptive feature and an important part of female reproductive health.

In addition, we did not characterize other T cell subsets that are seeded in the CVT after infection, such as CD4 T cells and Treg. Therefore, we did not conclude whether these subsets also underwent progressive loss and differentiation after immunization. One difficulty in studying the CD4 and Treg responses in this context was our inability to identify antigen-specific cells. Past studies have characterized CD4 T cells that are specific to the HSV-1 glycoprotein D IPPNWHIPSIQDA epitope in C57Bl6 mice, using both transgenic gD-sepcific CD4 T cells (gDT-II cells) and I-Ab tetramers (Bedoui et al., 2009; Cabrera-Perez et al., 2015; Hor et al., 2015; Kato et al., 2015). This epitope differs by one amino acid in HSV-2, possibly creating issues with applying these tools to HSV-2 infection. However, we still tried to take advantage of these tools by using gDT-II cells and a strain of *Listeria* engineered to express IPPNWHIPSIQDA. We encountered several issues applying these tools, including the fact that most gD tetramer+ cells in gDT-II donor mice were actually CD3+ CD4- CD8-, making CD4 T cell enrichment difficult. In addition, we found that gDT-II cells did not reliably expand after infecting mice with HSV-2, and we could not detect responses with gD tetramer or gD peptide stimulations after vaginal HSV-1 or HSV-2 infection. Overall, in our hands, the IPPNWHIPSIQDA epitope was insufficiently immunodominant to allow us to reliably use these tools to track an antigen-specific population of CD4 T cells in the mouse vagina. Perhaps future studies can employ different tools or characterize new epitopes that would better enable the long-term tracking of CD4 T cells after HSV-2 infection in mice. In addition, recent (Soerens et al., 2016) and ongoing studies in the Lund lab continue to study the bulk CD4 and Treg compartments in the cervicovaginal tissue and their role in HSV-2 infection.

In our study of the T cell infiltrate into human genital herpes lesions, a major limitation is our small sample size. Though trends were relatively consistent across participants, our estimation of means and variability was still hindered by the fact that we had only six participants in the main study, six control samples, and three participants in the T cell activation and exhaustion portion of the project. Using these samples, we measured between 22 and 27 flow cytometry parameters at 6-8 timepoints, resulting in ~200 unique immune cell phenotypes per participant, not including the many combinations of co-expressed flow cytometry markers that may provide deeper phenotypic information. This level of phenotyping across so few participants represents both a data analysis and data interpretation challenge. Though shutdowns related to the 2020 pandemic were partially responsible for our small sample size, intensive longitudinal biopsy studies are, by definition, difficult and expensive to conduct and represent a challenging commitment for study participants. With a higher sample size, we may have been able to employ mathematical modeling approaches to explore the idea that expanded Treg populations were actively restraining local proliferation of CD4 and CD8 T cells. Our and others' long (24 week) periods of follow-up have established that these tissue sites seem to return to quiescence by 6-8 weeks after lesion formation (Zhu et al., 2009). Perhaps future studies could increase sample sizes by decreasing the length of follow-up, instead dedicating resources to increasing the number of participants.

We employed a biopsy daisy chain strategy (Figure 3.2) to repeatedly sample a small region of skin from a single HSV-2 lesion event. This method has the advantage of allowing the longitudinal assessment of small areas of skin, but also relies on the assumption that each daisy chain node is equivalent. In reality, the immune response to HSV-2 is probably extremely targeted, and may differ in size, composition, and phenotype from node to node. This is a limitation of the design that has no clear solution: it is not possible to safely and ethically biopsy an entire ulcer and

repeat biopsies of the exact same location would still not measure the same response each time given that tissue and cells were repeatedly removed. This limitation can be partially addressed through the use of inbred mouse models, in which each mouse is assumed to mount a roughly identical response, allowing for the sampling of a whole tissue site at multiple timepoints.

One goal of our study was to assess whether repeated HSV-2 reactivation eventually drives T cell exhaustion among the Trm compartment. We did not observe strong evidence of T cell exhaustion, but this conclusion has limitations related to sample size and the techniques/markers used. The presence or absence of T cell exhaustion in genital HSV-2 lesions could be further explored by isolating biopsy-derived T cells and determining whether they retain the ability to respond to *ex vivo* restimulation. Infection of a biopsy explant could be one method to evaluate this question while leaving the Trm compartment as untouched as possible. Guinea pigs can serve as a model of HSV-2 reactivation, but studies of T cell exhaustion in guinea pig models may be impeded by the limited understanding of guinea pig T cell exhaustion and the availability of antibodies targeting exhaustion markers. HSV-2 does not reactivate after vaginal infection of C57Bl6 mice, but perhaps reactivation could be simulated by repeatedly challenging the same animals with wildtype HSV-2 after initial immunization with HSV-2 TK-. Finally, as transcriptional and epigenetic signatures of T cell exhaustion in humans become increasingly well-defined (Abdelsamed et al., 2020; Boland et al., 2020; Szabo et al., 2019a), modern approaches such as single cell RNA or bisulfite sequencing with oligo-tagged antibodies might be able to robustly determine the presence or absence of exhausted T cells in human genital herpes lesions.

4.3 Final Thoughts

Cellular immunologists divide the cells they study into subsets. In reality, the lines between these subsets are often blurred. Depending on the context, Trm may be defined by their location

in non-lymphoid tissue, their surface-expressed proteins, their functions, and their recirculation patterns, but each definition has caveats. Over the last five years, new reports have challenged each of these definitions. Trm can actually form in the secondary lymphoid organs as well as the non-lymphoid tissues (Beura et al., 2018b; Böttcher et al., 2015). Surface marker expression is inconsistent and depends not just on the tissue of origin (Steinert et al., 2015), but also on the method and context of priming (our data). Cells that strongly resemble Trm can be isolated from human blood (Klicznik et al., 2019) and Trm in mice can give rise to so-called "ex-Trm" that rejoin the circulation (Fonseca et al., 2020). Given these and many other examples of cells that span subset definitions or migrate between subsets, the lack of developmental plasticity in one setting does not necessarily mean that developmental plasticity is impossible. I believe it is important to keep an open mind about a T cell's function and identity.

T cell subsetting, especially in humans, relies heavily on flow cytometric analysis of protein expression, and many proteins that are used to identify one subset can in fact be expressed by many subsets under certain conditions. For example, CD25 and Foxp3 can be used to identify Treg, but are also expressed by non-Treg CD4 T cells upon activation (Kmieciak et al., 2009). CD69 is a common Trm marker, but also widely known and used as a marker of T cell activation (Castellanos et al., 1997). Eomes expression is associated with T cell exhaustion (reviewed in (McLane et al., 2019)), but Eomes is also expressed by non-exhausted memory T cells and Trm precursors (Banerjee et al., 2010; Mackay et al., 2015) and is necessary for appropriate memory T cell survival and re-expansion. Loss of TCF-1 combined with PD-1 expression is a marker of irreversible T cell exhaustion in chronic infection (Im et al., 2016), but this phenotype is also observed among Trm that presumably retain functionality (Hombrink et al., 2016; Wu et al., 2020). Preventing or reversing T cell exhaustion by mechanisms such as checkpoint blockade has been

highly successful in cancer immunotherapy (reviewed in (McLane et al., 2019)), and ongoing research is exploring how to prevent the development of T cell exhaustion in engineered T cells designed to detect and eliminate tumors (Lynn et al., 2019). Given that activated T cells, resident-memory T cells, and exhausted T cells seem to share certain features, future research should explore whether modifications to prevent T cell exhaustion also affect the ability of engineered T cells to infiltrate into and persist within solid tumors in non-lymphoid tissues.

In closing, this project aimed to deepen our understanding of genital tract T cell responses. While antibody-based vaccines have been widely successful for many pathogens, vaccines for HSV-2 and other sexually transmitted infections may require a T cell component in order to achieve efficacy. We have found that the mouse and human cervicovaginal mucosa and genital skin contain unique and diverse T cell populations. The lifespans and characteristics of these T cells are driven by tissue-intrinsic factors as well as additional variables like the time since immune priming and the type and route of immunization or infection. Given the high burden of disease attributable to pathogens that initially infect the genital tract, we hope that these data will inform future attempts to induce protective mucosal immune responses via immunization.

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