The Development and Function of Memory Regulatory T Cells

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{Reg}) are a cell lineage that develops in the thymus and exits to the periphery, where they represent 5-10% of the peripheral $CD4^+$ T cell population. Phenotypically, they are characterized by the expression of the cell surface markers CD25, as known as the IL-2 receptor alpha chain, glucocorticoid-induced tumor necrosis factor receptor (GITR), and cytotoxic T-lymphocyte antigen-4 (CTLA-4), as well as forkhead box P3 (Foxp3), a transcription factor considered to be the most specific T_{Reg} marker. Functionally, T_{Reg} cells are defined by their ability to suppress the activation of multiple cell types including $CD4^+$ and $CD8^+$ T cells, B cells, natural killer (NK) cells, and dendritic cells (DCs). Suppression can be achieved by the production of immunosuppressive cytokines or direct cell-to-cell contact, with these mechanisms directly affecting suppressed cells or indirectly affecting them by modulating antigen presenting cells (APCs). The suppressive abilities of T_{Reg} cells are crucial in maintaining dominant tolerance—the active, trans-acting suppression of the immune system for the prevention of autoimmune diseases. In addition to preventing autoimmune diseases, studies have also demonstrated critical roles for T_{Reg} cells in down-modulating anti-tumor immunity, suppressing allergic diseases, such as asthma, and achieving transplant tolerance. Recent studies have also demonstrated roles for T_{Reg} cells during pathogen infection, which will be the focus of this thesis.

Studies examining T_{Reg} cells during infection have largely focused on chronic infection models. These studies have shown that T_{Reg} cells can affect responses to pathogens in various ways that can be beneficial or detrimental for either the host or the

invading pathogen. In some infections, T_{Reg} cells downregulation effector responses, which can lead to pathogen persistence and, in some cases, concomitant immunity. T_{Reg} cell-mediated suppression can also reduce immunopathology at sites of infection, which can occur as a result of a vigorous anti-pathogen immune response.

In contrast to chronic infection, how T_{Reg} cells behave and function following acute infections remains largely unknown as, to date, very few studies have been conducted. Current work with acute infection models has indicated that T_{Reg} cells affect immune responses in some acute infection models, but not in all. Furthermore, the results of these studies have implicated that current approaches to examine T_{Reg} cells during acute infection by depleting the total T_{Reg} cell repertoire, as opposed to targeting pathogen-specific T_{Reg} cells, may not be ideal. Finally, it is unclear what happens to activated T_{Reg} cells following the resolution of infection.

Due to the lack of knowledge about the role of pathogen-specific T_{Reg} cells during acute infection, we sought to employ a different approach to address some of the outstanding questions in the field. Here, we utilized $CD4^+$ non-T_{Reg} and T_{Reg} cells from T cell receptor (TCR) transgenic mice that recognize a pathogen-specific epitope found in three different models of acute viral infection: recombinant vaccinia virus, recombinant adenovirus, and influenza. Using this model system, we were able to track pathogen-specific T_{Reg} cells following acute viral infection to determine their kinetics during the course of infection, as well as their influence on $CD4^+$ non-T_{Reg} cells during different times after infection. We also employed major histocompatibility complex (MHC) Class

II tetramer technology to track the fate of endogenous pathogen-specific T_{Reg} cells following infection with influenza.

Using these models systems, we show that pathogen-specific T_{Reg} cells can be activated and expand upon acute viral infections *in vivo*. The activated T_{Reg} cells then contract to form a "memory" pool after resolution of the infection. These "memory" T_{Reg} cells expand rapidly upon a secondary challenge, secrete large amounts of IL-10, and suppress excessive immunopathology, which is elicited by the expansion of non-T_{Reg} cells, via an IL-10-dependent mechanism. The work presented in this thesis reveals a previously unknown "memory" T_{Reg} cell population that develops after acute viral infections and may help design effective strategies to circumvent excessive immunopathology.

Dedication

I would like to dedicate this thesis to my husband, Parker Andrews. His support while I worked to complete my degree was critical to me staying the course and completing this goal. He moved to North Carolina, sigh unseen, so that I could pursue my dreams at Duke University. He has waited patiently when I told him my laboratory work would take one hour and then actually took three. He has been a shoulder to cry on and a hand to hold when I needed encouragement. We have grown together through this experience, and I look forward to all of our future endeavors together. You are best thing that has ever happened to me.

I would also like to dedicate my work to all of my nephews and nieces: Nico, Christian, Reyna, Aidan, Hannah, Riley, Stellan, and any future little guys/girls for me to spoil. I hope that my accomplishments exemplify that with hard work, any goal is attainable. I look forward to watching all of you reaching your own goals, and I will always be rooting for your success and happiness.

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List of Abbreviations

7-AAD	7-aminoactinomycin D
Ab	Antibody
Ad	Adenovirus
Ad-HA	Recombinant adenovirus expressing hemagglutinin
AICD	Activation-induced cell death
AMP	Adenosine monophosphate
APC	Allophycocyanin
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CCR6	C-C Chemokine receptor type 6
CD	Cluster of differentiation
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor

Ebi3	Epstein-Barr-virus-induced gene 3
ECM	Experimental Cerebral Malaria
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immunosorbent assay
Er	Estrogen Receptor
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HA	Hemagglutinin
HBSS	Hank's buffered salt solution
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
ICS	Intracellular cytokine staining
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneally
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
i.v.	Intravenously
LN	Lymph node

mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDCK	Madin-Darby canine kidney
MHC	Major Histocompatibility Complex
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed Death-1
PE	Phycoerythrin
PE-Cy5	Phycoerythrin cyanine 5
PerCP	Peridinyl chlorophyllin
PFU	Plaque-forming units
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
Runx-1	Runt-related transcription factor 1
rVV-HA	Recombinant Vaccinia Virus expressing HA
SD	Standard deviation
Sf	scurfy

SLE	Systemic lupus erythematosus
TCID	Tissue culture infectious dose
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Central effector T cell
TGF-β	Transforming growth factor-β
T_h1	T helper 1
TLR	Toll-like receptor
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TPLSM	Two-photon laser scanning microscopy
T_{Reg} cell	Regulatory T cell
VV	Vaccinia Virus
WT	Wild type

Acknowledgements

Reaching a goal as seemingly insurmountable as achieving a Ph.D. requires perseverance, hard work, and a devotion to learning the art of being a scientific researcher. However, attaining a Ph.D. is not a solo journey but requires a strong support network of mentors, colleagues, family, and friends. From the time I was a young child with a budding passion for science and throughout my time at Duke, I been lucky to be blessed with a wonderful support network, whose contributions to my success are immeasurable. I am truly grateful to all of these people and hope that I have contributed to their lives as they have mine.

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Chapter One: Introduction

Over one hundred years ago, Paul Ehrlich speculated that the immune system possesses the potential for "horror autotoxicus" or autoimmunity and that mechanisms must exist to circumvent this deleterious fate [1]. We now know that lymphocytes of the adaptive immune system have the unique ability to discriminate between foreign and self-antigens, allowing the body to successfully defend against pathogens while maintaining self-tolerance. Several processes that act both cell-intrinsically and cell-extrinsically maintain this delicate balance between autoimmunity and pathogen defense.

Ehrlich's postulations prompted research establishing that one of the mechanisms to avoid autoimmunity was the selective deletion of autoreactive T lymphocytes during their development in the thymus, in a process termed "clonal deletion" [2-4]. Here, self-reactive T lymphocytes are deleted in response to high-affinity major histocompatibility complex (MHC)/self-peptide and T cell receptor (TCR) interactions [5]. Despite this process, a small number of self-reactive T cells escape deletion and enter the periphery; these self-reactive cells are either eliminated physically by peripheral deletion or functionally by the induction of anergy [6]. Because these mechanisms act in a cell-intrinsic way, they are collectively termed "recessive tolerance." In addition to "recessive tolerance", there exists cell extrinsic mechanisms for tolerance executed by a relatively recently discovered subset of T cells, termed regulatory T cells (T_{Reg}), which are dedicated to the dominant, trans-active suppression of immune activation [7-10].

The existence of a unique subset of cells devoted to dominant tolerance was proposed thirty years ago following an experiment wherein stimulation with thymus-

dependent antigens led to the production of "suppressor cells", which could downregulate the immune response [11]. To expand this initial observation, further studies to characterize the phenotype and molecular mechanisms of suppressor cells were initiated. Overall, these experiments were inconclusive and at times, inaccurate, leading to the concept of suppressor cells to fall out of favor with prominent scientists calling their existence into question [12]. The final blow to the suppressor cells concept occurred when molecular studies failed to identify the genes encoding soluble antigen-specific factors that were thought to mediate suppression [13].

It was not until 1995 that the concept of suppressor cells was revived, when it was observed that thymectomy of neonatal mice led to the development of organ-specific autoimmunity, which could be prevented by the transfer of cells from the spleen [14]. Sakaguchi and colleagues demonstrated that the cells responsible for the prevention of autoimmunity was a population of $CD4^+$ T cells expressing high levels of the IL-2 receptor alpha chain (CD25) [15]. These seminal publications lead to a rebirth of the field of suppressor cells, which were reborn under the name "regulatory T cells." This thesis will focus on the naturally-occurring $CD4^+CD25^+$ T_{Reg} cells that develop in the thymus as a unique lineage of $CD4^+$ T cells (for a review of induced T_{Reg} cells see [16]).

1.1 Regulatory T Cells

T_{Reg} cells develop in the thymus as a subset of $CD4^+$ T cells that acquire their suppressive abilities as the result of a unique microenvironment. While all of the signals required for this microenvironment are unknown, studies using TCR transgenic mice that co-express their cognate antigen have suggested that T_{Reg} cell lineage development in the

thymus requires high affinity TCR stimulation [17-19] and is critically dependent on B7 costimulation [20, 21]. It is believed that these and potentially other signals lead to the expression of the transcription factor forkhead box p3 (Foxp3) [18, 22]. Foxp3, which is classified by its winged helix-forkhead DNA-binding domain, is a lineage-defining factor for T_{Reg} cells and has been demonstrated to be required for the development and maintenance of the T_{Reg} cell lineage [23, 24]. To date, it is unclear how Foxp3 regulates expression of T_{Reg}-associated genes, but there is evidence supporting a role for the transcription factors nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Runt-related transcription factor 1 (Runx1) [25-27].

In addition to the expression of Foxp3, T_{Reg} cells are characterized by the high level expression of CD25 [15, 23], cytotoxic T lymphocyte antigen-4 (CTLA-4) [28, 29], and glucocorticoid-induced tumor necrosis factor receptor (GITR) [30, 31]. Because these molecules can also be found on activated non-T_{Reg} cells, Foxp3 is considered the most specific marker for T_{Reg} cells in both mice [23, 24] and humans [32, 33]. While Foxp3 is highly restricted to the T_{Reg} cell lineage in mice, it is much more promiscuous in humans; transient expression of Foxp3 in human cells has been reported following TCR stimulation, but this expression does not confer suppressive functions [34]. This promiscuity complicates the identification of T_{Reg} cells in humans, necessitating the identification of additional markers for human T_{Reg} cell studies and therapies aimed at modulating T_{Reg} cells.

Once T_{Reg} cells develop in the thymus, they exit into the periphery where they represent between 5-10% and 2-4% of the $CD4^+$ T cell population in healthy mice and humans, respectively [35]. In the periphery, T_{Reg} cells perform a variety of functions by downmodulating immune responses. Early studies of T_{Reg} cells focused on their role in dominant tolerance—the active, trans-acting suppression of the immune system. These studies initiated when it was shown that thymectomy in neonatal mice three days after birth resulted in the development of organ-specific autoimmunity that could prevented by transferring syngeneic T cells from the spleen or thymus [14]. Further work by Sakaguchi *et al.* demonstrated that the cells responsible for preventing autoimmune disease in this model were $CD4^+$ $CD25^+$ T_{Reg} cells [15, 23]. Later studies further classified these cells as $Foxp3^+$ [23, 24, 36]**.** Additional work to understand the role of T_{Reg} cells in maintaining tolerance has been extended through the use of genetic models of T_{Reg} cell deficiency.

The importance of the T_{Reg} cell lineage in the maintenance of tolerance is exemplified by studies where genetic mutations or other manipulations that functionally delete T_{Reg} cells result in aggressive and fatal autoimmune disease. Mice with the spontaneous scurfy mutation (*sf*), which causes a frameshift mutation in the forkhead domain rendering Foxp3 nonfunctional, develop a fatal autoimmune disease characterized by severe lymphoproliferation and lymphocytic organ-infiltration ultimately leading to death three to four weeks after birth [37, 38]. In humans, an analogous mutation leads to a condition termed “immune dysregulation, polyendocrinopathy, enteropathy, X-linked” (IPEX), where patients display symptoms similar to those observed in *sf* mice [39-41]. Dysregulation of T_{Reg} cells has also been

associated with other autoimmune diseases including multiple sclerosis, type 1 diabetes, and rheumatoid arthritis [42-44]. In addition to autoimmune disease, T_{Reg} cells have been implicated in the development of allergic disease, such as asthma [45] and in the suppression of anti-tumor immunity [46], as well as during pathogen infection, which will be discussed at length in this thesis.

The diverse functions of T_{Reg} cells are a result of their ability to suppress multiple cells of the immune system, including non-T_{Reg} $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells (DCs), B cells, natural killer (NK) cells, NK T cells, macrophages, and mast cells [20, 47-53]. Currently, the exact mechanisms by which T_{Reg} cells suppress each of these cell types are not fully elucidated; moreover, it is unclear how these mechanisms of suppression cooperate together depending on the particular immune response. At present time, the proposed mechanisms for suppression can be loosely divided into two groups: suppression by secreted molecules acting in a short-range manner and suppression that is cell contact-dependant. Accumulating evidence suggests that contact-independent suppression can be mediated by anti-inflammatory cytokines, such as interleukin 10 (IL-10), IL-35, and transforming growth factor β (TGF-β) [54-57]. Traditionally, studies with TGF-β have focused on its role as a secreted cytokine, but some studies have suggested that membrane-bound TFG-β can contribute to cell-contact dependant suppression, as shown in a model of NK cell suppression [20, 56]. Current research is aimed at fully elucidating the exact cell type(s) that T_{Reg} cell-produced IL-10, IL-35 and TGF-β act upon, as multiple cells express receptors for these cytokines.

The inhibitory receptor CTLA-4 has been proposed to be one mechanism for contact-dependant suppression [58]. T_{Reg}-mediated suppression via CTLA-4 can occur by reducing the immunostimulatory functions of antigen presenting cells (APCs) through the downregulation of CD80 and CD86 (B7.1 and B7.2) on DCs or through the induction of indoleamine 2,3-dioxygenase (IDO) activity in DCs, an enzyme that catalyzes the conversion of tryptophan to immunosuppressive compounds [59, 60]. In addition to CTLA-4, other contact-dependent mechanisms include granzyme or perforin-dependent killing of both T and NK cells [61-63]. Further, it has been reported that T_{Reg} cells can modulate effector cells by transferring their high levels of cyclic adenosine monophosphate (cAMP) to non-T_{Reg} cells. The transferred cAMP can then act as a second messenger to inhibit IL-2 production and proliferation in non-T_{Reg} cells [64]. A similar mechanism has been observed with extracellular adenosine, which is converted on the surface of T_{Reg} cells by CD39/CD73 from extracellular nucleotides. The T_{Reg} cell-generated extracellular adenosine, which is a known potent anti-inflammatory molecule, then binds to adenosine receptors present on activated effectors cells to downmodulate their activation [65].

Studies using intravital two-photon laser scanning microscopy (TPLSM) have attempted to further elucidate the dynamics of T_{Reg} cell suppression *in vivo.* These studies have demonstrated that T_{Reg} cells suppress the immune response by preventing stable contact between DCs and non-T_{Reg} cells [66, 67], suggesting that *in vivo*, T_{Reg} cells may modulate APC function to suppress T cells. In these studies, clusters of DCs with non-T_{Reg} or T_{Reg} cells were found, but non-T_{Reg} and T_{Reg} cell interactions were not observed.

It is unclear whether this observation is specific to the draining lymph nodes, as other sites, such as peripheral target organs, have not been studied to date. Further, the mechanism by which T_{Reg} cells prevent stable DC: non-T_{Reg} cell contact has not been elucidated.

Further studies are required to more fully appreciate how suppression occurs *in vivo*, as well as the mechanisms of suppression that are utilized during pathogen invasion. Because many studies examining T_{Reg} cells during infection do not directly assess the mechanism(s) of suppression, it is unclear how T_{Reg} cells exert their effects during pathogen infection. Studies with *Leishmania major*, which is the most thoroughly studied pathogen with respect to T_{Reg} cells, have demonstrated that suppression occurs through both IL-10-dependent and yet undefined IL-10-independent mechanisms [68]. The observation that suppression occurs in both an IL-10-dependent and –independent manner has also been reported for *Schistosoma mansoni* infection [69, 70]. Additional work to identify the IL-10-independent mechanisms observed for both *Leishmania major* and *Schistosoma mansoni* infection, as well as work to further characterize mechanisms of suppression in other pathogen models are necessary, as it is unclear whether the suppression that occurs following an inflammatory response associated with infection is the same as what occurs in a less immunostimulatory environment.

1.2 Function of Regulatory T Cells in Chronic Infection

While initial studies with T_{Reg} cells focused on their role in maintaining tolerance, accumulating evidence indicates an important role for T_{Reg} cells in the control of immune responses to pathogens [71, 72]. One of the earliest implications for this role came from a

model of chronic infection with *Leishmania major* when it was demonstrated that IL-10, a cytokine associated with T_{Reg} cells, contributes to pathogen persistence [73]. Subsequent studies demonstrated that Leishmania-specific T_{Reg} cells found at sites of infection suppress effector T cells, by both IL-10-dependent and –independent mechanisms, resulting in incomplete pathogen clearance and the development of concomitant immunity [68, 74]. Additional studies in both experimental animal models and humans have shown that T_{Reg} cells can accumulate following multiple types of infections and modulate the immune response in a variety of ways that can be beneficial for both the host and the pathogen.

Studies in humans have demonstrated that T_{Reg} cells can expand following infection and accumulate at sites of infection. For example, T_{Reg} cells have been found in patients with parasitic infections (i.e. *Leishmania braziliensis* [75]), viral infections (i.e. hepatitis B [76]), fungal infection (i.e. *Paracoccidioides brasiliensis* [77]), and bacterial infection (i.e. *Helicobacter pylori* [78]). While T_{Reg} cells can be easily detected during human infection, studies to elucidate their role(s) are more limited because of the experimental constraints of working with patients. As such, studies to examine the effects of T_{Reg} cells during human infection are generally limited to *in vitro* suppression assays, whereby responder cells (non-T_{Reg}) are cultured with T_{Reg} cells in the presence of stimuli for activation (i.e. APC + anti-CD3 antibody or agonist peptide). When these cells are cultured together, T_{Reg} cells will suppress the proliferation of non-T_{Reg} cells, which can be experimentally measured. Using this method, the suppression of pathogen-specific effector cells by human T_{Reg} cells has been demonstrated in a variety of disease models,

including *Plasmodium falciparum* where the removal of T_{Reg} cells enhances *in vitro* non-T_{Reg} cell-mediated immune responses against the pathogen [79].

While *in vivo* evidence for T_{Reg} cell function during infection in humans is lacking, one study comparing an ethnic group with a naturally lower susceptibility to *Plasmodium falciparum* malaria than sympatric ethnic groups has shed some light. This study revealed that the group's resistance to malaria was not associated with classical malaria-resistance genes, but rather may be related to the fact that members of the resistant group have a propensity for functionally deficient T_{Reg} cells characterized by reduced RNA for genes associated with T_{Reg} cell function, as well as lower serum levels of TGF-β [80]. These data suggest a strong role for T_{Reg} cells in malaria resistance and provide a unique human system to further understand how T_{Reg} cells can affect the outcome of pathogen infection.

To more fully understand the roles of T_{Reg} cells during infection than can be garnered with human studies, murine models of chronic parasitic, bacterial, viral, and fungal infections have been utilized. These experiments have shown that the presence of T_{Reg} cells can suppress effector cells, preventing the immune system from mounting an effective immune response, which can lead to pathogen persistence. In some extreme cases, this can be detrimental to host survival, such as infection with a lethal strain of *Plasmodium yoelii* where T_{Reg} cells vigorously block an effective immune response to such an extent that depletion of T_{Reg} cells can actually improve host survival [81]. In what appears to be a similar mechanism, as infection with a hypervirulent, lethal strain of *Mycobacterium tuberculosis* proceeds, the number of effector T cells is reduced, and this

is associated with an increase in the presence of T_{Reg} cells [82]. Further studies are required to confirm that T_{Reg} cells are responsible for the decrease in effector cells and whether this is related to the lethality of this strain.

Multiple studies of chronic infection have demonstrated that, while the presence of T_{Reg} cells does not contribute to the pathogen's lethality, depletion of T_{Reg} cells leads to increased effector responses, supporting pathogen clearance. For example, in a murine model of ocular infection with herpes simplex virus 1 (HSV1), depletion of T_{Reg} cells leads to enhanced $CD4^+$ and $CD8^+$ expansion, effector function, and infiltration into the cornea [83, 84]. A similar enhancement of effector function has been observed in other models of parasitic, bacterial, fungal, and viral infection (for review see [85]). However, not all studies have demonstrated that T_{Reg} cells function to block effector activation. With *Mycobacterium tuberculosis* infection, depletion of $^+Foxp3^+$ T_{Reg} cells with a Thy1.1-depleting antibody in bone marrow chimeras where only $Foxp3^+$ cells were $Thy1.1^+$ resulted in significantly reduced bacterial burden in the lungs, but this reduction was not associated with an increase in pathogen-specific non-T_{Reg} cell effector function or proliferation. This seemingly paradoxical effect may be related to the low levels of antigen available for T cell activation, which has been reported for this *Mycobacterium tuberculosis* model [86]. However, using the same model of infection, Quinn *et al.* demonstrated that depleting T_{Reg} cells with an anti-CD25 antibody increased effector interferon-γ (IFN-γ) production but did not alter bacterial load [87] suggesting that the effects of T_{Reg} cell depletion that are observed in *Mycobacterium tuberculosis* may be related to the method of depletion utilized. This may be critical in this particular model of

infection because pathogen load is highly limiting to T cell expansion due to the inefficiency of antigen presentation [86].

While the presence of T_{Reg} cells can lead to pathogen persistence, this can actually be beneficial for the host. There are some infections, such as schistosomiasis and leishmaniasis, where pathogen persistence is necessary for protective immunity, termed concomitant immunity [88]. For example, the downregulation of an effector response by T_{Reg} cells leads to the inefficient clearance of the pathogen at the site of infection and subsequently, concomitant immunity during *Leishmania major* infection. This beneficial concomitant immunity is lost if T_{Reg} cells are removed [68]. A similar observation was made with a murine model of infection with the fungus *Candida albicans* where the presence of T_{Reg} cells was required for resistance to reinfection [89]. Additionally, similar results have been observed in infection with a low dose of HSV1 [83]. Further work is ongoing to determine whether T_{Reg} cells are important for concomitant immunity in human infections.

In addition to allowing for the development of protective immunity, T_{Reg} cells can benefit the host by reducing immune-mediated pathology that can occur as collateral damage during a strong anti-pathogen immune response. This role for T_{Reg} cells in protecting against immune-mediated damage has been demonstrated in models of pulmonary inflammation caused by *Pneumocystis* pneumonia, inflammatory eye lesions with HSV, liver pathology in *Schistosoma mansoni* infection, and stomach pathology with *Candida albicans* where depletion of T_{Reg} cells led to increased pathology in all disease models [69, 83, 89-91]. Evidence for T_{Reg}-mediated protection has also been

observed in humans, where it was recently demonstrated that the presence of T_{Reg} cells in chronic hepatitis C virus-infected patients was positively correlated with reduced liver fibrosis [92].

On the other hand, not all chronic models of infection support a role for T_{Reg} cells in the prevention of immunopathology. Paradoxically, in a model of *Plasmodium berghei* infection, removal of T_{Reg} cells actually resulted in reduced immunopathology in the brain [93]. This result was thought to occur because depletion of T_{Reg} cells significantly reduced both parasite burden and the recruitment of $CD8^+$ T cells into the brain [93]. In this model of experimental cerebral malaria (ECM), it appears as if the high parasite burden localized in the brain, as well as the potential exclusion of T_{Reg} cells from the brain during ECM [94], may make immunopathology an unavoidable consequence to combating *Plasmodium yoelii* infection.

Collectively, research with chronic infections indicates that there is a delicate balance between T_{Reg} and non-T_{Reg} cells, and any changes to this equilibrium can alter the outcome of a chronic infection. If T_{Reg} cell suppression is too vigorous, this can lead to poor pathogen clearance and possibly even host death. At the other end of the spectrum, without adequate suppression, non-T_{Reg} cells can cause collateral tissue damage due to an overzealous immune response. However, if non-T_{Reg} and T_{Reg} cells are balanced then this can lead to protective immunity without excessive immunopathology. Studies are underway to determine whether this balance can be exploited to modulate vaccine or disease outcomes.

1.3 Function of Regulatory T Cells in Acute Infection

Compared to studies of chronic infection, to date very few studies have been conducted to understand the functions of T_{Reg} cells during acute infection. The results of these studies have painted a contradictory picture for T_{Reg} cells during acute infection and highlight how different models of T_{Reg} cell depletion can lead to opposing results. Utilizing an anti-CD25 antibody to deplete T_{Reg} cells, it was shown that T_{Reg} cells do not affect the immune response or the disease outcome in acute *Pseudomonas aeruginosa* lung infection in mice [95]. Using a similar method of depletion, two independent studies examined the function of T_{Reg} cells during acute *Trypanosoma cruzi* infection. While one study found no role for T_{Reg} cells in this setting, the other study found only a limited role for T_{Reg} cells in affecting parasite load and disease mortality [96, 97]. The impact of T_{Reg} cells was largely dependent on the dose of *Trypanosoma cruzi* where mice receiving a lower dose displayed reduced parasitemia and mortality following T_{Reg} cell depletion, but these effects were no longer observed when higher numbers of parasites were used for infection [97]. The discrepancies between these two studies and between the responses from different parasite doses may be related to the relative inefficiency of T_{Reg} cell depletion by anti-CD25 antibodies observed during infection, as well as the potential depletion of effector T cells, which upregulate CD25 following activation [98, 99]. These pitfalls of depletion may become more apparent in models of acute infection because effector cells can express higher levels of CD25 during acute compared to chronic infection, which may render them more susceptible to anti-CD25-mediated depletion

[99]. Because of these caveats, newer mouse models that more selectively deplete $Foxp3^+$ T_{Reg} cells have been utilized to study the roles of T_{Reg} cells during acute infection.

To overcome the inherent problems of using anti-CD25 antibodies to study T_{Reg} cells during infection, *Foxp3*^{DTR} mice, which express the human diphtheria toxin receptor (DTR) under control of the Foxp3 promoter, have been used to study T_{Reg} cells during acute genital infection with HSV-2 [100]. In these mice, treatment with Diphtheria toxin (DT) allows for the rapid and efficient depletion of Foxp3-expressing cells, but not other murine cells because they are insensitive to DT-toxicity [101, 102]. The results of these experiments were quite unexpected; while T_{Reg} cell-depleted mice displayed increased activation of effector cells in the draining lymph nodes, they were unable to control genital HSV-2 infection [100]. This led to severe lesions and faster viral dissemination into the spinal cord, resulting in hindlimb paralysis. As a result, DT-treated $Foxp3^{DTR}$ mice succumbed to disease much earlier than T_{Reg} cell-sufficient mice [100]. This contradictory effect was owed to the fact that T_{Reg} cells modulate chemokine levels to ensure proper recruitment of immune cells to the site of infection [100]. This altered chemokine milieu resulted in delayed DC, NK cell, and T cell arrival at the site of infection in T_{Reg} cell-depleted mice, compared to T_{Reg} cell-sufficient mice [100]. This is not the first report that has implicated a role for T_{Reg} cells in cellular migration; in a model of acute respiratory syncytial virus infection, T_{Reg} cells were shown to alter $CD8^+$ T cell trafficking to the lung following infection, with T_{Reg} cell depletion leading to exacerbated disease severity [103].

While using Foxp3^{DTR} mice is a much more specific method to study the role of T_{Reg} cells during infection, experiments must be carefully controlled to ensure that they are properly interpreted. Depletion of T_{Reg} cells using this method leads to increased activation of T cells, an increase in the number and activation of DCs, and the production of inflammatory cytokines and chemokines even in the absence of infection [100, 101]. These effects may be a direct result of increased activation of autoreactive T cells, which are normally controlled by T_{Reg} cells, but may also reflect direct or indirect effects of T_{Reg} cells on other cells of the immune system, such as NK cells or DCs. Further, it is unclear whether the results observed after infection, in terms of the chemokine environment, are due to T_{Reg} cell-mediated effects on pathogen infection or the result of ongoing changes associated with total T_{Reg} cell ablation that affect the initiation of an early viral response. These changes highlight the catastrophic modifications that occur in the immune system when T_{Reg} cells are ablated and suggest that a more fine-tuned approach may be necessary to study T_{Reg} cell function during an immune response, to ensure that the effects observed are not related to induced autoimmunity.

These studies also raise the question as to whether suppression during infection is antigen-specific or whether it occurs as the result of a bystander effect of T_{Reg} cells responding to self-antigens. This is a critical point in deciding whether depletion of the polyclonal T_{Reg} cell population is the best method to study T_{Reg} cells during infection. Previous work to classify the T_{Reg} cell TCR repertoire has suggested some overlap between the TCRs of T_{Reg} and non-T_{Reg} cells, suggesting that T_{Reg} cells could recognize more than just self-antigens [104, 105]. Indeed, work by Suffia *et al.* demonstrated that

T_{Reg} cells found at the sites of *Leishmania major* infection proliferate in a pathogen-specific manner and that suppression *in vitro* is antigen-specific [74]. Additional studies with both mice and humans in other models of infection support these observations [106, 107]. While the antigen-specificity of suppression *in vivo* has been demonstrated in our laboratory in an autoimmune setting [108], further studies are necessary to demonstrate that the *in vivo* suppression observed during infection is antigen-specific. However, the current evidence supports the notion that T_{Reg} cell suppression during a pathogen response is directed at pathogen antigens, suggesting that studies examining T_{Reg} cell function during infection may benefit from a more fine-tuned approach.

The previous studies addressing the roles of T_{Reg} cells during acute infection have painted an incomplete and contradictory picture. These studies highlight the need to utilize different experimental models to more fully understand their roles. Additionally, data indicate that suppression during infection is pathogen-specific, further highlighting the need to examine pathogen-specific T_{Reg}, as opposed to depletion of the polyclonal population. To address these issues, the following experimental models were employed to examine pathogen-specific T_{Reg} cells during and following the resolution of acute viral infection with the results of this work presented in this thesis.

1.4 Experimental Models

1.4.1 Transgenic T Cell Receptor System

To address some of the outstanding questions on the roles of T_{Reg} cells during acute viral infection, we utilized $CD4^+$ T cells from transgenic mice that express a known TCR recognizing an I-E^d –restricted influenza hemagglutinin (HA) epitope. These TCR

transgenic cells have been previously used to study T_{Reg} cells in models of autoimmunity, as well as in allograft and tumor rejection [108-111]. TCR transgenic mice, such as the ones employed here, have been extensively used to study both $CD4^+$ and $CD8^+$ T cells in a variety of experimental models. They are a unique tool for studying the immune system because T cells of a known specificity can be transferred into recipient mice and tracked using congenic marker systems, such as Thy1.1 and Thy1.2 (CD90.1 and 90.2). The number of TCR transgenic cells that can be transferred can be significantly higher than that of the endogenous population for a given TCR specificity, which coupled with the tracking system, allows for detailed phenotypic and functional analysis of a specific cell population. Additionally, because the TCR specificity is known, viral or other models that express the cognate antigen can be employed to study an antigen-specific pathogen response. Using this approach has allowed us to examine the function of pathogen-specific T_{Reg} cells during and following the resolution of an acute infection, as opposed to examining the effects following total T_{Reg} cell ablation. For the purposes of these studies, $CD4^+$ TCR transgenic non-T_{Reg} and T_{Reg} cells were transferred into congenically-marked mice that were then infected with a variety of viral models to stimulate the TCR transgenic cells.

1.4.2 Viral Models to Study T_{Reg} cells during Acute Infection

For the purpose of this thesis, three viral models of acute infection that express HA were utilized: recombinant vaccinia-virus expressing HA (VV-HA), Adenovirus expressing HA (Ad-HA), and influenza (H1N1/PR8). All three of these viral models express HA and thus, can be coupled with TCR transgenic cells recognizing HA. To

address the goals of this thesis, these three viruses were utilized to stimulate TCR transgenic $CD4^+$ non-T_{Reg} and T_{Reg} cells *in vivo* to examine how T_{Reg} cells act, as well as how they influence non-T_{Reg} cells, during and following infection. Furthermore, to study the antigen recall response as an indicator of immunological memory, one viral model was administered for the initial response (prime), and another virus was administered for the antigen recall response (boost). Successive injections of the different viruses were done because it is not feasible to observe a memory T cell response when the same viral model is injected repeatedly into a mouse due to the generation of neutralizing antibodies.

1.4.3 Tracking Endogenous T_{Reg} cell Responses to Acute Viral Infection

In addition to tracking TCR transgenic cells, the response of endogenous T_{Reg} cells to acute viral infection was also examined to represent a more physiologically relevant quantity of pathogen-specific T_{Reg}. Recent advances have made it possible to track endogenous $CD4^+$ cells responses to a variety of infections and disease models using MHC Class II tetramers [112]. The tetramer is composed of four MHC Class II molecules that are bound to a specific peptide and conjugated with biotin. The four molecules are then linked together with fluorescently-labeled streptavidin to form a tetrameric structure. Because MHC Class II molecules are bound to the same specific peptide, the tetramer can bind more than one TCR on a given T cells, which gives the interaction a high enough avidity to be detected by Fluorescence-activated cell sorting (FACS). For the purposes of this thesis, we utilized a MHC class II tetramer presenting an HA epitope in conjunction with a model of intranasal influenza infection. Following

infection with influenza virus, the expansion of endogenous, pathogen-specific T_{Reg} cells was tracked with the tetramer. Because the relative abundance of naïve $CD4^+$ cells for a given specificity is quite low (20-200 cells), a method to enrich tetramer-positive $CD4^+$ T cells was also used [113].

1.5 Thesis Prospectus

T_{Reg} cells play a critical role in maintaining balance in the immune system to allow for the protection against pathogens without the induction of autoimmunity. There is clear evidence that T_{Reg} cells play important roles during chronic infection, where the presence of T_{Reg} cells controls effector immune responses, modulating disease outcome in a variety of ways that can be beneficial for both the host and invading microorganism. However, few studies have been done to examine T_{Reg} cells during acute infections, and those that have been done have raised multiple questions about their roles. First, it is still not entirely clear whether T_{Reg} cells affect the outcome of acute infections or whether they are important in only select acute infection models. Additionally, work will need to be done to further clarify how T_{Reg} cells modulate the outcome of acute infection to determine whether they modulate migration, as with genital HSV-2 infection or influence effector function and/or immunopathology, as observed in chronic infection models. Finally, studies using acute infection models can be used to determine what happens to activated T_{Reg} cells after clearance of an acute infection. Because of the caveats using depletion of the polyclonal T_{Reg} cell pool, these questions were addressed utilizing multiple viral models of acute infection coupled with TCR transgenic T cells to study pathogen-specific T_{Reg} cells.

With this in mind, the overall goal of this thesis was to understand how pathogen-specific T_{Reg} cells act during and following the resolution of infection. To accomplish this goal, we have utilized TCR transgenic T_{Reg} cells to track their response to three viral models. Additionally, to understand the role of T_{Reg} cells during acute infection, we examined how the presence of T_{Reg} cells during the initial stages of an immune response to an invading pathogen, as well as during a memory recall response, modulate the effector $CD4^+$ non-T_{Reg} cell response. This work addresses many of the outstanding questions on the roles of T_{Reg} cells during acute viral infections. Further, our work led to the discovery that "memory" T_{Reg} cells develop after the resolution of an acute viral infection. These "memory" T_{Reg} cells suppress the immunopathology elicited by non-T_{Reg} effector cell expansion during a recall response, through an IL-10-dependent mechanism. In summary, this thesis topic was addressed with the following chapter:

Chapter Three: How do T_{Reg} cells behave following acute infection, and specifically, how do they behave following the resolution of infection? And, what is the function of T_{Reg} cells during an acute viral infection?

Chapter Two: Materials and Methods

2.1 Mice

B10.D2 and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). 6.5 HA-TCR transgenic mice on the BALB/c background that express a TCR recognizing an I-E^d –restricted HA epitope (^{110}SFERFEIFPKE^{120}) were kindly provided by H. von Boehmer (Harvard University, Boston, MA). These mice were back-crossed onto the Thy1.1$^+$ B10.D2 background [108]. Mice expressing EGFP under the control of the endogenous Foxp3 promoter (Foxp3-GFP knock-in mice on the BALB/c background) were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were intercrossed with 6.5 HA-TCR transgenic mice to generate 6.5 HA-TCR Foxp3-GFP mice.

All mice utilized in these studies were between 8 - 12 weeks of age. Experimental procedures were performed in accordance with protocols approved by the Animal Care and Use Committee of the Duke University Medical Center.

2.2 Adoptive Transfer of HA-specific Transgenic T Cells

Naïve clonotypic HA-specific T_{Reg} and non-T_{Reg} CD4$^+$ T cells (Thy1.1$^+$) were prepared from 6.5 HA-TCR transgenic mice as described [108]. CD25$^+$ T cells were enriched by Magnetic-activated cell sorting (MACS) positive selection by staining with PE-conjugated anti-CD25, followed by anti-PE MicroBeads (Miltenyi Biotec). Selection of CD25$^+$ cells was done using a MACS column (Miltenyi Biotec) according to the manufacturer's instructions. Following this procedure, un-bound cells (CD25$^-$ -enriched)

were sorted gated on $6.5^+CD4^+CD25^-$ cells (non-T_{Reg}), and $CD25^+$- enriched cells were sorted gated on $6.5^+CD4^+CD25^+$ cells (T_{Reg}) with a FACSVantage (BD Biosciences) high-speed cell sorter. The purity of FACS sorted populations of cells was >98%. For some experiments, activated HA-TCR transgenic donor cells ($Thy1.1^+$) were enriched by staining with PE-conjugated anti-Thy1.1, followed by anti-PE MicroBeads, and selected using a MACS column. $Thy1.1^+$- enriched cells were sorted gated on $Thy1.1^+CD4^+$ cells, and the resulting populations had a purity >98%. After sorting, 1 x 10^5 cells (T_{Reg} or non-T_{Reg}) were transferred into recipient B10.D2 mice ($Thy1.2^+$) intravenously (i.v.) in 200μL HBSS.

2.3 Viruses

2.3.1 VV-HA

Recombinant vaccinia virus encoding HA (VV-HA) and recombinant E1-deleted adenoviruses encoding HA (Ad-HA) were previously described [114]. VV-HA was grown in TK-143B cells and purified from the cell lysate by sucrose banding. The titer of virus was determined by plaque forming assay on TK-143B cells. Mice were infected with 2 x 10^6 plaque-forming units (PFU) of rVV-HA intraperitoneally (i.p.) or 5 x 10^5 PFU intranasally.

2.3.2 Ad-HA

Ad-HA was grown in 293 cells (ATCC), purified by two rounds of CsCl density centrifugation, and desalted by gel filtration through a Sephadex G-25 column (PD-10

column, Amersham Bioscience). The titer of virus was determined by a plaque forming assay on 293 cells. Mice were infected with 2 x 10^9 PFU of Ad-HA intravenously.

2.3.3 Influenza

Influenza A/PR/8/34 (H1N1) was obtained from American Type Culture Collection (Manassas, VA). Immediately prior to intranasal administration of the influenza, mice were anesthetized with 3% isoflurane. After anesthesia, 40 µl of 4000 the 50% tissue culture infectious dose ($TCID_{50}$) of influenza in sterile PBS was then instilled intranasally.

2.4 Antibodies and Flow Cytometry

Antibodies for staining were PE, FITC, and APC-conjugated anti-CD4, PE and PerCP- conjugated anti-Thy1.1, APC-conjugated anti-IFN-γ, biotinylated anti-CD62L, PE-conjugated anti-CD44, PE-conjugated anti-IL10, Streptavidin-PeCy5, and PE-conjugated anti-CD25 (all from BD Biosciences). FITC-conjugated anti-Foxp3 was purchased from eBioscience. Anti-TCR-HA Abs (6.5) were purified and conjugated in our laboratory. Collection of flow cytometry data was carried out using a FACSCanto (BD Biosciences), and events were analyzed using FACSDiva Software (BD Biosciences).

The hybridoma for HA mAb, H36-4-5.2 (H36), was kindly provided by Dr. W. Gerhard (the Wistar Institute, Philadelphia, PA). The mAb was purified from hybridoma culture supernatant using a HiTrap™ Protein A column (Amersham Bioscience). The resulting antibody was labeled with the Alexa Fluor® 546 Protein Labeling Kit

(Molecular Probes) according to the manufacturer's instructions. Alexa Fluor® 488-conjugated anti-FITC was purchased from Molecular Probes.

2.5 Intracellular Staining

For intracellular Foxp3 staining, 1 x 10^6 cells were surface-stained, and then fixed/permeabilized using a cytofix/cytoperm kit (BD Biosciences) according to the manufacturer's instruction. Following fixation, cells were stained with FITC-conjugated anti-Foxp3. For intracellular IFN-γ and IL-10 staining, cells were cultured for 6 hours in the presence of 5 μg/ml GolgiPlug (brefeldin A; BD Biosciences) and 20 μg/ml of the I-E^d-HA peptide. For IFN-γ staining, following culture, cells were surface- stained with PeCy5-conjugated anti-CD4 and PE-conjugated anti-Thy1.1, fixed/permeabilized, and then cells underwent intracellular staining with APC-conjugated anti-IFN-γ and FITC-conjugated anti-Foxp3. For IL-10 staining, following culture, cells were surface-stained with APC-conjugated anti-CD4 and PE-conjugated anti-Thy1.1, fixed/permeabilized, and then cells underwent intracellular staining with PE-conjugated anti-IL-10 and FITC-conjugated anti-Foxp3.

2.6 In Vitro Suppression Assay

Naïve non-T_{Reg}, naïve T_{Reg}, and activated T_{Reg} cells were sorted according to the above protocol. Naïve non-T_{Reg} $CD4^+$ T cells (2×10^4, responders) were cultured with purified naïve or activated T_{Reg} cells (2×10^4, 1×10^4, or 5×10^3, suppressors) at different responder: suppressor ratios, in the presence of irradiated (3000 rad) B10.D2 splenocytes

(4×10^4, APC), and 10 μg/ml of the I-E^d-HA peptide. Incorporation of [^{3}H]thymidine (1 μCi/well) during the last 12 h of a 72 h culture was measured by scintillation counting.

2.7 Influenza Viral Titer

To determine the viral titer, bronchoalveolar lavage (BAL) fluid was collected from influenza-infected mice, and a plaque assay was performed as previously described by Lin et al. [115]. Briefly, the tracheas of euthanized mice were cannulated with an 18-gauge angiocath connected to a 1-ml syringe, and the lungs were flushed with a total of 2 ml of PBS. The lung tissue was then dissociated, washed with the same BAL fluid, centrifuged to remove cells, and the supernatant was collected. The viral titer was then determined by a standard plaque assay using Madin-Darby canine kidney (MDCK) cells (ATCC). BAL fluid was serially diluted using PBS with Ca^{2+} and Mg^{2+} and 0.1% BSA and then applied to a confluent monolayer of MDCK cells for 1 hr at 37°C. The diluted BAL fluid was removed and replaced with an overlay of MEME (Sigma) containing 1 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (Sigma), and 0.6% immunodiffusion agar (MP Biomedicals, Solon, OH). Plates were incubated for 3 days in a tissue culture incubator (37°C, 5% CO_2), and the resulting plaques were visualized with 1% crystal violet in 20% methanol. The total PFU for each lung were then calculated.

2.8 Kinetics of Endogenous HA-specific T Cells

Foxp3-GFP knock-in mice were infected with 4000 $TCID_{50}$ of influenza administered intranasally. At designated times following infection, the lungs were

harvested, and lung lymphocytes were isolated by digestion with collagenase as described below. Lung lymphocytes were stained with a MHC Class II tetramer (I-E^d, NIH Tetramer Core Facility, Atlanta, GA) presenting an HA epitope (^{110}SFERFEIFPKE^{120}) or, as a control, *Plasmodium chabaudi* merozoite surface protein (^{1157}ISVLKSRLLKRKKYI^{1171}) for 1 hr at 37°C. Lymphocytes were then stained with anti-PE MACS beads (Miltenyi Biotec) for 20 min on ice, and then tetramer-positive lymphocytes were selected using a MACS column. The tetramer-positive cells were then stained with APC-conjugated anti-CD4, PE-Cy5-conjugated anti-CD8 and anti-B220 (eBiosciences), and 7-aminoactinomycin D (7-AAD, Invitrogen). Cells were then analyzed by FACS. The number of viable selected cells was determined by trypan blue exclusion, and the total number of $CD4^+B220^-CD8^-$7-AAD^- HA-$Tetramer^+$ non-T_{Reg} ($Foxp3^-$) and T_{Reg} ($Foxp3^+$) cells was calculated.

2.9 Depletion of "Memory" T_{Reg} Cells Using Anti-Thy1.1 Antibody

The hybridoma for the depleting Thy1.1 mAb, (clone 19E12), was kindly provided by Dr. M. Bevan (University of Washington, Seattle, WA). The mAb was purified from hybridoma culture supernatant using a HiTrap™ Protein A column (Amersham Bioscience). For depletion, mice were injected i.v. with 200 µg anti-Thy1.1 depleting antibody two days prior to and on the day of antigen rechallenge.

2.10 Liver Histopathology and Immunohistochemistry

Liver tissues were harvested and prepared for paraffin and cryosections. Paraffin sections (5 µm) were stained with hematoxylin and eosin according to standard

procedures. Random sections were examined for histopathology in a blinded fashion using the criteria as we described previously for adenoviral hepatitis with minor modifications [116]. Each section was evaluated according to three independent criteria; within each criterion the severity of pathology was quantified based on a scale of 0 (no pathology) to 4 (severe pathology). The following three criteria were included: 1) periportal degeneration and focal necrosis; 2) intralobular degeneration and focal necrosis; and 3) periportal inflammation. Analyses were performed on three animals encompassing two sections for each animal.

Frozen sections (5 μm) were acetone-fixed and incubated in the presence of 10% Goat Serum (Vector Laboratories) to block non-specific binding. Sections were then stained with FITC- conjugated anti-CD4, followed by Alexa Fluor® 488-conjugated anti-FITC and Alexa Fluor® 546-conjugated H-36. Utilizing a Zeiss fluorescence microscope, the number of $CD4^+$ cells in each section was quantified by counting $CD4^+$ cells found in ten randomly selected fields of view.

2.11 Lung Histopathology and Immunohistochemistry

Lung tissue was perfused with Optimal Cutting Temperature Media (Tissue-Tek) according to standard procedures. Random sections were examined for histopathology in a blinded fashion. The severity of the pathology was evaluated according to two independent criteria on a scale of 0 (no pathology) to 4 (severe pathology). The two criteria included perivascular infiltration and peribronchial infiltration. For immunofluorescence, frozen sections were stained as above.

2.12 Isolation of Liver and Lung Lymphocytes

Lymphocytes were isolated from non-lymphoid tissues as described previously [117]. Briefly, liver tissue was homogenized and passed through a 70-µm-cell strainer. The single-cell suspension was resuspended in 10 ml of HBSS and centrifuged on a 5 ml Ficoll gradient (Amersham). Cells were harvested from the Ficoll gradient and washed twice with HBSS prior to analysis. For lung lymphocytes, lung tissue was digested with 50 U/ml collagenase (Sigma) for 1 hr at 37°C. Lung tissue was then homogenized and passed through a 70-µm-cell strainer. Red blood cells were lysed, and cells were washed with HBSS prior to analysis by FACS.

2.13 In Vivo Blocking of IL-10R, TGF- β, and CTLA-4

For *in vivo* blocking of IL-10R, TGF- β, and CTLA-4, mice were injected with blocking antibodies six hours before infection with VV-HA intranasally, as well as two days following antigen rechallenge. The following antibodies were used: 0.5 mg of anti-IL-10R (1B1.3A) mAb, which was purified from hybridoma cell culture supernatant (ATCC), 0.5 mg of anti-TGF-β (1D11) Ab (R&D Systems), and 0.25 mg of anti-CTLA-4 (UC10-4F10-11) Ab (BioXCell, Lebanon, NH), as well as proper IgG controls (rat, mouse, and hamster, respectively) all from Jackson ImmunoResearch Laboratories.

2.14 Real-time RT-PCR Analysis

Total RNA was isolated from purified cells using TRIzol reagent (Invitrogen Life Technologies), and cDNA was generated using a reverse transcription kit (Promega). Real-time PCR was performed using an iCycler (Bio-Rad) to measure SYBR green

incorporation. The following primer set was used for IL-10: 5'-CAACATACTGCTAACCGACTCCT-3' and 3'-TCACTCTTCACCTGCTCCAC-5'. Amounts of mRNA were normalized to β-actin RNA levels within each sample.

2.15 Statistical Analysis

Results were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test with 95% confidence bounds.

3. Chapter Three: The Development and Function of "Memory" Regulatory T cells after Acute Viral Infections

3.1 Introduction

Accumulating evidence has suggested an important role for T_{Reg} cells in the control of immune responses to pathogens [71, 72]. The overwhelming majority of these studies have focused on chronic infections where the presence of T_{Reg} cells controls effector immune responses affecting disease outcome in a variety of ways. By downmodulating the immune response, T_{Reg} cells can function to reduce immune-mediated pathology, as demonstrated in models of pulmonary inflammation caused by *Pneumocystis* pneumonia, inflammatory eye lesions with herpes simplex virus (HSV), and liver pathology in *Schistosoma mansoni* infection [69, 83, 90]. In addition to reducing immunopathology, the presence of T_{Reg} cells can reduce effector function and as a consequence, lead to pathogen persistence. In some cases, this can be detrimental to host survival such as infection with a lethal strain of *Plasmodium yoelii* where T_{Reg} cell deletion protects mice from death [81]. However, the presence of T_{Reg} cells can also be beneficial to the host by maintaining protective immunity. Specifically, in models of *Leishmania major* infection, Leishmania-specific T_{Reg} cells found at the site of infection prevent pathogen clearance, which is critical for the development of concomitant immunity [68, 74].

In contrast to chronic infections, how T_{Reg} cells behave and function during acute infections remains largely undefined. Here, we show that pathogen-specific T_{Reg} cells can be activated and expand upon acute infections with vaccinia virus (VV) or influenza virus

in vivo. Similar to non-T_{Reg} $CD4^+$ T cells, activated T_{Reg} cells undergo contraction to form a "memory" pool after resolution of the infection. These "memory" T_{Reg} cells can rapidly expand, secrete high levels of IL-10, and suppress the collateral tissue damage and inflammation elicited by recall expansion of non-T_{Reg} memory $CD4^+$ T cells. These results suggest that the development of the "memory" T_{Reg} cell population following an acute infection may help prevent excessive immunopathology during a recall response. Furthermore, we provide evidence that the ability of "memory" T_{Reg} cells to suppress memory non-T_{Reg} $CD4^+$ T cells during a recall response is dependent on IL-10.

3.2 Results

3.2.1 Pathogen-specific T_{Reg} Cells Are Activated and Expand upon VV Infection

To study the behavior of pathogen-specific T_{Reg} cells in response to an acute infection *in vivo*, we utilized a model of influenza hemagglutinin (HA)-specific T_{Reg} cells in response to infection with recombinant VV encoding HA (VV-HA). HA-specific T_{Reg} cells were derived from 6.5 HA-TCR transgenic mice ($Thy1.1^+$) that express a TCR recognizing an I-E^d-restricted HA epitope (^{110}SFERFEIFPKE^{120}) [18, 19, 108, 118]. We first characterized the activation and expansion of HA-specific T_{Reg} cells following VV-HA infection. HA-specific T_{Reg} cells ($6.5^+CD4^+CD25^+$) were purified from 6.5 HA-TCR transgenic mice by FACS sorting. To ensure purity of the HA-specific T_{Reg} cells, we analyzed Foxp3 expression in our post-sort populations. The sorted HA-specific T_{Reg} cell population was consistently >85% $Foxp3^+$ (data not shown).

1 x 10^5 Thy1.1$^+$, purified HA-specific T_{Reg} or non-T_{Reg} cells (6.5$^+$CD4$^+$CD25$^-$) were transferred into congenic B10.D2 mice (Thy1.2$^+$). Mice were subsequently infected with VV-HA (2 x 10^6 PFU) intraperitoneally or left uninfected as a control. 7 days after infection, splenocytes were analyzed for clonal expansion of the HA-specific T_{Reg} (Thy1.1$^+$CD4$^+$Foxp3$^+$) or non-T_{Reg} (Thy1.1$^+$CD4$^+$Foxp3$^-$) cell populations. 0.47% of total lymphocytes were clonotypic T_{Reg} cells (Figure 1A, B), which represented ~50- fold expansion compared with that of the control mice (Figure 1A). This expansion was less than that of the clonotypic non-T_{Reg} cells, which represented 2.9% of the total lymphocytes (~290- fold expansion) (Figure 1A, B). Despite expansion, very few clonotypic T_{Reg} cells produced IFN-γ, compared to the 24% of the non-T_{Reg} cell population that were IFN-γ$^+$ (Figure 1B). On the other hand, no Foxp3$^+$ cells were detected in the non-T_{Reg} cell population (Figure 1B). Similar degrees of expansion for both T_{Reg} and non-T_{Reg} cell populations were also found in other lymphoid tissues including mesenteric and peripheral lymph nodes (data not shown).

To further confirm that Foxp3$^+$ T_{Reg} cells are capable of expanding upon VV infection *in vivo*, we intercrossed HA-TCR transgenic mice with Foxp3-GFP knock-in mice, and similar results were obtained when 6.5$^+$CD4$^+$Foxp3-GFP$^+$ T_{Reg} cells were used for adoptive transfer experiments followed by infection with VV-HA (Figure 2).

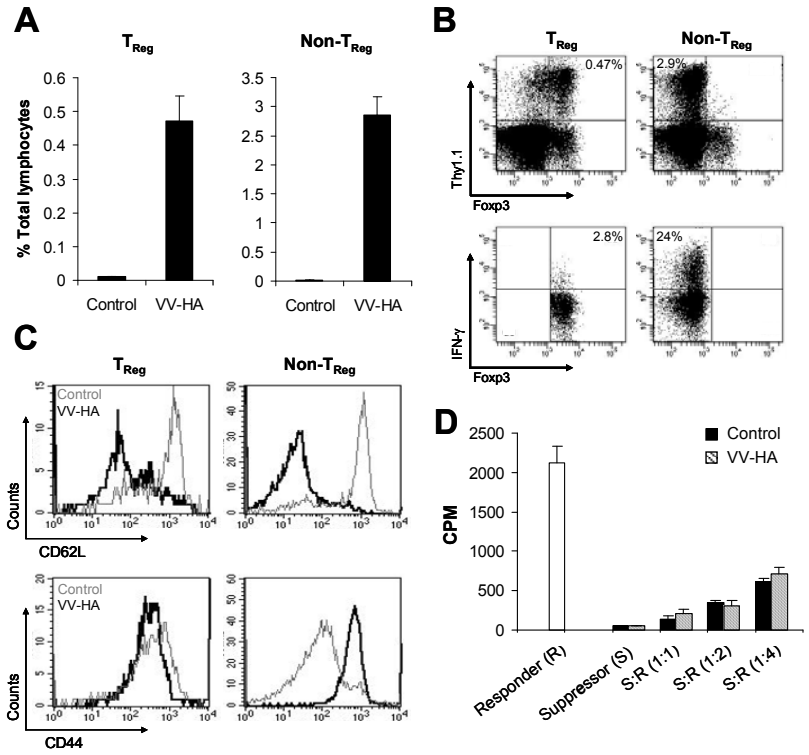

Figure 1: Activation and Expansion of Pathogen-specific T_{Reg} Cells upon an Acute Viral Infection *in Vivo*.

Purified naïve HA-specific T_{Reg} or non-T_{Reg} cells (Thy1.1$^+$) were transferred into B10.D2 mice (Thy1.2$^+$), which were subsequently infected with VV-HA or left uninfected as a control. 7 days after infection, mice were harvested for the following analyses. (A) Splenocytes were stained with anti-CD4, anti-Thy1.1, and anti-Foxp3, and the mean percentage of T_{Reg} (CD4$^+$Thy1.1$^+$Foxp3$^+$) or non-T_{Reg} cells (CD4$^+$Thy1.1$^+$Foxp3$^-$) among total lymphocytes was plotted with the standard deviations included. (B) Splenocytes were stained with anti-CD4, anti-Thy1.1, anti-Foxp3, and anti-IFN-γ. The quadrant percentage indicated is among total lymphocytes. (C) Splenocytes were stained with anti-CD4, anti-Thy1.1, anti-Foxp3, and anti-CD62L or anti-CD44 and subjected to FACS analyses. (D) 7 days after infection, activated HA-specific T_{Reg} cells were purified by FACS sorting. In addition, naïve T_{Reg} cells were also sorted from naïve 6.5 HA-TCR transgenic mice as the control. The activated (VV-HA) or naïve (Control) HA-specific T_{Reg} cells (Suppressor) were then mixed with naïve HA-specific non-T_{Reg} CD4$^+$ T cells (Responder) at the indicated ratios of suppressors (S) to responders (R) and cultured in the presence of irradiated APC pulsed with 10μg/ml of the I-E^d-HA peptide. Cultures were labeled with [3H]thymidine and harvested for scintillation counting. Results are expressed as the mean CPM ± SD. Results are representative of three independent experiments.

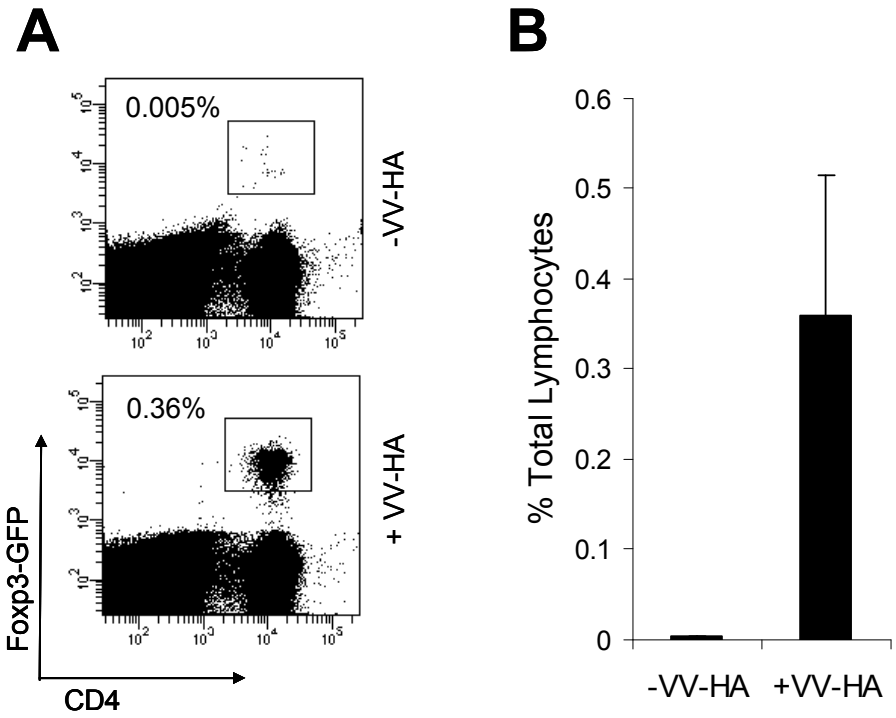

Figure 2: Expansion of Pathogen-specific Foxp3-GFP$^+$ T_{Reg} Cells upon Viral Infection *in Vivo*.

Purified Foxp3-GFP$^+$ TCR-HA$^+$ T_{Reg} cells were transferred into BALB/c mice, which were subsequently infected intraperitoneally with VV-HA (+VV-HA) or left uninfected (-VV-HA) as a control. 7 days after infection, splenocytes were harvested and stained with anti-CD4. (A) The percentage of Foxp3-GFP$^+$ donor T_{Reg} cells among CD4$^+$ T cells is shown. (B) The mean percentage of T_{Reg} cells among CD4$^+$ T cells with the standard deviations is indicated. Results are representative of three independent experiments.

We next analyzed the phenotype of clonotypic T_{Reg} cells after VV-HA infection. Similar to HA-specific non-T_{Reg}, HA-specific T_{Reg} cells downregulated CD62L compared to naïve controls (Figure 1C). However, unlike non-T_{Reg} $CD4^+$ T cells, which upregulated CD44 upon infection (Figure 1C), HA-specific T_{Reg} cells expressed high levels of CD44 expression even before VV-HA infection, and no obvious changes were observed after infection (Figure 1C). This is consistent with the notion that T_{Reg} cells are antigen-experienced as their selection in the thymus requires intermediate to high affinity TCR:peptide ligand interactions [19, 119]. Collectively, these results show that pathogen-specific T_{Reg} cells can be activated and expand following an acute viral infection. To determine if activated T_{Reg} cells maintain suppressive function, we purified activated T_{Reg} cells seven days after VV-HA infection by FACS sorting and then compared their suppressive capacity to that of naïve T_{Reg} cells purified from 6.5 HA-TCR transgenic mice using an *in vitro* suppression assay. Indeed, we found that similar to naïve T_{Reg} cells, the activated T_{Reg} cells suppressed the proliferation of naïve HA-specific non-T_{Reg} $CD4^+$ T cells in response to HA peptide-pulsed irradiated naïve splenocytes (Figure 1D), suggesting that despite activation and expansion, these activated T_{Reg} cells retain suppressive function.

3.2.2 Activated T_{Reg} Cells Undergo Contraction to Form a "Memory" Population

We next examined the fate of the activated T_{Reg} cells following the peak of clonal expansion at day 7. Again, purified HA-specific T_{Reg} or non-T_{Reg} cells were transferred into congenic B10.D2 mice followed by infection with VV-HA. At different days after

infection, splenocytes were analyzed for the percentages of clonotypic T_{Reg} or non-T_{Reg} cell populations. The majority of the activated pathogen-specific T_{Reg} cells underwent contraction after resolution of the infection, and only a small fraction (~5%) survived the contraction to form a stable "memory" population by day 28, which persisted through day 50 (Figure 3A). This was similar to the formation of non-T_{Reg} memory $CD4^+$ T cells *in vivo* (Figure 3A). Based on homing characteristics and effector functions, at least two subsets of memory T cells have been described for both $CD4^+$ and $CD8^+$ T cells [120]: Central memory cells (T_{CM}) that express CD62L and effector memory cells (T_{EM}) that lack CD62L. Indeed, non-T_{Reg} memory $CD4^+$ T cells displayed both the $CD62L^{lo}$ (T_{EM}) and $CD62L^{hi}$ (T_{CM}) populations. However, the "memory" T_{Reg} cells were almost entirely $CD62L^{lo}$ corresponding to a T_{EM} phenotype (Figure 3B).

One central hallmark of memory T cells is rapid recall expansion upon a secondary antigen challenge [121]. To determine whether the "memory" T_{Reg} cells also possessed this salient memory quality, mice that had received clonotypic T_{Reg} cells and were infected with VV-HA 50 days before, were rechallenged with recombinant adenovirus encoding HA (Ad-HA) intravenously. 4 days after rechallenge, "memory" T_{Reg} cells rapidly expanded (~10- fold expansion compared with that of non-boosted mice; Figure 3C, D). This fold expansion was similar to that of the non-T_{Reg} memory $CD4^+$ T cells (Figure 3C, D). Thus, these pathogen-specific "memory" T_{Reg} cells can also rapidly expand upon a secondary challenge. Taken together, these results suggest that a stable population of T_{Reg} cells with memory characteristics can develop after resolution of the infection.

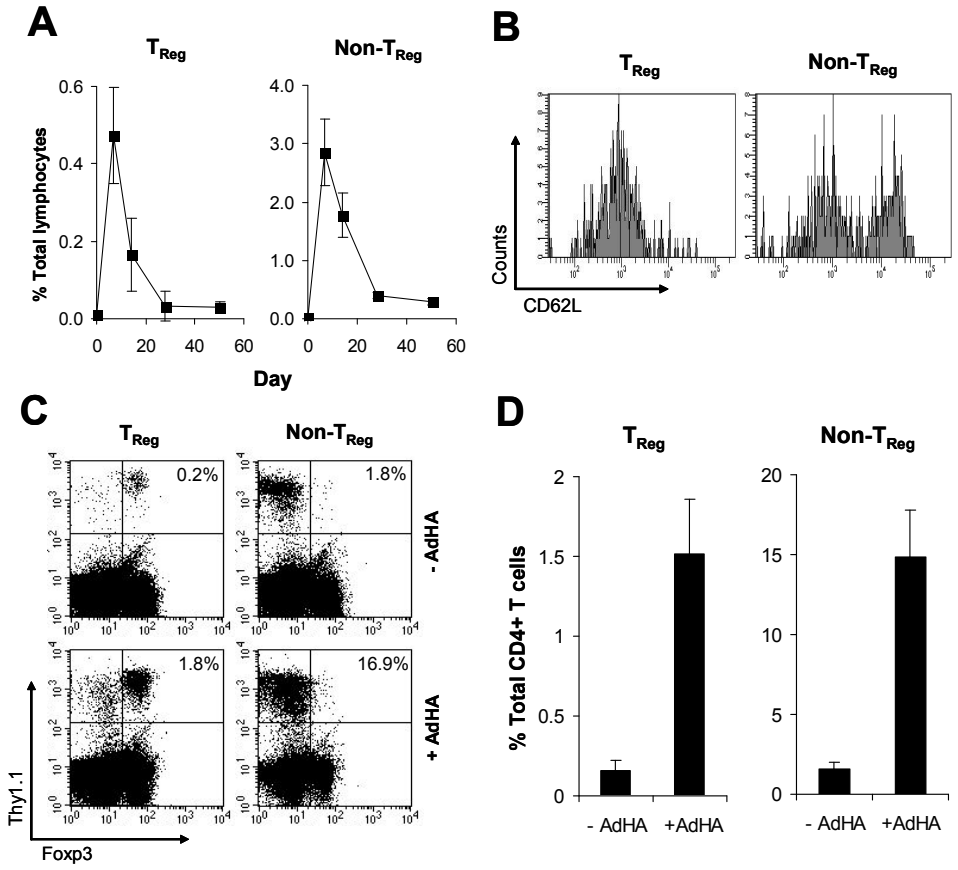

Figure 3: Activated Pathogen-specific T_{Reg} Cells Undergo Contraction to Form a "Memory" Population after Resolution of Infection.

Purified naïve HA-specific T_{Reg} or non-T_{Reg} cells (Thy1.1$^+$) were transferred into B10.D2 mice (Thy1.2$^+$), which were subsequently infected with VV-HA or left uninfected as a control. (A) 7, 14, 28, and 50 days after infection, splenocytes were stained with anti-CD4, anti-Thy1.1, and anti-Foxp3, and the mean percentage of T_{Reg} (CD4$^+$Thy1.1$^+$Foxp3$^+$) or non-T_{Reg} cells (CD4$^+$Thy1.1$^+$Foxp3$^-$) among total lymphocytes was plotted with the standard deviations included. (B) 50 days after infection, splenocytes were stained with anti-CD62L, anti-CD4, anti-Thy1.1, and anti-Foxp3. Events were gated on CD4$^+$Thy1.1$^+$Foxp3$^+$ (T_{Reg}) or CD4$^+$Thy1.1$^+$Foxp3$^-$ (non-T_{Reg}). (C-D) 50 days after infection, mice were rechallenged with Ad-HA (+AdHA) or left uninfected as a control (-AdHA). 4 days after rechallenge, splenocytes were stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The percentage of CD4$^+$Thy1.1$^+$Foxp3$^+$ (T_{Reg}) or CD4$^+$Thy1.1$^+$Foxp3$^-$ (non-T_{Reg}) among total CD4$^+$ T cells is indicated (C). The mean percentage of T_{Reg} and non-T_{Reg} cells within the CD4$^+$ T cell gate was plotted with the standard deviations included (D). Results are representative of three independent experiments.

3.2.3 "Memory" T_{Reg} Cells Suppress Non-T_{Reg} $CD4^+$ T Cell Expansion upon Secondary Challenges

We next investigated the role of pathogen-specific T_{Reg} cells during the primary and recall responses to an acute infection. We co-transferred $Thy1.1^+$, HA-specific T_{Reg} (1×10^5) with non-T_{Reg} cells (1×10^5), or HA-specific non-T_{Reg} cells only (1×10^5) into congenic B10.D2 mice ($Thy1.2^+$), followed by infection with VV-HA. 7 days after infection, we found that despite the fact that the initial transfer was at a 1:1 ratio (non-T_{Reg}: T_{Reg}), clonotypic T_{Reg} cells represented only about 10% of the donor ($Thy1.1^+$) population in mice that had received both T_{Reg} and non-T_{Reg} cells (data not shown). This is most likely due to the differences in expansion observed between clonotypic T_{Reg} and non-T_{Reg} cells in response to VV-HA infection (Figure 1). We observed no significant difference ($p = 0.132$) in the expansion of non-T_{Reg} $CD4^+$ T cells with or without the presence of clonotypic T_{Reg} cells (13.2% and 15.9% of $CD4^+$ T cells, respectively; Figure 4A, B). Additionally, there was no significant difference in the percentage of IFN-γ^+ cells among clonotypic non-T_{Reg} cells with or without HA-specific T_{Reg} cells (data not shown).

Similarly, T_{Reg} cells did not significantly ($p = 0.174$) suppress the expansion of non-T_{Reg} cells when primed with Ad-HA (Figure 5). Furthermore, addition of twice as many T_{Reg} cells did not significantly ($p = 0.099$) reduce the expansion of non-T_{Reg} cells upon VV-HA infection (Figure 6).

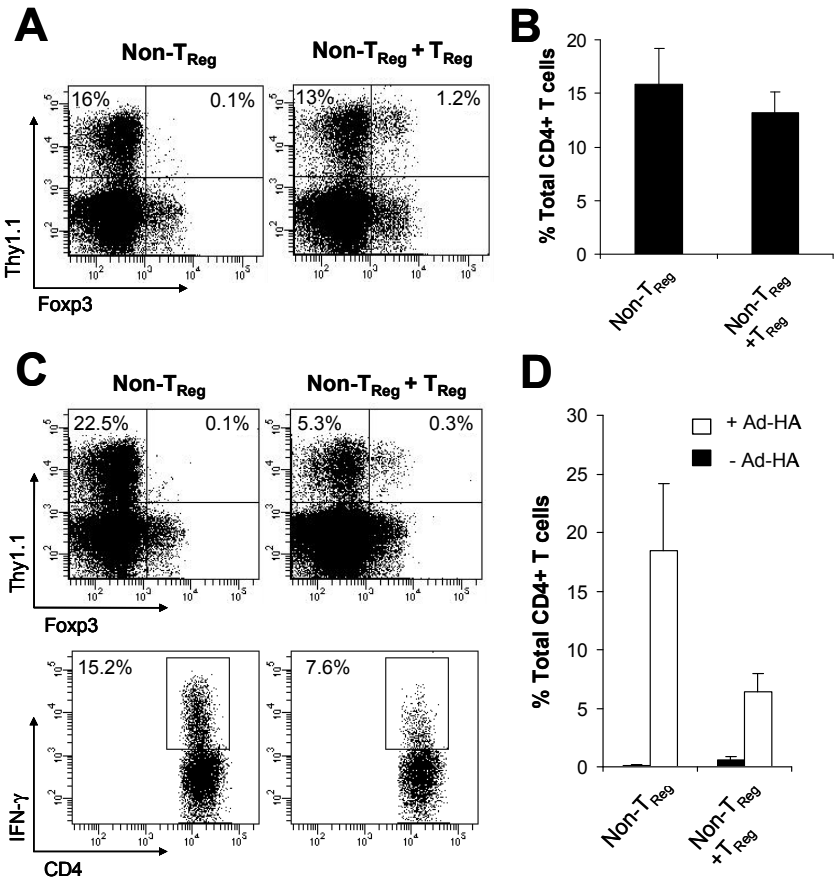

Figure 4: "Memory" T_{Reg} Cells Suppress the Expansion of Non-T_{Reg} Memory $CD4^+$ T Cells in Mice Primed with VV-HA and Rechallenged with Ad-HA.

Purified naïve HA-specific non-T_{Reg} cells with (T_{Reg} + Non-T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected with VV-HA. (A-B) 7 days after infection, splenocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The percentages of $CD4^+Thy1.1^+Foxp3^+$ and $CD4^+Thy1.1^+Foxp3^-$ within the $CD4^+$ T cell gate are indicated (A). The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate was plotted with the standard deviations included (B). (C-D) 50 days after infection, mice were rechallenged with Ad-HA (+AdHA) or left uninfected as a control (-AdHA). 4 days after rechallenge, splenocytes were harvested and analyzed for expansion and function. The percentages of $CD4^+Thy1.1^+Foxp3^+$ and $CD4^+Thy1.1^+Foxp3^-$ within the $CD4^+$ T cell gate are indicated (top panels). The percentage of IFN-γ^+ non-T_{Reg} cells among the $CD4^+Thy1.1^+Foxp3^-$ cells is indicated (bottom panels) (C). The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate was plotted with the standard deviations included (D). Results are representative of three independent experiments.

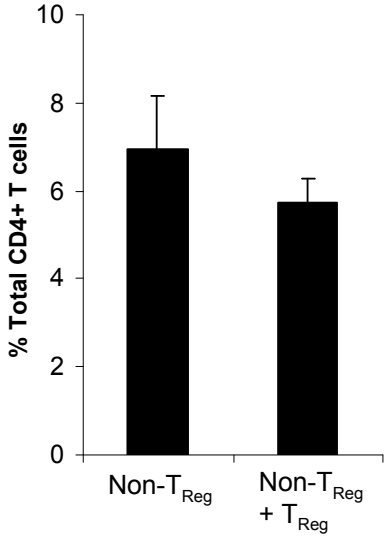

Figure 5: T_{Reg} Cells Do Not Suppress the Expansion of Non-T_{Reg} Cells Following Priming with Ad-HA.

Purified naïve HA-specific non-T_{Reg} cells with (Non-T_{Reg}+ T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected with Ad-HA. 7 days after infection, splenocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate is plotted with the standard deviations included.

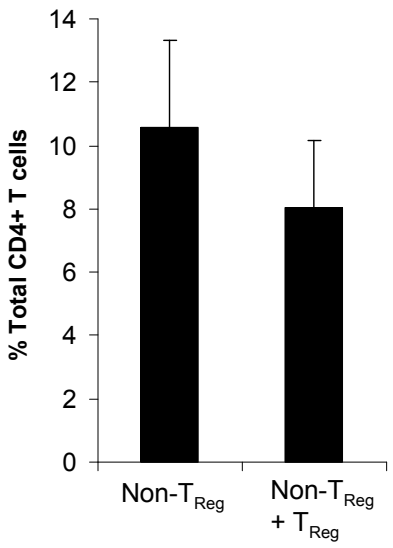

Figure 6: Addition of More T_{Reg} Cells Does Not Significantly Reduce the Expansion of Non-T_{Reg} Cells Following Priming with VV-HA.

1 x 10^5 purified naïve HA-specific non-T_{Reg} cells with (Non-T_{Reg} + T_{Reg}) or without 2 x 10^5 T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were subsequently infected with VV-HA. 7 days after infection, splenocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate is plotted with the standard deviations included.

Collectively, these results indicate that the presence of activated, pathogen-specific T_{Reg} cells does not affect the expansion and effector differentiation of non-T_{Reg} $CD4^+$ T cells during the primary response to VV-HA or Ad-HA.

We then evaluated whether "memory" T_{Reg} cells could suppress the recall response of non-T_{Reg} memory $CD4^+$ T cells. 50 days after infection with VV-HA, the above mice that had received either T_{Reg} cells with non-T_{Reg} cells, or non-T_{Reg} cells only, were rechallenged with Ad-HA. 4 days after boost infection, mice were analyzed for the expansion of non-T_{Reg} $CD4^+$ T cells. Massive recall expansion of non-T_{Reg} memory $CD4^+$ T cells was detected in the absence of "memory" T_{Reg} cells (Figure 4C, D). In contrast, the extent of recall expansion of non-T_{Reg} $CD4^+$ T cells was significantly ($p < 0.001$) reduced in the presence of "memory" T_{Reg} cells (Figure 4C, D). In addition, there was a reduction in the percentage of IFN-γ^+ cells among non-T_{Reg} $CD4^+$ T cells in the presence of "memory" T_{Reg} cells (Figure 4C). The differences in expansion were not due to differential contraction of non-T_{Reg} cells in the presence of T_{Reg} cells, as the percentage of non-T_{Reg} memory $CD4^+$ T cells was similar between both groups prior to antigen rechallenge (Figure 4D). Thus, "memory" T_{Reg} cells suppress the expansion and effector function of non-T_{Reg} memory $CD4^+$ T cells during an antigen recall response.

We next tested whether the observed function of "memory" T_{Reg} cells could be confirmed in a different model of acute viral infection. Here, we utilized a model of influenza viral infection that induces lung pathology. Again, we co-transferred $Thy1.1^+$, HA-specific T_{Reg} cells (1×10^5) with non-T_{Reg} cells (1×10^5) or HA-specific non-T_{Reg} only cells (1×10^5) into congenic B10.D2 mice ($Thy1.2^+$), followed by infection with

influenza virus (4000 $TCID_{50}$) intranasally. 7 days later, we evaluated the spleen (data not shown) and draining hilar lymph nodes for clonal expansion of HA-specific non-T_{Reg} and T_{Reg} cells. We observed no significant ($p = 0.375$) difference in the primary expansion of non-T_{Reg} $CD4^+$ T cells with or without the presence of clonotypic T_{Reg} cells (Figure 7A, B). In addition, there was no significant difference in the percentage of IFN-γ^+ cells among clonotypic non-T_{Reg} cells with or without HA-specific T_{Reg} cells (data not shown). Furthermore, the presence of T_{Reg} cells did not affect the expansion of non-T_{Reg} cells (Figure 8A, $p = 0.658$) or the viral load (Figure 8B, $p = 0.919$) in the lung during the primary response to influenza virus. Thus, similar to VV-HA or Ad-HA infection, the presence of activated, pathogen-specific T_{Reg} cells does not affect the expansion or effector differentiation of non-T_{Reg} $CD4^+$ T cells during the primary response to influenza virus.

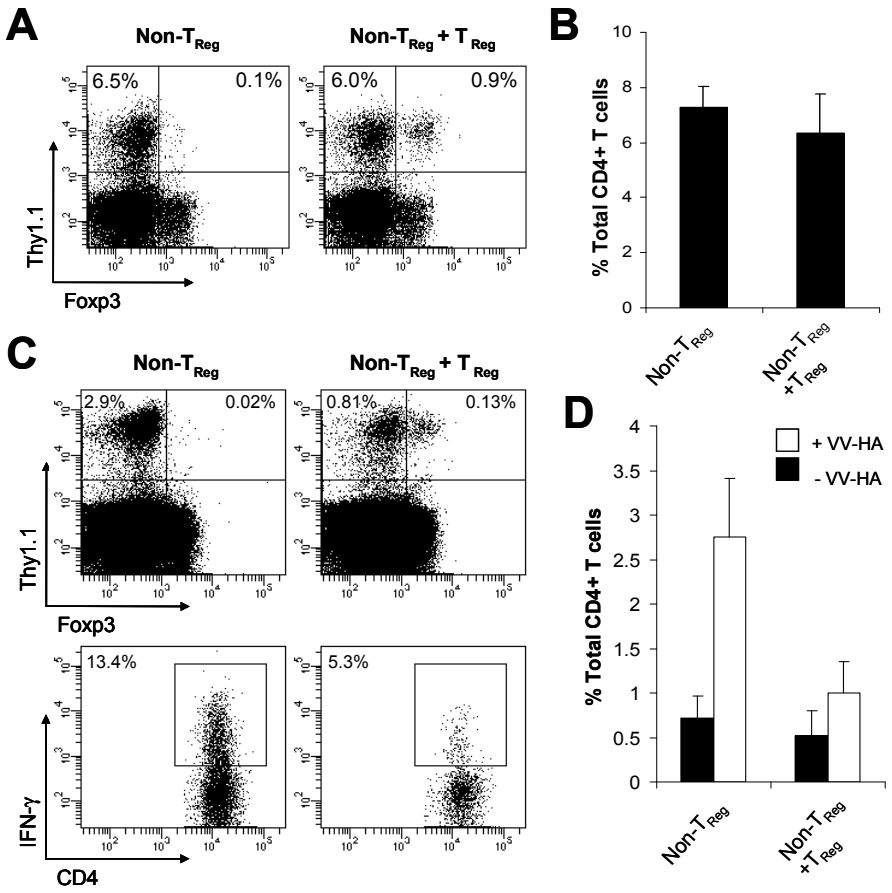

Figure 7: "Memory" T_{Reg} Cells Suppress the Expansion of Non-T_{Reg} Memory $CD4^+$ T Cells in Mice Primed with Influenza Virus and Rechallenged with VV-HA.

Purified naïve HA-specific non-T_{Reg} cells with (T_{Reg} + Non-T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected with influenza virus. (A-B) 7 days after infection, draining hilar lymphocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The percentages of $CD4^+Thy1.1^+Foxp3^+$ and $CD4^+Thy1.1^+Foxp3^-$ within the $CD4^+$ T cell gate are indicated (A). The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate was plotted with the standard deviations included (B). (C-D) 50 days after infection, mice were rechallenged with VV-HA (+VV-HA) or left uninfected as a control (-VV-HA). 5 days after rechallenge, draining hilar lymphocytes were harvested and analyzed for expansion and function. The percentages of $CD4^+Thy1.1^+Foxp3^+$ and $CD4^+Thy1.1^+Foxp3^-$ within the $CD4^+$ T cell gate are indicated (top panels), and the percentage of IFN-γ^+ non-T_{Reg} cells among $CD4^+Thy1.1^+Foxp3^-$ are indicated (bottom panels) (C). The mean percentage of non-T_{Reg} cells before or after VV-HA challenge within the $CD4^+$ T cell gate was plotted with the standard deviations included (D). Results are representative of three independent experiments.

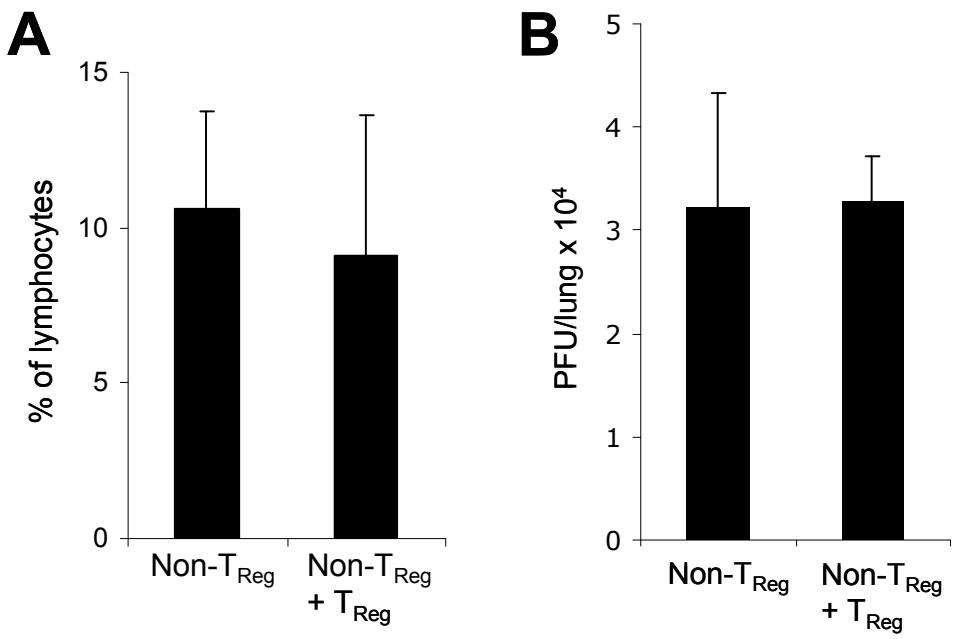

Figure 8: The Presence of T_{Reg} Cells Does Not Affect the Expansion of Non-T_{Reg} Cells or the Viral Load in the Lung during the Primary Response to Influenza Virus.

Purified naïve HA-specific non-T_{Reg} cells with (Non-T_{Reg}+ T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were then infected with influenza (4000 $TCID_{50}$) intranasally. (A) 7 days after infection, lung lymphocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The mean percentages of $CD4^{+}Thy1.1^{+}Foxp3^{-}$ cells among total lymphocytes are indicated. (B) 7 days after infection, bronchoalveolar lavage fluid was collected, and viral titers were determined by measuring the number of PFU in the lung of each mouse. The mean PFU/lung is shown. Results are representative of three independent experiments.

The influenza viral infection model also provided us with a physiologic setting to evaluate the formation of virus-specific "memory" T_{Reg} cells since HA is an immunodominant antigen of the influenza virus, which may induce high enough numbers of endogenous HA-specific T_{Reg} cells for us to detect using an MHC class II, HA-specific tetramer. To test this possibility, Foxp3-GFP knock-in mice were infected with 4000 $TCID_{50}$ of influenza virus intranasally. 7 days after infection, lung lymphocytes were analyzed for the presence of endogenous HA-specific T_{Reg} cells using an MHC Class II, HA-specific tetramer. Indeed, significant expansion of endogenous HA-specific $CD4^+$ T cells, of which about 8% are $Foxp3^+$ T_{Reg} cells, was detected in mice infected with influenza virus compared to the uninfected naïve controls (Figure 9A). No significant $tetramer^+$ cells were detected when an irrelevant tetramer was used for staining (Figure 9A), which further confirms the specificity of the HA-specific tetramer. Similar to non-T_{Reg} $CD4^+$ T cells, the majority of these virus-specific T_{Reg} cells underwent contraction to form a stable "memory" pool by day 21 (Figure 9B). These results indicate that similar to transgenic T cells, endogenous virus-specific T_{Reg} cells can expand and contract to form a stable "memory" population in response to an acute viral infection.

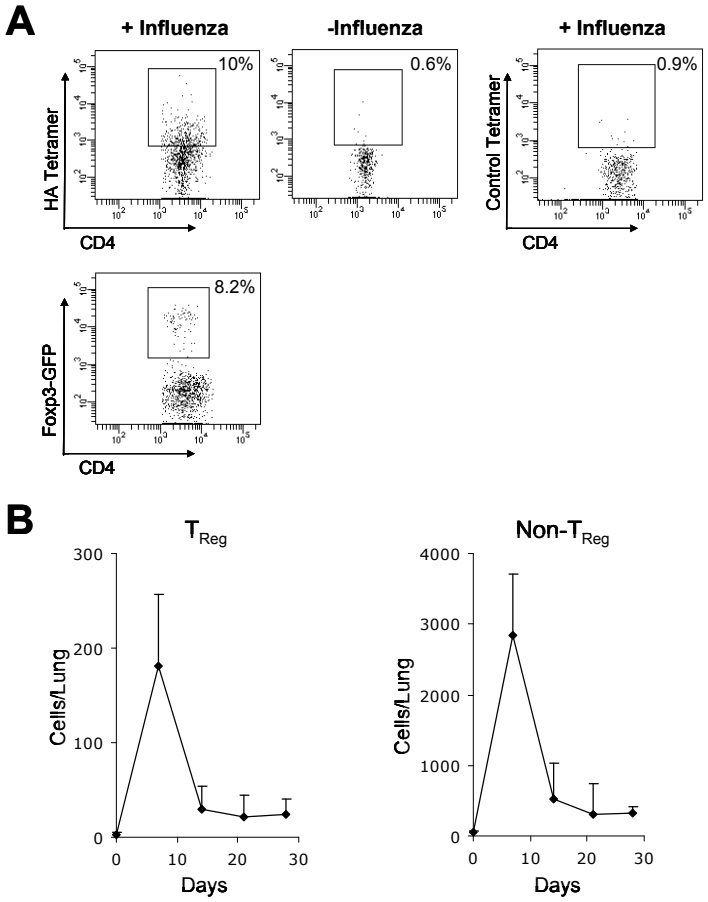

Figure 9: Endogenous Virus-specific T_{Reg} Cells Expand and Contract to Form a Stable Pool Following Influenza Infection.

Foxp3-GFP mice were infected with influenza (4000 $TCID_{50}$) intranasally, and the expansion of virus-specific non-T_{Reg} and T_{Reg} cells in the lungs was measured using an influenza HA-specific MHC Class II tetramer. At designated time points following infection, lungs were digested, and HA-specific endogenous $CD4^+$ T cells were stained with PE-conjugated HA-specific Class II tetramer, followed by MACS selection with anti-PE magnetic beads. Selected HA-specific T cells were stained with anti-CD8, anti-B220, anti-CD4, and 7-AAD for FACS analysis. (A) Lungs from naïve (-Influenza) or D7-infected (+Influenza) mice were stained with HA-tetramer or an irrelevant control tetramer and analyzed for the percentage of $tetramer^+$ cells among $CD4^+B220^-CD8^-$7-AAD^- cells (upper panel), and the percentage of Foxp3-GFP^+ T_{Reg} cells among HA-$tetramer^+$ CD4 T cells (lower panel). (B) The absolute number of endogenous HA-specific T_{Reg} (left) and non-T_{Reg} cells (right) per lung were quantified at 7, 14, 21, and 28 days following influenza infection. Results are representative of three independent experiments.

We next evaluated whether "memory" T_{Reg} cells formed following influenza priming would suppress activation of memory non-T_{Reg} cells after antigen rechallenge with VV-HA. 50 days after infection with influenza, the above mice that had received either T_{Reg} cells with non-T_{Reg} cells, or non-T_{Reg} cells only, were rechallenged with VV-HA (5×10^5 PFU) intranasally. 5 days after boost infection, mice were analyzed for the expansion of non-T_{Reg} $CD4^+$ T cells. The recall expansion of non-T_{Reg} memory cells was significantly ($p < 0.001$) reduced in the presence of "memory" T_{Reg} cells (Figure 7C, D). We also observed a significant reduction in IFN-γ^+ cells among clonotypic non-T_{Reg} cells in the presence of "memory" T_{Reg} cells (Figure 7C). Furthermore, this suppression of clonotypic non-T_{Reg} cell expansion was abrogated when mice were depleted of clonotypic "memory" T_{Reg} (Figure 10), confirming that the clonotypic "memory" T_{Reg} cells were responsible for the reduced expansion of clonotypic non-T_{Reg} $CD4^+$ T cells. Taken together, the results from two different models of acute viral infection support our conclusion that "memory" T_{Reg} cells suppress the expansion and function of non-T_{Reg} $CD4^+$ T cells during a recall response.

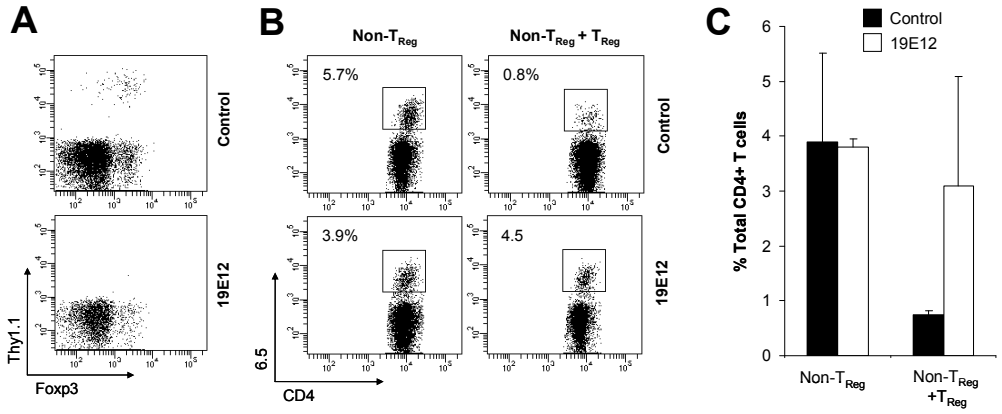

Figure 10: Clonotypic "Memory" T_{Reg} Cells Are Responsible for the Suppression on Clonotypic Non-T_{Reg} Cells during the Recall Response.

Purified naïve HA-specific non-T_{Reg} cells (Thy1.2$^+$) with (T_{Reg} + Non-T_{Reg}) or without Thy1.1$^+$ T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected intranasally with influenza (4000 $TCID_{50}$). 50 days after infection, mice were rechallenged with VV-HA intranasally. Two days prior to and on the day of the rechallenge, mice were treated with a Thy1.1-depleting antibody (19E12) or mouse IgG control antibody (Control) intravenously. 5 days following VV-HA rechallenge, lymphocytes from the draining hilar lymph nodes were harvested and analyzed by FACS. (A) Lymphocytes were stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The depletion of Thy1.1$^+$ "memory" T_{Reg} cells by the Thy1.1-depleting antibody is shown. (B-C) Lymphocytes were stained with anti-CD4 and anti-6.5. The percentages of 6.5$^+$ non-T_{Reg} cells among CD4$^+$ T cells are shown (B). The mean percentages of 6.5$^+$ non-T_{Reg} cells within the CD4$^+$ T cell gate were plotted with the standard deviations included. Results are representative of three independent experiments.

3.2.4 "Memory" T_{Reg} Cells Control the Extent of Immunopathology during Recall Responses

What then is the biological significance of "memory" T_{Reg} cell-mediated suppression on non-T_{Reg} $CD4^+$ T cells during a recall response *in vivo*? Because of the vigorous expansion of non-T_{Reg} $CD4^+$ T cells that occurred during a recall response, we hypothesized that "memory" T_{Reg} cell-mediated suppression may prevent the collateral damage that could result from such a robust response. To test this hypothesis, we examined immunopathology in mice with or without "memory" T_{Reg} cells. For the first model, we selected liver tissues to examine for histopathology because studies have shown that the liver is the primary target organ for adenoviral vectors [122]. Again, we co-transferred T_{Reg} cells with non-T_{Reg} cells, or non-T_{Reg} cells alone into B10.D2 mice, followed by VV-HA infection. 50 days after infection, mice were challenged with Ad-HA. 4 days after rechallenge, liver tissues were evaluated for histopathology. We found widespread severe immunopathology and tissue damage characterized by hepatocellular degeneration, focal necrosis, and periportal infiltration in mice that received only non-T_{Reg} $CD4^+$ T cells (Figure 11A, B). Immunohistochemical staining revealed that the portal inflammation mainly consisted of infiltrating $CD4^+$ T cells (Figure 11A, C). By contrast, overall immunopathology was diminished with a significant ($p < 0.001$) reduction of periportal infiltrating $CD4^+$ T cells in the presence of "memory" T_{Reg} cells (Figure 11).

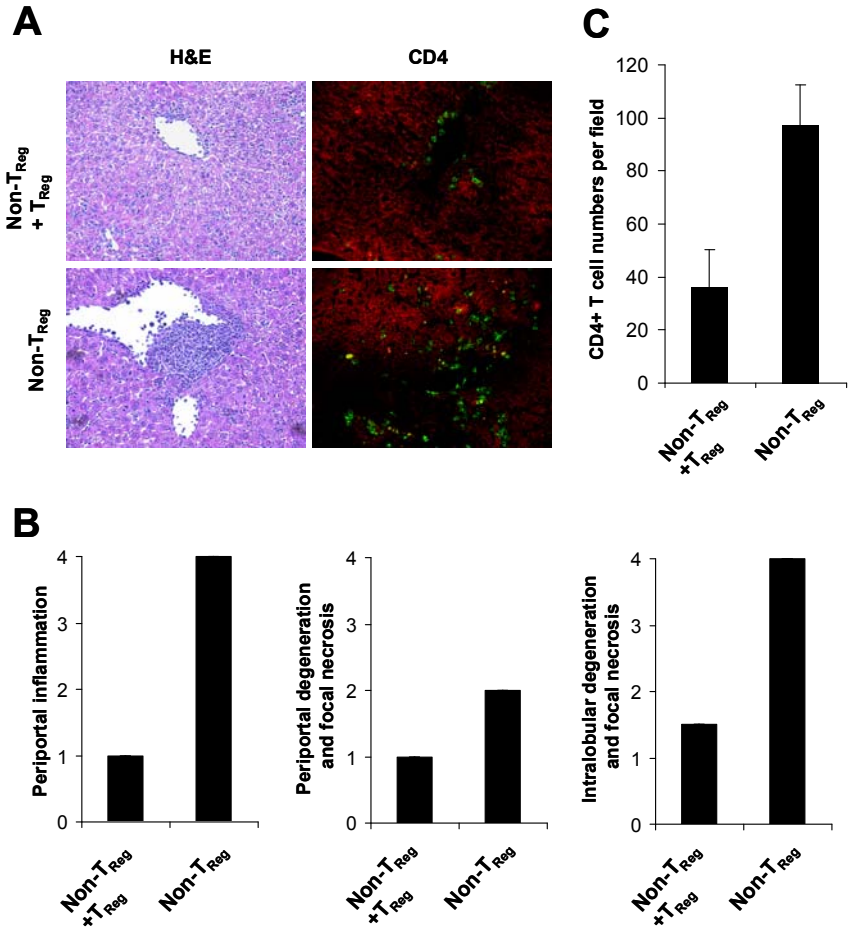

Figure 11: "Memory" T_{Reg} Cells Control the Extent of Liver Immunopathology during a Recall Response.

Purified naïve HA-specific non-T_{Reg} cells with (T_{Reg} + Non-T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected with VV-HA. 50 days after infection, mice were rechallenged with Ad-HA, and 4 days after rechallenge, liver tissues were harvested and examined for histopathology and infiltrating lymphocytes. (A) Paraffin sections were stained with H & E (left panel) and cryosections were stained with anti-CD4 (Green) and anti-HA (Red) by immunofluorescence. (B) Paraffin sections were stained with H & E and evaluated for evidence of pathological changes by light microscopic examination. Sections were characterized with respect to periportal inflammation, periportal degeneration and focal necrosis, and intralobular degeneration and focal necrosis. Extent of pathology was scored from 0 (no pathology) to 4 (severe pathology). (C) The mean numbers of $CD4^+$ T cells/ field of view were plotted with the standard deviations included. Results are representative of three independent experiments.

The recall response was accompanied by a significant accumulation of "memory" T_{Reg} cells in the liver (Figure 12). These results suggest that "memory" T_{Reg} cell-mediated suppression prevents the overwhelming immunopathology that can occur during a robust recall response. However, the expression of HA in the liver was similar in mice with or without "memory" T_{Reg} cells at day 4 post-rechallenge (Figure 11A), which became undetectable by day 10 post-rechallenge (data not shown), suggesting the presence of "memory" T_{Reg} cells, did not appear to affect the clearance of HA in the liver.

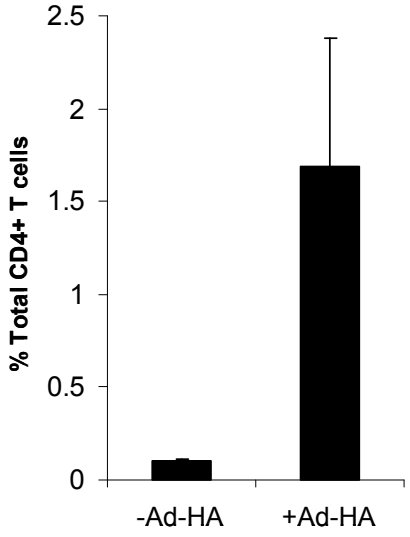

Figure 12: Accumulation of "Memory" T_{Reg} Cells at the Site of Infection Following Antigen Rechallenge.

Purified naïve HA-specific non-T_{Reg} and T_{Reg} cells were transferred into B10.D2 mice, which were infected with VV-HA intraperitoneally. 50 days after infection, mice were rechallenged with Ad-HA (+Ad-HA) or left uninfected as a control (-Ad-HA). 4 days after rechallenge, lymphocytes were harvested from the liver and analyzed for HA-specific T_{Reg} cells. The mean percentage of T_{Reg} cells within the $CD4^+$ T cell gate was plotted with the standard deviations included.

We then assessed the immunopathology in the second model of infection with influenza virus. Again, we co-transferred T_{Reg} cells with non-T_{Reg} cells, or non-T_{Reg} cells alone into congenic B10.D2 mice, followed by influenza infection. 50 days after infection, mice were challenged with VV-HA. 5 days after rechallenge, lung tissues were evaluated for histopathology. We found severe immunopathology and tissue damage characterized by perivascular and peribronchial infiltration mainly consisted of infiltrating $CD4^+$ T cells in mice that received non-T_{Reg} cells alone (Figure 13A). By contrast, overall immunopathology was diminished with a reduction of perivascular and peribronchial infiltration in the presence of "memory" T_{Reg} cells (Figure 13). Collectively, these results suggest that "memory" T_{Reg} cell-mediated suppression prevents the overwhelming immunopathology during a robust recall response.

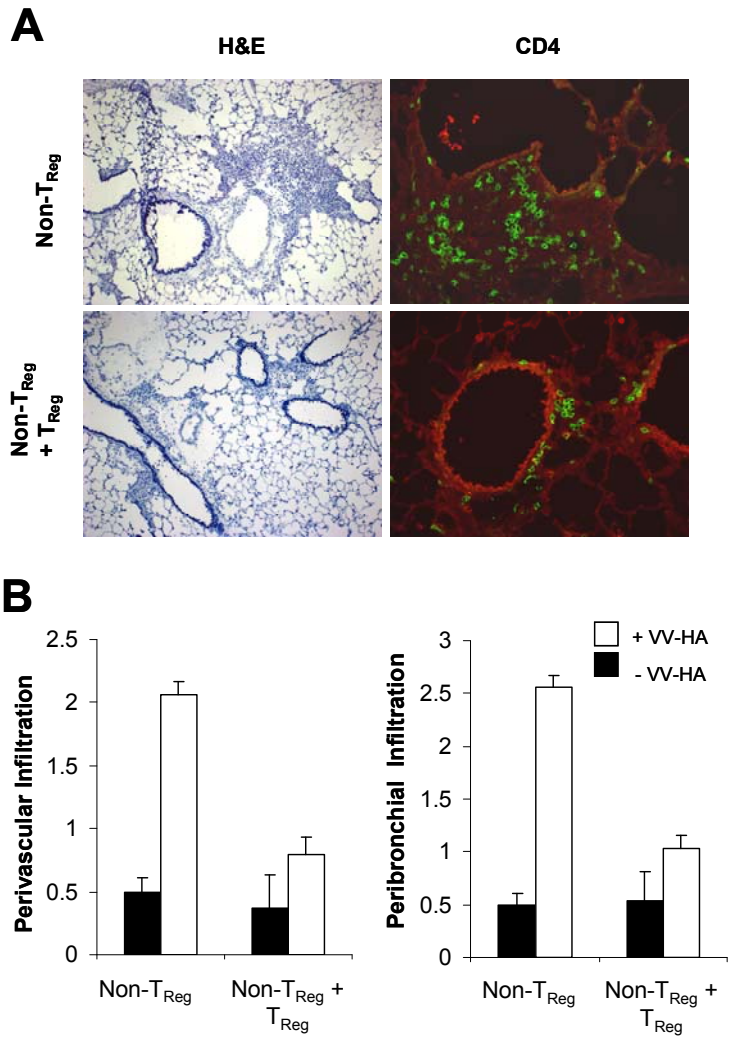

Figure 13: "Memory" T_{Reg} Cells Control the Extent of Lung Immunopathology during a Recall Response.

Purified naïve HA-specific non-T_{Reg} cells with (T_{Reg} + Non-T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice, which were infected with influenza virus. 50 days after infection, mice were rechallenged with VV-HA. 5 days after rechallenge, lung tissues were harvested and analyzed for immunopathology. (A) Cryosections were stained with hematoxylin (left panel) and with anti-CD4 (Green) and anti-HA (Red) by immunofluorescence. (B) Cryosections were stained with hematoxylin and evaluated for evidence of pathological changes by light microscopic examination. Sections were characterized with respect to perivascular and peribronchial infiltration. Extent of pathology was scored from 0 (no pathology) to 4 (severe pathology). The mean score for ten fields of view from at least three cryosections per group was graphed with the standard error included. Results are representative of three independent experiments.

3.2.5 Suppression by "Memory" T_{Reg} Cells is Mediated through IL-10

We next investigated what is responsible for "memory" T_{Reg} cell-mediated suppression on non-T_{Reg} $CD4^+$ T cells during the recall response. Since CTLA-4, IL-10, and TGF-β have been implicated in T_{Reg} cell-mediated suppression *in vivo* [123], we examined the effects of CTLA-4, IL-10, and TGF-β blockade on the recall response. We transferred HA-specific non-T_{Reg} cells with T_{Reg} cells into congenic B10.D2 mice, which then received influenza virus intranasally. 50 days following infection, mice were treated with anti-CTLA-4, anti-IL10 receptor (IL-10R), anti-TGF-β, or a control IgG, followed by a secondary challenge with VV-HA. 5 days later, mice were analyzed for the expansion of non-T_{Reg} $CD4^+$ T cells. We found that CTLA-4 neutralization had no effect on the expansion of non-T_{Reg} $CD4^+$ T cells or lung pathology in the presence or absence of "memory" T_{Reg} cells (data not shown). In contrast, IL-10R blockade led to significantly enhanced expansion of non-T_{Reg} $CD4^+$ T cells (Figure 14A, B) and exacerbation of lung pathology (Figure 14C) only in the presence of "memory" T_{Reg} cells, but not in the absence of "memory" T_{Reg} cells, suggesting that IL-10 is responsible for "memory" T_{Reg} cell-mediated suppression of non-T_{Reg} $CD4^+$ T cells. We further found that although quiescent "memory" T_{Reg} cells produced little IL-10, they produced much higher levels of IL-10 upon antigen rechallenge (Figure 14D, E). Although neutralization of TGF-β also led to enhanced expansion of non-T_{Reg} $CD4^+$ T cells and exacerbation of lung pathology in the presence of "memory" T_{Reg} cells, similar degrees of increased expansion and pathology were also found in the absence of "memory" T_{Reg} cells (data not shown), suggesting the effect of TGF-β blockade on the expansion of non-T_{Reg} $CD4^+$ T

cells and pathology is not mediated through "memory" T_{Reg} cells. Taken together, these data indicate that "memory" T_{Reg} cell-mediated suppression of non-T_{Reg} $CD4^+$ T cells and immunopathology during the recall response is mediated by IL-10.

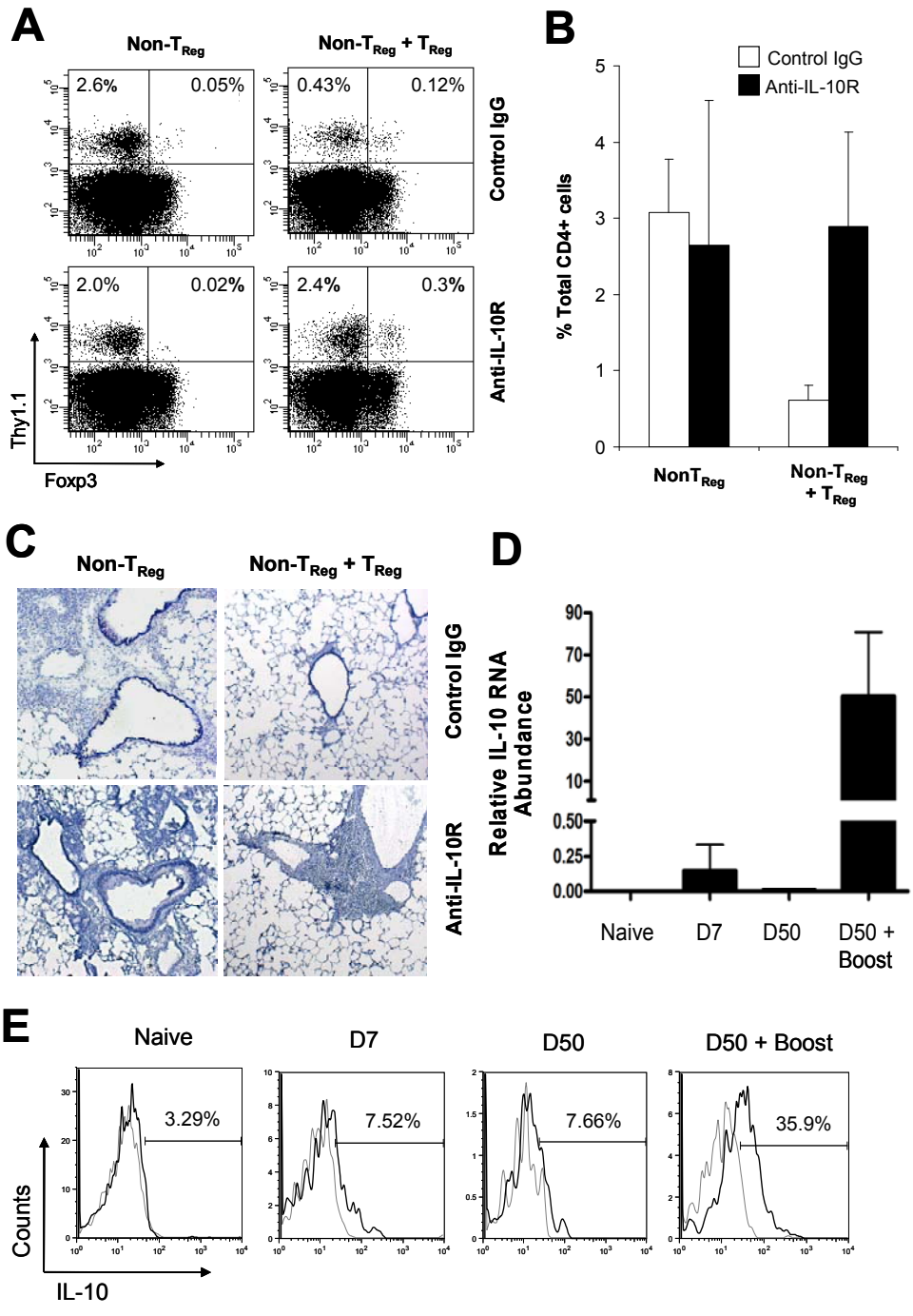

Figure 14: "Memory" T_{Reg} Cells Suppress the Expansion of Non-T_{Reg} Memory $CD4^+$ T Cells Via IL-10.

Figure 14: "Memory" T_{Reg} Cells Suppress the Expansion of Non-T_{Reg} Memory $CD4^+$ T Cells Via IL-10.

(A-C) Purified naïve HA-specific non-T_{Reg} cells with (T_{Reg} + Non-T_{Reg}) or without T_{Reg} cells (Non-T_{Reg}) were transferred into B10.D2 mice and infected with influenza virus. 50 days after infection, mice received 0.5 mg anti- IL-10R or the control IgG i.v. 6 hours prior to infection with VV-HA and again two days later. 5 days following rechallenge, draining hilar lymph nodes and lung tissue were harvested for analysis. Hilar lymphocytes were harvested and stained with anti-CD4, anti-Thy1.1, and anti-Foxp3. The percentages of $CD4^+Thy1.1^+Foxp3^+$ and $CD4^+Thy1.1^+Foxp3^-$ within the $CD4^+$ T cell gate are indicated (A). The mean percentage of non-T_{Reg} cells within the $CD4^+$ T cell gate was plotted with the standard deviations included (B). Cryosections of lung tissue were stained with hematoxylin (C). (D-E) Purified naïve HA-specific T_{Reg} cells ($Thy1.1^+$) were transferred into B10.D2 mice and infected with influenza virus or left uninfected (naïve). HA-specific T_{Reg} cells were analyzed 7 (D7) and 50 (D50) days post-infection, as well as five days after antigen rechallenge (D50+boost), which was done 50 days after initial infection. (D) HA-specific T_{Reg} cells were purified by staining pooled cells from the lymph nodes and spleen with anti-CD4 and anti-Thy1.1 to sort $CD4^+Thy1.1^+$ T_{Reg} cells. mRNA was isolated from naïve, D7, D50, and D50 + boost T_{Reg} cells, and the levels of IL-10 and β-actin were measured by real-time RT-PCR. The relative quantities of mRNA, normalized to β-actin, from a representative sample are indicated. (E) HA-specific T_{Reg} cells were stained with anti-CD4, anti-Thy1.1, anti-Foxp3 and anti-IL-10 (intracellular, black) or isotype control (gray). The percentages of $CD4^+Thy1.1^+Foxp3^+$ cells that are $IL\text{-}10^+$ within the $CD4^+$ T cell gate are indicated. Results are representative of three independent experiments.

3.3 Discussion

Previous work has demonstrated the importance of T_{Reg} cells in the regulation of immune responses during infection with a variety of pathogens [85]. However, the majority of this work has focused on chronic disease states, and the role of T_{Reg} cells during acute infections, and the fate of T_{Reg} cells following the resolution of the infection remains largely unknown. In this study, we have examined the kinetics and function of pathogen-specific T_{Reg} cells in two different models of acute viral infection. Our work reveals a previously unknown "memory" T_{Reg} cell population that develops following acute viral infection *in vivo*. We show that "memory" T_{Reg} cells suppress the expansion and effector function of non-T_{Reg} memory $CD4^+$ T cells upon secondary challenge. These "memory" T_{Reg} cells play a critical role in controlling excessive immunopathology that occurs during the recall expansion of non-T_{Reg} memory $CD4^+$ T cells upon secondary challenge. Furthermore, we demonstrate that these "memory" T_{Reg} cells secrete high levels of IL-10 upon antigen rechallenge and that the function of "memory" T_{Reg} cells is mediated by an IL-10-dependant mechanism.

Upon viral infections, non-T_{Reg} $CD4^+$ and $CD8^+$ T cells expand and then contract, with only a small fraction (5-10%) surviving to form the stable memory population [120]. Following infection with VV-HA, we observed that pathogen-specific T_{Reg} cells behave with similar kinetics to non-T_{Reg} $CD4^+$ T cells, albeit with a lower initial expansion. The "memory" T_{Reg} cells that survive the contraction phase can also respond rapidly to secondary antigen rechallenge. However, unlike non-T_{Reg} memory $CD4^+$ T cells that consist of both $CD62L^{lo}$, effector memory (T_{EM}) and $CD62L^{hi}$, central memory (T_{CM})

populations, these "memory" T_{Reg} cells display almost exclusively a $CD62L^{lo}$, T_{EM} phenotype. The expression of CD62L on T_{CM} cells allows them to home to LN efficiently and differentiate into effector cells upon antigen restimulation. In contrast, due to lack of LN homing receptors CD62L, T_{EM} cells migrate into non-lymphoid tissues and display immediate effector functions for the rapid control of invading pathogen. The T_{EM} phenotype of the "memory" T_{Reg} cells may allow them to migrate quickly into non-lymphoid tissues, such as liver and lung, to exert immediate control of collateral tissue damage [120]. Indeed, we found rapid accumulation of "memory" T_{Reg} cells in the liver upon antigen rechallenge (Figure 12).

Our observation that "memory" T_{Reg} cells suppress the expansion and effector function of non-T_{Reg} memory $CD4^+$ T cells and play a critical role in controlling excessive immunopathology during the recall response, further supports the growing body of evidence that T_{Reg} cells are essential in prevention of immunopathology in various models of bacterial, viral, parasitic, and fungal infections [69, 83, 89, 90]. Inhibition of immunopathology during a recall response may be of particular importance because of the rapid and robust expansion of memory non-T_{Reg} cells**.** Indeed, we observed much greater immunopathology in the liver or lungs during a recall response compared to a primary response (A.M.S. and Y.Y., unpublished observation).

Multiple mechanisms have been proposed for T_{Reg} cell-mediated suppression *in vivo*, including the production of immunosuppressive cytokines such as IL-10 and TGF-β, direct cell-cell contact, as well as inhibition of APC function [123]. Here, we have provided *in vivo* evidence that IL-10R blockade significantly reduces the ability of

"memory" T_{Reg} cells to suppress the recall expansion of non-T_{Reg} $CD4^+$ T cells, as well as the attendant immunopathology and that "memory" T_{Reg} cells produced high levels of IL-10 upon rechallenge, suggesting that IL10 is responsible for "memory" T_{Reg} cell-mediated suppression. It remains to be defined how IL-10 suppresses non-T_{Reg} $CD4^+$ T cell responses and whether other mechanisms of T_{Reg} cell-mediated suppression are also utilized. Thus, further work should address these important questions.

In this study, no obvious T_{Reg} cell-mediated suppression of the expansion of non-T_{Reg} $CD4^+$ T cells was detected during the primary response to VV, adenovirus or influenza *in vivo*. Since these activated T_{Reg} cells were able to suppress the activation of naïve T cells *in vitro*, the lack of suppression on non-T_{Reg} $CD4^+$ T cells by activated T_{Reg} cells *in vivo* likely suggests that these activated T_{Reg} cells might transiently lose suppressive capability due to direct TLR stimulation on T_{Reg} cells, and/or that non-T_{Reg} $CD4^+$ T cells have become refractory to T_{Reg} cell-mediated suppression due to the effect of pro-inflammatory cytokines, such as IL-6, produced during the primary response to VV infection as suggested previously [114, 124, 125]. Our results are in line with the observations in models of infection with Pseudomonas and Trypanosoma [95, 96] but are in contrast to the model of HSV infections [84, 126], as well as the model of peptide IFA immunization [127]. The reasons for the discrepancy may be due to different experimental models and conditions. In the studies with HSV infection, the polyclonal population of T_{Reg} cells was deleted as opposed to addition of antigen-specific T_{Reg} cells in our study, which could affect the overall suppressive capability of T_{Reg} cells. In addition, different infections and immunizations could elicit different inflammatory

milieu and/or innate immune responses, both of which have been suggested to play a role in overcoming T_{Reg} cell-mediated suppression [114, 124, 125].

We observed a striking difference in the suppressive ability of naïve or activated T_{Reg} cells during the primary response vs. "memory" T_{Reg} cells during the recall response. This difference is not due to changes in T_{Reg} cell frequency between the primary and recall responses, as the percentage of T_{Reg} cells among total antigen-specific T cells remains similar during the initial priming and the recall response. It is also unlikely this difference is due to different viruses used between the priming and recall response because VV has been used in both settings in our models. Since significantly higher levels of IL-10 was produced by "memory" T_{Reg} cells upon antigen rechallenge compared to the day 7 activated T_{Reg} cells, it is reasonable to suggest that the increased suppressive potential of "memory" T_{Reg} cells is due to the ability to produce markedly elevated levels of IL-10 upon rechallenge. Thus, there is a qualitative difference between memory and naïve T_{Reg} cells in the ability to suppress non-T_{Reg} cells, and this qualitative difference is reflected in their ability to rapidly produce high levels of IL-10 in response to antigen stimulation. The underlying mechanism leading to this qualitative gain by "memory" T_{Reg} cells requires further investigation.

In conclusion, we have shown that pathogen-specific T_{Reg} cells expand and contract following acute viral infections, with a small population developing into the "memory" T_{Reg} cell pool. While these pathogen-specific T_{Reg} cells do not significantly suppress the expansion of non-T_{Reg} cells during the primary response, "memory" T_{Reg} cells inhibit the expansion and effector function of memory non-T_{Reg} cells upon a

secondary challenge. The suppression by "memory" T_{Reg} cells is critical to the control of immunopathology that occurs during the recall response as a result of robust non-T_{Reg} cells expansion. Finally, we demonstrate that suppression by "memory" T_{Reg} cells is, at least in part, due to their ability to produce high levels of IL-10 during the recall response. Our work reveals a previously unknown "memory" T_{Reg} cell population that develops following acute viral infection *in vivo* and may help design effective strategies to circumvent excessive immunopathology during a recall response.

4. Chapter Four: Future Directions

In this thesis, we demonstrated that "memory" T_{Reg} cells develop following acute viral infection and that these cells vigorously suppress the expansion and effector function of non-T_{Reg} cells in an IL-10-dependent manner to protect against immunopathology. We initiated the studies in this thesis because previous work has established roles for T_{Reg} cells during chronic infections, but the functions that T_{Reg} cells may play during acute infection is unclear, particularly with acute viral infections. To address this gap in our knowledge, in this thesis we sought to more fully define how T_{Reg} cells modulate $CD4^+$ non-T_{Reg} cells during acute infection, as well as to discover what happens to activated T_{Reg} cells following the resolution of an acute viral infection. To achieve this goal, we utilized $CD4^+$ non-T_{Reg} and T_{Reg} cells from HA-TCR transgenic mice coupled with viral models expressing HA. These models enabled us to track pathogen-specific T_{Reg} cells over the course of infection and to determine how pathogen-specific T_{Reg} cells modulate effector $CD4^+$ non-T_{Reg} cell responses at different time points during infection. We found that, similar to non-T_{Reg} cells, pathogen-specific T_{Reg} cells expand, contract, and a small fraction survive to become a memory pool. Similar results were found using a MHC Class II tetramer to track endogenous HA-specific T_{Reg} cells following influenza infection. These "memory" T_{Reg} cells can rapidly expand following antigen rechallenge and possess an "effector-memory"-like phenotype.

Utilizing the TCR transgenic system also enabled us to define the function of T_{Reg} cells during an acute viral infection. We found that the presence of pathogen-specific T_{Reg} cells during priming does not affect non-T_{Reg} cell expansion or function. However, our

experiments reveal that "memory" T_{Reg} cells are capable of suppressing memory non-T_{Reg} cells during a recall response. Additionally, we provide evidence that the function of "memory" T_{Reg} cells may be to prevent collateral tissue damage that occurs during a robust recall response. Finally, our data suggest that "memory" T_{Reg} cell–mediated suppression is related to their ability to produce large amounts of IL-10 during the recall response. The results of these studies raise multiple questions about T_{Reg} cell biology during acute viral infection and open up the possibilities for numerous future studies, which will be discussed here.

4.1 The Kinetics of T_{Reg} Cells during an Acute Viral Infection

Our initial work to examine T_{Reg} cells during acute viral infection established their kinetics through the use of TCR transgenic T cells recognizing pathogen-specific T_{Reg} cells. Previous studies have examined the kinetics of the polyclonal T_{Reg} cell population following both acute and chronic infection models and found that T_{Reg} cells expand upon infection, such as with *Leishmania major* infection [68]. In support of these observations, we found that the percentage and absolute number of HA-specific T_{Reg} cells, as determined by tetramer staining, were higher in the lungs of influenza-infected Foxp3-GFP knock-in mice compared to naïve mice.

To more fully study the kinetics of T_{Reg} cells during infection, we purified HA-specific T_{Reg} cells and transferred them into congenically-marked mice, which were then infected with VV-HA. Using this system, we observed that T_{Reg} cells expanded, albeit to a lesser degree than non-T_{Reg} cells, with the peak of expansion occurring seven days following infection. It is unclear whether the lower initial expansion of T_{Reg} cells is due

to reduced proliferation compared to non-T_{Reg} cells following viral infection or due to reduced seeding after transfer. Previous reports demonstrated that only about 10% of input TCR transgenic non-T_{Reg} cells "take", but it is unknown whether this percentage is the same with HA-specific T_{Reg} cells [128, 129]. However, because our results examining endogenous HA-specific T_{Reg} cell expansion by tetramer staining showed similar fold expansion between non-T_{Reg} and T_{Reg} (approximately 60-fold increase over naïve), it is plausible that transferred T_{Reg} cells have a lower seeding efficiency compared to non-T_{Reg} cells. To definitively prove this, however, we would need to compare the seeding percentages of TCR-HA non-T_{Reg} and T_{Reg} cells following transfer. Furthermore, experiments to compare the proliferation of pathogen-specific non-T_{Reg} and $T_{Reg,}$ for example by using carboxyfluorescein succinimidyl ester (CFSE)-labeled cells, could be done to determine whether there is any difference in proliferation between the two cell types.

After this initial expansion, T_{Reg} cells contracted, and approximately 90% to 95% of the cells present at the peak of expansion died. Previous work with $CD4^+$ and $CD8^+$ non-T_{Reg} cells, in addition to the data on TCR-HA non-T_{Reg} provided here, demonstrated similar levels of contraction [130, 131]. The HA-specific T_{Reg} cells that survived the contraction phase rapidly expanded following antigen recall, a hallmark of immunologic memory. To our knowledge, this is the first report to definitively show that T_{Reg} cells are capable of becoming memory cells following infection. In support of this observation, it was recently demonstrated that influenza-specific T_{Reg} cells could be detected in the blood of healthy patients, suggesting pathogen-specific "memory" T_{Reg} cells may also

exist in humans [132]. Further work is required to confirm whether these influenza-specific T_{Reg} cells possess similar memory functions as the population we observe in mice.

Based on our observations of the kinetics of T_{Reg} cells following infection, future studies can be directed at understanding how contraction and memory formation of T_{Reg} cells after acute viral infection occurs to determine whether they proceed in a similar fashion as $CD4^+$ non-T_{Reg} cells. Current research suggests that $CD4^+$ T cell contraction is controlled by multiple antigen-dependent and independent mechanisms that initiate both apoptotic and non-apoptotic pathways of cell death [131, 133]. During contraction, activation-induced cell death (AICD), mainly proceeding through Fas/FasL interactions, contributes to the active initiation of cell death, while withdrawal of antigen and/or cytokines and growth factors represent more passive or spontaneous mechanisms leading to death [121, 131, 134]. It seems conceivable that there may be differences between $CD4^+$ non-T_{Reg} and T_{Reg} cell contraction because T_{Reg} have been reported to have different susceptibilities to AICD and unique growth factor requirements, such as IL-2, a cytokine implicated in contraction [131, 135, 136].

In addition to studies examining T_{Reg} cell contraction, work should be initiated to more fully understand memory development in T_{Reg} cells after pathogen infection because we observe that "memory" T_{Reg} cells are only found with a T_{EM} phenotype. In terms of $CD4^+$ non-T_{Reg} cell memory formation, much remains unknown about memory populations and their development. It is not clear whether memory $CD4^+$ T cells develop in a linear fashion (naïve to effector to memory) or arise in an alternative way, although

newer studies suggest that memory cells arise from IFN-γ-producing effector T cells [137, 138]. After memory T cells develop, they can be divided into two subsets: T_{EM} and T_{CM}. The relationship between these two lineages and how their developmental pathways may differ are unclear [117, 139]. However, recent work has suggested that the fate of a $CD8^+$ T cell memory precursor into either the T_{EM} or T_{CM} subset is dependent upon cytokines, such as IL-15 and IL-2, and antigen availability [140, 141]. Here, it was demonstrated that IL-2 signaling promotes a T_{EM} phenotype, while IL-15 signaling promotes a T_{CM} phenotype [140]. This difference between T_{EM} and T_{CM} development in $CD8^+$ T cells suggests that our observation that "memory" T_{Reg} cells are only found with a T_{EM} phenotype may be related to their responsiveness to IL-2. Thus, work should be initiated to examine IL-2 versus IL-15 responsiveness and how this affects memory development in pathogen-activated T_{Reg}, as well as non-T_{Reg} cells, to determine whether the observations in $CD8^+$ T_{EM} and T_{CM} cell development are applicable to $CD4^+$ T cells. Such work is not only critical to learn about T_{Reg} cell biology but also to understand more about memory T cell populations overall, which is critical for vaccine development.

4.2 The Role of T_{Reg} Cells during the Primary and Recall Response of an Acute Infection

One of the main goals of this thesis was to define the function of T_{Reg} cells during acute infection. Collectively, the existing studies that have explored this role have been inconclusive, as some previous studies have indicated T_{Reg} cells modulate acute infections, while others have demonstrated a role for T_{Reg} cells. To address this question, we transferred TCR transgenic $CD4^+$ non-T_{Reg} cells with or without T_{Reg} cells, which

enabled us to examine whether the addition of pathogen-specific T_{Reg} cells would affect non-T_{Reg} cells at different time points during infection. We used this experimental approach because nearly all of the previous studies examining T_{Reg} cells during infection in murine models have utilized depletion of the polyclonal T_{Reg} cell population, generally through the use of a CD25-depleting antibody or with *Foxp3*^{DTR} mice. The use of pan-T_{Reg} cell depletion may not be an ideal model to study T_{Reg} cells during infection, as previous work has indicated that T_{Reg} responses to infection can be pathogen-specific [74]. With this approach, we demonstrated that the addition of pathogen-specific T_{Reg} cells does not significantly affect the expansion and/or effector function of $CD4^+$ pathogen-specific non-T_{Reg} cells during the initial response to acute viral infection. On the other hand, the presence of HA-specific T_{Reg} cells affected both the expansion and effector function of non-T_{Reg} cells during a memory recall response.

Because of the potential caveats associated with transferring a relatively larger number of T_{Reg} cells of the same antigen-specificity, compared to what would be present in the endogenous setting, we also examined endogenous HA-specific T_{Reg} cells using MHC Class II tetramer technology. With this approach, we were able to show that the kinetics of endogenous HA-specific T_{Reg} cells were similar to TCR transgenic T_{Reg} cells, with the cells expanding following infection and then contracting to form a stable pool, of presumably, memory cells. While it would be ideal to extend these studies to determine whether these surviving cells expand rapidly following secondary infection, because of the neutralizing antibodies that develop during influenza priming, we would not expect that infecting mice with a second dose of the same virus would lead to a potent memory

response. To circumvent this problem, we determined whether secondary vaccination with VV-HA would lead to an appreciable memory response, but we did not detect one (data not shown), presumably because HA is not an immunodominant epitope in this viral model.

To extend the studies with endogenous T_{Reg} cells and complement our work with TCR transgenic T_{Reg} cells, we are currently performing experiments to determine the role of endogenous T_{Reg} cells during priming; while we would like to examine the role of endogenous "memory" T_{Reg} cells during the recall response, we presently do not have a system to assess this. Whereas these experiments would expand our findings to a more physiologically relevant setting, we are much more experimentally constrained than with transgenic cells. Ideally, we would want to measure the expansion and function of endogenous HA-specific non-T_{Reg} cells with or without the presence of endogenous HA-specific T_{Reg} cells, but we do not have the ability to specifically deplete HA-specific T_{Reg} cells. One approach would be the transfer of endogenous HA-specific non-T_{Reg} cells with or without T_{Reg} cells into naïve mice. However, this is not feasible because the number of HA-specific naïve T_{Reg} cells is very low, and it has been reported that tetramer binding can contribute to cell death and/or phenotypic changes as the result of TCR crosslinking [142]. Additionally, in this experimental setup, we could not rule out the involvement of the endogenous T_{Reg} cell population.

To circumvent these experimental challenges, we are currently performing experiments to deplete the polyclonal T_{Reg} cell population prior to and during the initial response to influenza infection. Here, Foxp3-GFP knock-in mice will be treated with an

anti-CD25 antibody, as previously described, followed by infection with influenza [114]. We will track the depletion of T_{Reg} cells using GFP, as well as monitor the HA-specific non-T_{Reg} cell response using a MHC Class II tetramer to determine whether ablation of T_{Reg} cells affects the non-T_{Reg} cell response to the immunodominant HA epitope. While the effect of T_{Reg} cell depletion on the $CD4^+$ T cell response to influenza is unknown, previous work demonstrated that depletion increased the number of IFN-γ-producing $CD8^+$ T cells specific for known influenza epitopes [143]. Thus, these experiments will add to our knowledge about the role of T_{Reg} cell-mediated suppression of $CD4^+$ T cells during influenza infection.

The results from the aforementioned experiments with Foxp3-GFP knock-in mice will provide another line of evidence to more fully understand the role of T_{Reg} cells during the priming stage of acute viral infection. However, we are aware that there are multiple caveats to these experiments. One area of potential concern is the inefficient depletion of $Foxp3^+$ T_{Reg} cells, which has been previously reported during acute infection [98]. Because we are using Foxp3-GFP knock-in mice, we will be able to easily track T_{Reg} cell depletion using GFP to ensure effective depletion during influenza infection. Another potential problem is the depletion of non-T_{Reg} cells that express CD25, which has been reported to be upregulated following activation, particularly during certain acute infection models [99]. We can determine whether this is the case by examining if non-T_{Reg} $CD4^+$ T cells rapidly upregulate CD25 during the initial phases of influenza infection in the absence of anti-CD25 treatment. If anti-CD25 treatment is too inefficient

or is found to deplete activated $CD4^+$ non-T_{Reg} cells, then it would be feasible to use $Foxp3^{DTR}$ mice to selectively delete T_{Reg} cells.

In addition to these caveats, because we are depleting the polyclonal T_{Reg} cell population instead of adding in antigen-specific T_{Reg} cells, we may observe disparate results compared to our TCR transgenic non-T_{Reg} and T_{Reg} cell transfer studies. If we determine that depletion of the polyclonal T_{Reg} cells population during influenza priming has an effect on non-T_{Reg} cell expansion, this would not nullify the results observed with the TCR transgenic system, but rather suggest that suppression during priming may not be antigen-specific and that perhaps we did not observe suppression in the TCR transgenic system because there were not enough T_{Reg} cells present. This seems unlikely, however, because the addition of more T_{Reg} cells such that the ratio of T_{Reg} to non-T_{Reg} cells at day seven was much higher than what is physiologically present did not lead to significant suppression. If we observed suppression with T_{Reg} cell depletion, this could also indicate that depletion of the polyclonal T_{Reg} cell population causes changes to multiple aspects of the immune system, irrespective of infection status, which could modulate the course of pathogen infection without any true T_{Reg} cell-mediated, pathogen-specific suppression. Indeed, it is has been previously reported that T_{Reg} cell depletion leads to profound changes in pro-inflammatory chemokines and cytokines in the absence of infection [100].

4.3 Defining What Dictates whether Effectors Are Suppressed

Our observation that T_{Reg} cell-mediated suppression does not modulate TCR-HA $CD4^+$ non-T_{Reg} cells during priming supports previous data indicating no or a minimal

role for T_{Reg} cells during acute infections [95-97]. Collectively, these results indicate that during an acute infection either T_{Reg} cells temporarily lose their suppressive abilities, effectors become less susceptible to suppression in an inflammatory environment, or a combination of the two. To date, studies have been initiated to define the signals that dictate whether T_{Reg} cells are suppressive and whether non-T_{Reg} cells are vulnerable to suppression, but further work is needed to fully understand the interplay between these two possibilities and how they modulate responses to infection.

With respect to changes on T_{Reg} cells following infection, it has been reported that T_{Reg} cell function can be modulated by cytokines, co-stimulatory or co-inhibitory receptors, and through Toll-like receptors (TLRs), which belong to the family of pattern recognition receptors (PRRs) [144]. Suppression can be abrogated following direct T_{Reg} cell stimulation of TLRs (for review see [145]). TLRs recognize pathogen-associated molecular patterns (PAMPs), which are molecules associated with pathogens, including viral nucleic acids or bacterial cell wall components [146]. While it has been demonstrated that T_{Reg} cells express various TLRs, how direct TLR signaling on T_{Reg} cells has not been fully elucidated and remains debated. Some studies have suggested that TLR2 or 8 stimulation abrogates the suppressive capacity of T_{Reg} cells, while others have shown that TLR4 and 5 can act to enhance suppression [145]. Because many of these studies have been conducted *in vitro*, additional studies are necessary to fully tease apart the intricacies of TLR signaling on T_{Reg} cell function *in vivo*. In addition to direct TLR stimulation on T_{Reg} cells, TLRs can also affect T_{Reg} cells through an indirect mechanism

via stimulation of DCs; this causes DCs to express IL-6, which blocks the ability of T_{Reg} cells to suppress [124].

With respect to *in vivo* function of TLRs during infection, the role of TLR2 and the outcome of *Candida albicans* infection, an infection model modulated by T_{Reg} cells, is the most studied [89, 147]. Using WT T_{Reg} cells transferred into TLR2-deficient mice, it was shown that systemic administration of TLR2 ligands during *Candida albicans* infection resulted in levels of fungal outgrowth similar to mice that did not receive T_{Reg} cells, suggesting that direct TLR2 stimulation *in vivo* blunts T_{Reg} cell-mediated suppression during fungal infection [125]. This conclusion is not without controversy, however, as it was recently suggested that direct TLR2 stimulation on T_{Reg} cells results in increased survival, but does not affect suppressive capacity [148]. Thus, further work to define how TLR signaling affects T_{Reg} cells *in vivo* during infection is needed. Our model system may provide a means to analyze how TLR2 signaling modulates T_{Reg} cells because work in our laboratory has established that VV stimulates TLR2 [149, 150].

In addition to an abrogation of suppression on T_{Reg} cells, it is possible that effector $CD4^+$ non-T_{Reg} cells also become refractory to suppression following infection. The idea that effectors cells can become refractory to suppression initiated from animal models of autoimmunity, including diabetes models and mice deficient in the E3 ubiquitin ligase Cbl-b that showed no defects in T_{Reg} cells but rather had effector T cells that were resistant to T_{Reg} cell-mediated suppression [151-153]. A similar observation has been made in humans, where effector T cells from patients with diabetes, systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) have been reported to be

resistant to suppression [154-156]. The mechanisms that have been proposed for this resistance are similar to the mechanisms proposed for the abrogation of T_{Reg} cell suppression, including stimulation by TLRs, co-stimulatory or co-inhibitory receptors, and cytokines [157]. With TLRs, CpG DNA, which is recognized by TLR9, has been shown to directly stimulate effector T cells *in vitro* and abrogate their ability to be suppressed; it should also be noted that CpG can also stimulate T_{Reg} cells to abrogate their suppressive capacity [158]. Because the expression of specific TLRs on non-T_{Reg} and T_{Reg} cells overlaps, further work to assess the role of direct TLR stimulation on both of these cells types is necessary [144].

Much of the research done to-date on the role of co-stimulatory and co-inhibitory receptors and cytokines has yet to delineate whether they function to abrogate the suppressive abilities of T_{Reg} cells and/or desensitize non-T_{Reg} cells to suppression. The receptors implicated include GITR, OX40, 4-1BB, and Programmed death-1 (PD-1) [157, 159-161]. Work has also implicated the following cytokines, which can act directly or indirectly on T cells, in determining whether T_{Reg} cells can suppress or whether effectors are susceptible to suppression: IL-1, IL-6, IL-12, IL-21, IL-2, and IL-15 [124, 162-166]. Future studies are required to tease apart the intricacies of how TLRs, cytokines, and co-stimulatory and co-inhibitory receptors modulate T_{Reg} cell-mediated suppression and effector T cells responsiveness to suppression. Additionally, experiments to examine how these molecules can modulate effectors responses during acute viral infection, as well as other acute infection models, are warranted.

The differences we observe between priming versus the secondary results raise questions about why suppression occurs only in the memory response. There is a definitive qualitative difference between naïve and "memory" T_{Reg} cells in that they are able to rapidly produce large quantities of IL-10 following antigen recall, suggesting that "memory" T_{Reg} cells do not lose the ability to suppress following recall. It is not clear whether this enhanced functional ability on T_{Reg} cells is the only reason why we observe suppression or whether non-T_{Reg} cells become more susceptible to suppression during the recall response as well. Because the cytokine milieu, co-stimulatory/co-inhibitor signals, and TLRs present may be different during the recall, as opposed to the primary response, memory non-T_{Reg} cells may receive a distinctive combination of signals affecting their ability to be suppressed. Further, work with $CD4^+$ memory cells has indicated that their activation threshold is different quite than naïve T cells, suggesting that they may respond differently to the stimulatory environment during the recall response [167-169]. Further work is necessary to more fully define whether non-T_{Reg} cells become more susceptible to suppression during a recall response or whether the suppression we observe during the boost response is due to the high amount of IL-10 production associated with "memory" T_{Reg} cells. Overall, further work is necessary to understand the interaction between the suppressive ability of T_{Reg} cells and the ability of $CD4^+$ non-T_{Reg} cells to be suppressed during both priming and the recall response.

4.4 Further Defining "Memory" T_{Reg} Cells Phenotypically

One area of research that warrants future studies is to further understand the differences between naïve and "memory" T_{Reg} cells. We are specifically interested in

further exploring phenotypic differences to identify markers of "memory" T_{Reg} cells, which would enable their identification in the endogenous setting in both humans and mice. The ability to identify "memory" T_{Reg} cells is critical for extending studies to learn about their properties and functions, as well as to determine how to utilize "memory" T_{Reg} cells for therapeutic purposes, which will be discussed later.

To initiate these studies, we will examine the expression of markers that have previously been described on a subset of T_{Reg} cells termed "effector/memory-like," which possess increased suppressive capacity over other T_{Reg} cell subsets. This population was first described when it was found that T_{Reg} cells can be divided into subsets based on the expression of the integrin $\alpha_E\beta7$ (CD103), with the $CD103^+$ population showing a stronger regulatory capacity *in vitro* and *in vivo* [170]. Further work demonstrated that the $CD103^+$ subset preferentially migrates to inflammatory sites and has an effector/memory phenotype characterized by higher levels of CD69, an activation marker, and a larger percentage of cells that have downregulated CD62L (L-selectin), a homing receptor involved in the migration of T cells into lymph nodes [171]. Work with CD103 in T_{Reg} cells during infection has demonstrated that CD103 is required for T_{Reg} cell retention at the sites of *Leishmania major* infection [172].

In addition to CD103, the expression of the C-C Chemokine receptor type 6 (CCR6) can be used to distinguish "effector/memory-like" T_{Reg} cells [173, 174]. C-C CCR6 is a chemokine receptor that binds to CCL20 and β-defensin, which are associated with inflammation [175]. It is expressed on a variety of cells, including DCs, B cells, and memory T cell subsets, with CCR6 expression in $CD4^+$ T cells marking only those cells

that are antigen experienced [176, 177]. $CCR6^+$ T_{Reg} cells have a phenotype similar to activated T cells, including upregulated CD69 and downregulated CD62L. While there is some overlap between the expression of CCR6 and CD103 in T_{Reg} cell populations, subsets that are single positive for either receptor exist, and the potential difference(s) between their functions are unknown [173].

Along with CD103 and CCR6, $CD39^+$ human "effector/memory-like" T_{Reg} cells have been described [178]. CD39 is an ectonucleotidase that hydrolyzes extracellular adenosine triphosphate (ATP) to adenosine monophosphate (AMP), which can then be further hydrolyzed by CD73 to produce extracellular adenosine, a potent anti-inflammatory molecule [179]. Both CD39 and CD73 have been shown to contribute to T_{Reg} cell-mediated suppression [65]. By examining the expression of CD39 in human T_{Reg} cells, it was found that T_{Reg} cells can be divided into two populations: those expressing CD39, which also express CCR6, a known marker of "effector/memory-like" T_{Reg} cells, and those that do not express CD39 or CCR6. Further highlighting the importance of this observation, patients with multiple sclerosis, a disease associated with T_{Reg} cell dysregulation, displayed significantly reduced numbers of $CD39^+$ T_{Reg} cells [42, 178]. Analysis of polyclonal murine T_{Reg} cell populations demonstrated that the majority of $Foxp3^+$ T_{Reg} cells express CD39, but that activated T_{Reg} cells upregulate CD39 expression and that only activated T_{Reg} cells can hydrolyze ATP [178]. These results suggest that further studies are necessary to quantify CD39 expression during the different phases of pathogen infection and highlight the need to extend these studies to understand whether

functional changes in ATP hydrolysis are associated with pathogen-experienced T_{Reg} cells.

In addition to examining the expression of these known markers of "effector/memory-like" T_{Reg} cell, novel markers could be identified using microarray analysis to compare the expression of a wide-array of genes from naïve, day 7 activated, "memory" and "memory" + boost T_{Reg} cells. We are currently developing methods to enable us to isolate sufficient quantities of quality RNA from the relatively rare population of "memory" T_{Reg} cells to be able to perform such a microarray. The results of these studies would allow us to identify markers that can isolate endogenous "memory" T_{Reg} cells in both mice and humans, which is critical to being able to further study this subset and potentially harness "memory" T_{Reg} cells therapeutically.

4.5 Further Defining "Memory" T_{Reg} Cells Functionally

In addition to identifying what phenotypic markers distinguish naïve and "memory" T_{Reg} cells, further understanding the function of "memory" T_{Reg} cells during the recall response is critical to being able to harness them for therapeutic purposes. A striking difference we observed between naïve and "memory" T_{Reg} cells was their ability to produce large amounts of IL-10 following antigen recall, and the production of this cytokine appeared to be one mechanism of "memory" T_{Reg} cell-mediated suppression. Because of the critical role for IL-10 during the memory response, future studies should focus on understanding how IL-10 modulates the effector response. Our use of an IL-10R neutralizing antibody to determine the role of IL-10 during the recall response does not reveal which cell type(s) IL-10 acts upon, whether through direct stimulation of non-T_{Reg}

cells or indirect modulation of effector $CD4^+$ T cells. Previous work has demonstrated that IL-10 can act on APCs and macrophages to inhibit T helper 1 (T_h1) immune responses [180]. However, IL-10 can also act directly on $CD4^+$ T cells to inhibit their proliferation and cytokine production [181-183].

To examine the cell type(s) responding to "memory" T_{Reg} cell-produced IL-10, an *in vitro* suppression assay is a logical initial experiment. For this experiment, HA-specific T_{Reg} cells from the recall response would be sorted and mixed with WT or IL-$10R^{-/-}$ APCS, WT or IL-$10R^{-/-}$ HA-specific naïve non-T_{Reg} cells, and the I-E^d-HA peptide. Proliferation, or the lack thereof, of non-T_{Reg} cells would then be measured by [^{3}H]thymidine incorporation. Because it has previously been reported that IL-10 does not contribute to *in vitro* suppression with naïve T_{Reg} cells, it will be important to determine whether IL-10 is secreted by boosted T_{Reg} cells during the course of the suppression assay, which can be done by testing the culture supernatant with an enzyme-linked immunosorbent assay (ELISA) [108]. Given that boost T_{Reg} cells produce such large quantities of IL-10, we anticipate these *in vitro* studies will allow us examine the cell types it acts upon. Regardless of the results of these *in vitro* experiments, it would be ideal to extend these studies to the *in vivo* setting.

To complement studies *in vitro*, the cell type(s) that respond to T_{Reg} cell-produced IL-10 can be studied *in vivo*. For these studies, it will be necessary to utilize a system whereby the IL-10R can be depleted a selected time points to ensure that only the effects of IL-10 during the recall response are studies; an estrogen-receptor-based (Er-based) Cre system to selectively deplete IL-10R prior to boost would be one way to accomplish this.

To use this system for the goal at hand, the expression of Er-Cre would need to be restricted to only the cell type(s) of interest either by placing Er-Cre under the control of a cell-restricted promoter or through the transfer of Er-Cre$^+$ T cells. While these experiments would be technically challenging, they would define how boost T_{Reg} cell-produced IL-10 modulates the effector CD4$^+$ T cell non-T_{Reg} cell response.

In addition to further defining how IL-10 modulates immune responses, future studies should also focus on how IL-10 is rapidly upregulated in memory cells. Work with other T cell memory populations offers some clues into a possible mechanism to explain rapid cytokine production. Work with CD8$^+$ T cell memory cells has demonstrated that epigenetic modifications to enhance transcription of cytokine genes underlie the ability of memory CD8$^+$ T cells to rapidly produce IFN-γ upon antigen recall without the need for multiple cellular divisions [184, 185]. To examine whether epigenetic modifications occur at the IL-10 locus, DNA methylation, histone modifications and chromatin remodeling of the regulatory regions of IL-10 can be compared between naïve, day seven activated, "memory", and "memory" + boost HA-specific T_{Reg} cells. There are multiple tools available for this analysis, including chromatin immunoprecipitation (ChIP), nuclease hypersensitivity assays and DNA methylation analysis [186, 187].

In addition to further understanding IL-10 regulation during the recall response, we are investigating whether "memory" T_{Reg} cells utilize different methods of suppression other than IL-10. To this end, we are currently examining other known methods of suppression not examined in this thesis, including IL-35, perforin, granzyme,

cAMP, and adenosine production. IL-35 is a recently described cytokine that is a heterodimer of Epstein-Barr-virus-induced gene 3 (Ebi3, which encodes IL-27β) and IL-12α, which are both expressed in T_{Reg} cells [54]. Mice deficient in either of these genes showed functionally defective T_{Reg} cell suppression *in vitro*, and Ebi3 or IL-12α-deficient T_{Reg} cells failed to cure IBD *in vivo* [54]. Further work is ongoing to elucidate the mechanisms of IL-35 suppression.

In addition to IL-35, perforin and granzyme-mediated killing is also a mechanism of T_{Reg} cell suppression. Perforin is a cytolytic protein that, once released from intracellular granules, creates pores in a target cell's plasma membrane and at high concentrations can lead directly to cell death. The creation of these pores facilitates the entry of granzymes into the target cells, which can then initiate apoptosis [188]. Studies examining perforin and granzyme as a mechanism of T_{Reg} cell-mediated suppression have yielded disparate results with some studies showing a role for killing of NK and T cells by perforin alone, granzyme alone or through both [61-63]. Thus, additional studies are required to fully define this mechanism of suppression and to determine whether T_{Reg} cells utilize it during pathogen invasion.

Two other recently described methods of suppression are the production of cAMP or extracellular adenosine, which are both immunosuppressive compounds. T_{Reg} cells are known to express high levels of CD39 and CD73, which are ectonucleotidases that sequentially hydrolyze ATP into AMP and adenosine. Extracellular adenosine can then bind to adenosine receptors present on effector T cells, APCs, and myeloid cells to exert direct and indirect immunosuppressive effects on effector T cells [189]. In a similar

manner, cAMP, which is generated intracellularly in T_{Reg} cells especially following activation, is transferred to effector T cells via gap junctions to inhibit cell activation, proliferation, and cytokine production [64, 190]. Similar to IL-35, perforin, and granzyme, it has not been studied whether these suppression mechanisms act during pathogen infection.

We are currently testing the expression levels of IL-35, perforin, granzyme, CD39, and CD73 by Real-time RT-PCR comparing expression between naïve, day seven activated, "memory", and "memory" + boost T_{Reg} cells. For cAMP, we will need to utilize an assay that detects levels of cAMP, as well as possibly examining the expression of adenylyl cyclase 7, which was recently shown to be responsible for T_{Reg} cell cAMP metabolism [191]. In addition to these methods, we anticipate that our microarray analysis, described above, will reveal previously unknown suppression mechanisms. If any of these methods identify molecules that are upregulated in "memory" T_{Reg} cells or in T_{Reg} cells following antigen recall, we will initiate further tests to examine protein expression, for example by FACS analysis, as well as confirm function by *in vitro* and *in vivo* suppression assays.

In addition to examining other potential mechanisms of suppression, we should investigate whether "memory" T_{Reg} cells suppress other cells of the immune system, as our studies focus on only direct or indirect suppression of $CD4^+$ non-T_{Reg} cells. Previous work has demonstrated that T_{Reg} cells can suppress a variety of other cells of the immune system, including DCs, B cells, NK cells, NK T cells, macrophages, and mast cells [20, 47-53]. In terms of acute infection models, previous work has demonstrated that T_{Reg}

cells modulate $CD8^+$ T cell recruitment into the lungs, as well as $CD8^+$ T cell effector function during acute respiratory syncytial virus infection [103]. With current systems in our laboratory, it is feasible to examine whether HA-specific T_{Reg} cells affect HA-specific $CD8^+$ non-T_{Reg} cells. For these experiments, Clone 4 HA-TCR transgenic mice that express a TCR recognizing a K^d-restricted HA epitope (^{518}IYSTVASSL^{526}) would be transferred with or without HA-specific T_{Reg} cells, and the resulting $CD8^+$ T cell response during both priming and the recall response would be monitored as in the studies shown in this thesis [192]. The results from these experiments would add to our knowledge on the total effect of T_{Reg} cells on T cells.

4.6 Harnessing Pathogen-specific T_{Reg} Cells Therapeutically

The research done on T_{Reg} cells over the last fifteen years has opened up multiple possibilities to utilize T_{Reg} cells for therapeutic purposes to ameliorate autoimmunity, enhance anti-tumor immunity, prevent graft rejection, and more recently, modulate the outcome of infection [193]. In terms of harnessing pathogen-responsive T_{Reg} cells therapeutically, there are three main areas that have been proposed and are actively being explored: 1) To deplete T_{Reg} cells to boost anti-pathogen immunity, 2) To deplete or prevent the induction of T_{Reg} cells to establish protective immunity, 3) To expand, either physically or functionally, T_{Reg} cells to prevent collateral damage to tissues. While these goals have yet to be attained clinically, they offer exciting possibilities for future treatments.

With any of these potential therapies, care will need to be taken to maintain the balance between immunity to pathogens and autoimmunity. In terms of depleting T_{Reg}

cells to boost anti-pathogen immunity or establish protective immunity, it may not be best to deplete the polyclonal population in humans, as it has been demonstrated that total ablation of T_{Reg} cells can lead to autoimmunity [101]. An approach to therapeutically diminish T_{Reg} cells function may have reduced side effects. Along these lines, success has been found in murine infection models by targeting molecule utilized by T_{Reg} cells for suppression, such as IL-10 and CTLA-4 [73, 194, 195]. Work is also underway to determine how to expand T_{Reg} cells, whether it is done *ex vivo* or *in vivo*, with some studies using activated APCs and microbial products, such as flagellin [196, 197].

Because of the potential concerns with blocking the polyclonal T_{Reg} cell population, therapies specifically aimed at pathogen-specific T_{Reg} cells may be more effective and could avoid potentially damaging side effects. To this end, it will be critical to more fully understand the epitopes utilized by T_{Reg} cells during infection and whether the hierarchy of epitopes between effector $CD4^+$ non-T_{Reg} and T_{Reg} cells are the same. If the dominant epitopes utilized by non-T_{Reg} and T_{Reg} cells differ, then this could potentially be exploited to specifically deplete T_{Reg} cell-dominant epitopes without disrupting effector non-T_{Reg} cells. Thus, while there are multiple possibilities for using T_{Reg} cells therapeutically, further work needs to be done to ensure that the balance between autoimmunity and pathogen clearance is not deleteriously altered.

Specifically, our work to define the functions of "memory" T_{Reg} cells demonstrating their potent suppressive abilities may provide a good target for these therapeutics. Because our work defines "memory" T_{Reg} cells as a population that can assist in avoiding immunopathology, applications of expanding this population of

"memory" T_{Reg} cells for the prevention of collateral tissue damage require further studies. To this end, studies should focus on phenotypically identifying "memory" T_{Reg} cells in both murine and human populations, perhaps thorough Real-time RT-PCR or microarray approaches described earlier and determine ways to specifically expand this population.

4.7 Final Conclusions

In summary, our knowledge about the functions of T_{Reg} cells in the immune system has grown tremendously since the days of the ill-fated "suppressor cell." This expanding knowledge has revealed unique roles for T_{Reg} cells during pathogen invasion, especially in models of chronic infection. This thesis aimed to further our understanding of how T_{Reg} cells modulate acute infection outcomes. Work towards this aim led to the description of a previously unknown "memory" T_{Reg} cell population that develops after acute viral infections. This work, as well as the future projects described here to further define the "memory" T_{Reg} population, may help design effective strategies to circumvent excessive immunopathology.

While there is further work to be done to examine T_{Reg} cells during pathogen infection, the previous studies described here and the work in this thesis indicate that there is great therapeutic potential in modulating T_{Reg} cells during pathogen invasion. By elucidating the role of T_{Reg} cells during pathogen infection more fully, as well as understanding the ways in which pathogens modulate T_{Reg} cell function, their potential for vaccine development or therapeutics to treat infection can be harnessed. Overall, research examining T_{Reg} cells during infection has suggested that the relationship between T_{Reg} cells, invading microorganisms, and the resulting immune response is one

filled with various ententes. These nuances will need to be considered in any therapeutic intervention.

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Biography

Ana M. Sanchez was born in Chandler, AZ on April 22, 1982 to Carmen and Carlos Sanchez. Upon graduation from high school, Ana attended Arizona State University in Tempe, AZ. Here, she was awarded at B.S. in Microbiology, with a minor in Mathematics in May 2004. Following her time at ASU, Ana began her graduate education at Duke University in August 2004, completing her degree in December 2010. During her time as a research scientist, she has written the following book chapters and articles:

Sanchez AM, Huang X and Yang Y. The Development and Function of "Memory" Regulatory T Cells Following Viral Infection. *In preparation.*

Sanchez, AM and Yang Y. The Role of Natural Regulatory T cells in Infection. *Immunological Research (In press).*

Yang Y and **Sanchez AM**. (2008) Modulation of Regulatory T Cells in Cancer Immunotherapy. *Recent Developments in Immunology*. 97-117. ISBN: 978-81-7895-375-5.

Rogers KA, Rogers AB, Leav BA, **Sanchez AM**, Vannier E, Uemats S, Akira S, Golenbock D, Ward HD. (2006) MyD88-Dependent Pathways Mediate Resistance to *Cryptosporidium parvum* Infection in Mice. *Infection and Immunity*. 74: 549-56.

In addition to these scholarly works, Ana received Minority Supplemental Funding from the National Institutes of Health from May 2006 until May 2010. She was also awarded two minority travel scholarships to attend Keystone Symposia meetings in February 2005 and 2007, as well as a Duke University Graduate Student Travel scholarship in February 2007.