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Regulatory T cell contributions to mucosal antiviral immunity

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Abstract

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Regulatory T cells (Tregs) prevent autoimmunity and limit immunopathology using a variety of suppressive mechanisms, yet their roles during an immune response against pathogens remains unclear. Following intravaginal herpes simplex virus (HSV) type 2 infection, mice lacking Tregs fail to control viral replication within the infected tissue, pointing to a role for Tregs in facilitating productive immune responses. CD4 T cells are necessary for successful anti-HSV-2 immune responses in mice; as such we examined the role of Tregs in the generation of HSV-2-specific CD4 T cells. Using adoptive transfer of T cell receptor transgenic CD4 T cells into Treg-sufficient or Treg-depleted mice prior to HSV-2 infection, we found that Tregs are required for timely accumulation of HSV-2-specific CD4 T cells within the infected tissues. Further, Tregs

are critical for appropriate trafficking of dendritic cells (DCs) from the vaginal mucosa to the dLN, which results in fully effective CD4 T cell priming, activation, and ultimately migration to the infected tissues. Finally, using CTLA-4 conditional knockout mice, we demonstrate that in the context of HSV-2 infection, Tregs require CTLA-4 to allow for proper trafficking of migratory DCs. Taken together, our data highlight the critical role of Tregs in proper potentiation of adaptive immune responses to microbial infection.

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ABBREVIATIONS

ANOVA:	Analysis of variance
APC:	Antigen-presenting cell
CCL:	Chemokine (C-C motif) ligand
CCR:	C-C chemokine receptor
CD:	Cluster of differentiation
cDNA:	Complimentary DNA
Cre:	Cre recombinase
CTLA-4:	Cytotoxic T-lymphocyte-associated protein 4
DC:	Dendritic cell
dLN:	Draining lymph node
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DT:	Diphtheria toxin
DTR:	Diphtheria toxin receptor
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
FACS:	Fluorescence-activated cell sorting
FITC:	Fluorescein isothiocyanate
Flox:	Flanked by LoxP
Foxp3:	Forkhead box P3
gD:	Glycoprotein D
HBSS:	Hank's balanced salt solution

HSV:	Herpes simplex virus
IL:	Interleukin
IFN:	Interferon
KO:	Knockout
MHC:	Major histocompatibility complex
NK:	Natural killer
OCT:	Optimal cutting temperature
Ova:	Ovalbumin
PBS:	Phosphate-buffered saline
Rag:	Recombination-activating gene
RNA:	Ribonucleic acid
RSV:	Respiratory syncytial virus
TCR:	T cell receptor
TK:	Thymidine kinase
TLR:	Toll-like receptor
Treg:	Regulatory T cell
qRT-PCR:	Quantitative reverse transcription polymerase chain reaction
WT:	Wild type

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Chapter 1: Introduction

1.1: Immune tolerance

A primary purpose for the immune system is to prevent diseases that can be caused by the destructive actions of pathogenic microorganisms. To accomplish this goal, the immune system must be able to recognize when a potential threat is present in the host and then carry out whatever steps are necessary to either limit destructive action by the pathogen, or eliminate the pathogen from the host completely.

The vast number of different microorganisms that have the potential to cause disease creates a clear challenge for the immune system to overcome if it is going to be able to recognize and act against all potential pathogens. This challenge is made all the more dramatic by the different rates of evolutionary change between the host and the microorganism. Humans are, of course, the primary host of interest in immunology research, and they evolve over the course of many decades; whereas microorganisms that can cause human disease can evolve over the course of days, or even hours (Acevedo et al., 2014; Cuevas et al., 2015). This means that even if an immune system could have developed a fixed set of strategies to precisely recognize the array of possible pathogenic microorganisms, the pathogens would be able to evolve evasion mechanisms far faster than the host could evolve new strategies for recognition. Indeed, the innate system does rely on an array of different receptors that have evolved to recognize broadly conserved patterns that are found on microorganism but not host cells. While the innate immune system is capable of limiting pathogen growth at the beginning of an infection, it is not always sufficient to clear the infection and prevent the pathogen's spread. To fully combat pathogenic microorganisms, precise recognition is required.

For this considerable challenge to be overcome, the immune system had to evolve such that it contains not only a series of fixed pathogen recognition strategies, but also the ability to create an enormous combination of new recognition potential *de novo* within an individual host. This strategy is realized in the adaptive immune system, consisting primarily of B cells and T cells, each of which expresses on its surface a unique receptor that allows each cell to recognize a highly specific structure. Each of these receptors is developed through the random combination of pre-existing stretches of DNA that come together to code for a unique B cell receptor or T cell receptor (TCR) (Fugmann et al., 2000; Hozumi and Tonegawa, 1976). This elegant system therefore allows a host to produce a vast array of different receptors that collectively have the potential to recognize all potential pathogens.

With this system, however, comes the risk that the adaptive immune system will develop antigen receptors that have the ability to target not only pathogens, but also self-antigens, or antigens that are found on harmless microorganisms. Both of these scenarios can cause unneeded damage to, or even death of the host. The study of immune tolerance focuses on understanding how, despite its potential, a successful immune system focuses its attacks only on pathogens that have the potential to cause harm.

Within a host, immune tolerance is achieved both by the regulation of the production of adaptive immune cells, which is called central tolerance, and also by continued regulation of mature cells as they circulate through the host, which is called peripheral tolerance. Central tolerance is enforced in the bone marrow and thymus, where B cells and T cells develop, respectively. As the adaptive cells go through the semi-random rearrangement process of producing antigen receptors, those cells expressing receptors that recognize self antigens with too high of an affinity are pruned (Klein et al., 2014). However, because any given antigen

receptor has the potential to cross-react with several different antigens, it would be essentially impossible to fully eliminate all self-reactive clones without leaving a significant "hole" in the immune repertoire that could be exploited by potential pathogens to avoid detection. Therefore, many T cells and B cells exit the bone marrow and thymus with some ability to recognize, and potentially target self or innocuous antigens (Yu et al., 2015; Wardemann et al., 2003). To deal with this, the immune system uses an array of different strategies in the periphery to ensure that immune responses only target potential threats.

Many of these strategies are cell intrinsic to the potentially self-reactive immune cells, but suppression is also enforced onto the potentially self-reactive cells by antigen-presenting cells, as well as a distinct group of suppressive T cells (Walker and Abbas, 2002).

1.2: Regulatory T cells as a suppressive cell

A long standing idea that a population of suppressive cells may contribute to peripheral tolerance (Gershon and Kondo, 1970; Cunningham, 1975; Allison et al., 1971) gained acceptance in the mid 1980's to mid 1990's after a series of studies identified peripheral CD25+ CD4 T cells as a sub-population of CD4 T cells that actively suppress the immune system and prevent the targeting of self or innocuous antigens (Sakaguchi et al., 1995; 1985; Powrie and Mason, 1990).

While this served as an important breakthrough in demonstrating the existence of a population of suppressive CD4 T cells, CD25 was an imperfect marker to use for studying the details of these cells, because CD25 can be expressed on a variety of different cell types, including effector T cells after activation.

Following up on studies that suggested Foxp3 mutations in both humans (Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001) and mice (Lyon et al., 1990; Kanangat et al.,

1996; Blair et al., 1994; Clark et al., 1999; Brunkow et al., 2001) can lead to multi-organ autoimmunity, the search for a more precise marker for the population of suppressive T cells resulted in a few different groups identifying Foxp3 as the key transcription factor that drives the development of these suppressor cells (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003). By this time, these cells were generally regarded as a unique lineage of CD4 T cells called regulatory T cells (Tregs) (Suri-Payer et al., 1998; Fontenot et al., 2005). Since their acceptance as a legitimate lineage of CD4 T cells, Tregs have been studied extensively. Particular progress has been made in understanding how Tregs develop and in understanding what mechanisms they use to maintain homeostasis.

Commitment to the Treg lineage can come about in the thymus or in the periphery. In both cases, expression of Foxp3 is necessary, although not fully sufficient to induce Treg commitment (Sugimoto et al., 2006; Hill et al., 2007). As Tregs develop, they both begin to express Foxp3 and acquire a characteristic pattern of hypomethylation that favors expression of genes commonly expressed by Tregs. The methylation pattern can be passed onto daughter cells during mitosis, allowing for the stability of the Treg fate through cell proliferation (Ohkura et al., 2012; Zheng et al., 2010). The precise cues that initiate commitment to the Treg lineage are not completely clear, but TCR stimulation is involved. Foxp3 expression is induced in the thymus in response to strong recognition of MHC plus peptide (Hsieh et al., 2012; Jordan et al., 2001; Kawahata et al., 2002) and the Treg specific hypomethylation pattern is induced following extended contact between the TCR and MHC plus peptide (Ohkura et al., 2012). In the periphery, TCR stimulation in a tolerogenic environment favors Treg differentiation (Chen et al., 2003) and imprints Tregs that develop in the periphery with a unique methylation pattern (Zheng et al., 2010). The result of this development is a population of Tregs that have a wide array TCR

specificities that recognize both self and foreign peptides (Shafiani et al., 2013; Hsieh et al., 2004; 2012; Pacholczyk et al., 2006; Suffia et al., 2006). As a population, then, Tregs can use a variety of different mechanisms to suppress unwanted immune responses throughout the host against a huge variety of potential targets.

Precisely how Tregs suppress effector cells is still under investigation, but several key mechanisms are known (Campbell and Koch, 2011). Within tissues, Tregs can produce the anti-inflammatory cytokine IL-10 to counteract the presence of pro-inflammatory cytokines such as IFNγ (Rubtsov et al., 2008). Of particular importance within secondary lymphoid organs, Tregs also constitutively express CTLA-4. CTLA-4 is a surface protein that can bind to the co-stimulatory molecules CD80 and CD86 on the surface of antigen presenting cells (APCs) with very high affinity (Collins et al., 2002). CTLA-4 mediated binding to CD80 and CD86 can both block effector T cells from gaining access to co-stimulation and lead to the removal of the co-stimulatory molecules from the surface of the APC, leaving the APC less able to provide signal two to an effector T cell (Qureshi et al., 2011; Walker and Sansom, 2011). The importance of CTLA-4 for Treg function has been demonstrated using a conditional knockout system. Mice in which Tregs alone are unable to express CTLA-4 develop fatal auto-immunity with many of the same symptoms that develop in mice that fully lack Tregs (Wing et al., 2008). Armed with these, and several more, mechanisms of suppression, mature Tregs respond to different risks by expressing various homing receptors so that they can migrate to where they are needed to maintain or restore homeostasis (Koch et al., 2009; Zheng et al., 2009; Chaudhry et al., 2009; Campbell and Koch, 2011; Campbell, 2015). Thus, while more is certain to be discovered, much is already known about what molecular tools Tregs use to localize to where they are needed and suppress unwanted immune response.

One key tool developed to study the importance of Tregs in maintaining homeostasis is the Foxp3^{DTR} mouse model. In these mice, transcription of Foxp3 drives the expression of the human diphtheria toxin receptor (DTR). While all mouse cells express the mouse version of DTR, the human DTR has a higher affinity for diphtheria toxin (DT), and thus the only cells susceptible to DT-mediated death upon injection of a low dose of DT in these transgenic mice are Foxp3-expressing cells. Despite attempts to demonstrate Foxp3 expression in cell types other than Tregs, it is widely-accepted that in mice, Foxp3 is reliable marker of Tregs without promiscuous expression in other cell types (Chen et al., 2008; Chang et al., 2005; Zuo et al., 2007; Manrique et al., 2011; Kim et al., 2009; Liston et al., 2007; Put et al., 2012; Rudensky, 2011). Foxp3 is also very stable within Tregs (Rubtsov et al., 2010). Because of this, the Foxp3^{DTR} mouse model can be used to precisely target Tregs for removal in an adult mouse (Kim et al., 2007).

1.3: Regulatory T cells during infections

The appreciation that Tregs are always present and capable of suppressing unwanted immune responses was a major breakthrough in our understanding of how allergies and autoimmunity are prevented despite the presence of T cells and B cells that could initiate unwanted and damaging immune responses. However, the constant presence of Tregs also raises the question as to how Tregs allow, when appropriate, an immune response against a pathogen and what role they play throughout the subsequent immune response.

Some early studies suggested that Treg-mediated suppression can be overcome when dendritic cells (DCs) with a high enough level TLR stimulation present antigens to effector cells (Pasare and Medzhitov, 2003). Concurrently, however, other studies were demonstrating that Tregs expand in response to infection (Suvas et al., 2003; Belkaid et al., 2002). This suggested

that Tregs are not simply prevented from suppressing an anti-pathogen immune response by being switched off, but rather play a dynamic role in shaping how the immune response develops.

An early breakthrough study showed that an immune response against *Leishmania major* that develops without Tregs present is more robust than the immune response that develops when Tregs are present (Belkaid et al., 2002). Thus, the absence of Tregs allows for the clearance of the parasite. Interestingly, in this model, fully functioning immune memory depends on the persistence of antigen, so the sterilizing immunity that can be achieved when Treg activity is disrupted prevents the establishment of proper memory; so Treg-mediated parasite persistence ends up benefitting both the host and the parasite.

A similar, although less well-noted, study using a model of pneumonia induced by *Pneumocystis carinii* was published just before the study looking at *L. major* infection. *P. carinii* causes a persistent infection in mice that can lead to immune-mediated pneumonia. When chronically infected Rag-/- mice received a transfer of effector CD4 T cells, the pathogen load was decreased, but pneumonia was induced, presumably because of unrestrained activity of the effector CD4 T cells. In contrast, when chronically infected Rag -/- mice received a transfer of both effector CD4 T cells and Tregs, the parasite load was not significantly affected, but the mice were also largely protected from pneumonia (Hori et al., 2002), showing that Tregs play a key role in limiting the degree of immunopathology that is sustained during an immune response.

Shortly after, another study looked at the ability of mice to control parasitemia following infection with *Plasmodium yoelii*, a rodent model of malaria. In line with both the *L. major* and *P. carinii* models, mice that were depleted of Tregs prior to infection were better able to control parasitemia, a phenotype that correlated with a more robust anti-parasite T cell response

(Hisaeda et al., 2004).

In addition to studies using parasite models, other studies looked at the role of Tregs during an ocular infection with HSV-1. Broadly in line with the parasite studies, mice lacking Tregs during an immune response against HSV-1 mounted a stronger anti-viral response that resulted in the clearance of HSV-1 more quickly than in Treg-sufficient mice. However, along with this alacrity came increasing ocular lesions; foci of tissue damage that are caused by the immune response against the virus (Suvas et al., 2003; 2004).

Using a model of chronic Friend virus, another group showed that virus-specific CD8 T cells are suppressed by Tregs (Dittmer et al., 2004). When Treg function was diminished, the effector CD8 T cells were capable of significantly reducing the viral load, showing that Tregs can maintain their suppression long into a chronic infection and that the result of such suppression is increased pathogen load.

These animal model studies were complemented by a variety of studies in humans. Various different groups presented evidence that the immune responses against cytomegalovirus, *Plasmodium falciparum*, human immunodeficiency virus, hepatitis B virus, and hepatitis C virus may all be suppressed by Tregs (Aandahl et al., 2004; Franzese et al., 2005; Kinter et al., 2004; Cabrera et al., 2004; Rushbrook et al., 2005; Boettler et al., 2005; Walther et al., 2005).

1.4: Dominant model explaining the role of Tregs during immune responses

Collectively, these early studies were not focused on explaining precisely how the anti-pathogen immune response overcomes Treg mediated suppression to initiate an immune response. They did, however, suggest that in the absence of Tregs, an anti-pathogen immune response develops with similar qualitative properties to the immune response that develops with

Tregs present, but that Tregs are needed to control the size and strength of the immune response, thereby limiting immunopathology.

This view was put forth in a several reviews between 2004 and 2007 (Belkaid, 2007; Belkaid and Rouse, 2005; Rouse and Suvas, 2004; Mittrücker and Kaufmann, 2004). The general concept can be summarized using a modified figure from a 2005 review written by Yasmine Belkaid and Barry Rouse (Belkaid and Rouse, 2005) (**Fig. 1**).

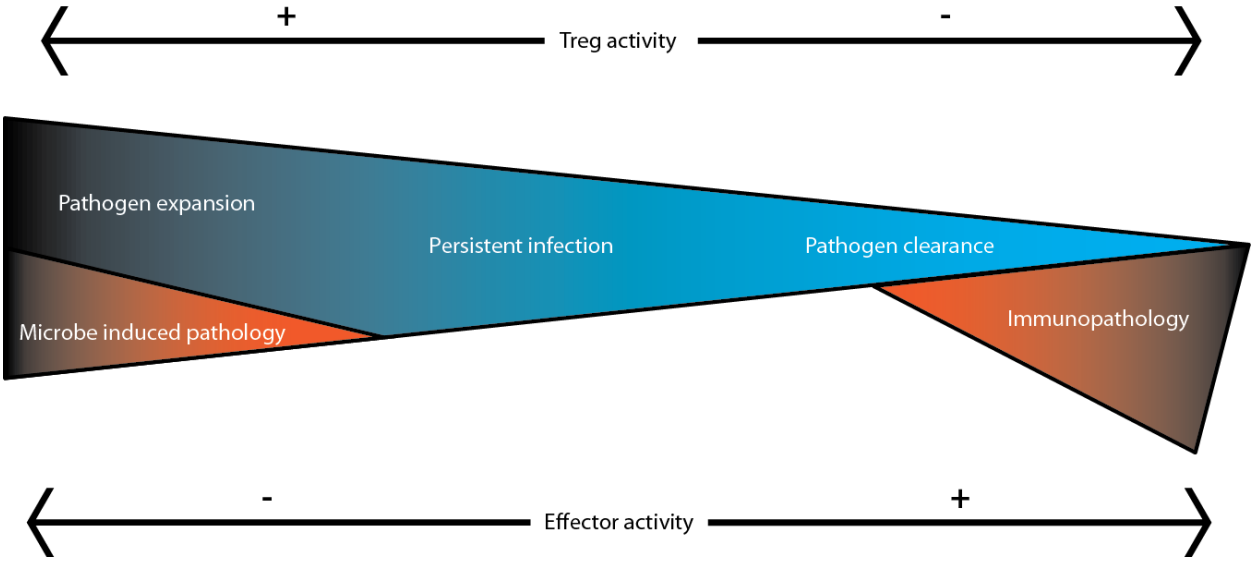

Figure 1: Schematic representation of the dominant view of the roles played by Tregs in the context of an infection

Treg activity is inversely related to effector activity. Increasing Treg activity causes decreased effector activity, which can favor pathogen expansion and microbe-induced pathology. Decreasing Treg activity favors increased effector activity, which makes pathogen clearance more likely, but also increases the risk of immunopathology. The figure is adapted from a review written by Yasmine Belkaid and Barry Rouse (Belkaid and Rouse, 2005).

In general, early research into the role of Tregs in immune control of infections demonstrated that Treg activity is inversely related to effector cell activity and that the balance between these two aspects of the immune response have a significant impact on the outcome of an immune response targeting a potential pathogen. If Treg activity is too strong, the weakened effector cell response favors pathogen expansion and an increased risked of microbe-induced pathology. If, however, Treg activity is too weak, the correspondingly strong effector cell response may clear the pathogen with more efficiency, but also has the potential to induce unacceptable amounts of collateral damage to self. Thus, Tregs primary role during an infection is to help calibrate the effector response such that pathogen expansion is controlled without the accumulation of unsustainable amounts of damage inflicted on the host by the actions of effector cells. Of note, an underlying assumption of this model is that Tregs have relatively little affect on the initiation of an anti-pathogen immune response, but rather that they primarily modulate the robustness of the immune response once it has initiated.

Over time, more potential pathogens were studied, including *Mycobacterium tuberculosis* (Scott-Browne et al., 2007), influenza A virus and vaccinia virus (Haeryfar et al., 2005), *Schistosoma mansoni* (Taylor et al., 2006; Baumgart et al., 2006), *Aspergillus fumigatus* (Montagnoli et al., 2006), and others (Belkaid and Tarbell, 2009; Berod et al., 2012). Collectively, they continued to generally support the concept presented in Figure 1.

1.5: Evidence of unrecognized roles for Tregs during infections

In 2008, however, a study suggested that such a paradigm is not always the case. The general model presented in Figure 1 suggests that an immune response that develops in the absence of Tregs should be more robust than the immune response that develops in the presence of Tregs. If this is true, then pathogen clearance should come about at least as quickly when the

immune response develops without Tregs because of the more robust response. In the case of intravaginal HSV-2 infection, this prediction is not true. Rather, mice that were experimentally depleted of Tregs prior to infection suffered from a significantly higher viral burden at the infected tissue. The virus also spread into the central nervous system more quickly in Treg-depleted mice, which in the case of intravaginal HSV-2, leads to earlier death in Treg-depleted mice (Lund et al., 2008). A comparable phenotype has also been noted following a liver infection with lymphocytic choriomeningitis virus (Lund et al., 2008), a lung infection with respiratory syncytial virus (RSV) (Ruckwardt et al., 2009; Fulton et al., 2010), and an oral infection with both *Candida albicans* (Pandiyan et al., 2011) and *Citrobacter rodentium* (Wang et al., 2014).

Different mechanisms have been suggested to explain why Treg depletion can sometimes allow for greater pathogen expansion. In the case of RSV, it was suggested that antigen-specific T cells fail to target the infected tissue as efficiently in Treg-depleted mice (Ruckwardt et al., 2009; Fulton et al., 2010). In the case of oral infection with *C. albicans* or *C. rodentium*, it was suggested that the presence of Tregs is needed for the development of a Th17 skewed response, which is essential for full anti-pathogen activity (Pandiyan et al., 2011; Wang et al., 2014). In the case of intravaginal HSV-2 infection, it was suggested that Tregs are needed to coordinate the early trafficking of innate immune cells such as NK cells, Plasmacytoid DCs, and classical DCs and that the dysregulation of these early effector cells allows for uncontrolled replication of the virus.

Importantly, however, in the case of intravaginal HSV-2 infection, mice depleted of Tregs prior to infection begin to die by the expected peak of the adaptive phase of the immune response, which limited the potential to explore how the antigen-specific T cell response develops in Treg-depleted mice. Indeed, unlike the majority of the studies reviewed thus far, the

antigen-specific T cell response was not studied following intravaginal HSV-2 infection. Thus, despite an understanding that the immune response following HSV-2 infection is less effective when initiated in the absence of Tregs, little was known about how Treg depletion affects the antigen-specific T cell response.

1.7: Mouse model of herpes-simplex virus type 2 intravaginal infection

In humans, primary genital infection with HSV-2 is initiated in genital epithelial cells that support viral replication. After an initial lytic phase of infection, the progeny virions that are released from epithelial cells can either infect further epithelial cells, or infect cells of the enervating sacral ganglia. Once the virus has entered the nervous system, the virus is maintained in a latent state, but is capable of reactivating and re-infecting genital epithelial cells and causing localized lesions (Shin and Iwasaki, 2013).

Clinical isolates of HSV-2 have been used to study the virus, but their usefulness for *in vivo* mouse studies is somewhat limited because mouse infection with wild type virus is lethal (Spang et al., 1983; Jones et al., 2000). HSV-2 ΔKpn was first developed as a non-lethal model of intravaginal HSV-2 infection in 1984. It is a particularly useful model because mice infected with HSV-2 ΔKpn clear the initial infection and are subsequently protected from future challenge with the normally lethal wild type HSV-2 virus (McDermott et al., 1984). As such, because of studies using both this and other similarly attenuated virus strains (Jones et al., 2000), both the primary and secondary immune response following intravaginal HSV-2 ΔKpn has been well studied.

By the mid 1990's it was demonstrated that CD4 T cell-dependent production of IFNγ at the infected tissue is a key component of viral control after primary HSV-2 ΔKpn infection. Two

waves of IFNγ are observed within the infected tissue; the first is dependent on NK cells and peaks at two days after infection while the second is evident at four days after infection and is dependent on CD4 T cells (Milligan and Bernstein, 1995; 1997). CD4 T cell-mediated production of IFNγ was implicated as being vital for viral control as depletion of CD4, but not CD8 T cells prior to infection results in delayed clearance of the virus and blocking of IFNγ beginning at three days after infection has a similar effect (Milligan and Bernstein, 1997).

In 2003, a study was published that demonstrated the importance of CD11b+, migratory, sub-mucosal DCs for CD4 T cell priming after intravaginal HSV-2 infection. These migratory DCs are found below the epidermis prior to infection, but migrate to the junction between the dermis and the epidermis following infection and collect viral antigen from infected epithelial cells. Importantly, the DCs do not become infected themselves, so they do not carry viable virus with them when they migrate from the infected tissue to the dLN. Indeed, no viral DNA can be detected within the dLN following HSV-2 infection, suggesting that virus is not transported by DCs to the dLN, nor does it travel as free virus in the lymph. As such, the CD11b+ migratory DCs are essential for anti-HSV-2 T cell priming after intravaginal HSV-2 infection. After collecting viral antigen at the infected tissue in the first hours after infection, these DCs migrate from the infected tissue to the dLN by around day two after infection. The first evidence of antigen-specific T cell priming in the dLN can be seen by day three after the infection (Zhao et al., 2003; Iwasaki, 2003).

After being primed in the dLN, CD4 T cells migrate to the infected tissue where they can first be found three to four days after infection. The peak infiltration of CD4 T cells is seen at about six days after infection. CD8 T cells do migrate to the infected tissue, but only after CD4 T cells have “licensed” their entry. This dynamic, coupled with data suggesting that depletion of

CD8 T cells does not significantly affect viral control, places CD4 T cells as the primary adaptive immune cell of interest in this HSV-2 model (Nakanishi et al., 2009; Milligan and Bernstein, 1997).

After migrating to the infected tissue, T cells are induced to produce IFNγ following recognition of viral peptide presented in the context of MHC II by a population of inflammatory monocytes. These monocytes enter the infected tissue starting at around day three post infection following a gradient of CCR2 ligands that are induced in the infected tissue by the local production of type 1 interferons (Iijima et al., 2011).

The key aspects of the immune response following intravaginal infection with HSV-2 are summarized visually in Figure 2.

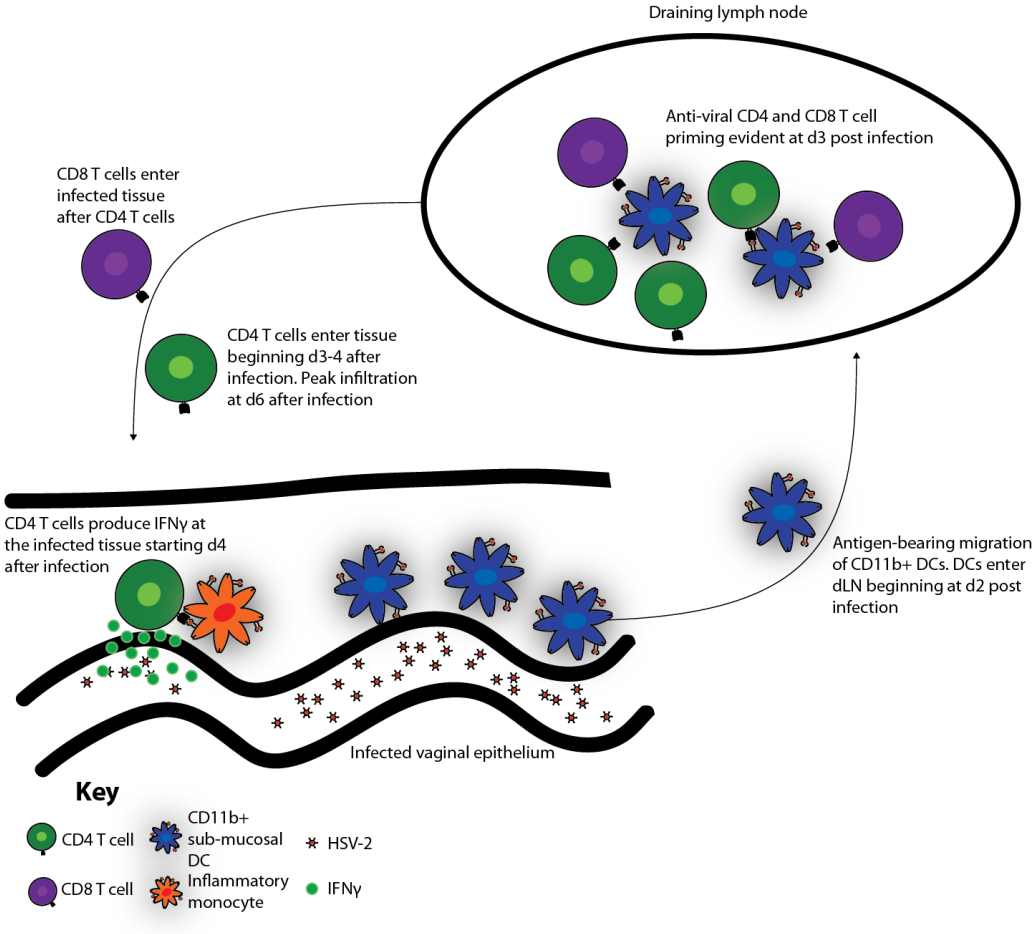

Figure 2: Visual summary of key aspects of the immune response following intravaginal HSV-2 infection

The virus initially infects vaginal epithelium cells. Migratory CD11b+ sub-mucosal DCs collect antigen from the epithelial layer, migrate to the dLN, and mediate T cell priming. After being activated in the dLN, CD4 T cells first enter the infected tissue starting three to four days post infection. CD8 T cells enter the infected tissue only after CD4 T cells have done so. Once in the infected tissue, T cells are induced to produce IFNγ after stimulation by inflammatory monocytes in the tissue that present viral antigen. The IFNγ produced by T cells is important in mediating viral control.

1.8: Primary aim

The primary aim of this thesis was to assess what role Tregs play in the antigen-specific CD4 T cell response following HSV-2 infection. The immune response following intravaginal HSV-2 infection is not as effective when it is mounted in the absence of Tregs (Lund et al., 2008). This observation suggests that the dominant model for the role played by Tregs during anti-pathogen immune responses is not true in all cases, but that their role may vary depending on the specific infection being studied. Changes in the early, innate immune response against HSV-2 have already been noted. This thesis, however, aimed to use a well-characterized model of attenuated intravaginal HSV-2 infection to extend analysis towards understanding the role of Tregs in the antigen-specific CD4 T cell response following intravaginal HSV-2.

The data presented suggest that when Tregs are not present at the time of infection, the adaptive immune response is not more robust, but actually dampened. In mice lacking Tregs, fewer antigen-specific CD4 T cells enter the infected tissue, proper CD4 T cell priming is dysregulated, and the migration of antigen-bearing DCs to the dLN is decreased. Collectively, these data suggest that the presence of Tregs is, in certain contexts, critical for more than modulating the magnitude of an immune response following an infection, but rather is needed to successfully initiate and target an immune response against an invading pathogen.

Chapter 2: Results

2.1: Regulatory T cells promote local adaptive immunity following non-lethal intravaginal HSV-2 infection

Previous studies utilized wild-type HSV-2, which is lethal in mice and leads to death in Treg-depleted mice as early as day six post-infection (Lund et al., 2008). Therefore, we first wanted to validate the use of HSV-2 186Δkpn (McDermott et al., 1984; Jones et al., 2000) as a viable model to study the adaptive immune response following intravaginal HSV-2 infection. As expected, mice depleted of Tregs prior to intravaginal HSV-2 infection showed delayed clearance of the virus from the infected tissue (**Fig. 3a)**, but the infection was not lethal regardless of Treg status at the time of infection, making the attenuated strain of HSV-2 a good model with which to study the role of Tregs in the development of the adaptive immune response against the virus.

One observation from earlier work that suggested a difference in the adaptive immune response within the infected tissue was that despite the influx of bulk CD4 T cells at day four post-infection, there was no localized increase in IFNγ levels (Lund et al., 2008). In wild type mice, the influx of antigen-specific CD4 T cells into the infected tissue beginning at day four causes localized production of IFNγ, a step that is necessary for viral control (Milligan and Bernstein, 1995; 1997).

We also saw significantly less IFNγ production at four days post infection in the infected tissue of Treg-depleted mice that were infected with the attenuated strain of HSV-2 (**Fig. 3b**). Therefore, we used this model to explore what role Tregs play in the development of the antigen-specific CD4 T cells response following intravaginal HSV-2 infection.

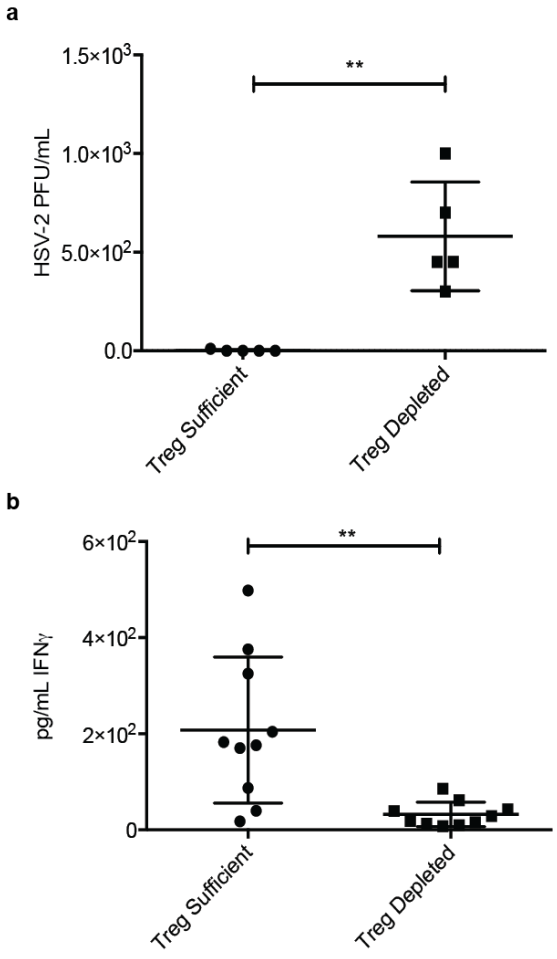

Figure 3: Elevated viral burden and diminished adaptive immune response following vaginal HSV-2 infection in the absence of Tregs

Foxp3^{DTR} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.0 x 10^6 PFU of HSV-2 186ΔKpn. A) Viral titer from vaginal washes of mice infected for 6d. B) Concentration of IFNγ within vaginal washes of mice infected for 4d. All data come from 2-3 experiments with 4-5 mice per group. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an unpaired, two-way t test. **: $p \leq 0.01$. Error bars show standard deviation.

2.1: Inflammatory monocytes enter the infected tissue normally in the absence of Tregs

One possible explanation for the decreased level of IFNγ in the infected tissue at four days after infection would be a failure of inflammatory monocytes to enter the tissue and present viral antigen to the incoming CD4 T cells. In a wild type mouse, inflammatory monocytes enter the infected tissue following a gradient of CCR2 ligands that are induced in the infected tissue by local type 1 interferon production. Once in the tissue, inflammatory monocyte-derived DCs are needed to induce the wave of IFNγ that is produced by CD4 T cells and is normally evident in the tissue beginning at day four post infection (Iijima et al., 2011). Since Treg-depleted mice produce less type 1 interferon at the infected tissue shortly after infection than wild type mice (Lund et al., 2008), we hypothesized that, as a result, fewer inflammatory monocytes are able to enter the tissue. To test this hypothesis, we crossed Foxp3^{DTR} mice with CCR2 reporter mice (Hohl et al., 2009). Fluorescent labeling of CCR2 expressing monocytes allowed us to analyze the influx of these cells into the infected tissue in both Treg-sufficient and Treg-depleted mice. Contrary to our hypothesis, there was no discernable difference in the number of monocytes that had entered the infected tissue in Treg-sufficient of Treg-depleted mice (**Fig. 4a**). Thus, we turned our attention to other possible explanations for the lack of IFNγ present in the infected tissue of Treg-depleted mice.

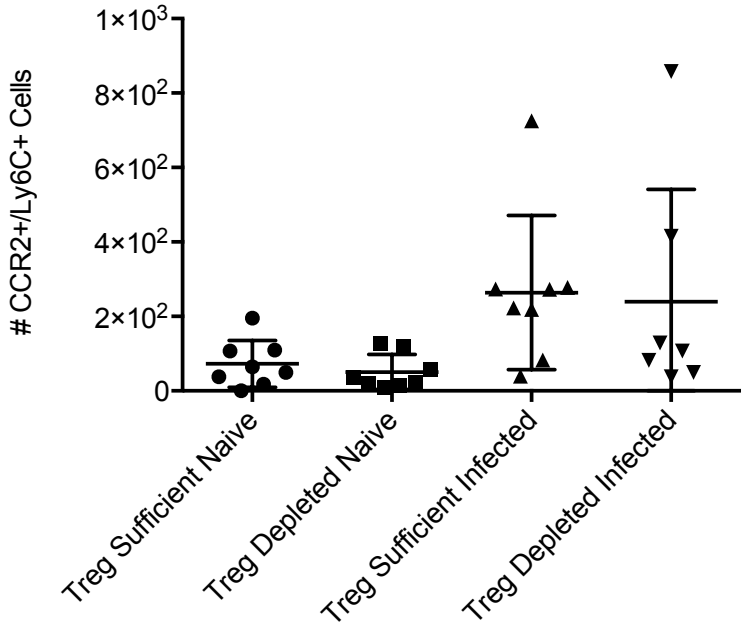

Figure 4: Inflammatory monocytes enter the infected tissue normally in the absence of Tregs

Foxp3^{DTR} x CCR2^{GFP} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. The number of CCR2/Ly6C+ inflammatory monocytes that were present in the vaginal tract 4 days post-infection as determined by flow cytometry. All data are taken from at least two similar, independent experiments with 4-5 mice per group. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups. No significant differences were seen.

2.2: HSV-2-specific CD4+ T cells fail to accumulate in the vagina in the absence of Tregs.

Because IFNγ produced by CD4 T cells is a key component of the adaptive response against HSV-2, and we observed decreased vaginal IFNγ in the absence of Tregs at a time when this critical anti-viral cytokine is produced by lymphocytes rather than NK cells (Milligan and Bernstein, 1997)(**Fig. 3b**), we hypothesized that Tregs are critical for protective HSV-2-specific CD4 T cell responses within the infected tissues.

We initially attempted to enumerate antigen-specific T cells by stimulating cells *ex vivo* with inactivated HSV to produce IFNγ. This technique proved unworkable, however, because many cells from naïve, Treg-depleted mice produced cytokines following stimulation with inactivated virus. This high level of background cytokine production made it impossible to differentiate cells that truly recognize viral antigen from cells that simply came out of a strongly pro-inflammatory environment. Thus, we instead relied on the transfer of TCR transgenic cells that express a TCR with known specificity (Kearney et al., 1994).

To assess the migration of HSV-2-specific CD4 T cells to the infected tissue in mice lacking Tregs at the time of infection, we transferred congenically marked, TCR transgenic gDT-II cells that recognize an HSV glycoprotein D epitope (gD290-302) (Bedoui et al., 2009; Gebhardt et al., 2011) into Foxp3^{DTR} mice, treated the mice with DT to deplete Tregs, and subsequently tracked the transferred cells' migration into the infected vaginal tract six days post-infection, as this is the expected peak of CD4 T cell infiltration (Nakanishi et al., 2009) (**Fig. 5a**). As expected, Treg-sufficient mice showed an influx of CD4 T cells into the vaginal tract following infection and this CD4 T cell response was dominated by the congenically labeled gDT-II cells. Conversely, while Treg-depleted mice showed an influx of bulk CD4 T cells into the vaginal tract following infection, the transferred gDT-II cells represented a minute fraction of

the CD4 T cell pool (**Fig. 5b-c**). Similarly, the total number of gDT-II cells that migrated to the infected tissue in Treg-sufficient mice was significantly higher than the number of gDT-II cells that migrated into the infected tissue of Treg-depleted mice (**Fig. 5d**). The significant decrease in the number of gDT-II cells entering the infected tissue supports the possibility that the bulk CD4 T cells that are present in Treg-depleted mice are not bona fide anti-HSV-2 T cells, but rather that they may have been activated because of cross-reactivity following Treg depletion and then pulled into the vaginal tract because of the virus-induced inflammation. This suggests that mice lacking Tregs at the time of HSV-2 infection are unable to properly combat HSV-2 in the infected tissue with an appropriate CD4 T cell response.

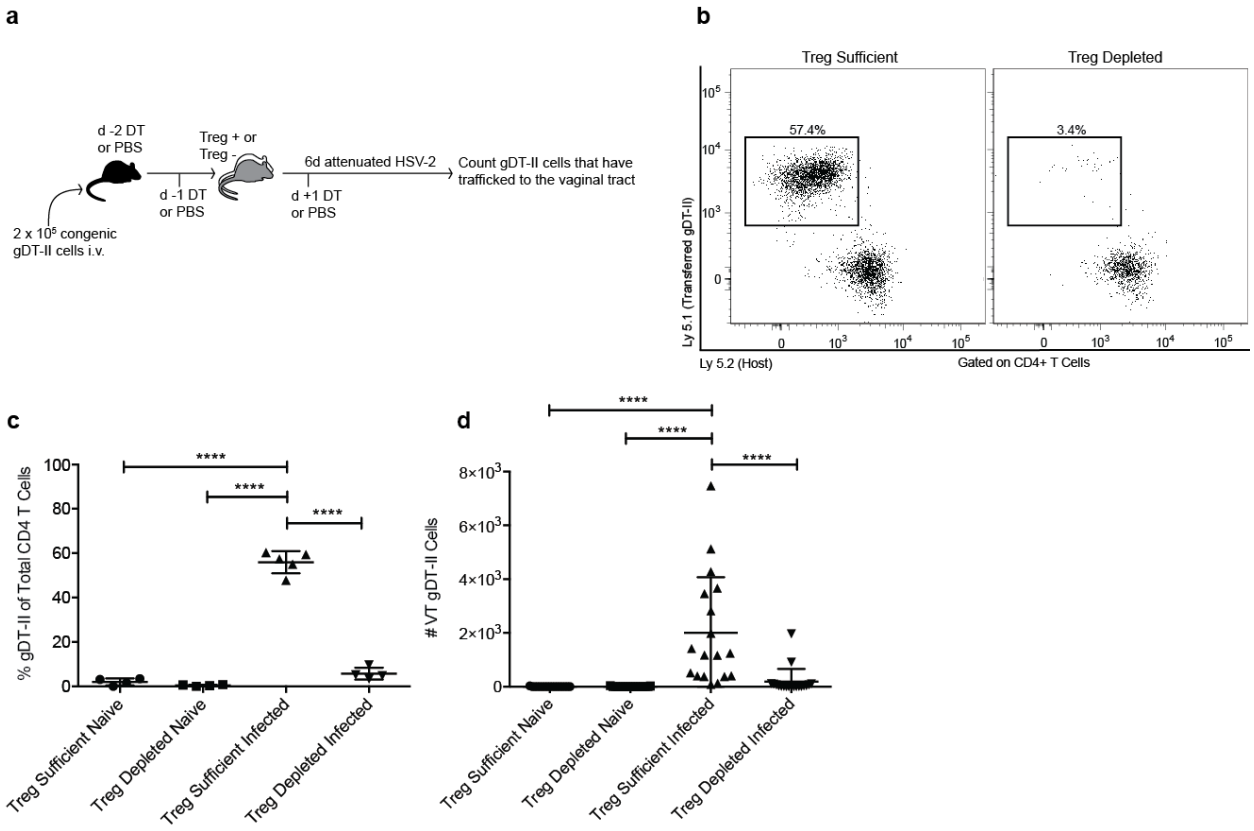

Figure 5: HSV-specific CD4 T cells fail to accumulate in the vagina in the absence of Tregs

A) Experimental setup. *Foxp3*^{DTR} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. B) Representative flow plots showing gDT-II cells in the vaginal tract of Treg-sufficient and Treg-depleted mice 6d after intravaginal HSV-2 infection. Samples were gated on CD4 T cells and the plots show Ly5.1 (transferred gDT-II cells) versus Ly5.2 (host cells). C) Representative data showing the fraction of total vaginal tract CD4 T cells consisting of antigen-specific gDT-II cells. D) Representative data showing the number of gDT-II cells recovered from mouse vaginal tracts. All data are taken from or representative of at least two independent experiments with 4-5 mice per group. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups. ****: $p \leq 0.0001$. Error bars show standard deviation.

2.3: Treg presence during T cell priming is critical for subsequent accumulation of antigen-specific CD4 T cells in the infected tissues.

We next examined the blood of Treg-depleted mice to determine if there is an equal share of circulating gDT-II cells six days after infection as compared to Treg-replete mice. Similar to the vaginal tract, mice lacking Tregs at the time of infection had significantly fewer gDT-II cells circulating in the blood (**Fig. 6a**), suggesting that the decreased entry of gDT-II cells into the vaginal tract was likely caused by inefficient activation, clonal expansion, and lymph node egress of the cells as opposed to the inability to properly target the vaginal tract with activated cells from the blood. To test this idea, we delayed administration of DT until three days after infection. At this time point, T cell priming is underway, although very few cells have yet migrated to the infected tissue (Nakanishi et al., 2009). When we delayed Treg depletion to a time at which T cell priming and activation is largely completed and cells have entered the migration stage, gDT-II cells were as prevalent in the blood of Treg-depleted mice as in Treg-sufficient controls (**Fig. 6b**). Similarly, the number of gDT-II cells entering the infected tissue was essentially unaffected by the absence of Tregs during this phase (**Fig. 6c**). Together, these data suggest that the curtailed number of gDT-II cells entering the vaginal tract of mice lacking Tregs at the time of infection (**Fig. 5**) is not due to a migration defect of T cells in Treg-depleted mice, but points to the potential necessity of Tregs during the initial T cell priming phase.

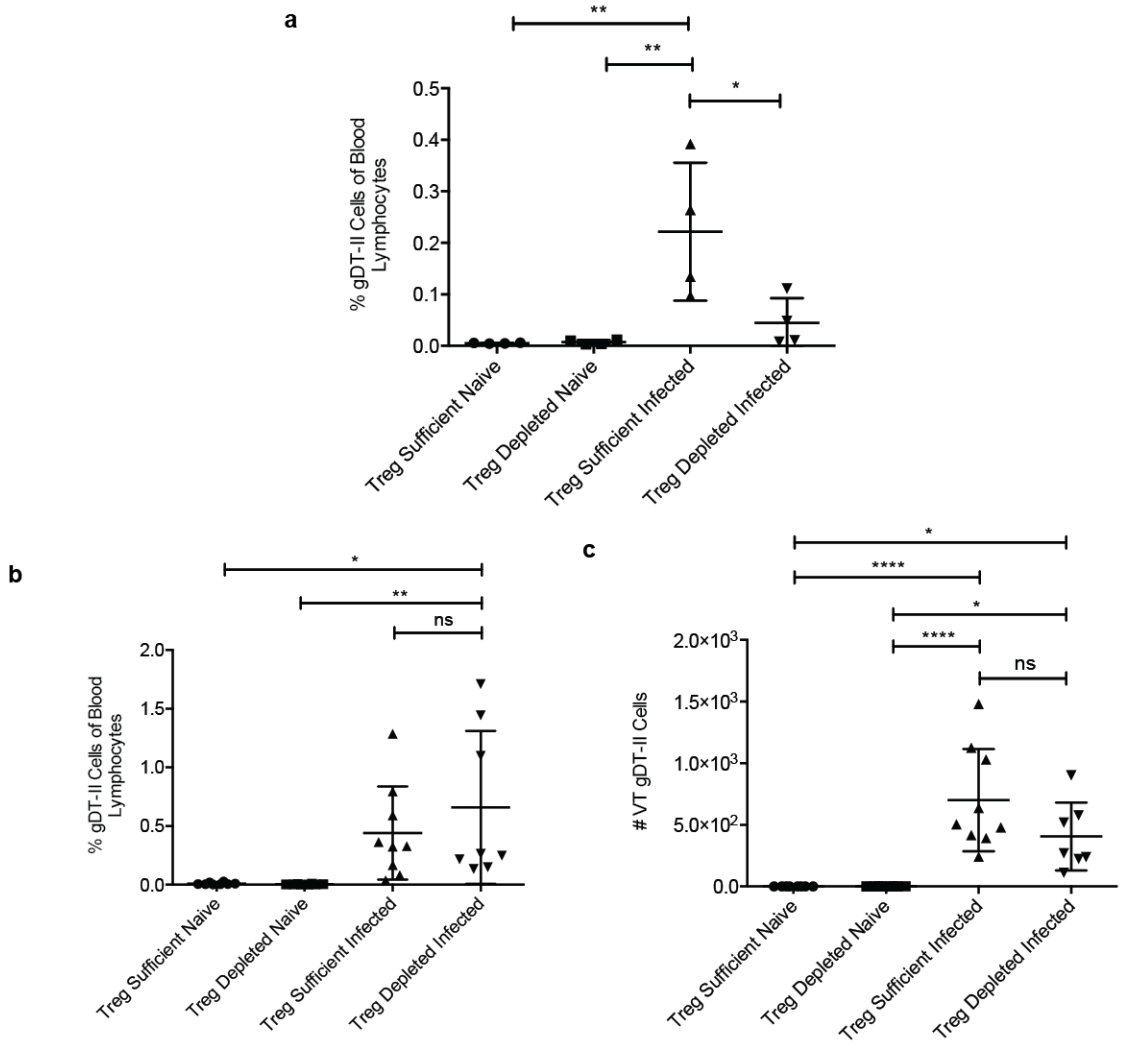

Figure 6: Absence of Tregs in the post-priming phase does not diminish antigen-specific CD4 T cell accumulation in the vagina

Mice were depleted of Tregs at days -2, 1, and +1 (A) or at days +3 and +4 (B and C) relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. A) The fraction of gDT-II cells in the blood as a percent of total blood lymphocytes 6d after infection when Tregs were depleted prior to infection. B) The fraction of gDT-II cells in the blood as a percent of total blood lymphocytes 6d after infection when Tregs were depleted during the post-priming, migration phase of the immune response. C) The number of gDT-II cells recovered from the vaginal tracts of mice 6d after infection when Tregs were depleted during the post-priming, migration phase of the immune response. All data come from or are representative of at least 2 independent experiments with 4-5 mice in each group. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups. *: $p \leq 0.05$, **: $p \leq 0.01$, ****: $p \leq 0.0001$. Error bars show standard deviation.

2.4: Tregs are needed for proper antigen-specific CD4 T cell priming

To test the hypothesis that Tregs are important for proper antigen-specific CD4 T cell priming, we transferred antigen-specific CD4 T cells into Foxp3^{DTR} mice, depleted the mice of Tregs, infected the mice intravaginally with HSV-2 and then measured the activation of the transferred cells three days post-infection, a time when T cell priming has just begun in this model (Zhao et al., 2003) (**Fig. 7a**). In these experiments, we used TCR transgenic OT-II cells, which recognize a peptide from the chicken ovalbumin (OVA) protein, and infected the mice with an attenuated strain of HSV-2 that expresses OVA (Dobbs et al., 2005). As a control, HSV-2 186ΔKpn lacking OVA expression was used for infection ("no OVA" group), enabling comparison of OT-II activation in the absence of cognate antigen but under otherwise similar *in vivo* conditions. OT-II cells transferred into Treg-sufficient mice infected with HSV-2-OVA showed increased expression of both CD69 and Nur77 three days post-infection, suggesting that antigen recognition had occurred, thereby triggering T cell activation (**Fig. 7b-c**). OT-II cells transferred into Treg-depleted, HSV-2-OVA infected mice, however, showed very little upregulation of these markers. These data suggest that the transferred OT-II cells in the Treg-depleted mice did not "see" antigen and therefore were not activated as efficiently as the OT-II cells in Treg-sufficient mice. In addition, we found significantly more OT-II cells in the dLNs of Treg-sufficient mice as compared to Treg-depleted mice three days post-infection, likely because the OT-II cells in the Treg-sufficient mice had begun to divide more efficiently than the same cells in the Treg-depleted mice (**Fig. 7d**). Together, these data suggest delayed or impaired CD4 T cell priming in Treg-depleted mice and are consistent with the delayed entry of antigen-specific cells into the infected tissue (**Fig. 5**) and subsequent delay in viral clearance (**Fig. 3**).

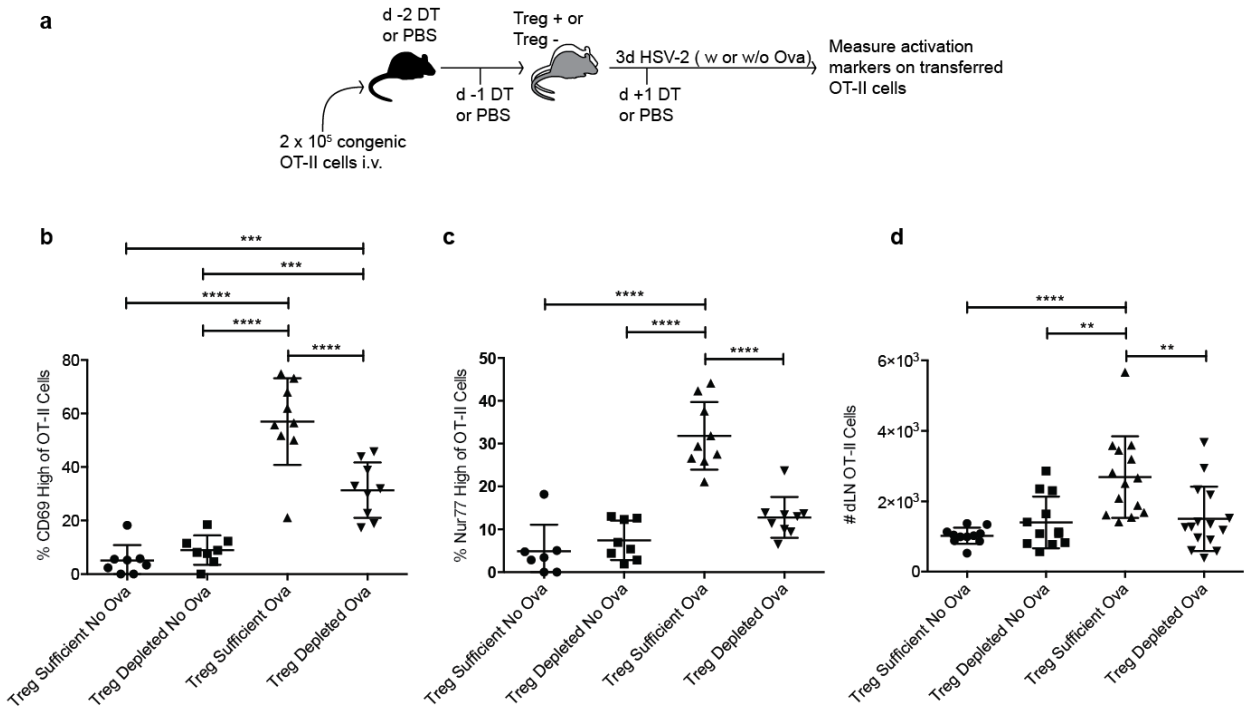

Figure 7: Antigen-specific CD4 T cell activation and priming following HSV-2 infection are compromised in the absence of Tregs

A) Experimental setup. *Foxp3*^{DTR} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn expressing OVA (OVA groups), while no OVA groups were infected with the same quantity of HSV-2 186ΔKpn. B) Percent of transferred OT-II cells showing increased expression of CD69 in the dLN of 3d infected mice. C) Percent of transferred OT-II cells showing increased expression of Nur77 in the dLN of 3d infected mice. D) The number of OT-II cells recovered from the dLN of mice 3d after infection. All data come from 2-3 experiments with 4-5 mice in each group. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups. **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$. Error bars show standard deviation.

2.5: Tregs are needed to maintain homeostatic production of CCL21.

CD11b+ sub-mucosal DCs that migrate from the HSV-2-infected tissues to the dLNs present antigen to naïve CD4 T cells and mediate effector T cell activation (Zhao et al., 2003). Since antigen-specific CD4 T cells in Treg-depleted mice did not activate efficiently in response to infection, we hypothesized that this could be in part caused by a dysregulation in the migration of the antigen-bearing sub-mucosal DCs. This possibility was further supported by previous work demonstrating an early drop in dLN production of CCL21 following Treg depletion (Lund et al., 2008). Migratory DCs express CCR7 to follow a gradient of CCL21 into the T cells zone of a dLN, thereby enabling them to present cognate antigen to T cells (Weber et al., 2013; Braun et al., 2011). As such, we reasoned that altered CCL21 production in the absence of Tregs could affect DC migration subsequent to HSV-2 infection. Thus, we confirmed that CCL21 production in the dLN was decreased in Treg-depleted mice infected with attenuated HSV-2 (Lund et al., 2008; Mueller et al., 2007). Indeed, qRT-PCR analysis coupled with ELISA and immunohistochemistry revealed a significant drop in CCL21 production in response to Treg depletion or HSV-2 infection within two days of the infection (**Fig. 8a-c**). This suggests that one key aspect of the homeostasis that is maintained by Tregs is the continual production of CCL21, a chemokine that is needed to coordinate DC and T cell contact in the dLN. In the context of decreased CCL21 production induced by Treg depletion, we next sought to determine how Treg depletion affects the migration of antigen-bearing DCs from the infected tissue into the dLN.

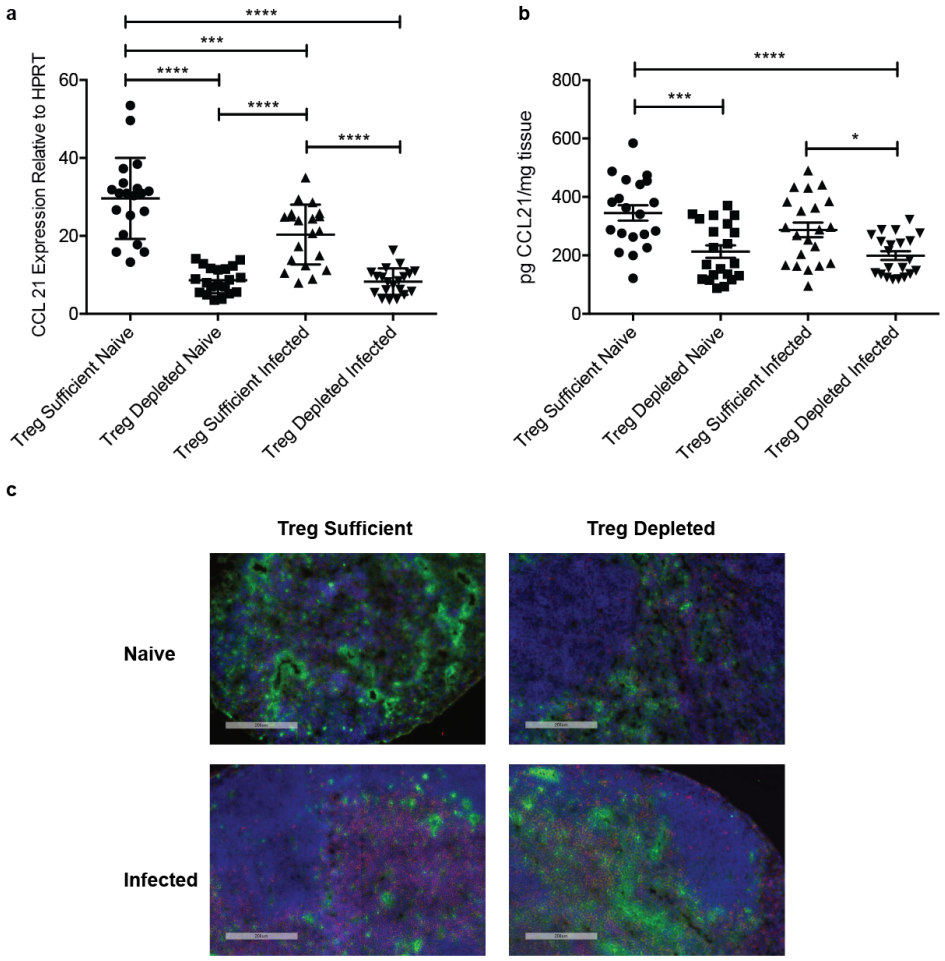

Figure 8: Tregs are necessary to maintain homeostatic expression of CCL21

Foxp3^{DTR} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. A) Expression of CCL21 in the dLN of Treg-sufficient or Treg-depleted mice 2d after infection determined by qPCR analysis. Data are shown relative to the housekeeping gene HPRT. B) Level of CCL21 protein in the dLN of Treg-sufficient or Treg-depleted mice 2d after infection determined by ELISA. Data are normalized to the weight of each tissue. C) Representative images of CCL21 and CD5 protein stained in dLN of Treg-sufficient or Treg-depleted mice. Green: CCL21, Red: CD5, Blue: DAPI nuclear stain. All data come from or is representative of at least 2 similar, independent experiments with 4-5 mice in each group that showed similar results (a and b) or 1 experiment with multiple stained sections (c). Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups.*: $p \leq 0.05$, ***: $p \leq 0.001$ ****: $p \leq 0.0001$. Error bars show standard deviation.

2.6: Tregs are critical for appropriate trafficking of dendritic cells from the vaginal mucosa to the dLN

To test the possibility that fewer migratory DCs traffic from the infected tissue to the dLN, we labeled DCs within the vaginal tract by applying a solution of FITC to the vaginal canal prior to infection with HSV-2. As previously demonstrated (Lee et al., 2009), this procedure does not result in drainage of FITC itself to the dLN. The mice were infected for two days before their dLNs were analyzed for FITC+ DCs that had originated in the vaginal tract (**Fig. 9a**). Significantly fewer FITC+ sub-mucosal DCs entered the dLN following infection in Treg-depleted mice as compared to Treg-sufficient mice, suggesting that the migration of antigen-bearing DCs from the infected tissues to the dLN is dysregulated in the absence of Tregs (**Fig. 9b-c**).

To further assess the entry of viral antigen into the dLN of Treg-depleted mice, we tested the ability of migratory DCs from the dLN of infected mice to present viral antigen to CD4 T cells. Mice were infected for two days with HSV-2 prior to isolating MHC II-high migratory DCs from the dLNs. The sorted migratory DCs were then co-cultured *ex vivo* with CD4 T cells isolated from the dLNs of WT mice infected with HSV-2 for five days and thus enriched for endogenous HSV-2-specific CD4 T cells. Subsequent to *ex vivo* co-culture, levels of IFNγ in the culture supernatant were measured by ELISA as a readout for the ability of the sorted DCs to present *in vivo*-acquired antigen to the anti-HSV-2 CD4 T cells (**Fig. 9d**). Consistent with the FITC labeling experiment, migratory DCs sorted from Treg-depleted mice induced less IFNγ production from anti-HSV-2 CD4 T cells, despite equal numbers of these cells used in culture (**Fig. 9e**). As expected, only MHC II-high, migratory DCs were capable of

inducing INFγ *ex vivo* (**Fig. 9e**) (Zhao et al., 2003). When the culture was supplemented with heat-inactivated HSV-2 virus *ex vivo*, DCs from both Treg-depleted as well as Treg-sufficient dLNs were fully capable of presenting antigen to anti-HSV-2 CD4 T cells (**Fig. 9e**). Indeed, consistent with previous data showing increased expression of co-stimulatory molecules such as CD80 on DCs in the lymph nodes of Treg-depleted mice (Kim et al., 2007), DCs sorted from Treg-depleted mice that were supplemented with HSV-2 antigen *ex vivo* induced more IFNγ production than the same population of cells sorted from Treg-sufficient mice (**Fig. 9e**). This suggests that the decreased production of IFNγ in the co-cultures where antigen was acquired *in vivo* was not the result of functionally-impaired DCs in the Treg-depleted group, but rather that the population of DCs sorted from Treg-depleted mice contained a smaller fraction of HSV-2-antigen-bearing cells that had entered the dLN following the infection.

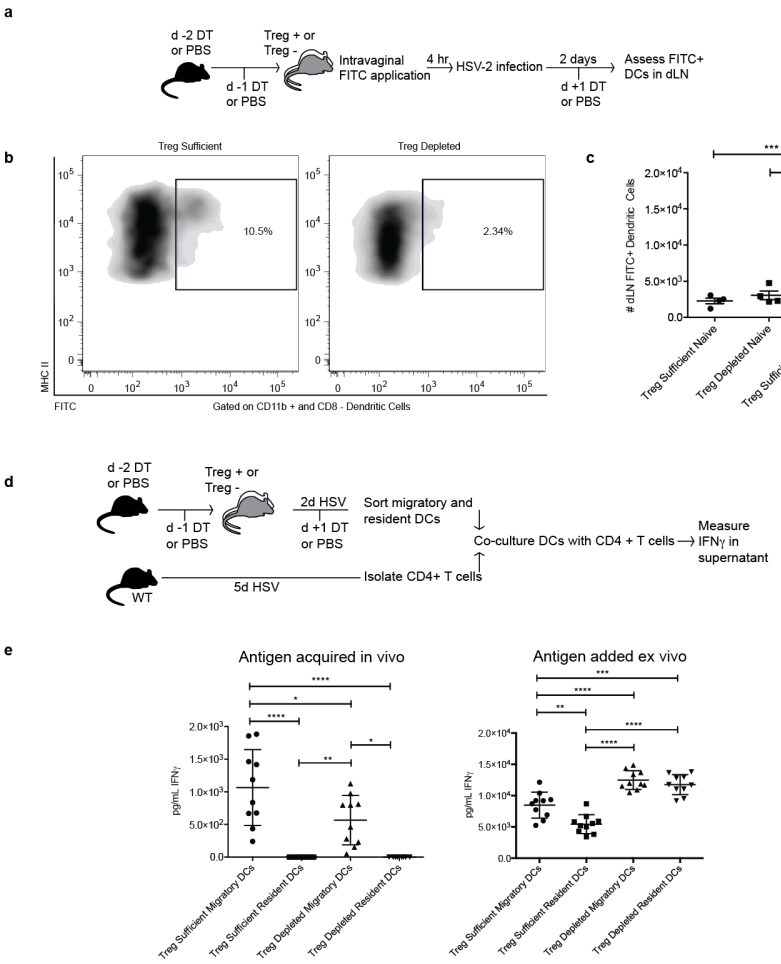

Figure 9: Dendritic cell trafficking from the vaginal mucosa is impaired in the absence of Tregs

Foxp3^{DTR} mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. A) FITC painting experimental layout. B) Representative flow plots showing the fraction of CD11b+/CD8- DCs in the dLN that stained positive for FITC 2d after infection. Cells were first gated on MHC II/CD11c+/CD11b+ and CD8- cells. C) Representative data showing the number of FITC+ DCs that had entered the dLN from the infected vaginal tract 2d after the mice were infected. D) *Ex vivo* antigen presentation experimental layout. E) The amount of IFNγ produced by anti-HSV-2 CD4 T cells after 60h of co-culture with DCs sorted from Treg-sufficient or Treg-depleted mice 2d after infection. On the left, cells were cultured without addition of exogenous antigen, whereas on the right, cells were provided HSV-2 *ex vivo* during culture. All data come from or is representative of at least 2 similar, independent experiments with 4-5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups.*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ ****: $p \leq 0.0001$. Error bars show standard deviation.

2.7: CCL21 down-regulation occurs independent of IFNγ production in Treg-depleted mice following intravaginal HSV-2 infection

As previously stated, we measured expression of CCL21 in the dLNs and found depressed levels in mice depleted of Tregs (**Fig. 8**). Therefore, we hypothesized that there may be insufficient levels of these chemoattractants to lure migratory DCs into the dLN in the absence of Tregs. IFNγ production within the dLN is known to decrease CCL21 expression (Mueller et al., 2007). The resulting decreased expression of CCL21 was also shown to affect the priming of CD8 T cells during a subsequent immune response, similar to what we have observed with CD4 T cell priming (**Fig. 7**). Thus, we postulated that excessive IFNγ present in the dLN of Treg-depleted mice could be driving the decreased levels of CCL21. To test this hypothesis, we bred IFNγ KO mice onto the Foxp3^{DTR} background. However, despite the absence of IFNγ in these mice, Treg depletion and HSV-2 infection still caused a decrease in CCL21 production in the dLN (**Fig. 10a**), suggesting that the drop is not wholly caused by IFNγ production in the dLN.

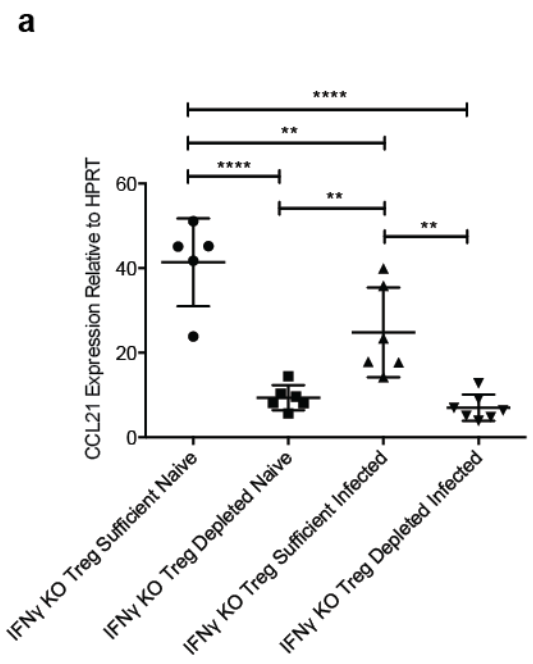

Figure 10: IFNγ does not affect CCL21 production in the dLN following Treg depletion and intravaginal HSV-2 infection

Foxp3^{DTR} x IFNγ$^{KO/KO}$ mice were injected with DT on days -2, -1, and +1 relative to the day of infection. Alternatively, Treg-sufficient mice were injected with PBS instead of DT. Mice were infected intravaginally with 1.88 x 10^5 PFU of HSV-2 186ΔKpn, while naïve groups were left uninfected. A) Expression of CCL21 in the dLN of Treg-sufficient or Treg-depleted IFNγ KO mice 2d after infection determined by qPCR analysis. Data are shown relative to the housekeeping gene HPRT. All data come from or is representative of at least 2 similar, independent experiments with 2-5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an ordinary one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups, **: $p \leq 0.01$, ****: $p \leq 0.0001$. Error bars show standard deviation.

2.8: A mixed bone marrow chimera strategy that allows for acute depletion of wild type Tregs and expansion of CTLA-4 conditional knockout Tregs

We next turned to the functional mechanism by which Tregs facilitate entry of antigen-bearing DCs into the dLN following vaginal HSV-2 infection. CTLA-4 is constitutively expressed by Tregs and is well known to be involved in multiple pathways of Treg-mediated suppression. Specifically, CTLA-4 can compete with CD28 for co-stimulatory molecule binding to APCs, as well as physically remove CD80 and CD86 from the surface of APCs, thereby limiting co-stimulation available for T cells (Qureshi et al., 2011; Walker and Sansom, 2011). *CTLA-4*$^{flox/flox}$ x *Foxp3*^{Cre} mice begin to show signs of multi-organ lymphocyte infiltration and disease at 7 weeks of age (Wing et al., 2008), demonstrating the importance of CTLA-4 expression by Tregs for the maintenance of immune homeostasis, but also presenting a challenge in using these mice in studies with further manipulations due to their early spontaneous disease. Thus, we used a mixed bone marrow chimera strategy to allow for a targeted depletion of CTLA-4 on Tregs in adult mice. Lethally irradiated *Foxp3*^{DTR} mice were re-constituted with equal parts bone marrow from Foxp3^{DTR} mice and either CTLA-4$^{flox/flox}$ x *Foxp3*^{Cre} mice or CTLA-4$^{WT/WT}$ x Foxp3^{Cre} mice (**Fig. 11a**). This allowed for the development of mice that maintain a population of fully functional Tregs (from Foxp3^{DTR} host and donor cells) that could be depleted upon administration of DT, leaving behind only Tregs that either lack CTLA-4 expression or act as Foxp3^{Cre} control cells (**Fig. 11b-c**).

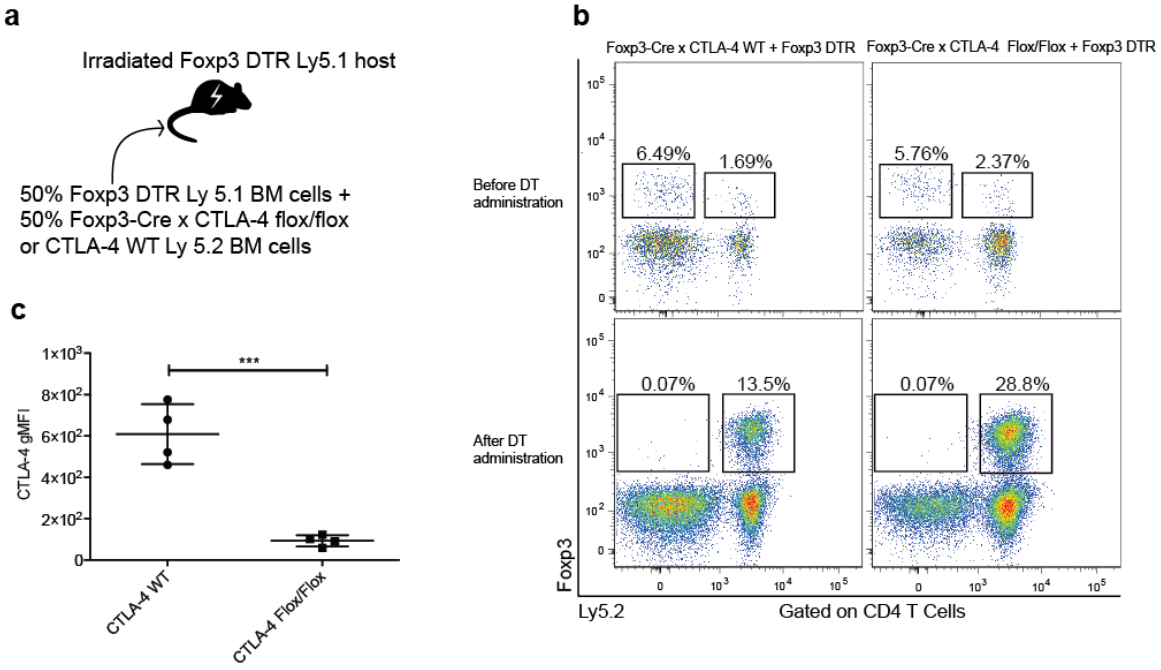

Figure 11: Mixed bone marrow chimeras consisting of Foxp3^{DTR} cells and Foxp3 Cre x CTLA-4 $^{flox/flox}$ cells allow for acute depletion of Foxp3^{DTR} Tregs and expansion of CTLA-4 conditional knockout Tregs

A) Mixed bone marrow chimera setup. B) Representative flow plots from blood (before DT administration) or lymphoid tissue (After DT administration) showing two congenically distinct Treg populations in mature bone marrow chimeras. Mixed bone marrow chimera mice were injected with DT on days -2, -1, and 1 relative to the day of infection to deplete the fully functional Foxp3^{DTR} Tregs in each mouse. Following DT administration, Ly 5.1 Foxp3^{DTR} Tregs are no longer present and the populations of Foxp3^{Cre} x CTLA-4$^{WT/WT}$ or CTLA-4$^{flox/flox}$ Ly 5.2 Tregs have expanded. C) Expression of CTLA-4 on Tregs measured on the day of harvest, when all fully competent, Foxp3^{DTR} Ly 5.1 cells have been eliminated. All data come from or is representative of at least 2 similar, independent experiments with 4-5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using an unpaired t test (c). ***: $p \leq 0.001$. Error bars show standard deviation.

2.9: CTLA-4 expression by Tregs is critical to promote proper dendritic cell migration from the infected tissues in the context of HSV-2 infection

When these chimeric mice were used to isolate DCs for our *ex vivo* T cell antigen presentation assay (**Fig. 12a)**, we found that similar to Treg-depleted mice, DCs from HSV-2-infected mice with CTLA-4 deficient Tregs were less able than DCs from CTLA-4 WT mice to induce IFNγ production from T cells without addition of exogenous HSV-2 (**Fig. 12b**). Thus, it appears that Tregs are needed to facilitate normal DC migration through a CTLA-4-dependent mechanism.

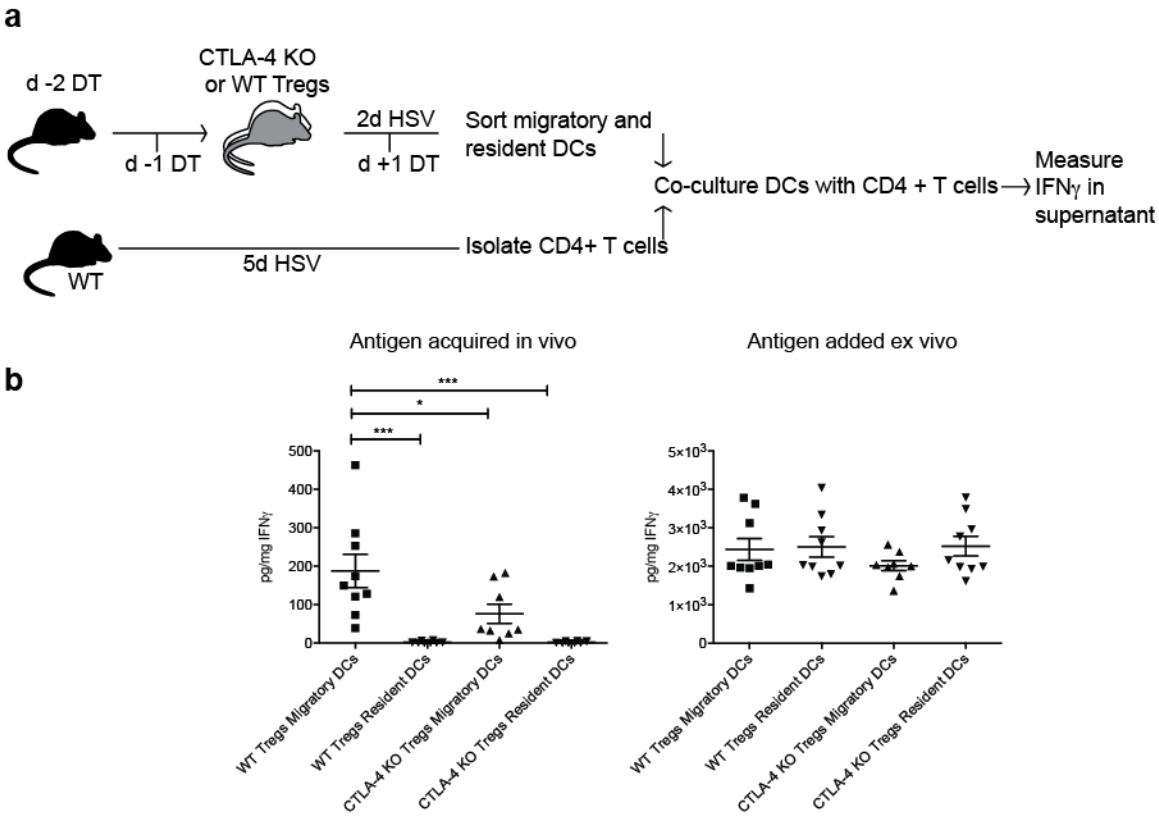

Figure 12: CTLA-4 Expression on Tregs is needed to promote proper migration of antigen-bearing dendritic cells from the infected tissue to the dLN

A) Experimental layout. Mixed bone marrow chimera mice were injected with DT on days -2, -1, and 1 relative to the day of infection to deplete the fully functional Foxp3^{DTR} Tregs in each mouse. B) Amount of IFNγ produced by anti-HSV-2 CD4 T cells following 60 hours of co-culture with DCs sorted from mice with either wild type or CTLA-4 conditional KO Tregs 2d after infection. On the left, cells were cultured without addition of exogenous antigen, whereas on the right, cells were provided HSV-2 *ex vivo* during culture. All data come from or is representative of at least 2 similar, independent experiments with 4-5 mice in each group that showed similar results. Each data set was first screened for outliers using the ROUT test with a Q value of 0.2%. Statistical significance was then determined using a one-way ANOVA followed by a Tukey test to compare the mean of each group with the mean of all other groups. *: $p \leq 0.05$. Error bars show standard deviation.

2.10: Summary of Findings

The data presented are consistent with a model wherein the depletion of Tregs leads to a series of changes within the lymph node, including decreased expression of CCL21. Subsequent to that drop, antigen-bearing DC migration is diminished, which, in the context of intravaginal HSV-2 infection, contributes to inefficient antigen-specific CD4 T cell priming, expansion, and migration to the infected tissue. Therefore, the data point to a previously unrecognized role for Tregs in maintaining the immune system in a state where it is capable of priming an antigen-specific CD4 T cell response when appropriate (**Fig. 13**).

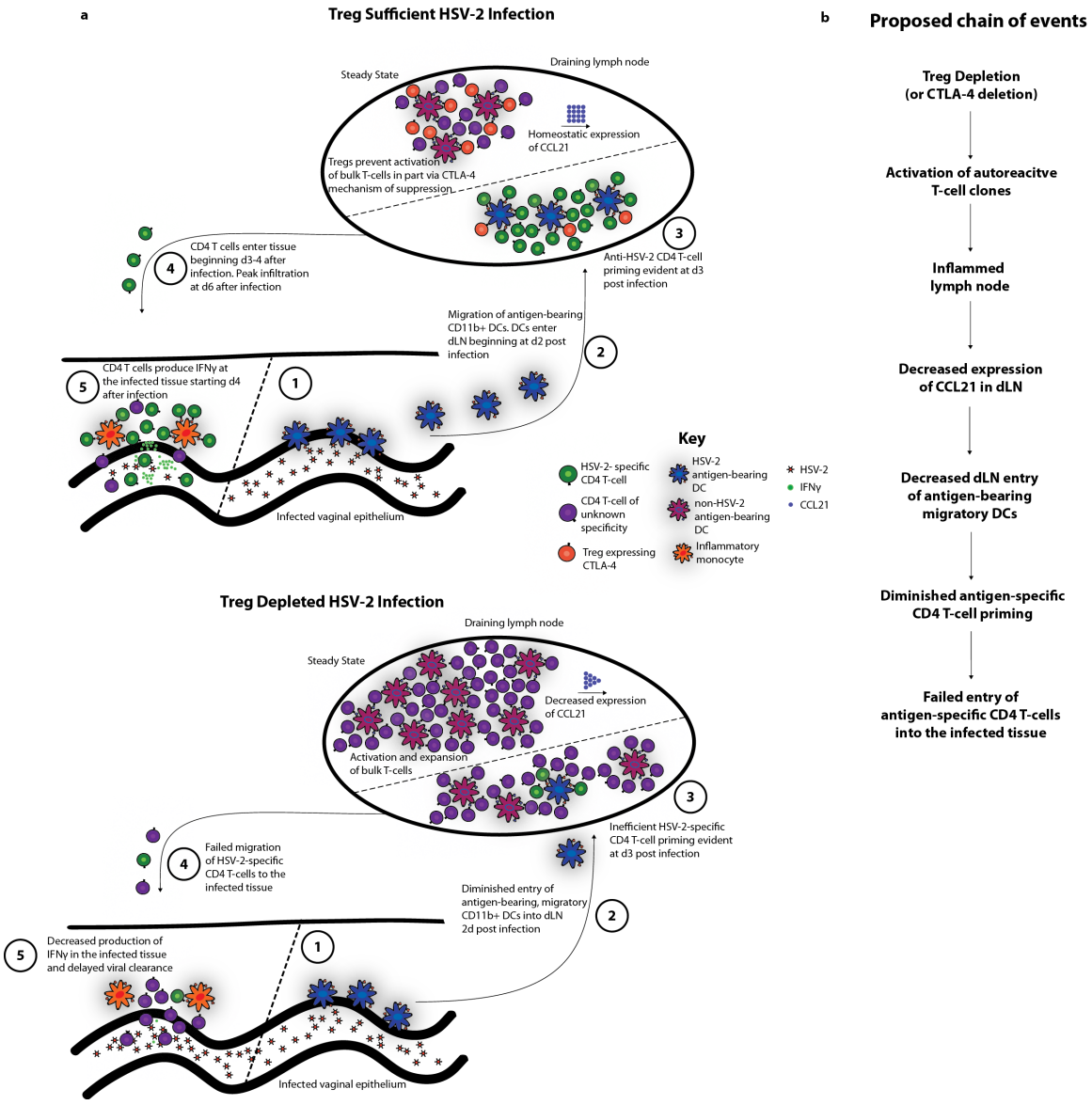

Figure 13: Visual Summary of Findings

A) Visual summary of the immune response against HSV-2 in both a Treg-sufficient (top) and Treg-depleted (bottom) mouse. Acute changes that are induced by Treg depletion lead to important differences throughout the developing immune response against HSV-2. B) The proposed chain of events involving the phenotypes presented throughout the dissertation.

Chapter 3: Discussion

3.1: Primary conclusions

The dominant model for the role played by Tregs during infections suggests that they are important primarily to dampen the anti-pathogen immune response to limit the amount of collateral damage to self-tissue that is sustained during the immune response (**Fig. 1**). Building on previous works that have suggested that this model is not appropriate for all immune responses, the work presented here implicates Tregs as being crucial in facilitating the antigen-specific CD4 T cell response following intravaginal HSV-2 infection, a phenotype that has not been seen in other models. In the absence of Tregs, migratory DC entry into the dLN is dysregulated (**Fig. 9**), antigen-specific CD4 T cell priming is inefficient (**Fig. 7**), and ultimate migration of antigen-specific CD4 T cells to the infected tissue is dramatically curtailed (**Fig. 5**). Furthermore, this work suggests that CTLA-4 is an important molecule used by Tregs to facilitate migratory DC entry into the dLN after intravaginal HSV-2 infection (**Fig. 12**). Collectively, these data provide evidence for a previously unrecognized role for Tregs in facilitating the proper initiation of an antigen-specific T cell response against a pathogen.

3.2: Further questions

The most immediate unanswered questions concerning the data presented are how mechanistically connected each presented phenotype is to each other, and how, collectively, they are precisely caused by the depletion of Tregs.

Treg depletion led to a decrease in CCL21 in the dLN, but the precise steps connecting Treg depletion to decreased CCL21 are not clear. The hypothesized link between bulk production of IFNγ in the dLN causing the drop in CCL21 was not supported by experiments using IFNγ KO mice crossed with Foxp3^{DTR} mice (**Fig. 10**). A similar mechanism involving different cytokines, such as IL-1 or IL-12 may explain the drop in CCL21 (Mueller et al., 2007), in which case similar experiments could be done to suggest a mechanism. Alternatively, since the Treg depletion results in pleiotropic effects, it may be that the elimination of any one single gene, as was attempted with IFNγ, would not prevent the drop in CCL21 production because multiple Treg depletion induced changes contribute to the phenotype. If this is the case, then perhaps blocking the action of a combination of cytokines, such as IFNγ, IL-1, and IL-12 would allow for homeostatic production of CCL21 despite Treg depletion.

Similarly, while the drop in CCL21 very likely contributes to the decreased entry of antigen-bearing DCs into the dLN following infection, since we were unable to restore CCL21 to wild type levels despite Treg depletion in IFNγ KO mice, the data leave open the possibility that other mechanisms are involved in the modulation of DC entry into the dLN.

The evidence is compelling that antigen-bearing DCs derived from the infected tissue are essential for CD4 T cell priming in this model. As such, the observed decrease in antigen-bearing DCs in the dLN is a likely mechanism to explain the inefficiency of antigen-specific T cell priming phenotype that was seen after Treg depletion. We have not, however, demonstrated that other factors may not also be involved.

Indeed, there is evidence, in the case of CD8 T cells, that Tregs can influence T cell priming by preventing the engagement of low affinity T cell clones with antigen-bearing DCs. When Tregs are depleted, low affinity CD8 T cells engage with antigen-bearing DCs for longer durations and are more able to cross the threshold into an activated state (Pace et al., 2012). One could imagine a similar dynamic occurring in the intravaginal HSV-2 model. Not only does Treg depletion cause fewer antigen-bearing DCs to enter into the dLN, but once they do arrive, they are not engaging primarily with the high-affinity anti-pathogen T cells that are needed for viral control, but are rather engaging with multiple T cell clones that have relatively little or no affinity for viral antigens. This dynamic could be particularly important when viral antigen entry into the lymph node is relatively limited, as is the case following intravaginal HSV-2 infection in general, and in the context of Treg depletion in particular.

Such a description is speculative, but could in theory be tested using tools similar as those used by Pace *et al* (Pace et al., 2012). If accurate, then Treg depletion would be expected to lead to an increased number of T cells that bind with low affinity to a virus-peptide loaded TCR tetramer compared to Treg-sufficient mice. Similarly, intra-vital imaging would be expected to show that TCR transgenic T cells with known high affinity for viral antigen would have a diminished competitive advantage compared to other T cell clones in terms of their ability to engage with viral-antigen-bearing DCs. While these experiments are theoretically possible, they would be technically challenging. Steps necessary to perform them include developing a CD4 T cell tetramer specific for HSV, as well as the fine tuning of the protocol necessary to image fluorescently labeled cells in

the inguinal lymph node while T cell priming is underway after intravaginal HSV-2 infection.

To more narrowly address the connection between decreased antigen entry into the dLN and less efficient antigen-specific T cell priming, a similar infection model could be used that, unlike intravaginal infection, allows for viral antigen to move freely in the lymph to the dLN. Previous studies have shown that while intravaginal HSV infection does not lead to free antigen moving to the dLN, HSV infection that is initiated with a sub-mucosal needle injection of the virus within the vaginal tissue does result in free antigen entering the dLN (Lee et al., 2009). Thus, the amount of antigen entering the dLN following needle injection of virus would presumably be less affected by changes in DC migration. Because of this, the importance of antigen entry into the dLN for the potential downstream affect of diminished antigen-specific T cell priming could be tested. If the Treg depletion-induced affect on priming is dependent on a paucity of antigen-bearing DCs, then priming should be restored to at least wild type levels in Treg-depleted mice infected through a needle injection. If, however, T cell priming is affected by mechanisms independent of antigen-entry into the dLN, then an effect would still be expected in Treg-depleted mice infected through a needle injection.

Finally, the near complete absence of transferred antigen-specific CD4 T cells that migrate to the infected tissue is likely to be at least partly caused by the diminished priming of antigen-specific CD4 T cells after infection (**Fig. 6**). However, Tregs may also be somewhat important for the successful migration of primed T cells to the infected tissue. We tested this hypothesis by delaying Treg depletion until after T cell priming. When we did so, gDT-II migration to the tissue was largely restored, in line with the idea

that Treg absence during priming is important for the observed phenotype. There may be some trend, however, towards reduced entry of gDT-II cells into the vaginal tract even when Treg depletion was delayed (**Fig. 6**). Although the trend did not rise to the level of significance, further experiments with greater statistical power may reveal some contribution for Tregs during the migration phase of the HSV-2 infection. This would be somewhat in line with what has been seen during RSV infection (Ruckwardt et al., 2009; Fulton et al., 2010).

Another primary question raised by the data in this thesis is how generalizable the data is to other pathogens. As emphasized in the introduction, the dominant model for the role of Tregs during an infection places little emphasis on their importance for the initiation of an anti-pathogen immune response, but rather focuses on their importance in modulating the magnitude of the immune response once generated. That model is based on several different infection models and it will be important to determine what aspects of the intravaginal HSV-2 model account for Tregs being important for the proper initiation of the anti-viral immune response.

One particular aspect of the immune response following intravaginal HSV-2 response that may be noteworthy is the relative scarcity of antigen that enters the dLN and the requirement for migratory DCs to carry the antigen to the dLN. Following a systemic infection, antigen would be expected to be abundant in the spleen, and possibly other secondary lymphoid organs. In some localized infections, antigen can move to the lymph node in the lymph, without being transported by DCs (Lee et al., 2009). In other localized infections, antigen only moves to the lymph node when carried by migratory DCs, but the antigen is transferred to lymphoid resident DCs for presentation to T cells

(Allan et al., 2006; Bedoui et al., 2009). In the first two of these cases, T cell priming may not be affected by Treg depletion because there is no shortage of antigen entering the lymphoid organs to mediate T cell priming. In the last scenario, the antigen is transferred to a population of DCs that is known to be particularly activated following Treg depletion, and may therefore be able to mediate sufficient T cell priming even with less antigen available as a result of diminished migratory DC entry into the dLN. Similarly, somewhat different populations of DCs patrol different tissues, and Treg depletion may be particularly impactful for vaginal tract CD11b+ DCs for reasons that are yet to be elucidated (Iwasaki, 2007).

3.3: Implications

Independent of these ongoing questions, the data presented in this thesis suggest that in some circumstances, Tregs are important not just in limiting an anti-pathogen immune response, but in facilitating a targeted immune response when appropriate. This insight has several implications.

Vaccination relies on presenting the immune system with antigens that are fundamentally harmless, but in a context that still triggers a memory inducing immune response. One strategy that has been proposed to create the context for the immune system to treat harmless antigens as a threat has been to couple vaccination with some sort of therapy that limits Treg activity in the hope of creating a pro-inflammatory environment (Berod et al., 2012). The data presented in this thesis suggest that while this may be a viable strategy during some vaccination routes, it may prove ineffective with other routes. Indeed, depending on the details of antigen presentation, the strategy could actually cause a decrease in priming of antigen-specific CD4 T cells.

Similarly, during cancer and chronic infections, it has been proposed that therapies targeting Tregs, or important molecules expressed by Tregs such as CTLA-4, might induce a more robust targeting of the cancerous cells or chronic pathogen (Fehervari and Sakaguchi, 2005), facilitating the elimination of these respective threats. While these benefits might be realized, the data in this thesis also suggest the potential for unintended consequences from such a strategy, as individuals with diminished Treg function may be less able to target an immune response against some potential pathogens.

3.4: Concluding remarks

Collectively, the data presented in this thesis significantly enhances our knowledge about how the presence of Tregs affects the development of the adaptive immune response following intravaginal HSV-2 infection. Contrary to what would have been predicted using the rationale of the dominant paradigm in the field, the antigen-specific CD4 T cell response targeting HSV-2 does not initiate properly when Tregs are absent at the time of infection. Fewer antigen-specific CD4 T cells target the tissue, antigen-specific priming is less efficient, and the migration of antigen-bearing DCs from the infected tissue to the dLN is dysregulated. Thus, the role of Tregs during infections is not only to restrict the magnitude of an immune response to limit immunopathology, but also, in some cases, to maintain the immune system in a state where it is poised to mount a pathogen-targeted immune response when appropriate.

Methods

Mice

C57BL/6 mice, 6-8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in filtered cages. IFNγ-deficient mice and OT-II mice (purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, respectively), gDT-II mice (Bedoui et al., 2009; Gebhardt et al., 2011)(kindly provided by Drs. Andrew Brooks (The University of Melbourne) and David Leib (Dartmouth College)), CCR2 Reporter mice (Hohl et al., 2009) (kindly provided by Dr. Tobias Hohl, Memorial Sloan-Kettering Cancer Center) and *Foxp3*^{DTR} (Kim et al., 2007)and CTLA-4$^{flox/flox}$ x *Foxp3*^{Cre} mice (kindly provided by Dr. Alexander Rudensky, Memorial Sloan-Kettering Cancer Center) were bred onsite at the animal facility at Fred Hutchinson Cancer Research Center (FHCRC). All animal experiments were approved by the FHCRC Institutional Animal Care and Use Committee. The Office of Laboratory Animal Welfare of the National Institutes of Health (NIH) has approved the FHCRC's Animal Welfare Assurance (#A3226-01), and this study was carried out in strict compliance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory.

Bone marrow chimeras:

Foxp3^{DTR} Ly 5.1 mice were irradiated with 900 Rads of irradiation one day before being reconstituted with bone marrow cells collected from the femurs and tibiae of Foxp3^{DTR} Ly 5.1 mice mixed with an equal number of cells from either CTLA-4$^{flox/flox}$ x Foxp3^{Cre} or CTLA-4$^{WT/WT}$ x Foxp3^{Cre} Ly 5.2 mice. Bone marrow cells were flushed with PBS from the

bone marrow using a small gage needle, red blood cells were lysed using a solution of deionized water supplemented with NH_4Cl, $KHCO_3$, and Na_2-EDTA, and then a single cell suspension was prepared by forcing cells through a 100 μM filter. Cells were counted, equal numbers of the appropriate cells were mixed together, and then 5 million total cells suspended in HBSS were injected intravenously into each recipient mouse. Bone marrow chimeras were allowed to rest for at least three months prior to being used in any experiment.

Infections

Mice were injected subcutaneously with 2 mg of Depo Provera (Greenstone) 5 to 7 days prior to being infected with HSV-2. At the time of infection, mouse vaginal tracts were swabbed with calcium alginate tipped swabs before 1.88×10^5 - 1.0×10^6 PFU of virus was applied to the vaginal tract in a 10 μL volume. HSV-2 was propagated on and titered by plaque assay on Vero cells. All infections used HSV-2 186 ΔKpn (Jones et al., 2000) (kindly provided by Dr. David Knipe, Harvard Medical School) or TK- HSV-2 expressing OVA (Dobbs et al., 2005) (kindly provided by Dr. Gregg Milligan, University of Texas Medical Branch at Galveston).

Vaginal Washes

Vaginal washes were collected from mice by swabbing vaginal canals with a solution of PBS supplemented with $MgCl_2$ 6 H_2O, $CaCl_2$ 2 H_2O, heat inactivated FBS, and glucose (titration buffer)-soaked calcium-alginate tipped swabs, followed by washing the vaginal canals with 50 μL of titration buffer, followed by a final swab with a dry calcium-

alginate tipped swab. The tips of both swabs as well as the 50 μL of titration buffer used for washing were stored in a final volume of 1 mL titration buffer. Samples were collected on ice and either assayed immediately or stored at -80C. Viral titer of vaginal washes was determined by plaque assay on Vero cells. IFNγ concentration in vaginal washes was determined using the Ready-Set-Go ELISA kit (eBioscience) according to manufacturer's instructions.

Antibodies

The following antibodies were used in this study: anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-Ly 5.1 (clone A20), anti-Ly 5.2 (clone 104), anti-Nur77 (clone 12.14), anti-CD69 (clone H1.2F3), anti-CD11c (clone N418), anti-MHC II (clone M5/114.15.2), anti-CD11b (clone M1/70), anti-CTLA-4 (clone UC10-4B9), anti-Foxp3 (clone FJK-16s), anti-Ly6C (clone HK 1.4) (all from eBioscience) and anti-CD3 (clone 145-2C11) (from eBioscience and BD Biosciences)

Regulatory T cell depletion

In all experiments, *Foxp3*^{DTR} mice were injected intraperitoneal with 30 μg/kg of Diphtheria toxin (DT) (EMD Millipore) dissolved in PBS upon first injection and 10 μg/kg of DT for all subsequent injections. Treg-sufficient mice were *Foxp3*^{DTR} mice injected with a matching volume of PBS. In experiments where Tregs were depleted prior to infection, DT injections were done on days -2, -1, and +1 relative to the day of infection. In experiments where Tregs were depleted after T cell priming, DT injections were done on days +3 and +4 relative to the day of infection. In some experiments, Treg-

sufficient mice were a non-DTR expressing strain injected with DT, and no differences were noted upon using either Treg-sufficient control setup.

Tissue Collection and Processing

When lymph node or spleen lymphocytes were being isolated, a single cell suspension was prepared by forcing the tissue through a 100 μM filter into PBS. When DCs from the lymph node or vaginal tract cells of any type were being isolated, tissues were chopped into small pieces in the presence of DMEM medium supplemented with Collagenase D (Roche) and DNase I (Roche) then incubated in the Collagenase D/DNase I solution for 30 minutes at 37 degrees C. Tissue was then transferred to a 15 mL conical tube before being re-suspended in HBSS supplemented with EDTA and FCS then incubated for 5 minutes at 37 degrees C. Tissue was then forced through a 100 μM filter to prepare a single cell suspension. Blood was collected from the orbital sinus using a capillary tube and collected into PBS supplemented with EDTA. In spleen and blood samples, red blood cells were lysed using a solution of deionized water supplemented with NH_4Cl, $KHCO_3$, and Na_2-EDTA. All lymph node samples were counted using a hemacytometer for further enumeration of cell populations.

Cell Transfers

For all cell transfers, a single cell suspension of spleen and lymph node cells was prepared as described above. CD4 T cells were enriched using the Stem Cell Technologies CD4 T cell negative selection kit according to manufacturers instructions. CD4 T cells were re-suspended in HBSS at 1 x 10^6 cells/mL, and 200 μL was injected

intravenously into each mouse so as to transfer 2 x 10^5 antigen-specific CD4 T cells. Ly 5.2 expressing TCR transgenic cells were transferred into Ly 5.1 expressing *Foxp3*^{DTR} mice and Ly 5.1 expressing TCR transgenic cells were transferred into Ly 5.2 expressing *Foxp3*^{DTR} mice.

Cell sorting

For *ex vivo* co-culture experiments, DCs were sorted based on expression of CD11c and MHC II. After dissection of the draining iliac lymph nodes (dLN), cells were isolated as described above. After counting and normalizing the number, cells were plated onto a 96 well plate. Cells were blocked for Fc binding through incubation with anti CD16/32 antibody (clone 93) (eBioscience) for 10 minutes at 4 degrees C. Cells were then stained with anti-MHC II antibody (clone 11:M5/114.15.2) and anti-CD11c antibody (clone N418) (eBioscience) by incubating cells for 15 minutes at 4 degrees C. Cells were re-suspended in PBS supplemented with EDTA and forced through a Falcon filter-tipped FACS tube. Cells from dLN were sorted into two groups; an MHC II high/CD11c mid population of migratory DCs and MHC II mid/ CD11c + population of non-migratory DCs. All sorting was done on a FACSAria (BD Biosciences). A sample of cells taken from each population from each group was then run on the flow cytometer to confirm sorting efficiency. CD4 T cells that were already enriched using the CD4+ T cell negative selection kit (Stem Cell Technologies) were also stained using anti-CD4 (clone GK1.5) and anti-MHC II antibodies (eBioscience) and sorted into a population of CD4+ and MHC II – cells to ensure that the CD4 T cells were not contaminated with antigen presenting cells.

Flow Cytometry

Single cell suspensions from various tissues were prepared as described above. LN cells were counted and 2 x 10^6 cells were transferred to a 96 well plate for staining. All blood and vaginal tract cells were transferred to a 96 well plate for staining. Cells were first incubated in eFlour 780 fixable viability dye (eBioscience) diluted in PBS for 30 minutes then were blocked for Fc staining by incubating in anti-CD16/32 antibody (clone 93) (eBioscience) diluted in .5% FCS in PBS for 10 minutes at 4 degrees C. After washing, cells were stained using various combinations of antibodies listed above in 0.5% FCS in PBS for 15 minutes at 4C. Cells with no intracellular staining needed were then fixed by incubating in 1% paraformaldehyde for 10 minutes at room temperature before being suspended in PBS. Cells with intracellular staining targets were then incubated in fix/perm solution (eBioscience) for 30 minutes at 4 degrees C. After washing with permeabilization buffer (eBioscience), cells were re-suspended in a solution of various combinations of antibodies listed above suspended in permeabalization buffer (eBioscience). After a final washing, cells were re-suspended in PBS for analysis on an LSR II. For vaginal tract samples, cells were forced through a Falcon filter topped FACS tube and combined with CountBright beads (Molecular Probes) for enumeration. Data were then analyzed using FlowJo software (Treestar). Cell counts from LN samples were determined by multiplying appropriate cell populations fractions by the cell count determined by hemacytometer. Blood cell fractions were determined by multiplying appropriate cell population fractions together.

Ex vivo co-culture

DCs and CD4 T cells were sorted as described above. 2500 - 3000 DCs were then co-cultured with 100,000 CD4 T cells in 120 μL of volume in a 384 well culture plate. After 60 hours of co-culture at 37 degrees C, supernatant was assessed for IFNγ using the Ready-Set-Go ELISA kit (eBioscience) according to manufacturer's instructions. Where noted, *ex vivo* cultures were supplemented with 1 x 10^5 PFU of heat inactivated HSV-2.

FITC Painting

Four hours prior to infection, mouse vaginal tracts were swabbed with calcium-alginate tipped swabs before having 10 μL of 1% FITC (Sigma-Aldrich) in DMSO applied using a pipette. Mice were then infected as described above. LN cells were isolated as described above. FITC+ DCs were enumerated in the draining iliac lymph nodes two days after infection by staining cells as described above and gating on FITC+/CD11b+/CD8- DCs and multiplying the gate percentages by the number of lymphocytes in the sample as determined by hemocytometer count. The FITC positive gate was set using cells from a mouse that had DMSO without FITC applied to the vaginal canal prior to infection.

qPCR

Draining iliac lymph nodes were harvested directly into RNALater (Life Technologies) then transferred into mortal and pestle tubes filled with RLT lysis buffer (Qiagen). RNA was then isolated using the RNeasy Plus mini kit (Qiagen) according to manufacturers instructions. Any residual genomic DNA was then eliminated using the DNase away kit

(Invitrogen) according to manufacturers instructions. A cDNA library was then prepared using the RT kit (Applied Biosystems) according to manufacturers instructions. qPCR was performed on CCL21 and HPRT using TaqMan primers (Applied Biosysems) and 2x qPCR master mix (Applied Biosystems) diluted with water and 250 ng of RNA from each sample. Relative expression of CCL21 in relation to HPRT was then calculated for each sample using the delta Ct method.

CCL21 ELISA

Draining iliac lymph nodes were harvested directly into NP-40 lysis buffer (PBS supplemented with 150 mM NaCl, 1% NP-40, 50 mM Tris-Cl, and Complete mini, protease inhibitor cocktail (Roche Applied Science)). Tissue was weighed then homogenized using an electric homogenizer before being centrifuged for 10 minutes at 2000 rpm at 4 degrees C. The supernatant was then analyzed for CCL21 protein using an ELISA kit according to manufacturers instructions (R & D Systems). The amount of CCL21 protein in each sample was normalized to the weight of the tissue.

Immunohistochemistry

Draining iliac lymph nodes were snap frozen in OCT blocks before being sliced and mounted on microscope slides for staining. Specimens were then fixed by incubating in 2% formaldehyde in PBS for 15 minutes at room temperature before being washed three times with PBS. After fixing, specimens permeabilized by incubation in ice cold 100% methanol for 10 minutes at -20 degrees C before rinsing in PBS for 5 minutes at room temperature. Specimens were then incubated in a blocking buffer (PBS supplemented with 5% normal donkey serum and 0.3% Triton X-100) for 60 minutes at room

temperature. After removing blocking buffer, a solution of primary antibodies (CCL21 polyclonal goat IgG, CD5 monoclonal Rat IgG2a, or isotype controls (all from R & D Systems)) diluted in dilution buffer (PBS supplemented with 1% BSA and 0.3% Triton X-100) was applied to the specimens before incubation overnight at 4 degrees C. After primary antibody staining, specimens were rinsed 3 times with PBS then incubated in a solution of secondary antibodies (Donkey anti-goat IgG F(ab)2 fragment conjugated to Alexa-647 and Donkey anti-rat IgG F(ab)2 fragment conjugated to Rhodamine/TRITC (both from Jackson Immunoresearch)) diluted in dilution buffer for 2 hours at room temperature in the dark before being rinsed 3 more times with PBS. Specimens were then incubated with DAPI for 3 minutes at room temperature in the dark before a final 3 rinses with PBS. Specimens were then covered with Prolong Gold Anti-fade reagent (Molecular Probes) and a glass coverslip. Images were acquired on an Aperio image collector (Leica).

Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). All data sets were first screened for outliers using the ROUT test with a Q value of 0.2%. When comparing groups in experiments with more than two experimental groups, an ordinary one-way ANOVA followed by a Tukey test was conducted to determine statistical significance between groups. When comparing only two experimental groups, an unpaired, two-tailed, parametric t test was conducted to determine statistical significance. In all cases, a p value less than 0.05 was considered significant. Error bars show +/- standard deviation.

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VITA

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