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Type I Interferon Disrupts T Cell Development and Differentiation During an Established Persistent Infection

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics by

by

Ivan Osokine

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ABSTRACT OF THE DISSERTATION

Type I Interferon Disrupts T Cell Development and Differentiation During an Established Persistent Infection

by

Ivan Osokine

Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2014

Professor David G. Brooks, Chair

Persistent viral infection places a significant burden on global heath. Pathogens such as human immunodeficiency virus (HIV), and hepatitis B and C are able to replicate at high levels within their host, outpacing the immune response and ultimately leading to its attenuation. This attenuation is largely the result of host-derived suppressive factors produced in response to the chronic inflammation and immune-mediated damage to critical lymphoid organs accrued throughout the course of the immune response itself. The landscape of the persistently infected immune environment is thus profoundly altered from its naïve state, raising the possibility that new CD4 and CD8 T cell responses that develop and differentiate in the midst of persistent infection could function differently than T cell responses in a healthy environment.

Using lymphocytic choriomeningitis virus (LCMV) as a model system of persistent infection, we determined that *de novo* virus-specific T cell responses primed in the midst of persistent infection are impaired. Virus-specific CD8 T cells primed in the midst of persistent infection assume a terminally differentiated phenotype and form

defective peripheral effector memory, while virus-specific CD4 T cell responses fail to generate Th1 immunity, instead becoming almost exclusively T follicular helper cells (Tfh). This alteration in the T cell differentiation pattern was mediated by chronic type I interferon (IFN-I) signaling, and blockade of IFN-I signaling restored normal differentiation. Moreover, we observed that IFN-I signaling in the context of persistent LCMV infection led to profound thymic atrophy and disrupted the development of T cell precursors. Together, our data demonstrate several novel mechanisms by which IFN-I acts to modulate the immune response during persistent viral infection. Moreover, the data suggest that modulation of the IFN-I signaling pathway or its downstream targets could lead to treatments to purge persistent viral infection.

The dissertation of Ivan Osokine is approved.

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Donald B. Kohn,

Jerry Zack,

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University of California, Los Angeles

2014

This work is dedicated to Nicole, and to my parents, my friends, my colleagues, and my mentors. Your support has made this possible.

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The work presented in chapter 2 of this dissertation will eventually be put forth as a co-first author paper with Cameron Cunningham. Currently, myself, Cameron, Heidi Elsaesser, David Brooks, are listed as authors.

The work presented in chapter 3 of the dissertation is a first author paper recently accepted and currently in press at PNAS. The title and full list of authors is as follows: Ivan Osokine, Laura M. Snell, Cameron R. Cunningham, Douglas H. Yamada, Elizabeth B. Wilson, Heidi Elsaesser, Juan Carlos de la Torre and David G. Brooks. Type I interferon suppresses de novo CD4 Th1 immunity during an established persistent virus infection. PNAS. In Press.

The work presented in chapter 4 is being prepared as a short first author publication. I would like to thank Heidi Elsaesser and David Brooks for their help with the work.

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1. Osokine I, Hsu R, Loeb GB, and McManus MT. Unintentional miRNA ablation is a risk factor in gene knockout studies: a short report. PLoS Genet. 2008 Feb;4(2):e34.

2. Ivan Osokine, Laura M. Snell, Cameron R. Cunningham, Douglas H. Yamada, Elizabeth B. Wilson, Heidi Elsaesser, Juan Carlos de la Torre and David G. Brooks. Type I interferon suppresses de novo CD4 Th1 immunity during an established persistent virus infection. PNAS. In Press.

CHAPTER ONE

Introduction

GENERAL OVERVIEW

Persistent viral infections afflict over a tenth of the global population and cause significant morbidity and mortality, thus posing a great challenge for public health programs and biomedical research to alleviate the spread and severity of these diseases. Viral pathogens such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis b virus (HBV) replicate at high levels within their host and have evolved strategies to circumvent immune surveillance and control. Through their interaction with the host immune system and through maladaptive immune responses, these pathogens induce a broad state of immunosuppression and render the cells that would normally mediate their clearance dysfunctional[1,2]. Although certain mechanisms of immune evasion are specific to each individual virus, several conserved immunologic phenomena, broadly termed “exhaustion”, emerge and contribute to the failure to clear infection[2]. It is therefore necessary to study the interaction between persistent viral pathogens and the host immune systems to identify the mechanisms that lead to immune dysfunction and purge persistent viral infection.

Broadly, the immune system can be subdived into several distinct branches with unique functions to prevent or control microbial pathogens. Barrier organs such as the skin and mucosal surfaces provide an inhospitable environment to pathogen entry[3]. Those pathogens that are able to penetrate or colonize barriers encounter innate immune cells such as neutrophils, macrophages, and dendritic cells, which serve to control local infection and initiate the adaptive immune response[4]. Subsequently, the adaptive immune response, consisting of CD4+ and CD8+ T cells and B cells, helps mediate microbial clearance[5,6]. Proper function of adaptive immunity is vital for control of infection, and individuals with genetic (e.g. SCID) or acquired (e.g. HIV) deficiencies in T or B lymphocyte responses can easily succumb to fatal opportunistic infection. It is

therefore not entirely surprising that the impairment of T and B cell responses is central to the persistence of viral pathogens.

CD4+ and CD8+ T cells are key for proper immune function. Upon encounter with microbial products displayed by innate antigen presenting cells (APCs) on major histocompatibility (MHC) protein complexes (termed priming), a T cell is activated and undergoes a complex intracellular program that leads to rapid expansion, maturation, and antimicrobial function[7]. Additional signals during priming sent either by the APC or present within the immune environment are critical to direct T cells to the appropriate response for the situation. Upon activation, CD8+ T cells become cytotoxic and can sense infected host cells via the interaction of their T cell receptor (TCR) and MHC displaying microbial products[5]. This interaction leads to the release of cell death-inducing proteins that directly kill the infected host cell as well as proinflammatory cytokines such as interferon gamma that help bolster the immune response. CD4+ T cells have a wide array of potential functions depending on the signals received during priming. Broadly, they can transform into a multitude of lineages capable of orchestrating the immune response to a diverse group of pathogens (bacterial, viral, parasitic), may suppress other immune responses in appropriate contexts, or provide signals to help B cells produce antimicrobial antibodies[8].

However, certain pathogens are able to resist the adaptive immune response and establish persistence in their host. Persistent viruses cause dramatic alterations in the immune environment, including increased immunosuppressive signaling by factors such as IL-10 and PD-1[9,10], the disruption of lymphoid architecture, and alterations in the quality of APC signaling[11]. These changes limit the function of CD4+ and CD8+ T cells activated at the onset of persistent viral infection, and they progressively lose their ability to control viral replication. Recent work by our group and others has greatly advanced our understanding of this phenomenon of immune exhaustion, and more insights remain

before it can be fully understood and counteracted. Surprisingly, however, very little is known about T cell responses that are generated *de novo* during an ongoing persistent infection. Instead of developing in a healthy host and being activated at the earliest signs of infection, these T cells would be programmed, from the initial stages of their development to their activation by APCs, in a substantially altered immune environment[12]. This dissertation explores how an ongoing persistent infection affects T cell biology, from their earliest stages of development in the thymus to the responses that they generate to the either primary infecting pathogen or a challenge with different secondary pathogen. The studies described here should prove valuable not only to enhance our understanding of basic T cell biology, but also to illuminate how *de novo* T cell responses can balance the increasingly dysfunctional responses generated at the onset of persistent infection and inform therapeutic vaccination and hematopoietic stem cell engineering techniques.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS IS A MOUSE MODEL FOR PERSISTENT VIRAL INFECTION

Lymphocytic choriomeningitis virus (LCMV) is a small, negative RNA strand virus of the family *Arenaviridae* (Figure 1.1A). Although it can function as an opportunistic pathogen of immunocompromised human beings, it is primarily carried and spread by rodents such as the common house mouse, *Mus musculus*. First isolated by Charles Armstrong in 1933[13], LCMV became quickly employed as a tool to study immunologic phenomena using amenable small animals as hosts. This subsequently led to profoundly momentous discoveries such as immunologic tolerance, major histocompatibility complex (MHC) restriction, and the importance of cytotoxic lymphocytes (CTLs) in controlling viral infection[14,15,16]. Importantly, serial passaging of the virus through animal hosts and subcloning of viral isolates from different organs yielded viral clones that

produced very distinct immune responses and kinetics of infection[17]. One such isolate, Clone 13 (Cl-13) differed from its parent Armstrong strain of virus in only two amino acid positions, one in the polymerase gene and one in the glycoprotein gene; however, unlike the parent Armstrong strain, which causes an acute infection that is eliminated rapidly, Cl-13 causes a long-lasting persistent viral infection characterized by functional impairment of the immune response[18,19] (Figure 1.1B). Thus, the development of the persistent Cl-13 strain and its genetic similarity to its parent Armstrong strain provided a superlative model to study the immunologic phenomena that give rise to a persistent vs. an acute infection.

Studies utilizing LCMV Cl-13 yielded the finding that persistent infection leads to the rapid impairment of virus-specific T cell responses, a phenomenon termed T cell "exhaustion." Exhaustion manifests as a loss of responsiveness to viral antigen, including reduced production of critical antiviral and immunostimulatory cytokines IFNγ, TNFα, and IL-2, reduced proliferation, weaker CTL responses, and the loss of Th1 helper T cell responses[20]. Some T cell subsets that are robust in LCMV Armstrong infection are rapidly deleted in LCMV CL-13 infection, likely due to overstimulation via the TCR[1]. Additionally, Cl-13 infected animals produce weaker B cell responses and less effective antibodies. Recent evidence demonstrates that CL-13 also leads to the dysregulation of the innate immune system, leading to the emergence of immunoregulatory APCs that send inhibitory signals to other branches of the immune response[21]. Other cell types, such as regulatory T cells[22] or macrophages may also play a role[23]. Combined, this leads to a reduced immune mediated control of viral replication and the long-term persistence of the pathogen in the host.

LCMV Cl-13 does not kill infected cells on its own and does not infect lymphoid populations apart from dendritic cells. Thus, host factors that regulate immunity to persistent infection can be studied without any serious confounding effect by the virus

itself. LCMV Cl-13 thus proved an invaluable model of persistent infection and led to the elucidation of numerous positive and negative immune regulatory mechanisms. Indeed, persistent infection is host to a network of pro and anti-inflammatory secreted cytokines that maintain the balance between viral control and potentially lethal immunopathology. The immunosuppressive cytokines IL-10 and transforming growth factor β (TGF-β) are linked to T cell dysfunction during persistent infection, and blocking their signaling *in vivo* enhances control of infection[9,24]. On the other hand, cytokines such as IL-2, IL-7, and IL-21 promote the continuing function of the T cell response[25,26,27]. Additional signals are provided by cell surface receptors. During persistent infection, signaling through PD-1 potentiates T cell exhaustion and limits T cell proliferation and suvival [10,28], LAG-3 signaling inhibits cell cycle progression[12], and TIM-3 signaling may limit CD4 Th1 responses[29]. Thus, the persistently infected immune environment is greatly altered from its normal state, and both the immunosuppressive and pro-inflammatory signals present during this state may profoundly affect T cell development and function.

LCMV continues to be broadly utilized within the scientific community, particularly as many of the immunoregulatory mechanisms discovered in persistent LCMV infection have proven to be relevant in human persistent infections such as HIV and HCV[1,2]. Therapeutic interference with some of the factors and signaling pathways described above has led to enhanced clearance of persistent LCMV infection, and clinical trials are currently ongoing to extend these findings to develop immunotherapies for human persistent infections[1]. Our growing understanding of the immune environment during persistent LCMV infection and the numerous biological and genetic tools available to study and dissect the LCMV-specific response make this a particularly attractive model to study T cell development and function *in vivo*.

DEVELOPMENT OF T CELLS FROM EARLY HEMATOPOIETIC PRECURSORS OCCURS WITHIN THE THYMUS

The journey of a T cell from hematopoietic precursor to a mature cell is fraught with difficulty and peril. The goal of the host immune system is to generate mature T cells that have the ability to function and respond to a large array of potential microbial antigens. However, T cells that respond to self antigens could cause autoimmune pathology and must thus be eliminated before they can mature. The thymus is the primary site of T cell development in mammals, and is therefore responsible for providing the appropriate microenvironment to guide T cell progenitors to maturation as well as weeding out defective or self-reactive cells. In an adult individual, early T cell progenitors migrate to the thymus from the bone marrow via the blood[30]. Once settled in the cortico-medullary junction of the thymus, these cells, now called thymocytes, begin to receive signals through chemokines and cell surface protein interactions such as Delta/Notch that promote their commitment to the T cell lineage[31]. At this point, thymocytes are ready to undertake the next steps toward maturation (Figure 1.2).

Thymocytes can be subdivided according to their expression of CD4 and CD8 surface proteins, which serve as a useful indicator of their developmental progress. The most immature thymocytes express neither CD4 nor CD8 and are termed double negative (DN). Recombination activating gene 2 (RAG2) is activated during this stage and results in the rearrangement and expression of the β chain of the TCR[32]. Due to the necessity of producing a highly diverse TCR repertoire, RAG2-mediated DNA rearrangement does not always produce an in-frame, working transcript. However, DN thymocytes that perform a successful, functional rearrangement of TCRβ become CD4/CD8 double positive (DP) cells and begin rearranging the TCRα chain[33]. TCR$\alpha\beta$ forms the heterodimeric antigen receptor for T cells, and DP cells must express a functional receptor to survive at this stage of development. DP thymocytes interact with

MHC complexes thymic epithelial cells within the thymic cortex. Successful TCR/MHC interactions send pro-survival signals to DP cells and allow their continued maturation (a phenomenon termed positive selection), whereas DP cells that don't express an MHC binding TCR complex die of neglect[31]. Additionally, thymocytes expressing a TCR that binds too tightly to MHC receive apoptosis promoting signals and die, preventing the generation of self-reactive T cells. This latter mechanism, termed negative selection, continues to weed out self-reactive thymocytes after they mature into the CD4 or CD8 single positive (SP) stage and migrate into the thymic medulla[34]. The small minority of thymocytes that survive the rigorous checkpoints and selection mechanisms of the thymus exit into the periphery as mature, naïve T cells.

The thymus is responsible for providing the appropriate microenvironment for T cell development, and crosstalk between thymocytes and thymic epithelium is necessary for the survival and maintenance of both populations[34,35]. Chemokines expressed by thymic epithelium and thymus-resident dendritic cells and macrophages mediate appropriate thymocyte migration[31]. Lymphotoxins, glucocorticoids, thymic hormones, Delta ligands, and cytokines are among some of the factors that are produced in context-dependent fashion within the thymus and support thymocyte survival[36,37,38,39]. It is therefore not surprising that the complexity and sensitivity of this environment can be disrupted during microbial infection. Indeed, accelerated thymic atrophy is commonly seen in a number of viral (e.g. HIV, rabies), parasitic (e.g. *T. cruzii, Schistosoma mansoni*), and bacterial (e.g. *Francisella tularensis*) diseases[40]. Although the mechanisms are disease-specific, thymic atrophy secondary to infection typically manifests as massive apoptosis of the sensitive DP thymocyte population.

The mechanisms underlying thymic atrophy vary according to pathogen, but can involve increased glucocorticoid levels, tumor necrosis factor (TNF) signaling, microbial factors, changes in cytokine secretion, and direct damage to the thymic epithelial

network[41,42,43]. Furthermore, this disruption can result in the release of immature thymocytes into the periphery, thus bypassing negative selection and generating a potential autoimmune T cell repertoire[44,45]. Because proper thymic function is vital for generating a diverse peripheral T cell repertoire and restraining the formation of self-reactive T cells, it is critical to understand how persistent viral infection affects the thymus and the T cell development pathway. Using LCMV as a model of persistent infection, we explored the mechanisms underlying virus-induced thymic atrophy and the effect on developing T cell responses (Chapter 2).

CD4 T CELLS ORCHESTRATE IMMUNITY

T cells that emerge from the thymus circulate within the circulatory and lymphoid system as naïve cells without effector function. During their circulation, T cells enter lymphoid organs (e.g. lymph nodes and the spleen) for brief periods of time to survey local APCs for potential microbial antigens presented on MHC. If a naïve T cell encounters an antigen it recognizes through its TCR, and receives additional proinflammatory signals from the APC, it becomes activated. This cell subsequently undergoes effector differentiation and numerous rounds of division to produce a response capable of responding to the microbial challenge. In the case of CD4 T cells, this response is uniquely tailored and highly optimized to the biology of the invading pathogen. This is in consequence to the fact that CD4 T cells can initiate upon activation a diverse and distinct array of cellular programs, termed lineages, each of which can differentially coordinate various arms of the immune response[8]. The following section will focus on the diversity of CD4 T cell lineages, their function, and how they are affected by persistent infection.

As a whole, CD4 T cells can orchestrate virtually all other branches of the immune response. They provide help to B cells, resulting in sustained and more specific

antibody responses, sustain the function of CD8 T cell effectors, boost macrophage phagocytic and lytic function, recruit other leukocytes to sites of inflammation, help tailor the appropriate response to bacterial, viral, fungal, or parasitic pathogens, directly kill infected cells, and finally, can also serve to suppress undesired immune activation[6,8]. This wide array of functions is feasible because activated CD4 T cell responses fall into distinct groups, or lineages, that secrete different patterns of cytokines and home to different sites within the organism to mediate their response (Figure 1.3). This notion of separate CD4 T cell lineages was originally demonstrated with the identification of two separate mature CD4 T cell populations that produced different cytokines (IFNγ vs. IL-4) and had different functions[46]. It was subsequently shown that naive CD4 T cells could induced to differentiate into one of these lineages with appropriate culture conditions and cytokine signals[47,48]. Currently, a number of different lineages with unique cytokine, functional, and transcriptional profiles have been identified. T helper 1 (Th1) lineage cells produce IFNγ, TNFα, and IL-2, and induce more potent activity of macrophages and CD8 T cells[49,50]. Th2 lineage cells produce several cytokines such as IL-4, which induces IgE class switching in B cells, IL-5, which recruits eosinophils, and IL-13, which has anti-parasitic activity[51,52,53]. Th17 cells produce IL-17, IL-21, and IL-22, which are highly proinflammatory and help combat bacterial and fungal challenge[54]. The existence of another lineage, termed Th9, has been proposed recently. Th9 cells produce IL-9 and have been proposed to play a role in airway inflammation and tumor immunity[55]. The ability of a naïve CD4 T cell to differentiate into multiple lineages helps the immune system combat a wide array of pathogens.

Our understanding of CD4 T cell responses has only deepened recently with the identification of the induced regulatory T (iTreg) and follicular helper T (Tfh) cell lineages. iTregs are immunosuppressive CD4 effectors, and help mediate tolerance to self-antigen and to tune down existing immune responses via the secretion of IL-10 and TGF-β[56]. Of

note, iTregs form after the activation of a naïve CD4 T cell in the periphery under proper conditions, whereas a similar CD4 T regulatory population, natural regulatory T cells (nTregs) are generated in the thymus. Tfh cells express the CXCR5 chemokine receptor, which allows them to home to B cell follicles in lymphoid organs and provide B cell help[57]. However, Tfh cells are able to secrete Th1, Th2, or Th17 cytokines depending upon the inflammatory environment, and Tregs require the same molecular transcriptional programs as Th1, Th2, and Th17 cells for appropriate regulatory activity[8]. This indicates that there is overlap in the molecular programs between CD4 T cell effector lineages imparts a certain degree of potentially useful plasticity. Thus, naïve CD4 T cells can adapt to the appropriate immunological environment depending on the signals received.

The signals required for Th lineage specification are frequently closely linked to the signals that Th lineage cells produce themselves. For instance, Th1 cells produce and require IFNγ for efficient differentiation, whereas Th2 cells produce and require IL-4[8]. Lineage specification occurs when proper signals are sent through cell-surface receptors and initiate one of several distinct transcriptional programs. By far the most important mediators between signaling and downstream transcription events are the signal transducer and activator of transcription (STAT) proteins, which relay signals from cell surface receptors to the nucleus. Signaling through STAT1 and 4 proteins (triggered by IFNγ and IL-12) positively regulates the transcription factor Tbet and helps program Th1 responses[48,58]. STAT 3 promotes Tfh and Th17 formation[59,60], whereas STAT6 transmits IL-4 signals to program the Th2 cell fate through the transcriptional regulator GATA3[61]. IL-2 signaling via STAT5 contributes to the differentiation of multiple lineages, particularly Tregs, but also represses the transcriptional regulator Bcl6 and subsequent Tfh formation through the induction of transcriptional regulator Blimp1[62]. The overlap between cytokines required for Th lineage specification and the cytokines produced by

the lineage might help amplify the lineage response. Additional signals sent by APCs and other cell types via e.g. cell surface receptor interactions also help specify lineage fate. In this manner, different signals derived from immune and non-immune sources collaborate to guide the CD4 T cell response down the appropriate path.

CD4 T cell responses are critical to sustain immunity during persistent viral infection, and robust CD4 T cell responses correlate with enhanced control of both HIV and HCV[63,64]. In both diseases, as well as in LCMV, virus-specific CD4 T cell responses primarily differentiate into Th1 and Tfh effector lineages, although Th1 responses are gradually lost in favor of Tfh responses as infection progresses[65,66,67]. In the LCMV model of persistent infection, CD4 T cells are necessary to sustain antiviral B cell responses[65,68], consistent with their role as Tfh. Paradoxically, CD4 T cells have also been implicated in sustaining antiviral CD8 T cell responses, an effect mediated by the secretion of IL-21[25]. During persistent infection, IL-21 is primarily produced by Tfh cells[65], and it is therefore currently unclear whether Tfh cells play a non-canonical role in sustaining CD8 immunity in the absence of Th1 responses. It is also unclear whether antiviral CD4 T cells undergo exhaustion in the same manner as their CD8 T cell counterparts. In CD8 T cells, exhaustion is defined as the loss of proliferative potential, cytotoxicity, and production of IFNγ, TNFα, and IL-2. Although CD4 T cells during persistent infection proliferate slowly and lose the ability to secrete these cytokines[69], this may be representative of a shift from Th1 to Tfh immunity rather than true dysfunction. However, as demonstrated in this dissertation, interference with the molecular pathways underlying exhaustion can restore cytokine secretion and proliferation in CD4 T cells without affecting Th differentiation, suggesting that a combination of both Th1 loss and exhaustion contributes to the observed phenotype of CD4 T cells during persistent infection.

As described above, persistent infection drastically alters the immune environment, and CD4 T cells are especially sensitive to signals received during activation and priming, as they rely on these to instruct their future effector fate. In Chapter 3 of this dissertation, we explore how naïve T cells introduced into and primed in a persistently infected environment exhibit alterations from their normal differentiation program. In addition, we assess the mechanisms responsible for these changes and explore how to restore normal effector differentiation in CD4 T cells primed during persistent infection.

CD8 T CELLS RECOGNIZE AND KILL INFECTED CELLS

Similarly to CD4 T cells, new CD8 T cells that emerge from the thymus circulate within the blood and lymphatic tissue until they encounter microbial antigen presented on MHC calls I by an APC. Encounter with a stimulatory APC sends proliferative signals to the newly primed cell, resulting in a dramatic 500,000-fold expansion[70]. As with CD4 T cells, cytokine and cell surface receptor signals from the APC and the environment are necessary for proper CD8 T cell function. The high rate of proliferation is dependent upon an increase in cellular metabolic activity, which is in turn dependent on the activation of the target of rapamycin (TOR) complex[71]. Costimulatory signaling by the APC via B7 proteins, 4-1BB, CD27 or OX-40 can provide this effect[72], although sufficiently strong TCR signaling or APC-independent signaling through T cell pathogen-associated molecular pattern (PAMP) receptors such as toll-like receptor 2 (TLR2) may activate the mTOR pathway without costimulation[73]. Finally, pro-inflammatory cytokines such as IL-2, IL-12, IL-21, or IL-27 can contribute to expansion[5]. Overall, the early events of CD8 T cell priming ensure sufficient proliferation and expansion that is necessary for these cells to mediate their effector function.

After priming and expansion in the lymphoid organs, activated effector CD8 T cells (CTLs) home to sites of inflammation. The main role of a CTL is to recognize and kill host cells infected with an intracellular pathogen. Whereas the TCR of CD4 T cells engages with MHC class II, whose expression is restricted to APC populations and B cells, the CD8 T cell TCR is specific for MHC class I, which is expressed ubiquitously by most cells of the body[5,7]. MHC class I presents protein fragments derived from the cytoplasm, including pathogen antigens. TCR ligation on the CTL thus sends a signal that the interacting cell is infected, leading to the release of the perforin and granzyme by the CTL. Perforin forms pores within the target cell, while granzyme enters through the pores and induces cell death. Additionally, CTLs secrete IFNγ and TNFα, which increase local inflammation and produce other effector molecules such as cell surface receptor FAS, which enhance their killing activity. Many of the local functions of a CTL within the tissue, including additional proliferation, are enhanced by CD4 T cell responses and tissue-resident APCs[8]. Interestingly, in addition to their proinflammatory role, CTLs are also capable of producing the immunosuppressive cytokine IL-10 during some types of infection[74]. This effect is not due to the formation of a unique IL-10 secreting lineage of CD8 T cell effectors, but is rather a transient and reversible ability of normal CTLs to limit local immunopathology[75]. After an infection has been cleared, the majority of pathogen-specific CD8 T cells die, with 5-10% of the responders surviving for prolonged periods as memory CD8 T cells.

Unlike CD4 T cells, CD8 T cells do not differentiate into discrete lineages with specific and unique functions. There is however a lineage specification event that occurs early during CD8 T cell effector differentiation that determines whether the cell and its progeny will die after the pathogen is eliminated or form a long-lived memory population that can combat the pathogen in the case of re-infection (Figure 1.4A). The short-lived effector cells (SLEC) represent the majority of the CD8 T cell response, especially in the

peripheral organs, and have more potent killing and cytokine secretion activity than the memory precursor effector cell (MPEC) population, although this latter population is capable of CTL antimicrobial activity. The specification of an SLEC or MPEC lineage occurs early during development, possibly as early as the first division of a primed CD8 T cell (Figure 1.4B)[76]. However, extrinsic factors can modulate lineage commitment and control the size of the SLEC and MPEC response. IL-2 is a critical factor in controlling the balance of effector vs. memory precursor populations, with higher levels of IL-2 signaling strongly promoting SLEC differentiation while suppressing MPEC formation[77,78]. IL-2 signaling through its receptor activates Blimp1 expression via STAT5, which enhances effector-associated transcriptional regulators Tbet and Id2 while suppressing memory-associated transcriptional regulators Eomesodermin (Eomes) and Bcl6. This presents an interesting parallel to B cell differentiation, where the Blimp1/Bcl6 axis also controls effector and memory formation, as well as in CD4 T cells where the same axis specifies peripherally acting Th lineages vs. lymphoid-resident Tfh cells.

Interestingly, CD8 T cell exhaustion and effector vs. memory precursor differentiation may be intertwined during persistent infection. The enhanced expression of multiple inhibitory receptors such as PD1 on CD8 T cells is associated with SLEC populations. Although SLECs normally have very robust CTL activity, their expression of inhibitory molecules may make them more sensitive to downregulation in scenarios where the pathogen persists[2]. Indeed, animals haploinsufficient for Blimp1 were able to mount more effective antiviral responses and clear persistent infection faster than wild type mice, suggesting a link between the degree of SLEC differentiation and loss of function[79]. MPEC formation is also affected during persistent infection, with MPECs being generated during persistent infection but disappearing after several weeks. The resulting effector population that survives long-term during persistent LCMV infection is completely dependent on antigen stimulation for survival[80]. Unlike true memory cells,

which require IL-7 and IL-15 but no antigen stimulation for long-term survival, effector CD8 T cells removed from persistently infected animals and placed into an antigen-free environment rapidly die[1]. This demonstrates that persistent infection completely disrupts memory potential in antiviral CD8 T cell effectors, and that alternate mechanisms come into play to maintain an antiviral response in the face of persistent infection. Given that persistent infection alters the differentiation and function of CD8 T cell responses primed at the onset of infection, we also assessed CD8 T cell responses primed in the midst of persistent infection. Chapter 4 of this dissertation covers how priming during persistent infection alters CD8 T cell biology.

TYPE I INTERFERON MODULATES IMMUNE RESPONSES

Type I interferons (IFN) are a family of homologous cytokines that are secreted in response to infection or inflammation and help control viral infection, potentiate anti-tumor immunity, and modulate the immune system in a wide variety of infectious conditions[81,82]. There are many subtypes of type I IFNs, but they all bind the type I IFN receptor, which is broadly expressed on immune and non-immune cells; however, IFN-I may exert differential effects on a cell depending on the type of cell that receives the signal and the state of the immune environment. This is due to the ability of IFN-I signaling to activate a number of different intracellular programs, and the susceptibility of IFN-I signaling to crosstalk through other signaling pathways[81] (Figure 1.5A). Like many cytokines, IFN-I activates a STAT signaling cascade upon binding the INF-I receptor, with the primary signaling complex consisting of an activated STAT1/2 heterodimer complexed with IRF 9 (termed ISGF3)[81]. ISGF3 serves as a transcriptional activator, binding to elements in the promoters and enhancers of IFN-stimulated genes and supporting their expression. However, ISGF3 is not fully active on its own, and requires additional phosphorylation from the protein kinase C pathway[83], as well as the

expression of appropriate transcriptional co-activators and chromatin modifying proteins[84,85]. Additionally, IFN-I signaling may induce other STAT complexes, as well as STAT-independent cellular programs through C3G and p38[81]. This array of potential signaling pathways and the crosstalk between them underscores the complexity of IFN-I mediated signaling and explains how IFN-I can have a multitude of biological effects (Figure 1.5B).

The oldest known and best-studied effect of IFN-I is its ability to directly repress viral replication by inducing cellular factors that directly interfere with the transcription of viral genes and the formation of viral proteins[86]. However, it is becoming increasingly clear that IFN-I can modulate both innate and adaptive immune responses to alter their antimicrobial properties[87]. Mice deficient in IFN-I signaling are more susceptible to infection by a variety of pathogens, including LCMV, and demonstrate multiple defects in T and B cell responses[88]. Indeed, in the context of multiple infections, IFN-I helps stimulate adaptive immunity both through direct action on CD4 and CD8 T cells as well as indirect action on macrophages and DCs, resulting in more potent T cell responses[88,89,90]. In the case of CD4 T cells, IFN-I has been demonstrated to enhance Th1 lineage commitment through STAT4-mediated signaling, thus potentiating the CD4 T cell response to intracellular pathogens[91]. In certain models of infection, CD8 T cells require IFN-I for proper proliferation and effector function. It is therefore telling that many adjuvants used to potentiate vaccine effects depend on IFN-I signaling for proper function[87]. Finally, IFN-I is necessary for the activity of natural killer (NK) cells, which help control the replication of intracellular pathogens until the adaptive immune response is properly established[86]. Thus, IFN-I serves as a broad inflammatory and immunostimulatory signal in response to microbial infection and is necessary to guide a nascent immune response to purge infection.

Interestingly, recent research is increasingly beginning to uncover an immunosuppressive role for IFN-I in certain contexts (Figure 1.5B). IFN-I enhances the susceptibility of animals to a number of bacterial infections, including Listeria monocytogenes, Francicella tullarensis, and Mycobacterium tuberculosis[82]. This susceptibility is due to IFN-I-mediated apoptosis and dysregulation of innate phagocytic and APC populations and the suppression of Th17 and Th1 CD4 T cell responses[92,93]. In addition to suppressing effector CD4 T cell responses, IFN-I may also promote the generation of IL-10-producing regulatory T cells, which can exert additional immunosuppressive effects within an infected immune environment[94]. The ability of IFN-I to stimulate proinflammatory CD4 T cell responses in some cases[91] and suppress in others[93] is unclear; however, as stated above, the intracellular signaling programs initiated by IFN-I are susceptible to crosstalk from multiple signaling pathways. It is therefore reasonable to hypothesize that this differential effect is due to other factors within the immune environment in these disparate infectious scenarios. Perhaps the most striking example of this disparity is the clinical use of IFN-I to stimulate immune responses against hepatitis C infection, as well as to suppress T cell responses in autoimmune conditions such as multiple sclerosis[95,96]. Additionally, the duration and magnitude of IFN-I signaling may differentially affect target responses. For instance, low level exposure of lymphocytes to IFN-I leads to increased susceptibility to proinflammatory IFNγ signaling and increased IFNγ upon activation, whereas long lasting IFN-I exposure at high levels produces the opposite effect[82]. This may explain why elevated IFN-I levels in persistent infections such as HCV and HIV are associated with increasing levels of immune dysfunction, and why transiently blocking IFN-I signaling during persistent LCMV infection leads to enhanced clearance[97].

This dissertation explores the effect of IFN-I signaling during persistent infection, and how IFN-I affects the development and function of T cell responses emerging during

persistent viral infection. In chapter 2, we demonstrate that IFN-I has a disruptive effect on thymopoiesis, and in chapters 3 and 4, we explore how IFN-I changes the function of CD4 and CD8 T cell responses primed in the midst of persistent infection. Overall, our findings seem to suggest that IFN-I plays a critical role in regulating immunity during persistent viral infection.

AIMS OF DISSERTATION

This dissertation explores the mechanisms by which de novo T cell development, activation, and function occurs in the midst of persistent infection, and how these parameters differ from the naïve environment or the onset of infection. Chapter 2 assesses how persistent viral infection impacts thymopoiesis, including the effects on various stages of T cell development and how this may impact the seeding of the peripheral compartment with new virus-specific T cells. In chapter 3, we examine the activation and priming of naïve virus-specific CD4 T cells in a persistently infected immune environment, and how their subsequent differentiation and function is altered from virus-specific CD4 T cells activated at the onset of infection. Chapter 4 explores a similar question with virus-specific CD8 T cells. Overall, this dissertation sheds insights into the regulation of *de novo* immunity during an established persistent infection.

Figure 1.1: Lymphocytic choriomeningitis virus can cause acute or persistent infection.

(A) Schematic representation of lymphocytic choriomeningitis virus. LCMV is a small, enveloped arenavirus. The viral particle consists of surface glycoproteins (G), which serve as receptors for viral binding and entry, the viral genome, which is composed of two ambisense RNA strands bound to viral nucleoprotein, the L protein (a viral RNA polymerase that reverse-transcribes the viral genome into mRNA), and oligomeric Z protein which is necessary for budding and virion integrity.

(B) Schematic demonstrating infection by LCMV strains Armstrong and Clone 13. Armstrong causes a short-lasting acute infection that is rapidly cleared by robust CTL and CD4 helper responses, whereas Clone13 induces global immunosuppression and causes a long-lasting persistent infection.

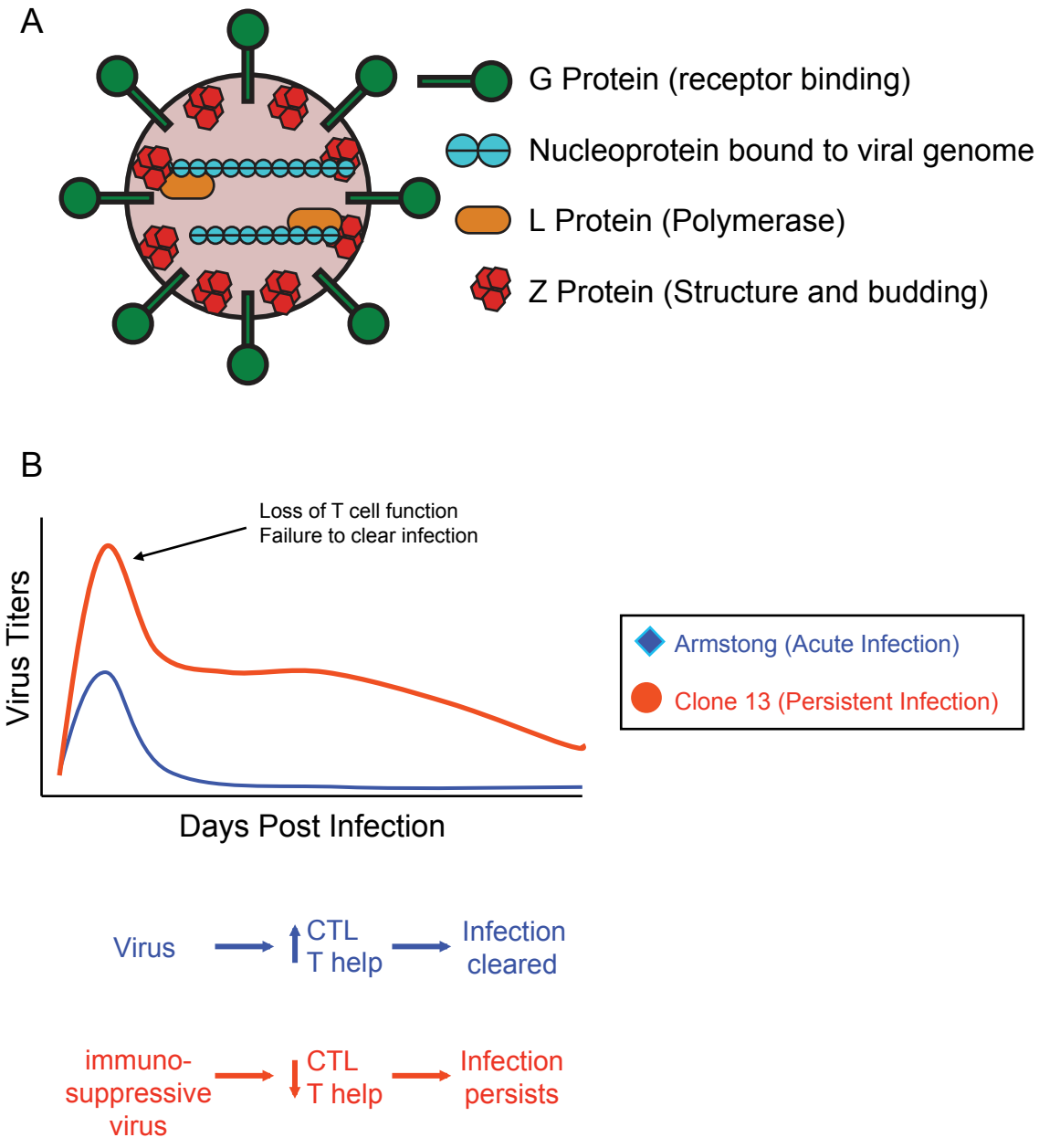

A
G Protein (receptor binding)
Nucleoprotein bound to viral genome
L Protein (Polymerase)
Z Protein (Structure and budding)
B
Loss of T cell function
Failure to clear infection
Virus Titers
Days Post Infection
Armstong (Acute Infection)
Clone 13 (Persistent Infection)
Virus
CTL
T help
Infection
cleared
immuno-
suppressive
virus
CTL
T help
Infection
persists

Figure 1.2: Hematopoietic precursors mature into T cells within the thymus.

Hematopoietic T cell progenitors enter into the thymus from the vasculature and migrate into the subcapsular region (left) to begin the developmental process. Once in the thymus, these progenitors become CD4-/CD8- double negative (DN) thymocytes and begin to rearrange a functional T cell receptor (TCR). DN thymocytes complete their TCR rearrangement while migrating into the thymic cortex (middle) where they begin to express the CD4 and CD8 co-receptor molecules to become CD4+/CD+ double positive (DP) thymocytes. Within the cortex, DP thymocytes interact with cortical thymic epithelial cells (cTEC), which promote their survival if they express a functional TCR (positive selection), or send death signals if the thymocytes bind too strongly and are potentially self-reactive (negative selection). Having passed the positive selection checkpoints in the cortex, thymocytes migrate into the medulla and lose expression of either CD4 or CD8, becoming CD4+ or CD8+ single positive (SP) thymocytes (no distinction between the two is made in this schematic). In the medulla, SP thymocytes interact with medullary thymic epithelial cells (mTEC), which express the AIRE transcription factor. AIRE allows mTECs to express proteins which would otherwise not be present within the thymus, thus allowing mTECs to weed out additional potentially self-reactive thymocytes. Thymocytes that survive the positive and negative checkpoints within the thymus or medulla exit the thymus as mature CD4 or CD8 T cells.

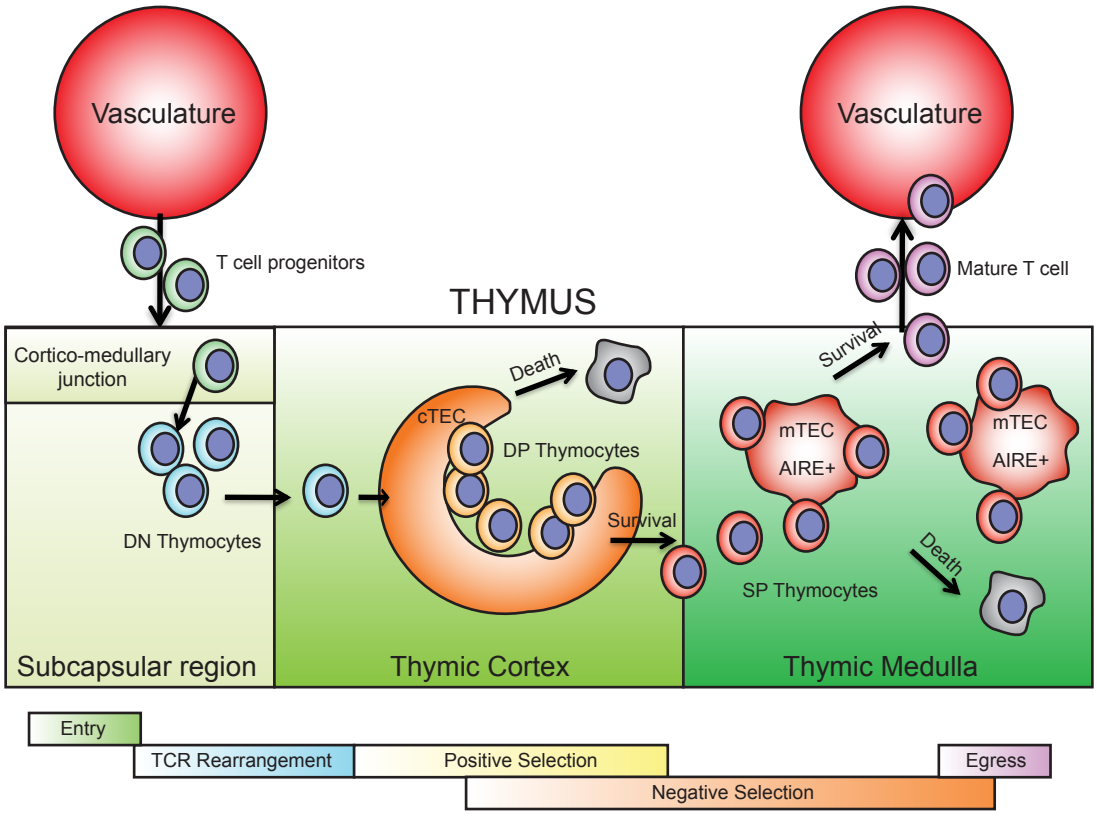

Vasculature
T cell progenitors
THYMUS
Vasculature
Mature T cell
Cortico-medullary junction
DN Thymocytes
Death
cTEC
DP Thymocytes
Survival
Survival
mTEC
AIRE+
mTEC
AIRE+
SP Thymocytes
Death
Subcapsular region
Thymic Cortex
Thymic Medulla
Entry
TCR Rearrangement
Positive Selection
Egress
Negative Selection

Figure 1.3: CD4 T cell effector function is guided by lineage differentiation.

A naïve CD4 T cell (center, gray) encounters an antigen presenting cell (center, red) and additional instructive signals that induce effector differentiation. The most well-studied CD4 T cell lineages are listed here, along with the typical profile of cytokines that they produce and their general function within an immune response.

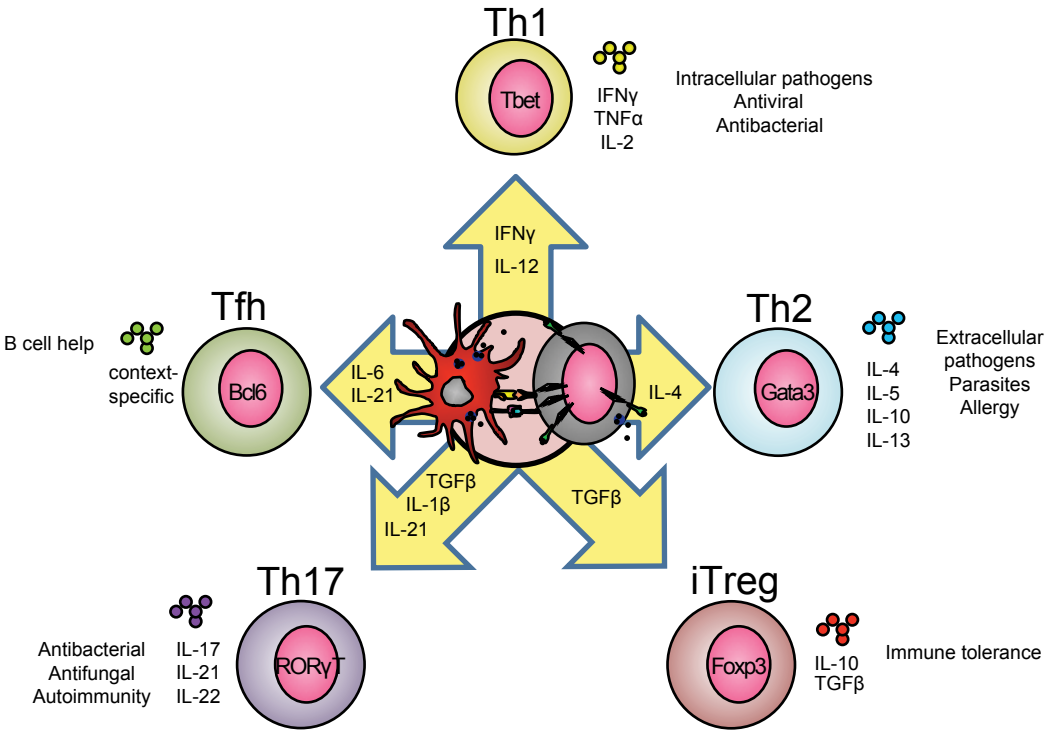

Th1
Tbet
IFNγ
TNFα
IL-2
Intracellular pathogens
Antiviral
Antibacterial
IFNγ
IL-12
Tfh
B cell help
context-
specific
Bcl6
IL-6
IL-21
IL-4
Th2
Gata3
IL-4
IL-5
IL-10
IL-13
Extracellular
pathogens
Parasites
Allergy
TGFβ
IL-1β
IL-21
TGFβ
Th17
Antibacterial
Antifungal
Autoimmunity
IL-17
IL-21
IL-22
RORγT
iTreg
Foxp3
IL-10
TGFβ
Immune tolerance

Figure 1.4: CD8 T cells commit to the effector or memory lineages.

(A) An activated naïve T cell divides to form short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). Although both have effector cytotoxic and cytokine production activity, the SLECs are more potent effectors. However, after termination of the immune response, the SLEC response contracts as SLECs undergo programmed cell death (apoptosis), whereas MPECs form long-lasting memory.

(B) Schema of CD8 T cell expansion and differentiation over the course of the immune response.

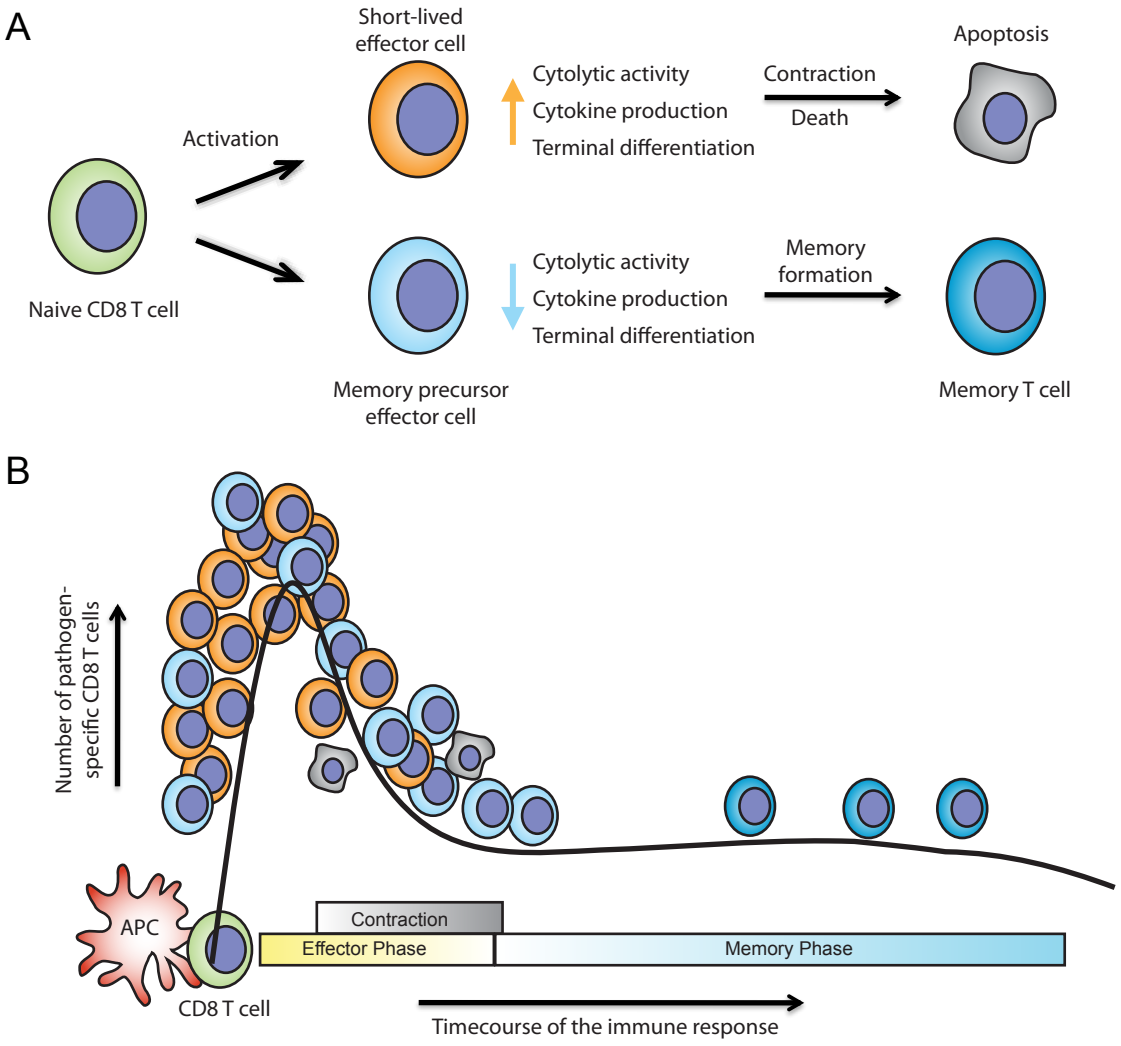
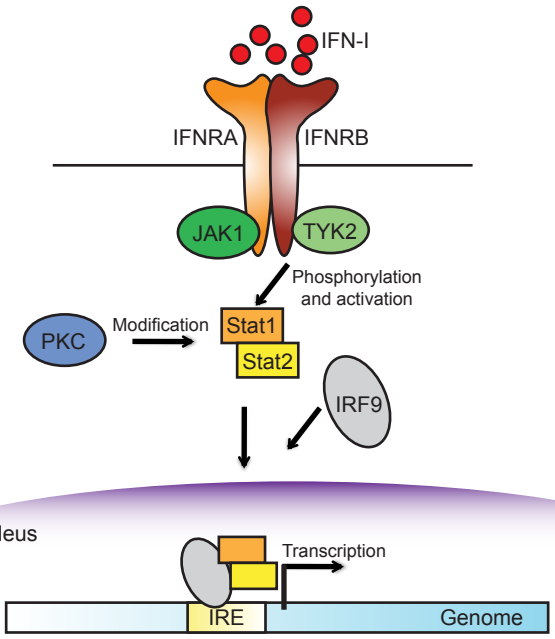

A
Short-lived effector cell
Apoptosis
Cytolytic activity
Cytokine production
Terminal differentiation
Contraction
Death
Activation
Naive CD8 T cell
Cytolytic activity
Cytokine production
Terminal differentiation
Memory formation
Memory precursor effector cell
Memory T cell
B
Number of pathogen-specific CD8 T cells
APC
CD8 T cell
Contraction
Effector Phase
Memory Phase
Timecourse of the immune response

Figure 1.5: Type I interferon signals mediate a variety of antiviral and immunomodulatory effects.

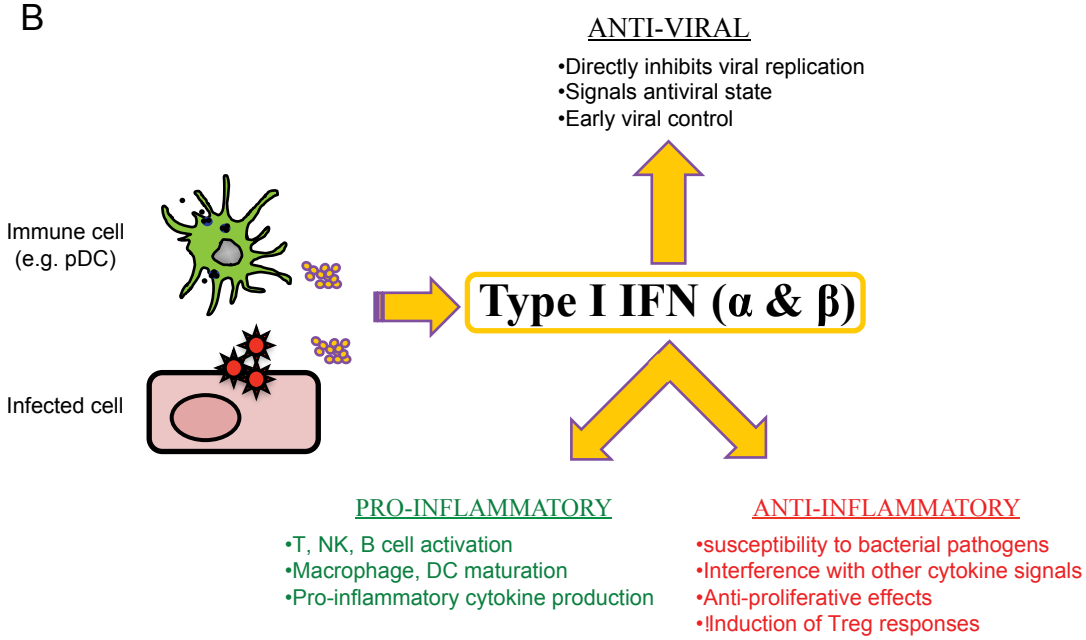
(A) Schematic representation of the main transcriptional complex induced by IFN-I signaling. IFN-I binds its receptor, which is a heterodimer consisting of the IFNRA and IFNRB chains. This activates two kinases (JAK1 and TYK2) associated with the receptor to phosphorylate the signal transducers STAT1 and STAT2, which bind each other. The STAT1 and STAT2 heterodimer subsequently binds IRF9 to form the transcriptionally active ISGF3 complex. ISGF3 translocates to the nucleus and binds interferon responsive elements (IRE) within the genome, and recruits other enhancers and chromatin modulation complexes (not shown) to initiate interferon-induced transcriptional programs.

(B) Schema of the sources and differential biological effects of IFN-I.

A


IFN-I
IFNRA
IFNRB
JAK1
TYK2
Phosphorylation
and activation
PKC
Modification
Stat1
Stat2
IRF9
Nucleus
Transcription
IRE
Genome

B


ANTI-VIRAL
•Directly inhibits viral replication
•Signals antiviral state
•Early viral control
Immune cell
(e.g. pDC)
Infected cell
Type I IFN (α & β)
PRO-INFLAMMATORY
•T, NK, B cell activation
•Macrophage, DC maturation
•Pro-inflammatory cytokine production
ANTI-INFLAMMATORY
•susceptibility to bacterial pathogens
•Interference with other cytokine signals
•Anti-proliferative effects
•Induction of Treg responses

CHAPTER TWO

CD8 T cells and type I Interferon signaling disrupt T cell development during persistent viral infection

ABSTRACT

The thymus is critical in generating and maintaining a diverse and functional T cell repertoire throughout the life of the host. A number of infectious pathogens, including persistent viral infections such as HIV, can trigger dramatic thymocyte depletion and induce thymic atrophy; however, the biological mechanism behind this depletion and the biological implications on thymic function are not well understood. In this study, we demonstrate that persistent, but not acute Lymphocytic Choriomeningitis Virus (LCMV) infection triggers a severe depletion of CD4+ CD8+ double positive thymocytes and induces a block in thymocyte development at the CD4- CD8- double negative stage. The thymic atrophy observed in persistent LCMV infection is dependent upon virus-specific CD8 T cell responses and type I interferon signaling. Importantly, although the thymic reconstitution occurred once virus-specific CD8 T cell responses exhausted, no new LCMV-specific CD8 T cells were generated. Thus, persistent viral infection negatively impacts thymic function and prevents the generation of new antiviral T cell responses.

INTRODUCTION

The thymus is the primary lymphoid organ responsible for the development and maturation of HSC-derived T cell progenitors into functional CD4+ and CD8+ T cells[31]. Interactions between developing thymocytes and elements of the thymic stroma, as well as resident antigen presenting cells (APC) eliminate clones bearing potentially harmful or non-functional T cell receptors (TCRs) while promoting the expansion and survival of thymocytes bearing potentially useful TCRs[34]. T cell precursors enter the thymus as CD4- CD8- double negative (DN) cells, and proceed to develop into CD4+ CD8+ double positive (DP) cells as they rearrange their TCR. This DP population undergoes positive and negative selection in the thymic cortex and surviving thymocytes further differentiate into CD4+ or CD8+ single positive (SP) cells, which undergo further rounds of instruction in the medulla to become mature T cells and exit the thymus[31]. The thymus is therefore critical in generating and maintaining a robust and functional pool naïve T cells and preventing self-reactive responses in the periphery.

Numerous infectious pathogens have a negative effect on thymic function, typically manifesting as a rapid and profound loss of thymic cellularity, particularly of the DP thymocyte population[40,42,44,98]. Although different pathogens induce thymic atrophy by different mechanisms, it is unclear what the overall implications of impaired thymic function are for the function of new T cells produced in the infected environment, diversity and maintenance of the T cell repertoire, and tolerance to foreign and self-antigens[40]. Importantly, therapies to combat persistent human infections, such as HIV may rely on the ability of the thymus to generate new antiviral immunity[99]. The use of hematopoietic stem-cell (HCS)-based approaches to rebuilt the immune response from the ‘ground up’ and control persistent infection have shown the promising ability to enhance the antiviral T cell pool and lead to improved control of HIV infection. However, the efficacy of this approach during an established persistent infection is unclear,

particularly following infection with viruses such as HIV that can infect and dramatically disrupt thymic architecture and deplete developing thymocytes. Even in situations wherein virus replication is controlled by effective antiviral therapies, low level of virus-replication and expression of viral peptides by thymic epithelial cells (TECs) and dendritic cells (DCs) in the thymus may lead to efficient depletion of virus-specific T cells through negative selection[31]. It thus becomes critical to understand the mechanisms by which persistent viral infection can affect the thymic function, and potential biologic and therapeutic consequences thereof.

Lymphocytic Choriomeningitis Virus (LCMV) is a model infectious agent that can induce an acute or persistent infection in mice. Infection with the LCMV variant Armstrong (Arm) induces robust T cell and B cell responses that eliminate the infection in 8-12 days leading to the generation of protective memory. In contrast, infection with LCMV-Clone 13 (Cl13) replicates to higher titers, outpacing the immune response leading to the expression of host-based regulatory factors and cell populations that suppress antiviral immunity facilitating persistent infection[1]. Multiple parallels between the immune response to LCMV and persistent viral infections in humans (e.g. HIV, Hepatitis B and C) make this infectious agent a powerful tool to dissect the immunological mechanisms of host-pathogen interaction[2]. Although it is known that LCMV can infect the thymic stroma and mediate life-long negative selection of virus-specific responses in congenitally infected mice[14], it is currently unknown how acute or persistent LCMV infection acquired during adulthood affects thymic function.

In this study we demonstrate that persistent, but not acute LCMV infection leads to profound thymic atrophy. Further, we determine that the virus specific CD8+ T cell response to LCMV within the thymus is responsible for the depletion of developing thymocytes and the dramatic reduction of thymic cellularity and function. Type I interferon signaling was also identified as a contributing factor to the destruction of the

thymus in this model. Most importantly, we found that no new virus specific CD4+ or CD8+ T cells were able to egress from the thymus of a persistently infected mouse. These data suggests that re-building the T cell repertoire through HSC adoptive transfer during a persistent viral infection will face substantial barriers that must be studied and overcome before such strategies can become feasible.

MATERIALS AND METHODS

Mice and virus

C57BL/6 (WT) mice were purchased from The Jackson Laboratory or the rodent breeding colony at University of California, Los Angeles. LCMV-GP_{33}-specific CD8 TCR transgenic (P14) mice have been described previously[100]. TNFR1 (JAX stock 003242), FasL-/- (JAX stock 001021), and OTI (JAX stock 003831) were purchased from The Jackson Laboratory. IFNR1-/- and IFNgR-/- mice were provided by Dr. Dorian McGavern. Adrenalectomized mice were obtained from The Jackson Laboratory and adrenalectomy was performed by Jackson surgical staff. All mice were housed under specific pathogen–free conditions. Mouse handling conformed to the experimental protocols approved by the University of California, Los Angeles Animal Research Committee (ARC). In all experiments the mice were infected i.v. via the retroorbital sinus with 2×10^6 plaque forming units (PFU) of LCMV Armstrong or LCMV Cl13. Virus stocks were prepared and viral titers were quantified as described previously[100].

Isolation and adoptive transfer of virus-specific T and B cells

LCMV-specific P14 cells were isolated from the spleens of transgenic mice by negative selection (StemCell Technologies). To assess virus-specific CD8 T cell migration into the thymus, 1,000 P14 cells were transferred i.v. into the retroorbital sinus prior to LCMV infection.

***In vivo* CD8 depletion, CD4 depletion, and NK cell depletion, and Type I Interferon Receptor (IFNR1) blockade**

To deplete CD8 T cells before LCMV infection, mice were treated i.v. with 500μg anti-CD8 antibody (BioXCell) 5 and 1 day(s) before infection and again on the day of infection. CD4 T cells were depleted with a similar strategy using 500μg anti-CD4

antibody (clone Gk1.5; BioXCell). NK cells depleted by injecting 500μg of anti Nk1.1 antibody (BioXCell) 4 and 1 day(s) before infection and days 2 and 5 post infection. Isotype control antibody was also used in control cohorts.

To block IFN-I signaling in vivo during persistent infection, mice were treated i.v. with 500μg anti-IFNR1 blocking antibody (clone MAR1-5A3; Leinco Technologies) or isotype control antibody 48 hours before infection and every 48 hours post infection through 9 days of infection.

Busulfan and Bone Marrow Transfer

C57BL/6 Ly5.2+ recipient mice were treated i.v. with 500μg anti-CD4 antibody (clone GK1.5; BioXCell) 4 days before infection with 2 x 10^6 pfu LCMV-Cl13. 30 days after infection, mice were treated i. p. with 30 mg/kg Busulfan (Sigma Aldrich) in 50% DMSO. Bone marrow was harvested from C57BL/6 Ly5.1+ donor mice and depleted of Lineage+ cells by negative selection on the autoMACS (Miltenyi Biotec). 4.5 x 10^5 Lineage negative stem cells were transferred i. v. into recipient mice 24 hours after Busulfan treatment.

Flow Cytometry

Analysis of immune subsets was performed by staining lymphocytes and thymocytes obtained from the spleen and thymus respectively ex vivo for the expression of CD4, CD8, CD25 (IL-2Rα), CD44, CD45.1 (Ly5.1), CD45.2 (Ly5.2), CD90 (Thy1.1). Peptide stimulations were performed as described previously[100]. Stimulated cells were stained intracellularly for the expression of IFNγ and TNFα. All flow cytometry antibodies were obtained from Biolegend, BD, or Ebiosciences. Endogenous virus-specific CD8 T cells

were visualized by staining with aGP$_{33}$ MHC tetramer (NIH tetramer core). Flow cytometric data was collected on the Facs Caliber and FacsVerse (Beckton Dickinson) and was analyzed using FlowJo (Treestar).

Statistical analysis

Student's *t* tests (two-tailed, unpaired) were performed using GraphPad Prism 5 software (GraphPad Software, Inc.).

RESULTS

Persistent LCMV infection rapidly induces severe thymic atrophy

LCMV can infect the thymus, but the impact of infection on thymic function is currently unknown. We therefore addressed how the acute (Arm) and persistent (Cl13) strains of LCMV affect the survival and development of thymocytes at multiple time points after infection. Both LCMV-Arm and Cl13 efficiently infect the thymus, but whereas LCMV-Arm is cleared within 9 days after infection, LCMV-Cl13 persists in the thymus at high levels up to 30 days after infection (Figure 2.1A). Although both acute and persistent infection induces a modest (~2-fold) drop in the numbers of developing thymocytes 5 days after infection, persistent, but not acute, LCMV infection leads to a dramatic loss of developing thymocytes by 9 days after infection (Figure 2.1B). This drop in cellularity is primarily due to the near-total loss of the CD4+ CD8+ double positive (DP) thymocyte population (Figure 1B and 1C). In contrast to LCMV-Arm infection, LCMV-Cl13 infected mice exhibited a slow and incomplete recovery of thymic cellularity, with the DP thymocyte population partially restored by 30 days post infection (Figure 2.1B and C). LCMV does not infect thymocytes (not shown) and therefore the thymic atrophy observed during LCMV-Cl13 infection is likely not due to the direct targeting and killing of thymocytes. Likewise, we estimate that the frequency of developing T cells bearing an LCMV specific TCR is less than a fraction of a percent, suggesting that thymic atrophy during LCMV-Cl13 infection in not a consequence of thymocyte loss due to negative selection by LCMV antigen-presenting TECs and DCs within the thymus. Overall, the profound thymic atrophy observed during persistent LCMV infection is primarily due to the loss of the DP thymocyte population.

The profound, long-term drop depletion of the DP thymocyte subset in the persistently infected thymus suggests disruption of T cell development at or before the DP stage. To

determine whether a developmental arrest occurs before the DP stage, we assessed the precursor CD4- CD8- double negative (DN) population. We observed that every subset of the DN population (DN1 (CD44+ CD25-), DN2 (CD44+ CD25+), DN3 (CD44- CD25+), and DN4 (CD44- CD25-), in their respective developmental order) experiences a large drop in total cellularity during Cl13 infection, with the greatest proportionate losses occurring within the DN2 and DN3 stages (Figure 2.2A and B). At Day 9 of Cl13 infection there is a substantial increase in the frequency of DN1 thymocytes within the DN compartment with correlating decreases in the DN2, DN3, and DN4 frequencies (Figure 2.2C). Although the initial loss of the DP population occurs through apoptosis at the DP stage (not shown), this data indicates that thymocyte development beyond the DN1 stage is not supported during Cl13 infection and replenishment of the DP population may not be occurring until the DN subset begins to recover.

Virus-specific effector CD8+ T cells home back into the thymus and mediate thymic atrophy

Peripheral effector CD8+ T cells are capable of homing back into the thymus to combat an infection and take up residence within the tissue as memory cells after pathogen clearance. To track and analyze the function of the peripheral virus-specific CD8+ T cell response within the thymus, we adoptively transferred LCMV-specific TCR-transgenic P14 cells prior to LCMV infection. We observed that virus-specific CD8+ T cells enter the thymus during both acute and persistent LCMV infection and are maintained over a long-term period (Figure 2.3A and B). Unlike LCMV Armstrong infection, LCMV Cl13 infects the thymus for an extended period (Figure 2.1), and maintenance of virus-specific responses during LCMV Cl13 infection is dependent on antigen signaling[1]. This indicates that virus-specific CD8 T cells continue to combat virus within the thymus throughout the course of persistent infection. As persistent infection progresses, virus-specific CD8 T

cells lose proliferative and cytolytic function, as well as the ability to secrete the antiviral cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), a phenomenon termed exhaustion[1]. We evaluated the function of thymic-resident virus-specific CD8 T cells and observed they also become exhausted during the course of persistent infection, losing the ability to secrete IFNγ and TNFα in stark contrast to thymic resident CD8+ T cells in acute infection (Figure 2.3C). Interestingly, exhaustion of thymic-resident virus-specific CD8 T cells correlates with the restoration of T cell development in the thymus (Figure 2.1). This raises the possibility that infection-induced thymic atrophy is dependent on virus-specific CD8 T cell function.

Because virus-specific CD8 T cells are capable of killing infected thymic stromal cells and disrupting the thymic microenvironment we sought to determine whether CD8+ T cells mediate thymic atrophy during persistent LCMV infection. By depleting peripheral CD8+ T cells with a CD8 T cell-depleting antibody before infection with LCMV-Cl13, we were able to establish a model of persistent infection that lacks a virus-specific CD8 T cell response. Treatment with the CD8 T cell depleting antibody removes CD8-expressing cells from the blood and peripheral lymphoid organs but does not perturb CD8-expressing developing thymocyte populations (data not shown). Strikingly, we observed that the elimination of peripheral CD8+ T cells prior to infection with Cl13 almost completely prevented the thymocyte loss observed during Cl13 infection (2.4A and B). This demonstrates that thymic atrophy during persistent infection is mediated by a CD8 T cell-dependent mechanism.

CD8 T cells can recognize viral antigen presented on MHCI, which triggers degranulation, killing of infected cells, and cytokine production. Alternatively, CD8 T cells may be activated nonspecifically by the inflammatory milieu. To determine whether

thymic atrophy occurred due to a virus-specific CD8 T cell-mediated mechanism, we infected OTI transgenic mice, which bear a CD8 restricted TCR specific for ovalbumin (OVA) and do not form a virus-specific CD8 T cell response upon LCMV infection. We found that OTI mice infected with LCMV-CL13 do not experience the massive thymic atrophy found in wild type mice (Fig 2.4C and D). Combined, our data indicates that virus-specific CD8 T cells are the primary mechanism driving thymic atrophy during LCMV-Cl13 infection.

To investigate the potential contribution of CD4 T cell and NK cell-mediated mechanisms to the thymic atrophy observed during persistent infection, we used depleting antibody treatments to eliminate CD4 T cells and NK cells prior to LCMV Cl13 infection. Lack of CD4 T cell or NK responses did not rescue the thymus from atrophy (Fig 2.5A and B), indicating that CD8 T cells are the primary immune mechanism responsible for thymocyte depletion and do not require CD4 or NK help. Virus-specific CD8+ T cells are capable of directly killing infected cells (via Perforin or FasL) or affecting the local micro-environment by the secretion of inflammatory cytokines such as IFNγ and TNFα. To dissect the mechanism of CD8+ T cell-mediated thymic atrophy, we evaluated the role of IFNγ, TNFα, Perforin, and FasL by infecting transgenic mice deficient in the aforementioned pathways. However, none of the investigated pathways made a significant individual contribution to thymocyte loss during Cl13 infection (Figure 2.5C-F). The data therefore suggests that a combination of redundant effector pathways mediates CD8+ T cell-dependent thymic atrophy.

Thymic atrophy is modulated by type 1 interferon

Type I interferon (IFN-I) is an immunomodulatory cytokine produced during numerous bacterial and viral infections, including LCMV infection. Importantly, IFN-I has been

reported to cause thymocyte death during HIV infection within a human fetal thymic organ culture, and our group demonstrated that blocking IFN-I at the onset of persistent infection resulted in diminished CD8+ T cell responses. We thus sought to identify the potential contribution of IFN-I to thymic atrophy during persistent infection. We observed that thymocyte loss in response to LCMV-Cl13 infection is significantly reduced in type I interferon receptor (IFNaRI-/-) knockout mice compared to wild type mice, although some thymic atrophy still occurred (Figure 2.6A and B). We subsequently blocked IFN-I receptor (IFNR) signaling early during infection in wild type mice and similarly observed a reduced level of thymic atrophy in anti-IFNR treated compared to isotype treated animals (Figure 2.6C and D). Our data thus identifies IFN-I signaling as an important component of thymic atrophy during persistent infection.

LCMV specific CD8 T cells cannot egress from an LCMV infected thymus

The restoration of thymopoiesis by day 30 after LCMV-Cl13 infection suggests that newly generated virus-specific T cells may now emerge from the thymus and be recruited into the ongoing antiviral response. To specifically assess de novo virus-specific T cell development we generated partial bone marrow chimeras using the chemotherapeutic agent busulfan. Used at a low dose (30 mg/kg) busulfan partially depletes the bone marrow hematopoietic stem cell niche without disrupting the peripheral immune compartment, allowing for the introduction and partial engraftment of donor stem cells without the disruption of an ongoing immune response. Uninfected mice or mice 30 days into persistent infection were treated with busulfan and given lineage-depleted bone marrow. T cell responses that develop from donor stem cells can be distinguished by the expression of congenic marker Ly5.1 but are otherwise genetically identical to the host. Because of the time required to achieve full peripheral

reconstitution from the donor stem cell compartment, mice were CD4 depleted prior to LCMV infection to produce a lifelong persistent infection that does not clear.

Despite a long-term reduction in thymic cellularity, donor stem cells introduced into persistently infected mice were able to populate the peripheral T cell compartment at the same rate as uninfected mice (Figure 2.7A). Donor-derived B cells, which do not require the thymus for development and thus serve as a control for donor stem cell engraftment, repopulated the periphery at a similar but slightly reduced rate in infected mice, leading to a slightly reduced overall peripheral donor-derived lymphocyte population in infected animals (Figure 2.7B). However, despite the robust donor-derived T cell reconstitution, donor-derived virus-specific CD8 T cells failed to develop (Figure 2.7B), suggesting that persistent viral infection of the thymus may be preventing the development of additional virus-specific responses by negative selection. Overall the data indicate that thymic function during persistent LCMV infection is eventually restored; however no new antiviral CD8+ T cell responses can emerge during persistent infection.

DISCUSSION

The thymic microenvironment is specifically adapted to promote the development of T cells with a potentially useful TCR repertoire and the suppression of autoimmune responses. It is therefore not surprising that disruption of thymic function can lead to severe autoimmunity and immunodeficiency[40,101]. Moreover, a number of viral, bacterial, and parasitic pathogens are known to induce thymic atrophy, affect thymocyte development, and promote the release of immature and autoreactive thymocytes into the periphery[40,44]. Indeed, thymic atrophy has been reported during HIV infection, and the generation of new T cells is impaired in viremic individuals compared to healthy individuals or recipients of antiretroviral therapy[98,102]. The importance of maintaining and generating a functional peripheral T cell repertoire, especially in individuals who may have lost part of their CD4 T cell compartment due to HIV, prompted us to use LCMV to examine how persistent viral infection affects T cell development and thymic function.

Acute LCMV infection has been previously shown to cause a mild degree of thymic atrophy by a mechanism possibly related to lymphotoxin hormone secretion and TNF receptor signaling (although the mechanistic data are, at best, correlative)[103]. In contrast, we demonstrated that persistent LCMV infection leads to a much more profound thymic atrophy, with over a 10-fold drop in total thymocyte numbers, a near-total loss of the DP thymocyte population, and a marked defect in thymocyte development past the DN1 stage. We presume that the loss of existing DP, DN2 and DN3 thymocytes occurs through apoptosis as in other models of infection such as HIV[104]; however, we can not rule out that the DP cells instead exit the disrupted thymus to seed the periphery. From a kinetic standpoint, a hematopoietic precursor requires several weeks of thymic education to develop into a mature peripheral T cell[31]. It is thus unlikely that the profound depletion of DP thymocytes is a consequence of the disappearance of the DN2 and DN3 early progenitors. The DN2 and DN3 as well as DP

populations are present at 5 days into an LCMV Cl13 infection and disappear around the same time by day 9. Moreover, the loss of any of these populations does not occur in the absence of virus-specific CD8 T cells. Together, this suggests that a common mechanism mediates the loss of the early DN thymocytes as well as the DP thymocytes simultaneously.

Throughout the process of TCR rearrangement and positive selection, thymocyte survival is crucially dependent on the thymic epithelium. Stromal hormones and cytokines such as thymulin and IL-7, as well as Notch signaling, promote DN cell survival and proliferation, while DP cell survival is dependent on hormones and direct contact with MHC on epithelial cells[31]. LCMV Cl13, but not LCMV Armstrong, is known to disrupt stromal cell networks within lymphoid organs via direct infection[105], and our data demonstrates that LCMV Cl13 establishes a long-term infection in the thymic stroma. Interestingly, within the lymphoid organs, disruption of stromal networks contributes to the suppression of the peripheral T cell effector response. Our data raise the possibility that a similar disruption in the thymus is contributing to the accelerated thymic atrophy and interference with T cell development. Although we do not directly assess the stromal network of the thymus, we do demonstrate that thymic atrophy is dependent on virus-specific CD8 T cell responses, which would target the infected epithelial network of the thymus. Similar dependence on CD8 T cells is observed in the stromal disruption of peripheral lymphoid organs[105]. It is interesting to note therefore that thymocyte development recovers at approximately the same rate as the lymphoid architecture in the periphery, and that this recovery correlates with the exhaustion of CD8 T cell responses. We thus suggest a model of LCMV-induced thymic atrophy where the infected epithelium is targeted by virus-specific CD8 T cells, leading to disruption T cell development, which gradually recovers as CD8 T cells lose their effector capacity and the epithelium is allowed to regenerate.

Although virus-specific CD8 T cells are the ultimate cause of thymic atrophy during persistent infection, the mechanisms by which CD8 T cells mediate this atrophy are still unclear. Using mice deficient for a number of CD8 effector pathways, we demonstrated that defects in CTL activity or secretion of IFNγ or TNFα could not rescue thymic atrophy on their own. Interestingly, the TNFR1 deficient mice that we infected cannot sense lymphotoxin in addition to TNFα, indicating that lymphotoxin is not critical for thymic atrophy during persistent LCMV infection. Further experiments need to be preformed to determine whether the above effector pathways are simply redundant, or whether other factors produced by CD8 T cells (e.g. MIP1α) mediate thymic disruption. Additionally, transfer of transgenic virus-specific CD8 T cells into LCMV-infected OTI mice should be preformed to determine whether antiviral CD8 T cells are sufficient as well as necessary for thymic atrophy during persistent infection.

CD8 T cell effector function is frequently dependent on IFN-I signaling during viral infection. Early IFN-I production is necessary for proper expansion and optimal effector function of CD8 T cells, and viral clearance can be protracted if the IFN-I pathway is inhibited[88,90]. It is therefore attractive to speculate that blockade of IFN-I signaling prevents thymic atrophy during persistent LCMV infection by downregulating CD8 T cell responses. However, studies of HIV and pathogenic SIV infection have demonstrated that IFN-I may directly impair T cell development and promote thymic atrophy[106,107]. Moreover, HCV patients treated with IFN-I demonstrate reduced thymic function as a direct consequence of treatment[108]. This suggests that IFN-I may act independently of the CD8 response or that virus-specific CD8 T cells potentiate the actions of IFN-I in the thymus. The latter is more likely based on our data; as mice deficient for virus-specific CD8 T cell responses still generate copious amounts of IFN-I early during infection yet experience no thymic atrophy, thus making it unlikely that IFN-I induces thymic atrophy on its own. Mixed bone marrow chimera experiments in which a

mix of OTI bone marrow and either INFAR deficient or wild type bone marrow is transferred into wild type or IFNAR deficient mice should be able to definitively elucidate how CD8 T cells and IFN-I interact to induce thymic atrophy.

Thymic atrophy during human persistent infection has been shown to negatively impact the generation of new peripheral T cell immunity, and disruption of thymic function by parasitic infection may impair negative selection within the thymus and lead to the release of autoreactive cells to the periphery. However, our data indicate that LCMV-mediated disruption of T cell development is transient despite a lifelong persistent infection. Although the thymus never regains the same cellularity observed prior to infection, all thymocyte populations are eventually restored to their normal frequency. Furthermore, T cell development and maturation proceeds at the same rate in naïve animals and animals more than 30 days in to a persistent LCMV infection. Furthermore, no new LCMV-specific CD8 T cells are produced after thymic recovery, indicating that developing LCMV-specific precursors are being weeded out by negative selection within the highly infected thymic stromal tissue.

This lack of any new virus-specific T cell responses may be specific to the ability of LCMV to infect thymic stromal cells and not generalizable to many other persistent infections. Indeed, T cell development in persistent murine polyomavirus infection is necessary to maintain a long-term antiviral pool[109]. The same may apply to human pathogens that do not target the thymus, for instance HCV. Lack of virus in the tissue may prevent CD8 T cell-mediated thymic atrophy during these infections, although IFN-I may still exert a disruptive effect. Interestingly, HIV viral loads are detectable within the thymus, although HIV infects lymphoid cells such as CD4+ thymocytes, dendritic cells, and macrophages rather than the epithelium. However, the presence of infected cells would still draw virus-specific CD8 T cells to the thymus, raising the possibility that HIV-induced thymic atrophy is mediated by similar mechanisms as LCMV. It is unclear

whether infection of lymphoid cells within the thymus by HIV would weed out new virus-specific T cell precursors with the same efficiency as we observe in LCMV and whether antiretroviral therapy is sufficient to solve this problem. The finding that blockade of IFN-I signaling can be therapeutic when administered late during persistent infection raises the possibility that it IFN-I blockade can be used both as an antiviral and pro-thymic therapy. Additionally, future studies focused on the period between the initial disruption of T cell development and recovery can yield more information on how thymic atrophy affects the peripheral T cell response and the consequences for control of persistent infection.

Figure 2.1. Persistent LCMV infection induces thymic atrophy and loss of DP thymocytes.

(A) Graph illustrates thymic viral load 5, 9, and 30 days after infection with LCMV-Arm or LCMV-Cl13.

(B) Flow plots illustrate the frequency of CD4/CD8 DN, SP, and DP thymocyte populations within the thymus of naïve mice and mice infected with LCMV Armstrong or LCMV Cl13 at 5, 9, 15, and 30 days post infection. Thymocyte subset frequency is listed as % of total thymocytes in the respective quadrant.

(C) Graphs illustrate the number of thymocytes within the thymus and the percent of thymocytes in the DP stage of development in uninfected mice and mice infected with LCMV Armstrong and LCMV Cl13.

(D) Mice were infected with LCMV-Arm or LCMV-Cl13 and sacrificed in parallel with naïve control mice at days 5 and 9 post infection. The double negative (DN) thymocyte compartment was analyzed based on their expression of CD25 (IL-2Rα) and CD44.

(E) The ratio of each DN population (DN1, DN2, DN3, and DN4) was determined

Data is representative of at least 2 experiments with 3-5 mice per group. * = $p<0.05$

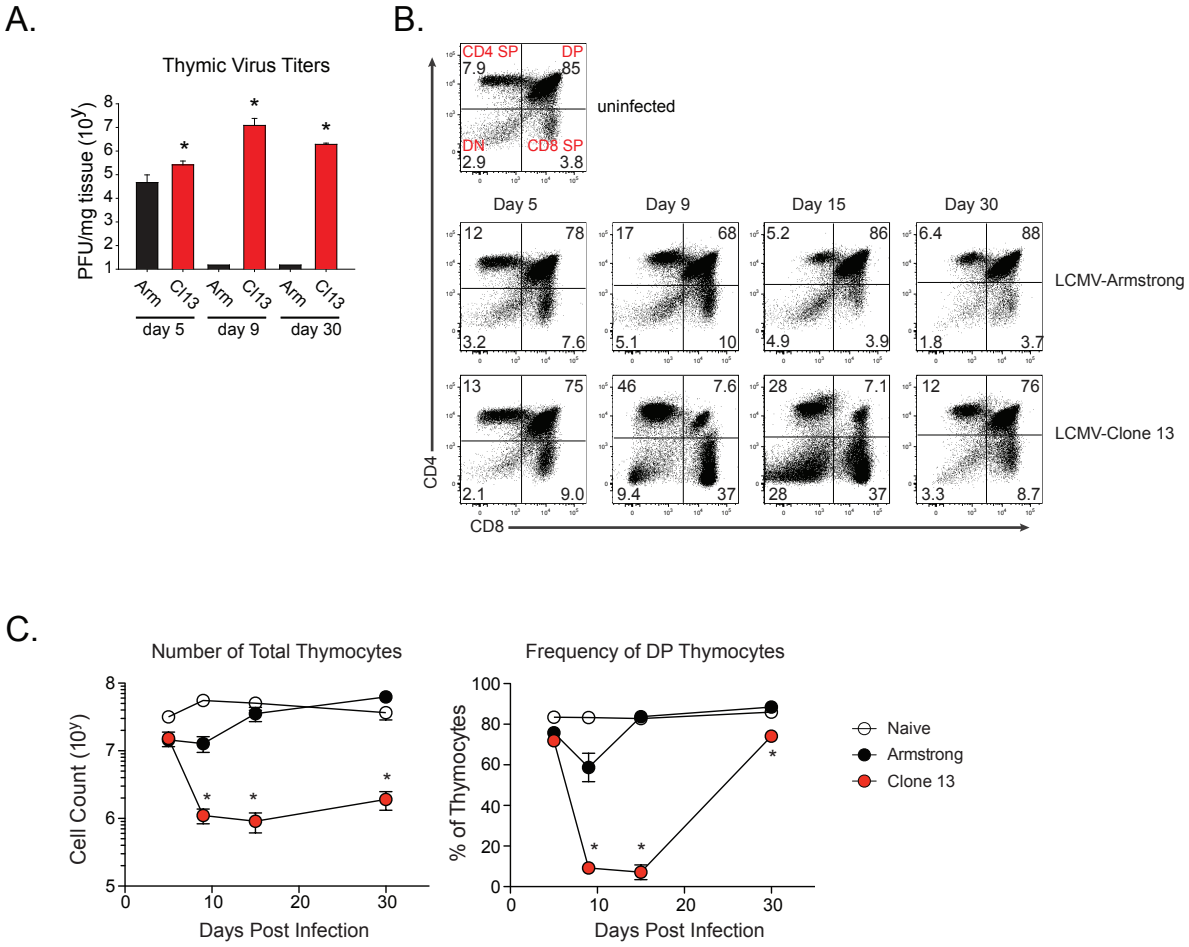

A.
Thymic Virus Titers
PFU/mg tissue (10^y)
Arm
Cl13
day 5
day 9
day 30
B.
CD4 SP
DP
DN
CD8 SP
uninfected
Day 5
Day 9
Day 15
Day 30
LCMV-Armstrong
LCMV-Clone 13
CD4
CD8
C.
Number of Total Thymocytes
Cell Count (10^y)
Days Post Infection
Frequency of DP Thymocytes
% of Thymocytes
Naive
Armstrong
Clone 13

Figure 2.2. T cell development is impaired at early stages during persistent viral infection.

(A) Flow plots illustrate the frequency of DN thymocytes in each stage of their development (DN 1-4) in naïve mice and mice infected with LCMV Arm and LCMV Cl-13 at 5 and 9 days after infection. DN thymocyte subset frequency is listed as % of total DN thymocytes in the respective quadrant.

(B) Graphs illustrate the number of total DN thymocytes within the thymus, and the number of thymocytes in each stage of DN development in naïve mice, and mice infected with LCMV Arm and LCMV Cl13 through 30 days after infection.

(C) Graphs illustrate the frequency of DN thymocytes in each stage of their development as (% of total thymocytes) in naïve mice and mice infected with LCMV Arm and LCMV Cl-13 at 5 and 9 days after infection.

Data is representative of 2 experiments with 3-5 mice per group. * = $p<0.05$

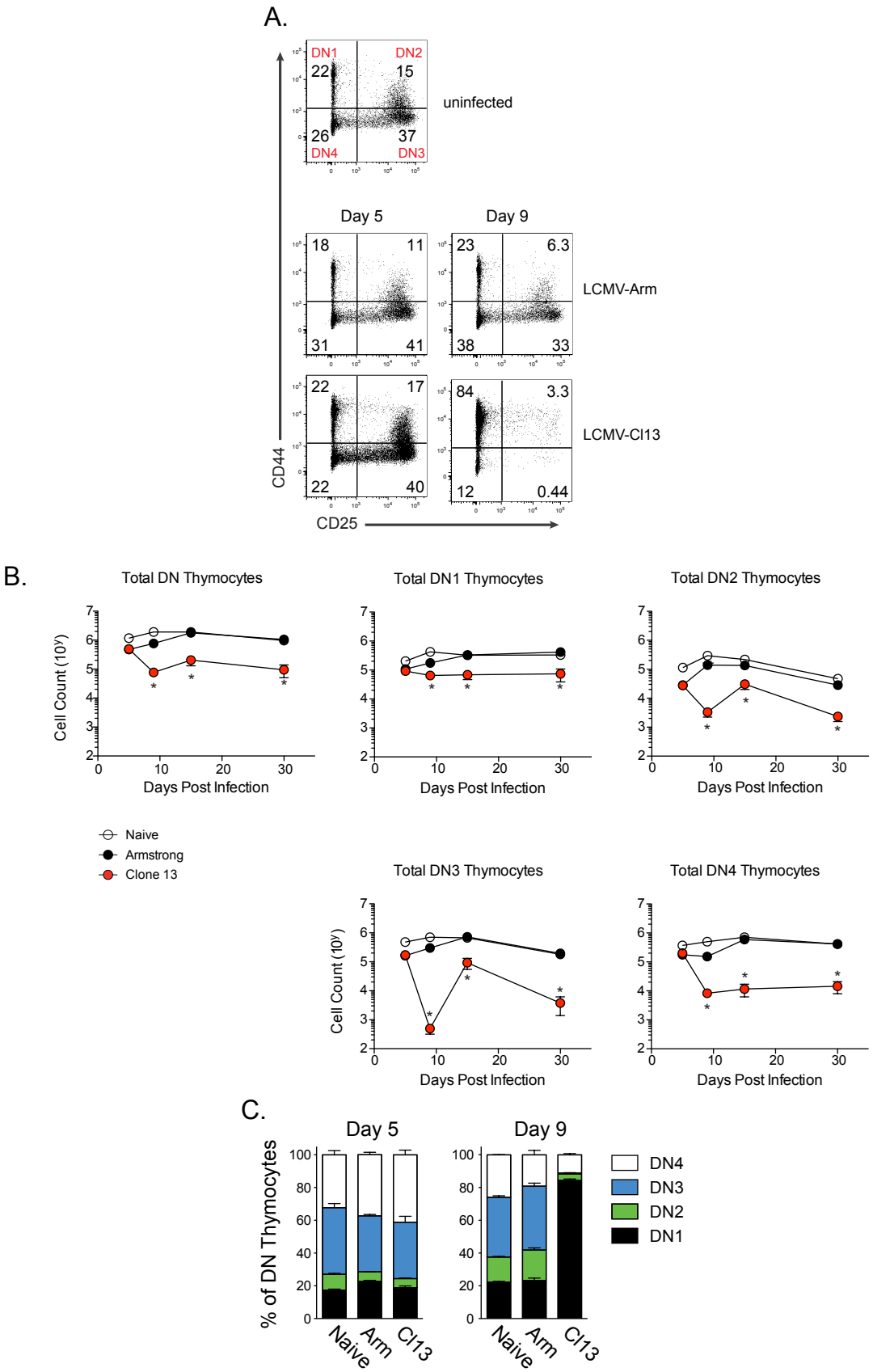

A.
DN1
DN2
22
15
26
37
DN4
DN3
uninfected
Day 5
Day 9
18
11
31
41
23
6.3
38
33
LCMV-Arm
22
17
22
40
84
3.3
12
0.44
LCMV-Cl13
CD44
CD25
B.
Total DN Thymocytes
Total DN1 Thymocytes
Total DN2 Thymocytes
Cell Count (10^y)
Days Post Infection
Naive
Armstrong
Clone 13
Total DN3 Thymocytes
Total DN4 Thymocytes
C.
Day 5
Day 9
% of DN Thymocytes
DN4
DN3
DN2
DN1
Naive
Arm
Cl13

Figure 2.3. Virus-specific CD8 T cells enter the thymus and perform anti-viral functions.

(A) Flow plots illustrate IFNγ and TNFα secretion by thymic-resident P14 cells 5, 9, and 30 days after LCMV Arm or LCMV Cl13 infection.

(B) Graph illustrates the total number of P14 cells within the thymus on the indicated day after LCMV Arm of LCMV Cl13 infection.

(C) Graph illustrates the frequency of thymic resident P14s that are polyfunctional (capable of producing both IFNγ and TNFα together after stimulation).

Data is representative of 2 experiments with 3-5 mice per group. * = $p<0.05$

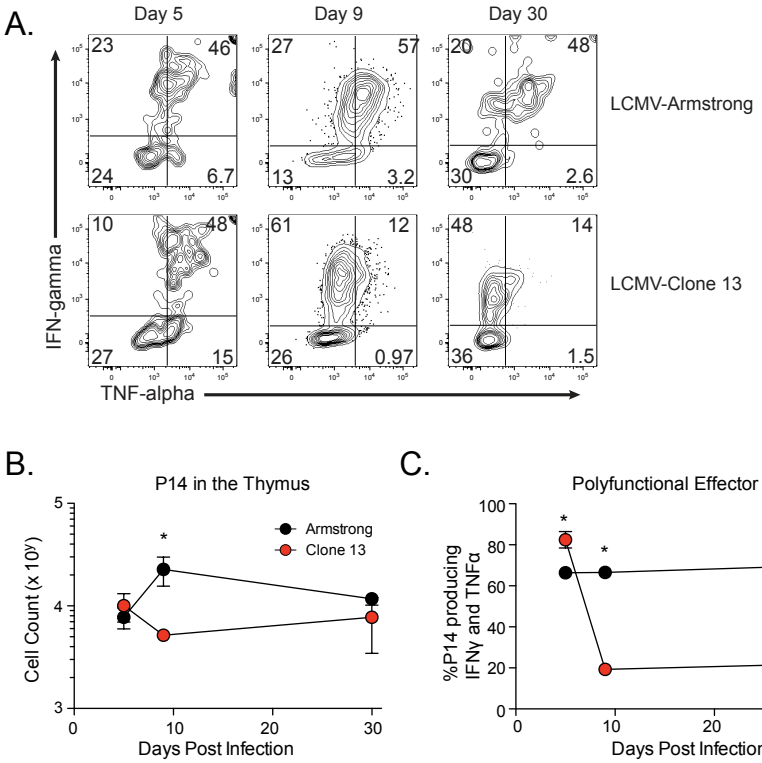

A.
Day 5
Day 9
Day 30
23
46
24
6.7
27
57
13
3.2
20
48
30
2.6
LCMV-Armstrong
10
48
27
15
61
12
26
0.97
48
14
36
1.5
LCMV-Clone 13
IFN-gamma
TNF-alpha
B.
P14 in the Thymus
Armstrong
Clone 13
Cell Count (x 10⁷)
Days Post Infection
C.
Polyfunctional Effector P14
%P14 producing IFNγ and TNFα
Days Post Infection

Figure 2.4. Virus-specific CD8 T cells are necessary for thymic atrophy during persistent infection.

(A and B) Mice were treated with an isotype control antibody (iso) or a CD8 T cell-depleting antibody (αCD8) prior to infection with LCMV-Cl13. Flow plots and graphs illustrate the number of total thymocytes and frequency of thymocyte subsets in mice infected with LCMV Cl13 for 9 days or naïve antibody-treated controls.

(C and D) Wild type or TCR transgenic OTI mice were infected with LCMV Cl13. Flow plots and graphs illustrate the number of total thymocytes and frequency of thymocyte subsets in wild type and transgenic mice infected with LCMV Cl13 for 9 days or naïve wild type and OTI controls.

Data is representative of 2 experiments with 4 mice per group. * = $p<0.05$

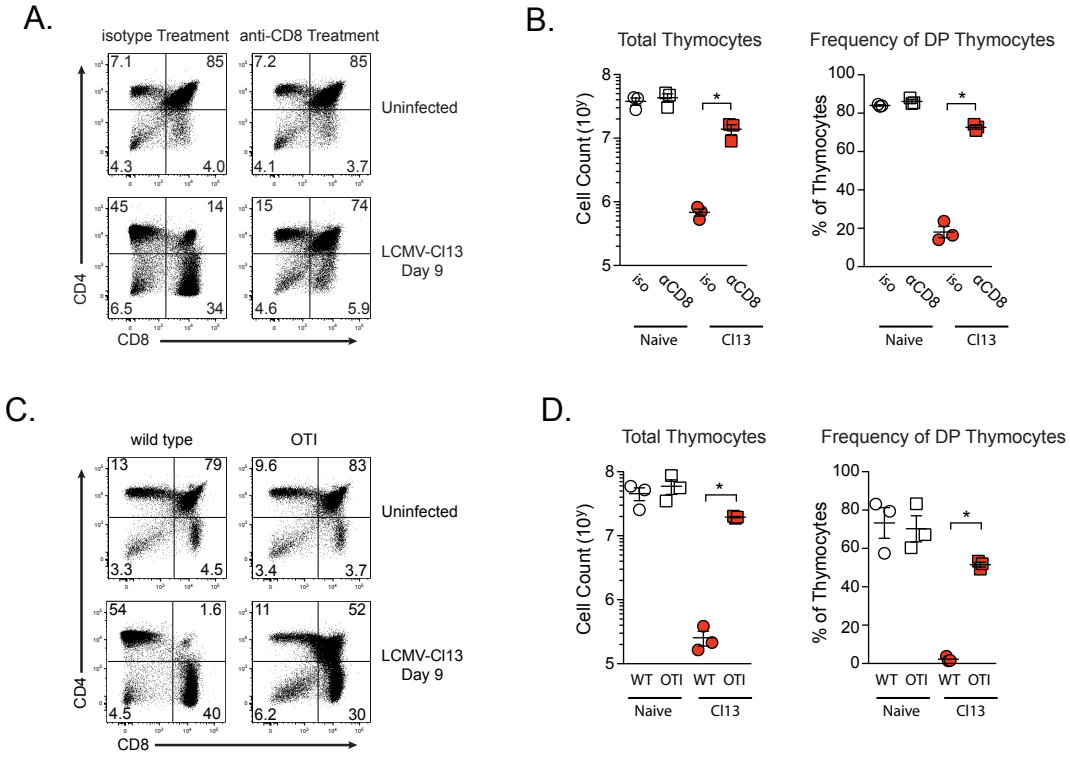

A.
isotype Treatment
anti-CD8 Treatment
7.1
85
4.3
4.0
7.2
85
4.1
3.7
Uninfected
45
14
6.5
34
15
74
4.6
5.9
LCMV-Cl13
Day 9
CD4
CD8
B.
Total Thymocytes
Frequency of DP Thymocytes
Cell Count (10⁷)
% of Thymocytes
iso
αCD8
Naive
Cl13
C.
wild type
OTI
13
79
3.3
4.5
9.6
83
3.4
3.7
Uninfected
54
1.6
4.5
40
11
52
6.2
30
LCMV-Cl13
Day 9
CD4
CD8
D.
Total Thymocytes
Frequency of DP Thymocytes
Cell Count (10⁷)
% of Thymocytes
WT
OTI
Naive
Cl13

Figure 2.5. CD4 T cells, NK cells, IFNγ, TNFα, Perforin, and FASL are not necessary for thymic atrophy during persistent LCMV infection.

(A) Mice were treated with an isotype control antibody (iso) or a CD4 T cell-depleting antibody (αCD4) prior to infection with LCMV-Cl13. Graphs illustrate the number of total thymocytes and frequency of DP thymocytes in mice infected with LCMV Cl13 for 9 days or naïve antibody-treated controls.

(B) Mice were treated with an isotype control antibody (iso) or an NK cell-depleting antibody (αNK1.1) prior to infection with LCMV-Cl13. Graphs illustrate the number of total thymocytes and frequency DC thymocytes in mice infected with LCMV Cl13 for 9 days or naïve antibody-treated controls.

(C-F) Graphs illustrate the number of thymocytes 9 days after LCMV Cl13 infection in interferon gamma receptor (IFNgR KO), TNF receptor (TNFR KO), Perforin, and FasL knockout mice compared to infected wild type controls.

Data is representative of 2 experiments with 3-4 mice per group. * = $p<0.05$

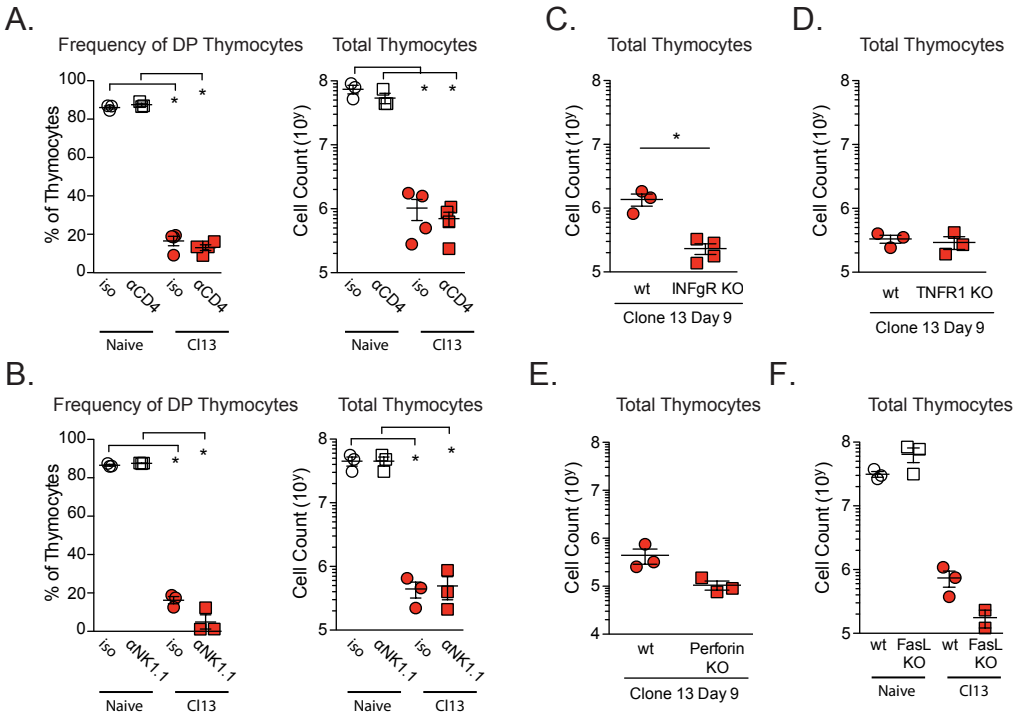

A.
Frequency of DP Thymocytes
% of Thymocytes
Iso
αCD4
Naive
Cl13
Total Thymocytes
Cell Count (10⁷)
B.
αNK1.1
C.
Total Thymocytes
wt
INFgR KO
Clone 13 Day 9
D.
TNFR1 KO
E.
Perforin KO
F.
FasL KO

Figure 2.6. Type I interferon mediates thymic atrophy during persistent viral infection.

(A and B) Flow plots and graphs illustrate the number of total thymocytes and frequency of thymocyte subsets in wild type or IFN-I receptor deficient (IFNAR KO) mice infected with LCMV Cl13 for 9 days.

(C and D) Mice were treated with an isotype control antibody (iso) or an antibody blocking IFN-I receptor signaling (αIFNAR) prior to infection with LCMV-Cl13. Flow plots and graphs illustrate the number of total thymocytes and frequency of thymocyte subsets in isotype or αIFNAR-treated mice infected with LCMV Cl13 for 9 days.

Data is representative of 2 experiments with 3-4 mice per group. * = $p<0.05$

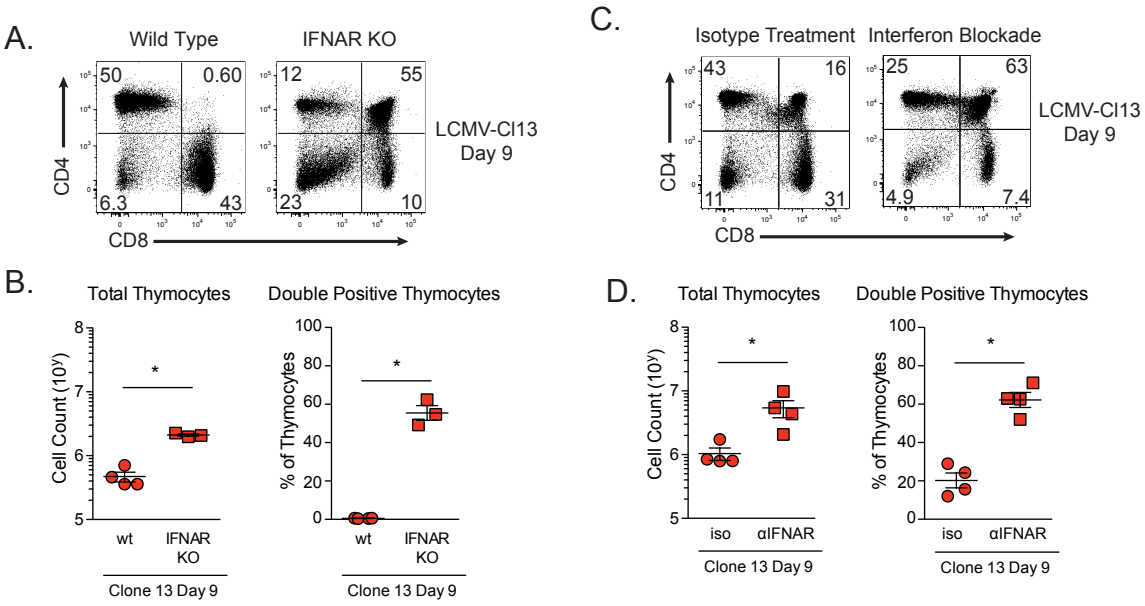
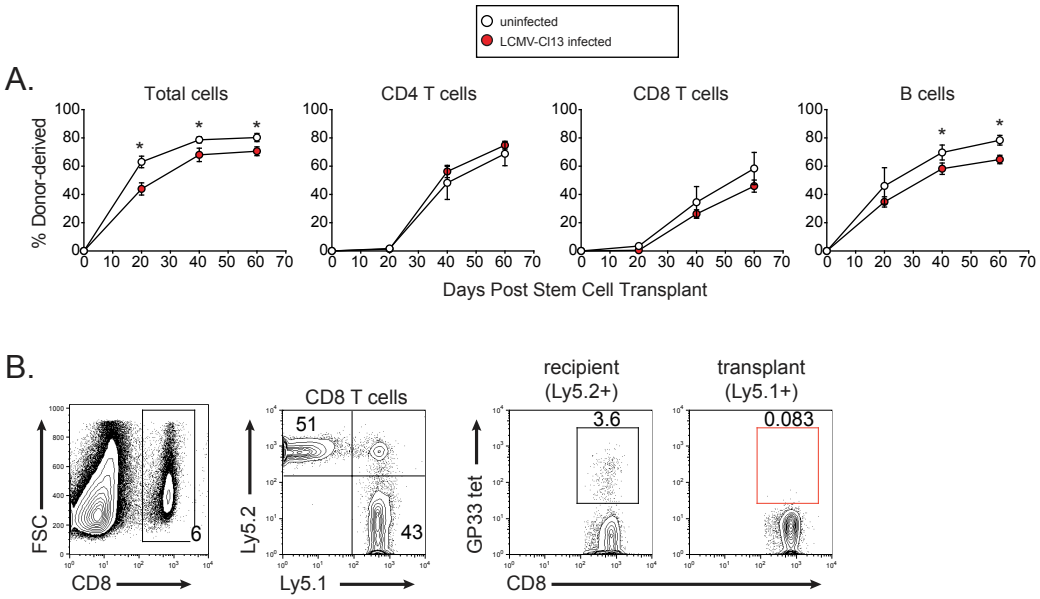

A.
Wild Type
IFNAR KO
50
0.60
6.3
43
12
55
23
10
CD4
CD8
LCMV-Cl13
Day 9
B.
Total Thymocytes
Double Positive Thymocytes
Cell Count (10^7)
% of Thymocytes
wt
IFNAR KO
Clone 13 Day 9
C.
Isotype Treatment
Interferon Blockade
43
16
11
31
25
63
4.9
7.4
CD4
CD8
LCMV-Cl13
Day 9
D.
Total Thymocytes
Double Positive Thymocytes
Cell Count (10^7)
% of Thymocytes
iso
αIFNAR
Clone 13 Day 9

Figure 2.7. No virus specific CD8+ T cells can emerge from a persistently infected thymus

(A) Graphs illustrate the rate of peripheral immune reconstitution after a busulfan-based stem cell transplant protocol. The rate of reconstitution is quantified as % of peripheral blood lymphoid cells that are derived from donor stem cells.

(B) Flow plots illustrate recipient and donor-derived peripheral CD8 T cell populations and recipient and donor-derived virus-specific CD8 T cell responses within the spleen nine weeks after stem cell transplant.

Data is representative of 2 experiments with 4-6 mice per group. * = $p<0.05$


A.
uninfected
LCMV-Cl13 infected
Total cells
CD4 T cells
CD8 T cells
B cells
% Donor-derived
Days Post Stem Cell Transplant
B.
FSC
CD8
6
CD8 T cells
Ly5.2
Ly5.1
51
43
recipient
(Ly5.2+)
transplant
(Ly5.1+)
GP33 tet
CD8
3.6
0.083

CHAPTER THREE

Type I interferon suppresses *de novo* CD4 Th1 immunity during an established persistent virus infection

ABSTRACT

CD4 T cells are central to orchestrate, sustain and potentially regenerate antiviral immunity throughout persistent viral infections. Although the evolving immune environment during persistent infection reshapes established CD4 T cell responses, the fate of naïve CD4 T cells primed in the midst of persistent infection is unclear. We demonstrate that in marked contrast to the onset of infection, virus-specific CD4 T cells primed during an established persistent infection fail to develop Th1 responses, to efficiently accumulate in peripheral tissues and rapidly and almost exclusively differentiate into T follicular helper cells. Consistent with lack of Th1 and heightened Tfh development, virus-specific CD4 T cells primed during the established persistent infection provide help to B cells, but only limited help to CD8 T cells. The failure of *de novo* Th1 generation and tissue distribution was mediated by chronic type I interferon (IFN-I) production and was effectively restored by blocking IFN-I signaling during CD4 T cell priming. Thus, we establish a new suppressive function of chronic IFN-I signaling and mechanism of immunoregulation during an established persistent virus infection.

INTRODUCTION

The majority of viruses stimulate robust and effective T cell responses that efficiently eliminate the infection; however, certain viruses are able to subvert host T cell control of viral replication and generate a persistent infection. Sustained CD4 T helper (Th) cell responses are a strong correlate of control and clearance of multiple persistent virus infections, including HIV and hepatitis C virus (HCV) infection in humans and lymphocytic choriomeningitis virus (LCMV) infection in mice[49,108]. CD4 T helper cells are central orchestrators of the immune response, and differentially activate diverse branches of innate and adaptive immunity to guide the appropriate response to an invading pathogen. In response to antigen stimulation, and combinations of costimulatory signals and cytokines encountered at the onset of infection, CD4 T cells develop into different Th subsets that preferentially stimulate and sustain CD8 T cells (Th1) or B cells (T follicular helper; Tfh), maintain tolerance to self-antigens (Treg), or mediate responses to extracellular, mucosal, or other challenges (Th2, Th17)[110]. In response to viral infections, CD4 T cells predominately develop into Th1 or Tfh cells[65,110]. Th1 immunity is characterized in CD4 T cells by the secretion of interferon gamma (IFNγ), TNF alpha (TNFα), and IL-2, and the ability to program memory CD8 T cell development in response to a cleared infection or to sustain residual CD8 T cell activity during persistent infection[49,50]. Tfh cells localize to the follicle via CXCR5 expression to mediate germinal center B cell and plasma cell differentiation though cell surface proteins as well as secreted cytokines such as IL-21[110]. Control of infection is critically dependent upon the correct orchestration of these responses.

At the onset of what will become a persistent LCMV infection, CD4 T cells initially generate a Th1 response, but these Th1 cells progressively develop into Tfh as infection progresses[65], indicating that CD4 T cell differentiation is continually modulated depending on the time of infection. This also suggests that CD4 T cell primed in the

midst of a persistent infection may develop differently than those activated at the onset of infection. It was recently shown that CD4 T cells can be primed during persistent infection[111,112], yet it is still unclear how the ongoing persistent infection alters *de novo* CD4 T cell priming, development and function. Importantly, a naïve T cell activated in an established persistent infection will encounter a substantially different immunologic environment than one primed at the onset of infection, most notably characterized by the disruption of lymphatic organ architecture, the immediate exposure to high levels of antigen and inflammatory and suppressive factors, as well as changes in the type and functional quality of antigen presenting cells (APC)[21,49]. Biologically**,** *de novo* T cell activation is likely required for diverse needs of the immune response during viral persistence, such as to balance attrition in response to many life-long persistent infections and to control escape mutations[113,114]. Therapeutically, recruitment and activation of naïve T cells will be required to stimulate *de novo* immunity through therapeutic vaccination, production of virus-specific and virus-infection resistant T cells by means of hematopoietic stem cell engineering, or other immune reconstitution approaches[115].

Given the broad immunologic implications that alterations in CD4 T cell differentiation could have on the antiviral immune response, we sought to understand the molecular, cellular, and effector development of CD4 T cell responses primed in the midst of persistent infection. Herein we demonstrate that virus-specific CD4 T cells primed in the midst of persistent infection are activated, but unlike their counterparts at the onset of infection, they almost entirely fail to form the Th1 effector responses traditionally associated with viral infections, instead rapidly becoming Tfh cells. Mechanistically, the defect in Th1 development is due to high levels of type I interferon (IFN-I) in the persistently infected immune environment, and blockade of IFN-I signaling effectively restores *de novo* Th1 differentiation. Ultimately, the failure to form Th1 coupled with

exclusive Tfh formation could have important effects toward the long-term control and treatment of persistent viral infections.

MATERIALS AND METHODS:

Mice and virus

C57BL/6 (WT) mice were purchased from The Jackson Laboratory or the rodent breeding colony at University of California, Los Angeles. B cell deficient μMT, Hen-egg lysozyme transgenic (Hel-tg), and CD11c-DTR mice were purchased from The Jackson Laboratory. Transgenic KL25 mice were provided by Daniel Pinschewer (Universite de Geneve). LCMV-GP_{61-80} specific CD4 TCR transgenic (SMARTA) and LCMV-GP_{33}-specific CD8 TCR transgenic (P14) mice have been described previously[100,116]. SMARTA mice deficient for the type I interferon receptor were generated by crossbreeding SMARTA mice with IFNR-/- mice (provided by Dr. Dorian McGavern). All mice were housed under specific pathogen–free conditions. Mouse handling conformed to the experimental protocols approved by the University of California, Los Angeles Animal Research Committee (ARC). In all experiments the mice were infected i.v. via the retroorbital sinus with 2×10^6 PFU of LCMV-Armstrong, LCMV-Clone 13, LCMV-M1 or LCMV-M2. Virus stocks were prepared and viral titers were quantified as described previously [100].

To generate an LCMV-Cl13 variant that could be recognized by the KL25 antibody we used reverse genetics approaches to rescue a recombinant Cl13 virus containing mutations within the GP1 coding region at I118L and S119N for LCMV-M1 and I118L, S119N and N121K for LCMV-M2.

Isolation and adoptive transfer of virus-specific T and B cells

LCMV-specific SMARTA cells, P14 cells, or TgKL25 B cells were isolated from the spleens of respective transgenic mice by negative selection (StemCell Technologies). All cell transfers were performed i.v. in the retroorbital sinus. To assess priming and differentiation of virus-specific CD4 T cells in the midst of persistent infection, we

transferred 5,000 SMARTA cells into either naive mice that were infected with LCMV-Cl13 1 hour later (early priming) or into mice that had been infected with LCMV-Cl13 21 days earlier. For experiments in which the mice were sacrificed at 60 hours after transfer, 250,000 SMARTA cells were transferred to enable detection at this early time point. For late priming during acute infection, 5,000 SMARTA cells were transferred into mice infected for 4 days with LCMV-Armstrong.

To assess viral escape from late primed responses, 5,000 SMARTA cells were transferred into mice 21 days after LCMV-Cl13 infection. Eight days later (day 29 post infection) 250,000 CFSE-labeled SMARTA cells were transferred into the same mice (i.e., a second SMARTA cell transfer) or into infection-matched mice that did not previously receive SMARTA cells at day 21 (control for effective proliferation). Proliferation was then assessed by CFSE dilution 60 hours after the second transfer.

To assess how late primed virus-specific CD4 T cells help B cells and CD8 T cell responses, 2-5x10^6 B cells from TgKL25 mice (containing ~1-3x10^5 KL25+ B cells) and/or 5000 P14 cells were transferred i.v. with or without 50,000 SMARTA cells. Cells were transferred into mice infected 30-45 days previously, and which were CD4 depleted prior to LCMV infection.

In vivo CD4 depletion, anti-CD28 treatment, PD1 blockade, IL-10 signaling blockade, and Type I Interferon Receptor (IFNR1) blockade

To deplete CD4 T cells before LCMV infection, mice were treated i.v. with 500μg anti-CD4 antibody (clone GK1.5; BioXCell) 4 days before infection and again on the day of infection. 30-45 days were allowed to pass before further experiments were performed to allow for the reconstitution of the endogenous CD4 T cell compartment.

To enhance CD28 mediated costimulatory signaling *in vivo*, mice were treated i.v. with 100μg of an anti-CD28 agonistic antibody (clone PV-1; BioXCell) or isotype

antibody control 4 hours after SMARTA cell transfer. To block PD1 or IL-10 signaling, we administered 250μg of anti-PD1 antibody or 250μg anti-IL-10R antibody or isotype control antibody 1 day before SMARTA cell transfer, and every 48 hours subsequently. To block IFN-I signaling in vivo during persistent infection, mice were treated i.v. with 500μg anti-IFNR1 blocking antibody (clone MAR1-5A3; Leinco Technologies) or isotype control antibody 1 or 2 days before SMARTA cell transfer (on day 19 or 20 of infection), and every 48 hours subsequently through day 27 or 28 after infection. For experiments where IFN-I signaling was blocked after virus-specific T cell priming, mice received isotype control antibody on day 20 and 22 of infection, and IFNR1 blocking antibody starting on day 24 of infection and every two days subsequently. To block IFN-I signaling at the onset of infection, animals were treated with isotype antibody or with anti-IFNR blocking antibody starting one day before LCMV-Cl13 infection and SMARTA cell transfer. Antibody treatment was continued every two days through day 7 after infection.

Isolation of peripheral organ and intestinal lymphocytes

Lymphocytes from spleen, inguinal, brachial/axillary, and mesenteric lymph nodes were isolated by mashing the organ through a 100 micron filter. For experiments in which intrahepatic or intestinal lymphocytes were analyzed, mice were perfused with 25 ml sterile PBS by intracardiac injection to remove blood from peripheral tissues. Intrahepatic lymphocytes were isolated by mashing the respective organ through a 100 micron filter and by subsequent centrifugation in 35% Percoll (GE healthcare).

To obtain intestinal lymphocytes, the gut was first resected and flushed with D10 medium to remove digestive matter. Subsequently, the gut was divided into small and large intestine, minced, digested with DTT (1mM in medium; Thermo Scientific) and filtered to isolate intraepithelial lymphocytes (IEL). To obtain lamina propria lymphocytes (LPL), the undigested fraction was further digested with Collagenase and DNAse I

(0.5mg/mL in medium; Roche), and filtered a second time. 40% / 80% Percoll gradient centrifugation was used to separate IEL and LPL from intestinal debris.

In vivo dendritic cell depletion

To deplete dendritic cells, CD11c-DTR mice were treated i.v. with 100ng diphtheria toxin in PBS (List Biological Laboratories) one day prior to SMARTA cell transfer. A second treatment was given one day after cell transfer. PBS vehicle was injected into undepleted controls.

RNA microarray and quantitative RT-PCR

5000 SMARTA cells were transferred into naïve mice that were immediately infected with LCMV-C13 (early priming) or mice infected with LCMV-Cl13 21 days earlier (late priming). Samples contained cells isolated from the spleens of 6 pooled mice per group.. Eight days after SMARTA transfer, early and late primed SMARTA cells were isolated by FACSorting using an Aria II (Beckton Dickinson). Post-sort purity was verified as >97% on the FacsVerse (Beckton Dickinson). RNA was isolated from the sorted cells using the RNeasy extraction kit (Qiagen). RNA levels were evaluated using an Agilent Bioanalyzer, labeled using the Ambion WT labeling kit, and hybridized to the Affymetrix Mouse Genes ST1.0 microarrays which were scanned and summarized using Affymetrix Expression Console and RMA16. Analysis of specific Th1 and Tfh gene products was based in part on Hale et al.[117]

For qRT-PCR, RNA was prepared from sorted early and late primed cells, or from peripheral blood mononuclear cells. Three to four biological replicates were obtained per group. RNA was normalized for input and amplified directly using the One-Step RT-PCR kit (Qiagen). PRDM1, GRAIL, MX1, OAS and HPRT were amplified using Applied Biosystems Assays-on-Demand TaqMan pre-made expression assays. mRNA

amplification was detected on the iCycler (Bio-Rad Laboratories). RNA expression was normalized to HPRT.

ELISA

To quantify LCMV-specific antibodies, LCMV-M2 was used to coat 96-well Maxisorp ELISA plates (Nunc) overnight. Plates were blocked with 3% BSA/ PBS/ 0.05% Tween20. Subsequently, serum isolated from the indicated mice was incubated on the LCMV-coated plates. Plates were washed and incubated with an HRP-labeled goat anti–mouse IgG antibody (Invitrogen), followed by the addition of o-phenylenediamine substrate in 0.05M phosphate citrate buffer. The reaction was stopped with 2N H_2SO_4, and the optical density values were read using an ELISA plate reader (Synergy 2; BioTek) at 490 nm. The concentration of antibody bound to plated virus was interpolated from a standard curve. The standard curve was generated from a serial dilution of purified mouse IgG (Invitrogen; 500–0.49 ng/ml) incubated on plates coated with goat anti–mouse IgG (Invitrogen).

KL25 hybridoma preparation and neutralization assays

KL25 B cell hybridomas were generated by isolating KL25+ B cells from TgKL25 mice. Binding of the purified KL25 antibody to LCMV-M1 and LCMV-M2 but not LCMV-Cl13 was confirmed by ELISA using plates coated with each individual virus. To determine KL25 neutralization activity against LCMV-Cl13, LCMV-M1, and LCMV-M2, each virus was individually pre-incubated with serial dilutions of KL25 antibody for 30 minutes and plaque assay performed on Vero cells. Assays were performed in triplicate.

Flow Cytometry

Analysis of immune subsets was performed by staining lymphocytes obtained from spleen, lymph nodes, liver, and intestinal fractions ex vivo for the expression of CD4, CD8, SLAM, CD122 (IL-2Rβ), CD25 (IL-2Rα), CD62L, IFNγ, TNFα, CD138, CD11c, MHCII (I-A/I-E), CD86 (B7.2) (BioLegend); CD45.1, Granzyme B (eBioscience); Thy1.1, Bcl6 (BD Biosciences). CXCR5 expression was assessed by staining with anti-CXCR5-biotin (BD Biosciences), followed by SA-BV (BioLegend). IL-21 cytokine expression was assessed by staining with a recombinant mouse IL-21R subunit/FC chimera (R&D Systems), followed by anti-Fc-PE (Jackson Immune Research). LCMV-specific KL25+ B cells were identified using antibodies against the heavy chain (clone IIIC) and idiotype (clone B2.5). CFSE dilution analysis was performed by incubating naïve SMARTA cells with 2.5 μM CFSE (Life Technologies). Flow cytometric data was collected on the FacsVerse (Beckton Dickinson).

Statistical analysis

Student's *t* tests (two-tailed, unpaired), and Mann-Whitney nonparametric tests (two-tailed, unpaired) were performed using GraphPad Prism 5 software (GraphPad Software, Inc.).

RESULTS

Virus-specific CD4 T cells primed in an established persistent infection experience an initial defect in effector differentiation

To investigate the dynamics of virus-specific CD4 T cell priming in the midst of viral persistence, we utilized the LCMV system. Infection with LCMV-Armstrong (Arm) induces robust CD4 and CD8 T cell responses that clear the virus within 8-10 days after infection. Conversely, the LCMV-Clone 13 (Cl13) variant replicates to substantially higher titers and rapidly elicits the expression of multiple host immunoregulatory factors that suppress the immune response to generate a persistent infection [17,118]. To determine how the environment during an established persistent infection affects *de novo* virus-specific CD4 T cell priming and differentiation, we transferred naïve LCMV-specific TCR-transgenic CD4 (SMARTA) T cells into mice that had been infected 21 days earlier with LCMV-Cl13. In parallel, naïve SMARTA T cells were transferred into naïve mice that were then infected with LCMV-Cl13, thus allowing a direct comparison of CD4 T cell priming at the onset and during an established persistent infection (Figure 3.1). Importantly, SMARTA transgenic cells behave similarly to their endogenous (i.e., host derived LCMV-GP_{66} tetramer+) CD4 T cell counterparts [62,69]. Herein, T cells primed at the onset of infection are termed "early primed," while T cells transferred into an established persistent infection are referred to as "late primed."

Sixty hours post transfer, early and late primed virus-specific CD4 T cells upregulate the activation marker CD44, proliferate, and expand to similar levels (Figure 3.2A), indicating priming and activation of naïve virus-specific CD4 T cells in the midst of a persistent infection. Depletion of dendritic cells (DC) in CD11c-DTR mice[119] before SMARTA transfer greatly reduced late primed CD4 T cell proliferation and expansion, indicating that DC are necessary for priming during persistent infection (Figure 3.2B and C). Following activation, early primed CD4 T cells down-regulate the lymph node

retention molecule CD62L and upregulate the IL-2 receptor alpha (IL-2Rα, CD25) and beta chains (IL-2Rβ, CD122), Granzyme B, and the Th1 and Tfh fate determining transcriptional regulators Tbet and Bcl6, whereas this was not observed in late primed cells (Figure 3.2D). Late primed virus-specific CD4 T cells did not develop into either of the Th1 ($SLAM^{hi}$, $IL\text{-}2R\beta^{hi}$, CXCR5-) or Tfh ($SLAM^{lo}$, $IL\text{-}2R\beta^{lo}$, CXCR5+) precursor populations evident in early primed CD4 T cells (Figure 3.2E), nor do they produce the critical antiviral/immunostimulatory cytokines IFNγ, TNFα, or IL-21 in response to antigen stimulation (Figure 3.2F). IL-2 is produced at low, but similar levels by both groups (9.3±1.0% of SMARTA (early priming) vs. 13.4±2.3% of SMARTA (late priming); p=0.16). These differences in differentiation were also observed 24 hours after priming, indicating the failure to undergo this initial differentiation program as opposed to accelerated kinetics of differentiation. Early and late primed CD4 T cells expressed the same levels of the transcription factor FoxP3 and Grail (not shown), indicating that they are not instead forming Tregs or becoming anergic. Thus, despite activation and proliferation, virus-specific CD4 T cells primed during an established persistent infection initially undergo an attenuated Th differentiation program.

Virus-specific CD4 T cells primed in the midst of an established persistent infection fail to generate Th1 cells.

To determine whether priming in the midst of persistent infection continues to inhibit Th differentiation, we sorted and performed microarray analysis on early and late primed virus-specific CD4 T cells at 8 days after priming, a time point coinciding with the peak of the early primed effector response [120]. At the population level, Tfh associated genes were increased in late priming, whereas the majority of Th1 associated genes were highly expressed in early primed cells (Figure 3.3A). Consistent with the RNA analysis,

the late primed virus-specific CD4 T cell response within the mesenteric, inguinal, and brachial/axillary lymph nodes and spleen was predominately Tfh (Figure 3.3B-D). Further, with exception of the inguinal lymph node, the absolute number of Tfh cells formed in each organ was not increased compared to early priming (Figure 3.3B-D). On the other hand, the number of Th1 cells was markedly reduced compared to early priming. Thus, our data indicate that an established persistent infection does not skew toward *de novo* Tfh formation *per se*, but instead that *de novo* Th1 development is not supported when priming is initiated in the midst of an ongoing persistent infection.

Transfer of physiologic numbers of virus-specific CD4 T cells in the midst of persistent infection did not accelerate viral control (Figure 3.3E), indicating that the lack of Th1 generation is not a result of accelerated viral clearance. Importantly, efficient activation and proliferation of naïve CD4 SMARTA T cells occurred when mice received a second transfer eight days after the first late-priming transfer, demonstrating that the lack of Th1 formation is not due to viral escape. Interestingly, the failure to generate Th1 cells was not observed when virus-specific CD4 T cells were transferred into an established acute LCMV-Arm infection (day 4 after infection; Figure 3.3F), indicating that the inability to efficiently generate Th1 cells is not simply due to viral infection, but rather is a property of the environment during an established persistent infection.

Functionally, late primed CD4 T cells produced decreased IFNγ and increased IL-21 compared to early primed CD4 T cells (Figure 3.4A) and expressed the master Tfh transcriptional regulator Bcl6 (Figure 3.4B), consistent with the formation of a Tfh response and decreased Th1 differentiation [62]. Unlike cells primed at the onset of acute LCMV-Arm infection, late primed Tfh differentiation did not require LCMV-specific B cells [110], or IL-6, as has been reported in other situations [60] (Figure 3.4C and D). Although it is possible that late primed cells receive autocrine IL-6 signals since the transferred cells were not IL-6 deficient, we did not detect increased IL-6 mRNA by microarray analysis or

IL-6 protein secretion after peptide stimulation. These data further support that virus-specific CD4 T cells form predominately a Tfh instead of Th1 response following priming during established persistent infection through mechanisms distinct from the Th1 to Tfh transition that occurs by early primed virus-specific CD4 T cells[65].

Since alterations in Th differentiation affect CD4 T cell homing and distribution [117,121], we next assessed whether the diminished Th1 formation in late primed virus-specific CD4 T cells led to changes in their tissue distribution. By day 8 after transfer late primed CD4 T cells had down-regulated the lymph tissue homing/retention molecule CD62L. Yet, corresponding to the lack of Th1 generation, the number of late primed virus-specific CD4 T cells in the liver were greatly reduced, and although they were present in the mesenteric lymph nodes, they were almost entirely absent from the gastrointestinal (GI) tract despite high virus titers in all organs (Figure 3.5). Thus, consistent with the lack of Th1 differentiation, virus-specific CD4 T cells primed in an established persistent infection were absent from multiple tissues and almost entirely fail to accumulate in the GI tract.

Late primed CD4 T cells help B cell responses

Tfh cells provide signals to B cells to mediate antibody secretion and direct cellular differentiation[110]. To test whether late primed CD4 T cells can help virus-specific B cells *in vivo*, we developed a system to introduce a traceable LCMV-specific B cell response into persistent infection. B cells from TgKL25 mice transgenically express the heavy chain of the KL25 antibody, and endogenous light chain rearrangement generates approximately 7-10% of naïve B cells expressing the KL25 antibody [122]. The KL25 antibody efficiently binds LCMV-WE [123], but not LCMV-Cl13 (Figure 3.6). To use the TgKL25 transgenic mice with LCMV-Cl13, we used reverse genetics to produce two recombinant Cl13 viruses containing mutations within its GP1 coding region facilitating

recognition by the KL25 antibody [123]. One viral variant termed LCMV-M1 is neutralized by KL25 and another termed LCMV-M2 is bound but not neutralized by KL25 (Figure 3.6). None of the mutations are in the LCMV-GP_{61-80} CD4 T cell epitope and they do not affect SMARTA cell recognition. Both LCMV-M1 and M2 replicate in vivo and suppress Th1 formation in the late priming situation analogous to WT LCMV-Cl13.

To determine the CD4 T helper capacity of late primed cells *in vivo*, we transferred transgenic LCMV-specific B cells (from TgKL25 mice) and/or transgenic LCMV-specific CD8 T cells (P14 cells) into mice persistently infected with LCMV-M2 and then with or without LCMV-specific CD4 SMARTA T cells. In these experiments mice were CD4 depleted prior to infection to generate a life-long viremic infection lacking endogenous LCMV-specific CD4 T cells and ensuring that all help is derived from the transferred virus-specific CD4 T cells. In the CD4 depleted model, late primed CD4 T cells failed to form Th1 cells or distribute to non-lymphoid organs (Figure 3.7). Late primed CD4 T cells did expand to greater levels in lymphoid organs (likely due to a larger available niche), although they did not lead to enhanced viral control (Figure 3.7). Importantly, transferred TgKL25+ B cells only expanded, differentiated into plasma cells and produced antibody when co-transferred with SMARTA cells (Figure 3.8A), consistent with the lack of pre-existing virus-specific CD4 T cells in CD4 depleted mice, and indicating that late primed CD4 T cells are capable of providing help to B cells *in vivo*. Similar results were observed using LCMV-M1. In the presence of late primed virus-specific CD4 T cells, TgKL25+ B cells and antibody production was maintained at least up to 40 days post transfer (Figure 3.8A and B). On the other hand, B cells from TgKL25 mice that were not specific for the viral glycoprotein were not enhanced long-term (Fig 3.8C), indicating that the sustained helper effect of late primed CD4 T cells on B cells is exerted via virus-specific interactions.

Although late primed CD4 T cells provided help for B cells, they did not increase the level of endogenous pre-existing LCMV-GP_{33-41} tetramer+ CD8 T cells by 8 days post transfer (in either undepleted or mice CD4 depleted prior to infection) and only induced a modest but unstained increase in co-transferred late primed virus-specific CD8 P14 T cells (Figure 3.8D). Further, transfer of virus-specific CD4 T cells did not enhance the cytokine expression of endogenous or co-transferred virus-specific CD8 T cells (Figure 3.8E). Taken together, these data indicate that upon transfer of physiologic numbers, late primed CD4 T cells are capable of providing B cell help but exert only minimal, short-term effects on the established or *de novo* CD8+ T cell response, consistent with their Tfh differentiation.

Increased costimulation restores some aspects virus-specific CD4 Tfh differentiation, but fails to induce a Th1 response.

CD28 costimulatory signaling mediated by B7.1 and B7.2 on DCs is critical for the activation of naïve CD4 T cells and the nature of the subsequent effector differentiation[124]. Since DCs in the midst of persistent infection expressed markedly less of the costimulatory molecule B7.2 compared to DCs at the onset of infection, we sought to determine whether boosting costimulatory signaling would restore Th1 differentiation. Mice infected with LCMV-Cl13 for 21 days received virus-specific CD4 SMARTA T cells and then 4 hours later were treated with an anti-CD28 agonistic antibody, which acts as a mimic of B7 signaling. Anti-CD28 stimulation induced late primed virus-specific CD4 T cells to down-regulate CD62L and upregulate Granzyme B, IL-2Rα, Tbet, and Bcl6 expression at 60 hours post priming, similar to early primed cells (Figure 3.9A). Despite the increase in IL-2Rα and Tbet expression at 60 hours, no increase in the Th1 precursors was observed, whereas early Tfh differentiation was restored (Figure 3.9B). Paradoxically, the failure to form Th1 precursors occurred despite copious secretion of

IL-2 by SMARTA cells from anti-CD28 treated animals (12.1±2.1% of SMARTA (late priming – isotype treatment) vs. 46.4±2.9% of SMARTA (late priming – anti-CD28 treatment); $p<0.05$). Furthermore, late primed virus-specific CD4 T cells treated with anti-CD28 failed to form a Th1 effector population 8 days after priming despite increased expansion (which was due to elevated numbers of Tfh) (Figure 3.9C). Thus, decreased expression of costimulatory molecules alters the initial activation of late primed virus-specific CD4 T cells, but does not induce Th1 differentiation.

Type I Interferon signaling inhibits *de novo* virus-specific Th1 formation during an established persistent infection.

IFN-I signaling remains active throughout persistent infection and chronic IFN-I signaling can suppress antiviral CD4 T cell responses [11,125]. To evaluate the role of IFN-I in modulating Th1 differentiation during persistent infection, we treated animals with an antibody that blocks IFN-I receptor (IFNR) signaling *in vivo* [11,125]. 60 hours post transfer, late primed CD4 T cells in anti-IFNR treated mice now down-regulated CD62L and upregulated IL-2Rα similar to levels observed in early priming. By day 8 post transfer, anti-IFNR blockade restored Th1 differentiation and the absolute number of late primed virus-specific CD4 T cells to the same level observed in early priming (Figure 3.10A). On the other hand, anti-IFNR blockade did not impair Tfh differentiation in late primed virus-specific CD4 T cells (Figure 3.10A), supporting that IFN-I signaling inhibits Th1 differentiation as opposed to skewing otherwise Th1 cells into Tfh.

Consistent with the restoration of Th1 immunity in the lymphoid organs, anti-IFNR blockade restored the frequency and number of late primed virus-specific CD4 T cells and Th1 development within the liver and small intestine to the same levels observed in early priming (Figure 3.10B and C). Anti-IFNR blockade also enhanced the capacity of late primed cells to produce IFNγ and TNFα, and did so to levels well above the

exhausted virus-specific CD4 T cell responses observed at the onset of infection (Figure 3.10D). Unlike during the established persistent infection, anti-IFNR blockade during priming at the onset of LCMV-Cl13 infection (i.e., early priming) did not increase the magnitude of the virus-specific CD4 Th1 response (Figure 3.11), indicating that IFN-I signaling plays temporally disparate roles in modulating virus-specific CD4 T cell differentiation as persistent infection progresses, and that it can be blocked to systemically restore *de novo* Th1 differentiation and cytokine expression.

To determine whether IFN-I acts directly on late primed CD4 T cells to inhibit Th1 formation, we transferred IFNRα deficient virus-specific CD4 T cells into persistently infected animals. In this experiment all components of the persistently infected immune environment except for the transferred virus-specific T cells are able to respond to IFN-I signaling. IFNR deficiency on virus-specific CD4 T cells did not increase Th1 differentiation (Figure 3.12A), indicating that IFN-I does not act directly on virus-specific CD4 T cells to repress the Th1 response. Interestingly, unlike at the onset of infection wherein direct IFN-I signaling is critical for the survival of virus-specific CD4 T cells (Figure 3.12B), late primed virus-specific CD4 T cells were present at equal amounts regardless of IFNR expression, thus again highlighting the disparate role of direct IFN-I signaling on CD4 T cells at the onset and in the midst of persistent infection. IFN-I blockade during persistent infection can reverse the exhaustion of T cell responses generated at the onset of infection[11,125]. To assess whether mechanisms that contribute to T cell exhaustion during persistent infection are responsible for repressing Th1 formation during late priming we blocked IL-10 and PD1 signaling but did not see notable differences in CD4 T cell differentiation (not shown). This indicates that T cell exhaustion and the defect in *de novo* Th1 formation are mediated by separate mechanisms.

To determine whether IFN-I is acting during virus-specific CD4 T cell priming and programming to repress Th1 formation or whether ongoing IFN-I signaling after priming continues to suppress Th1 differentiation, we blocked IFNR signaling 60 hours after cell transfer (once priming has already occurred; Figure 3.2). Anti-IFNR treatment rapidly curtails IFN-I signaling and within one day, IFN-I gene expression was inhibited (Figure 3.12C). Whereas blocking IFN-I signaling prior to priming fully facilitated Th1 differentiation, anti-IFNR blockade 60 hours after transfer did not significantly increase Th1 differentiation in lymphoid or peripheral organs (Figure 3.12D). Thus, our data demonstrate that IFN-I signaling represses *de novo* virus-specific CD4 Th1 generation during the priming interactions in an established persistent infection.

DISCUSSION

It is becoming clear that in addition to its critical antiviral functions, ongoing IFN-I signaling can be detrimental to the immune response, potentiating many of the immune dysfunctions associated with persistent virus infections [11,125,126]. In this report, we now identify a new suppressive mechanism associated with chronic IFN-I signaling specifically during an established persistent virus infection. Unlike at the onset of what will become a persistent LCMV infection where IFN-I signaling does not affect Th1 differentiation; in the established persistent infection, IFN-I signaling specifically prevents *de novo* Th1 generation. Despite a higher signature of IFN-I gene expression in late primed virus-specific CD4 T cells, the suppression of Th1 development is not mediated by direct IFN-I signaling by the CD4 T cells themselves and similar numbers of IFNR-/- and WT virus-specific CD4 T cells are generated. On the other hand, at the onset of infection, IFNR signaling directly by CD4 T cells is critical for their survival and IFNR-/- virus-specific CD4 T cells are almost entirely deleted by 8 days of infection. Thus, our data establish a bifurcation in the role of IFN-I signaling on the immune environment and CD4 T cells themselves as persistent infection progresses.

IFN-I signaling at the time of priming in the established persistent infection rapidly suppresses Th1 differentiation and Th1 markers are never observed (i.e., 60 hours after transfer). However, the initial expansion and survival (i.e., total number) of *de novo* primed virus-specific CD4 T cells observed 60 hours after transfer is the same at the onset and in the established persistent infection, demonstrating that the cells that would become Th1 are initially activated and present in early and late priming. Yet by day 8, cells that would have become Th1 fail to continue to differentiate and are absent, whereas Tfh differentiation is not dramatically affected. Thus, IFN-I signaling during an established persistent virus infection does not block activation of cells that would become Th1 or skew cells that would become Th1 into Tfh cells, but instead inhibits the

differentiation of activated T cells into Th1 effectors. Moreover, the ability to restore Th1 differentiation by blocking IFN-I is lost when anti-IFNR blockade is initiated 60 hours after virus-specific CD4 T cell transfer, confirming that IFN-I signals suppress the differentiation of Th1 cells in the initial priming interactions, without affecting their initial expansion. Interestingly, exposure to IFN-I under similar circumstances induces the differentiation of bystander CD8 T cells into a memory-like state with some effector properties (Marshall et al, JI 2012). As in our system, this effect on differentiation was not due to direct IFN signaling to the T cell, but rather was mediated indirectly, possibly through an APC, suggesting that IFN-I controls adaptive immune responses by modulating key components of innate immunity. Ultimately modulating secondary mechanisms downstream of IFN-I signaling may enable restoration of Th1 potential without entirely abolishing the IFN-I system.

Our group recently demonstrated that blockade of IFN-I in the midst of persistent infection helps revitalize the immune response and leads to faster clearance of infection[11]. It is possible that the mechanisms underlying the restoration of immunity and the restoration of Th1 development during late priming are related. However, the suppression of Th1 differentiation during late priming was not dependent on IL-10 or PD1 signaling. Furthermore, our data demonstrates that developmental fate of late primed CD4 T cells is programmed during a discrete time window during priming, whereas exhaustion occurs after prolonged and repeated exposure to viral antigen and inhibitory signals[1]. This suggests that disparate mechanisms regulate exhaustion of T cell responses primed at the onset of infection and the differentiation of T cell responses primed in the midst of infection. However, this does not rule out that there may be some mechanisms in common as well. Indeed, IFN-I enhances the presentation of costimulatory markers of APCs and may render them more potent. Although enhancing costimulation by anti-CD28 agonistic antibody was not sufficient to restore Th1

development during late priming, anti-CD28 treatment did help late primed cells overcome some of their early developmental defects and restored the expansion of late primed virus-specific CD4 T cells to levels seen in early priming. Thus, boosting costimulation partially mimics the effects of IFN-I blockade, and suggests that this may be a piece of the mechanism by which IFN-I blockade restores CD4 T cell development during late priming.

One of the functions of the B7 costimulatory signal is to promote IL-2 secretion by CD4 T cells undergoing priming[127]. Indeed, we saw greatly increased IL-2 secretion in late primed cells treated with anti-CD28. IL-2 signals through STAT5 to upregulate the expression of Blimp1, which promotes Th1 effector differentiation while suppressing Tfh development[62,128]. We thus find it somewhat curious that anti-CD28 treatment did not restore Th1 differentiation during late priming, and can only speculate that other mechanisms suppress the transmission of IL-2 signals or the function of Blimp1 during late priming.

CD4 T cell responses established at the onset of persistent viral infection rapidly develop both Th1 and Tfh immunity. However, as infection progresses the virus-specific CD4 Th1 cells are redirected toward Tfh[65]. In addition to this, our new data indicate that the suppression of *de novo* Th1 differentiation coupled with ongoing *de novo* Tfh generation may also explain the enlarged Tfh effector pool seen during many established persistent infections [65,66,67,129]. The sustained ability to continue to produce Tfh responses likely has benefits for the host, as Tfh are necessary to control a persistent infection[65] and the new Tfh are able to sustain B cell responses. On the other hand, an expanding Tfh accumulation may ultimately lead to dysregulation of B cell development, hypergammaglobulinemia, and the formation of auto-antibodies associated with persistent virus infections [130,131].

Th1 cells are associated with enhanced control of multiple persistent viral infections [25,132,133,134,135,136]. The inability to replenish the diminishing Th1 response in the face of the progressive transformation of previously activated CD4 T cells toward Tfh [65] or to help previously primed or *de novo* primed virus-specific CD8 T cells, could have a dramatic effect on the long-term control of virus infection by severely focusing the range of CD4 T cell help and failing to sustain CD8 T cell responses or other immune subsets (e.g. DC, macrophages). Recently, Aubert *et al.* demonstrated that transfer of high numbers ($4x10^6$) of naïve virus-specific CD4 T cells into persistent LCMV infection could enhance the pre-existing (exhausted) virus-specific CD8 T cell and B cell responses[111]. However, in our experiments, we only observed a moderate initial increase in previously established (exhausted) CD8 T cell responses that was not sustained and no decrease in virus titers. Differences in the amount of transferred virus-specific CD4 T cells (5000 vs. 4 million) likely account for this discrepancy and suggests that endogenously generated *de novo* CD4 T cell immunity may not produce a sufficiently strong Th1 response to help dysfunctional or *de novo* primed virus-specific CD8 T cells during viral persistence.

Consistent with the inability to generate Th1 immunity, distribution of newly primed CD4 T cells in non-lymphoid sites of virus replication is greatly limited, particularly in the GI tract. The reduced ability of *de novo* primed virus-specific CD4 T cells to reach peripheral tissues, could lead to the failure to reconstitute these sites as an infection progresses; thus, compounding immunodeficiency and creating viral sanctuaries during persistent infections. As a result, persistently infected individuals may become more susceptible to virus-escape variants, secondary infections and re-infection in those organs. Ultimately, if new CD4 T cell responses recruited to balance CD4 T cell attrition or combat viral escape mutants could not generate a new Th1 component and distribute to tissue reservoirs of infection, it would leave a hole in the CD4 T cell response and

further debilitate control of viral replication. Since CD4 T cells have the potential to direct and sustain multiple types of immune responses in multiple tissues, future therapeutic strategies should consider the alterations in *de novo* CD4 T cell differentiation and how to appropriately overcome them.

Figure 3.1: Overview of the experimental strategy

Naïve virus-specific CD4 SMARTA T cells were transferred into mice infected for 21 days with persistent LCMV-Cl13 (late priming) or into uninfected mice that were immediately infected with LCMV-Cl13 (early priming). Cohorts were sacrificed in parallel at multiple time points post cell transfer.

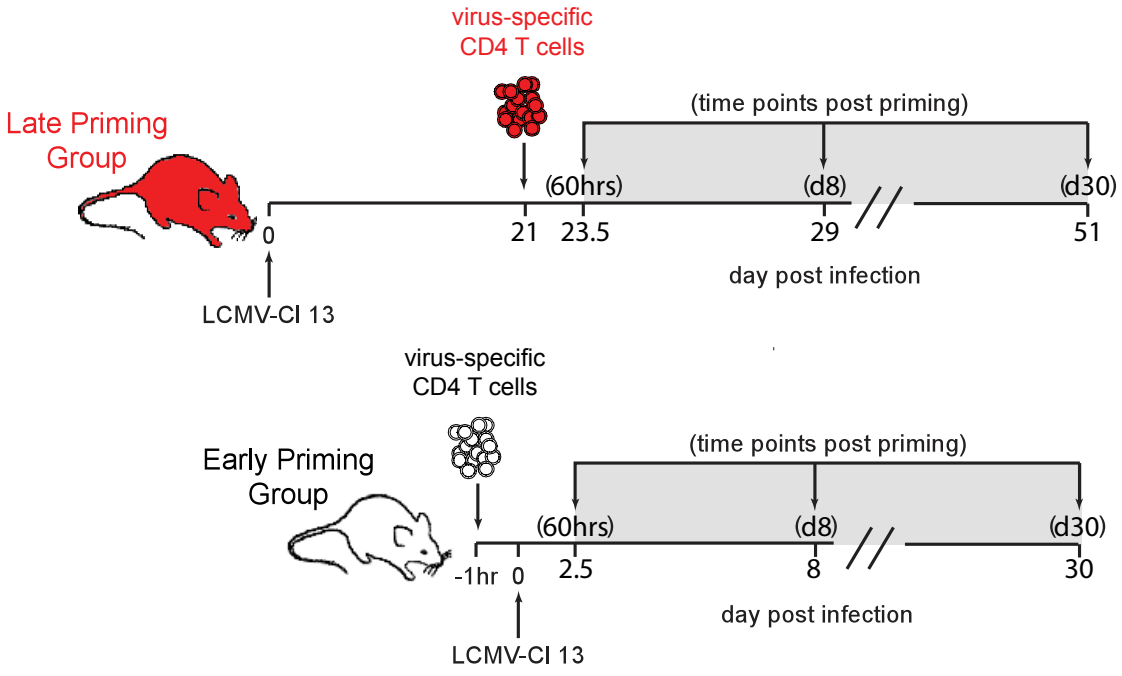

virus-specific
CD4 T cells
Late Priming
Group
(time points post priming)
(60hrs)
(d8)
(d30)
0
21
23.5
29
51
day post infection
LCMV-Cl 13
virus-specific
CD4 T cells
Early Priming
Group
(time points post priming)
(60hrs)
(d8)
(d30)
-1hr
0
2.5
8
30
day post infection
LCMV-Cl 13

Figure 3.2 Late primed CD4 T cells are activated and proliferate, but undergo a delay in differentiation.

(A) CFSE-labeled virus-specific CD4 SMARTA T cells were transferred into recipient mice and spleens isolated 60 hours after transfer. Early priming (E; white histogram); Late Priming (L; red histogram); Gray histogram (CFSE labeled SMARTA cells injected into naïve recipients that were not infected with LCMV).

(B) On day 20 after infection, CD11c-DTR transgenic mice were treated with PBS or diphtheria toxin (DT) to deplete dendritic cells, and CFSE-labeled naïve SMARTA cells were transferred 24 hours later. 24 hours after transfer, mice were again given DT or PBS. Mice were sacrificed 60 hours post transfer. The graph indicates the number of CD11c+, $MHCII^{hi}$ dendritic cells in the spleen of PBS or DT treated mice 36 hours after final treatment. Red, PBS control; Brown, DT treatment.

(C) Flow plots demonstrate CFSE dilution and the graph indicates the number of SMARTA cells 60 hours after transfer in the spleen in PBS (red) or DT (brown) treated mice. Gray, SMARTA cells transferred into naïve mice not subsequently infected with LCMV.

(D) Expression of the indicated protein and Blimp1 mRNA on early (E; white) and late primed (L; red) virus-specific CD4 SMARTA T cells 60 hours after priming. Endogenous CD4 T cells are shown (gray). Numbers on plots quantify percent of cells within gate (where applicable) or geometric fluorescence intensity of the population.

(E) Expression of Th1 ($SLAM^{hi}$, $CXCR5^{lo}$) and Tfh ($SLAM^{lo}$, $CXCR5^{hi}$) phenotypic proteins at 60 hours post priming.

(F) Percentages indicate IFNγ+, IL-21+, or IFNγ+/ IL-21+ double positive SMARTA cells following ex vivo peptide stimulation.

*=$p<0.05$, ns=not significant. Data is representative of 5 independent experiments with 3-5 mice per group.

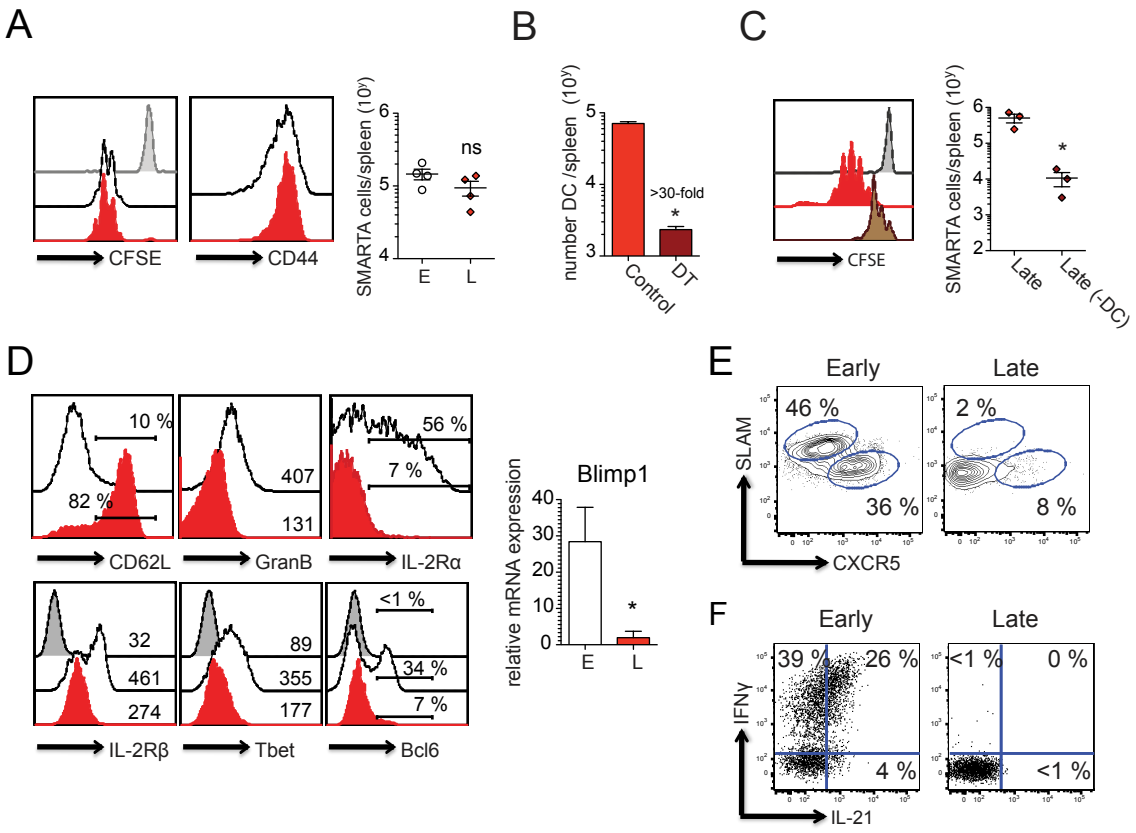

A
CFSE
CD44
SMARTA cells/spleen (10⁶)
ns
E
L
B
number DC /spleen (10⁵)
>30-fold
*
Control
DT
C
CFSE
SMARTA cells/spleen (10⁶)
*
Late
Late (-DC)
D
10 %
82 %
407
131
56 %
7 %
CD62L
GranB
IL-2Rα
32
461
274
89
355
177
<1 %
34 %
7 %
IL-2Rβ
Tbet
Bcl6
Blimp1
relative mRNA expression
*
E
L
E
Early
Late
SLAM
CXCR5
46 %
36 %
2 %
8 %
F
Early
Late
IFNγ
IL-21
39 %
26 %
4 %
<1 %
<1 %
0 %
<1 %

Figure 3.3. Late primed CD4 T cells generate Tfh, but have greatly diminished Th1 responses.

(A) mRNA expression was compared by microarray analysis in late vs. early primed SMARTA cells sorted 8 days after transfer. Graph displays the relative mRNA expression, with elevated values on the y-axis indicating increased expression in late primed virus-specific CD4 T cells and decreased values on the y-axis representing increased expression in early primed SMARTA virus-specific CD4 T cells.

(B-C) Flow plots illustrate Th1 ($SLAM^{hi}$, $CXCR5^{lo}$ or $SLAM^{hi}$, IL-$2R\beta^{hi}$) and Tfh ($SLAM^{lo}$, $CXCR5^{hi}$) differentiation in early primed and late primed virus-specific T cells 8 days after priming within the mesenteric lymph nodes and the spleen.

(D) Flow plots illustrate Th1 ($SLAM^{hi}$, IL-$2R\beta^{hi}$) differentiation by virus-specific CD4+ SMARTA cells 8 days after early or late priming within pooled brachial/axillary lymph nodes and in the inguinal lymph nodes. Graphs indicate number of Th1 and Tfh SMARTA cells within the organs

(E) Mice were infected with LCMV Cl13 and received SMARTA cells (red diamonds) or no cells (open circles) 21 days after infection. Graph illustrates plasma viral titers. LOD = limit of detection.

(F) Naïve SMARTA cells were transferred into mice that were immediately infected with acute LCMV-Armstrong (Arm - Early) or mice that had been infected with LCMV-Armstrong 4 days previously (Arm -Late). Flow plots demonstrate Th1 formation ($SLAM^{hi}$, CXCR5-) in splenic SMARTA cells 8 days after transfer and the graph shows absolute number of Th1 SMARTA cells in the spleen. *=$p<0.05$, ns=not significant. Data is representative of 3-6 independent experiments with 3-5 mice per group

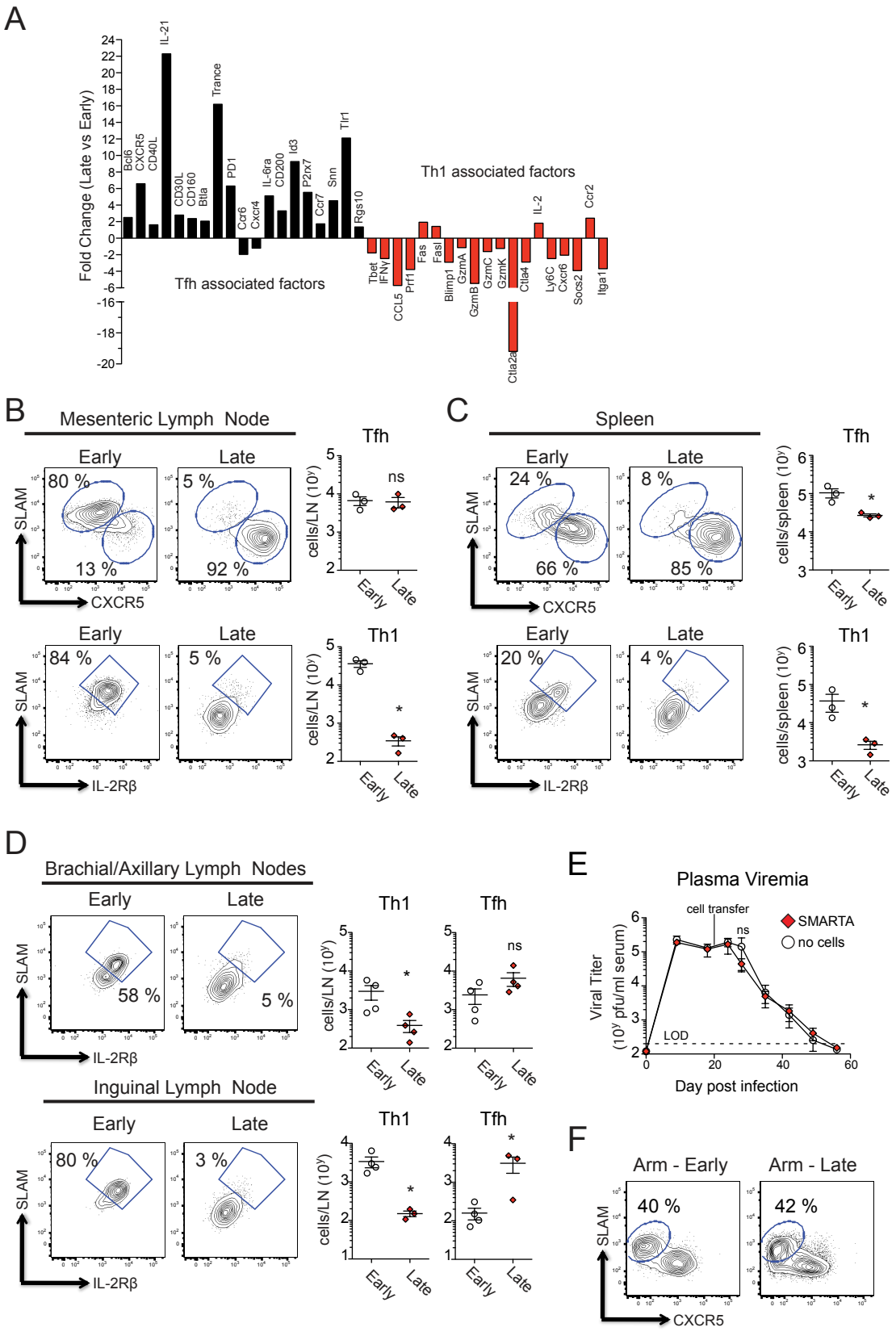

A
Fold Change (Late vs Early)
Tfh associated factors
Th1 associated factors
Bcl6
CXCR5
CD40L
IL-21
CD30L
CD160
Btla
Trance
PD1
Ccr6
Cxcr4
IL-6ra
CD200
Id3
P2rx7
Ccr7
Snn
Tlr1
Rgs10
Tbet
IFNγ
CCL5
Prf1
Fas
Fasl
Blimp1
GzmA
GzmB
GzmC
GzmK
Ctla2a
Ctla4
IL-2
Ly6C
Cxcr6
Socs2
Ccr2
Itga1
B
Mesenteric Lymph Node
Early
Late
80 %
13 %
5 %
92 %
SLAM
CXCR5
Tfh
ns
cells/LN (10^y)
84 %
5 %
IL-2Rβ
Th1
*
C
Spleen
24 %
66 %
8 %
85 %
20 %
4 %
cells/spleen (10^y)
D
Brachial/Axillary Lymph Nodes
58 %
5 %
Inguinal Lymph Node
3 %
E
Plasma Viremia
cell transfer
SMARTA
no cells
Viral Titer
(10^y pfu/ml serum)
LOD
Day post infection
F
Arm - Early
Arm - Late
40 %
42 %

Figure 3.4. Late primed virus-specific CD4 T cells form Tfh responses without LCMV-specific B cells or IL-6.

(A) IFNγ and IL-21 production by SMARTA cells on day 8 after priming following *ex vivo* peptide stimulation.

(B) CXCR5 and Bcl6 expression in SMARTA cells after early priming with LCMV-Arm, LCMV-Cl13, or late priming during an LCMV-Cl13 infection. Dashed line is included to facilitate a comparison of Bcl6 expression between the cohorts.

(C-D) Naïve SMARTA cells were transferred 21 days after LCMV-Cl13 infection into C57BL/6 wildtype or **(C)** B cell transgenic mice encoding an antibody specific to hen egg lysozyme (Hel-tg) that have B cells, but do not express anti-LCMV-specific B cells, or **(D)** IL-6-/- mice. Flow plots illustrate Th1 ($SLAM^{hi}$, CXCR5-) and Tfh ($SLAM^{lo}$, CXCR5+) differentiation by virus-specific CD4 SMARTA T cells in the spleen 8 days after priming. Graphs represent the absolute number of Tfh and Th1 SMARTA cells.

*=$p<0.05$; ns=not significant.. Data is representative of 2-4 independent experiments with 3-5 mice per group.

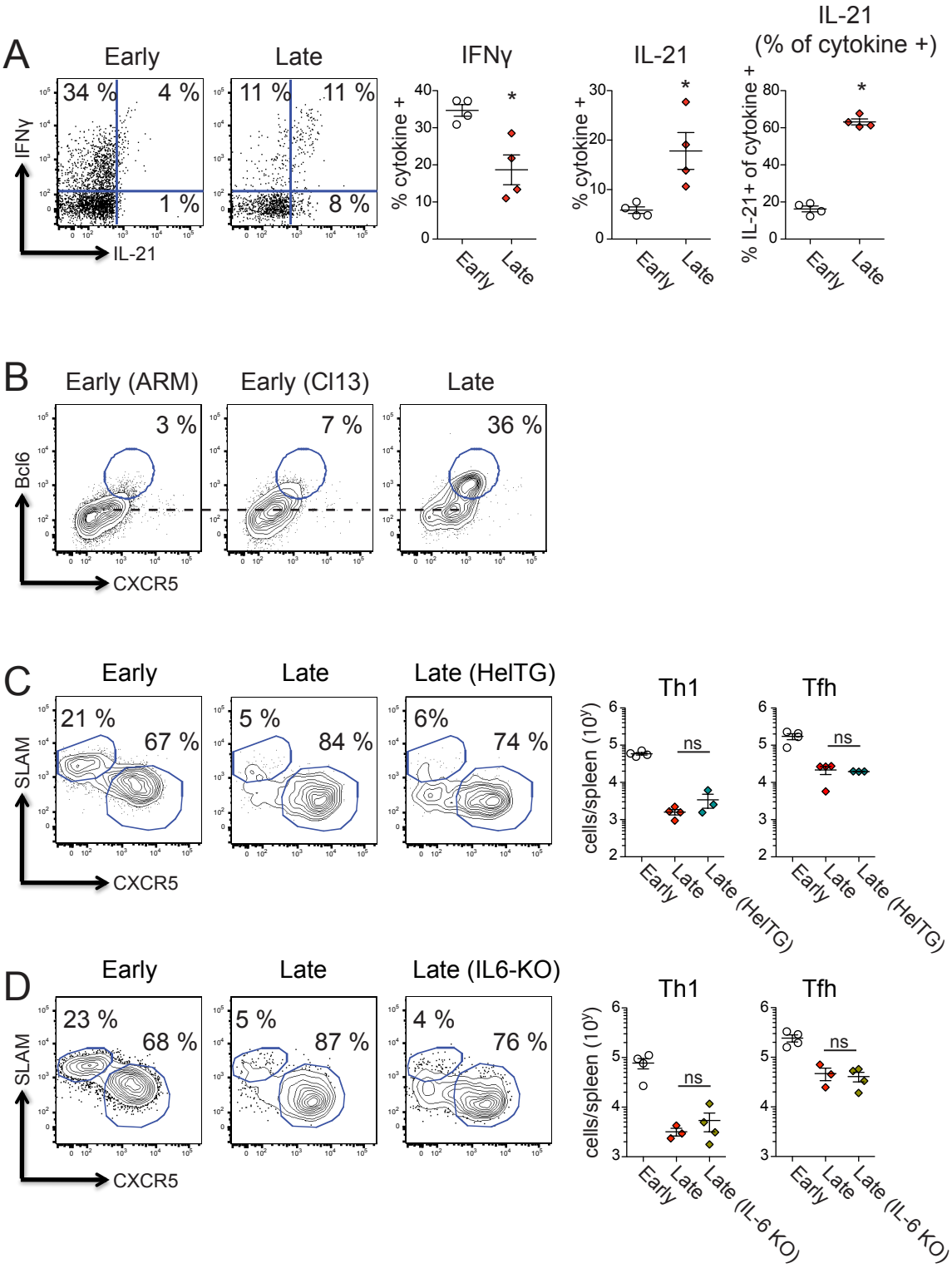

A
Early
Late
IFNγ
IL-21
34 %
4 %
1 %
11 %
11 %
8 %
IFNγ
% cytokine +
IL-21
% cytokine +
IL-21
(% of cytokine +)
% IL-21+ of cytokine +
Early
Late
B
Early (ARM)
Early (Cl13)
Late
3 %
7 %
36 %
Bcl6
CXCR5
C
Early
Late
Late (HelTG)
21 %
67 %
5 %
84 %
6%
74 %
SLAM
CXCR5
Th1
Tfh
cells/spleen (10⁷)
ns
Early
Late
Late (HelTG)
D
Early
Late
Late (IL6-KO)
23 %
68 %
5 %
87 %
4 %
76 %
SLAM
CXCR5
Th1
Tfh
cells/spleen (10⁷)
ns
Early
Late
Late (IL-6 KO)

Figure 3.5. Late primed virus-specific T cells are present at lower levels within peripheral organs.

(A) Total number of SMARTA cells within the liver, and the small and large intestine 8 days after early or late priming.

(B) Viral titers (plaque forming units/ gram tissue) in spleen, inguinal lymph nodes, liver, and small and large intestine on day 8 and day 29 after infection. The times represent the equivalent of 8 days after early or late priming.

*=$p<0.05$; ns=not significant.. Data is representative of 2-4 independent experiments with 4 mice per group.

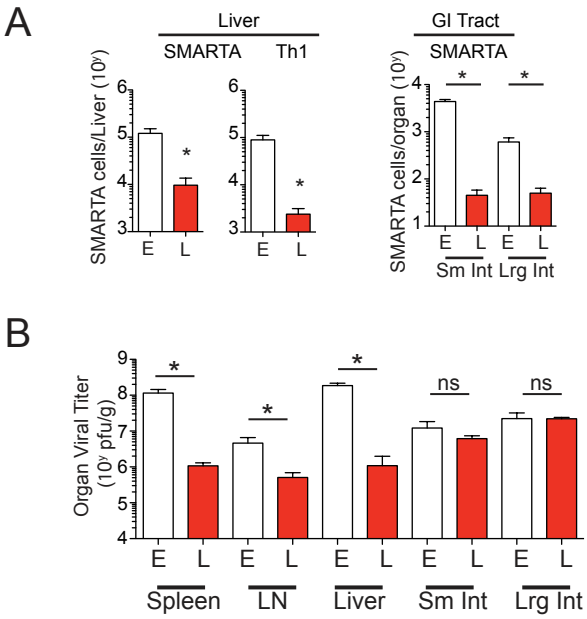

A
Liver
SMARTA
Th1
SMARTA cells/Liver (10⁵)
GI Tract
SMARTA
SMARTA cells/organ (10⁵)
E
L
Sm Int
Lrg Int
B
Organ Viral Titer (10⁵ pfu/g)
ns
Spleen
LN
Liver
Sm Int
Lrg Int

Figure 3.6. Binding and neutralization of LCMV-isolates by KL25 antibody.

(A) Neutralization of LCMV-Cl13 (black), LCMV-M1 (red), and LCMV-M2 (green) by the KL25 antibody. Graphs demonstrate the number of viral plaques as a percentage of no antibody control.

(B) A fixed concentration of KL25 antibody was co-incubated with media alone (blank), with $2x10^6$ plaque forming units of LCMV-C13, or with $2x10^6$ plaque forming units LCMV-M2. Antibody binding was determined by ELISA and is displayed as optical density units (ODU).

*=$p<0.05$ Data is representative of 2 independent experiments.

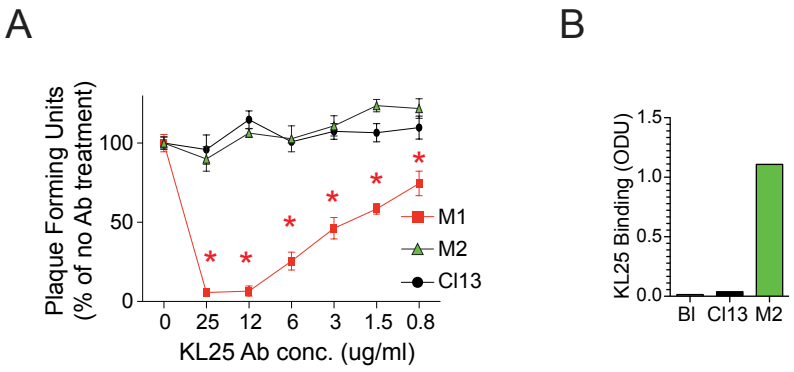

A
B
Plaque Forming Units
(% of no Ab treatment)
100
50
0
0 25 12 6 3 1.5 0.8
KL25 Ab conc. (ug/ml)
M1
M2
Cl13
KL25 Binding (ODU)
1.5
1.0
0.5
0.0
Bl Cl13 M2

Figure 3.7. Pre-existing antiviral CD4 T cell responses do not affect the late primed Th1 defect.

(A) Th1 and Tfh generation in early and late primed cohorts and in a cohort of mice that were CD4 depleted prior to infection with LCMV-Cl13 and received SMARTA cells 30 days after infection. Flow plots and graphs illustrate the percent of Th1 and Tfh SMARTA cells and the number of SMARTA cells within the spleen 8 days after transfer.

(B) Mice were CD4 depleted prior to LCMV infection. Thirty days after infection mice either received SMARTA cells or no cells and plasma virus titers quantified on days 8 and 30 after transfer (38 and 60 days after infection).

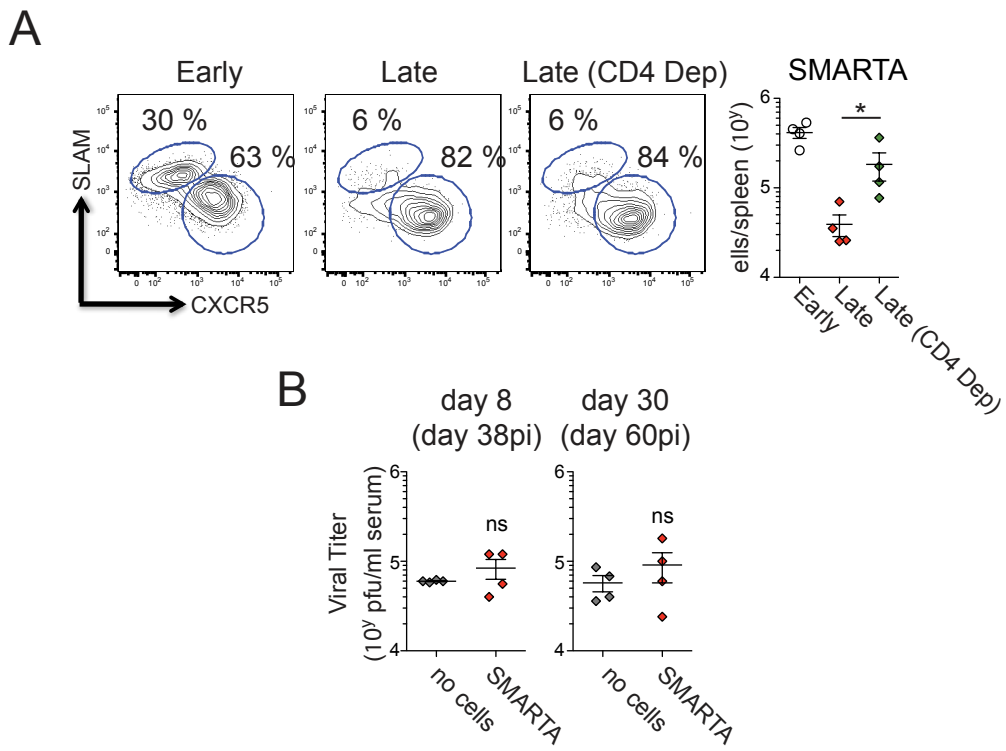

A
Early
Late
Late (CD4 Dep)
SMARTA
30 %
63 %
6 %
82 %
6 %
84 %
SLAM
CXCR5
ells/spleen (10^y)
*
Early
Late
Late (CD4 Dep)
B
day 8
(day 38pi)
day 30
(day 60pi)
Viral Titer
(10^y pfu/ml serum)
ns
ns
no cells
SMARTA
no cells
SMARTA

Figure 3.8. Virus-specific CD4 T cells primed during established persistent infection help B cell responses, but do not provide enhanced help to non-LCMV-specific B cells or LCMV-specific T cells

(A) Plasma LCMV-specific IgG levels on the indicated day following transfer of SMARTA cells alone (gray), TgKL25 B cells alone (blue), or SMARTA cells and TgKL25 B cells (red). Cells were transferred into mice infected for 30 days with LCMV M2. Mice were CD4 depleted prior to infection.

(B) Graphs demonstrate the expansion of adoptively transferred transgenic KL25+ B cells (+/- SMARTA cell transfer) 8 days and 40 days after transfer.

(C) Non-LCMV-specific, splenic donor B cells from TgKL25 mice (i.e., heavy chain positive, anti-idiotype antibody staining negative) were quantified numerically on day 8 and 40 after cell transfer.

(D) Graphs indicate the number of endogenous (pre-existing) LCMV-GP_{33-41} tetramer+ CD8 T cells and transferred virus-specific CD8 P14 T cells with or without SMARTA cell co-transfer.

(E) Flow plots and graph illustrate IFNγ+ secretion by virus-specific P14 cells 8 days after transfer alone or with SMARTA cells. Transfer was performed into persistently infected mice that were CD4 depleted prior to infection.

*=$p<0.05$. ns=not significant. Data is representative of 4 independent experiments with 4-5 mice per group.

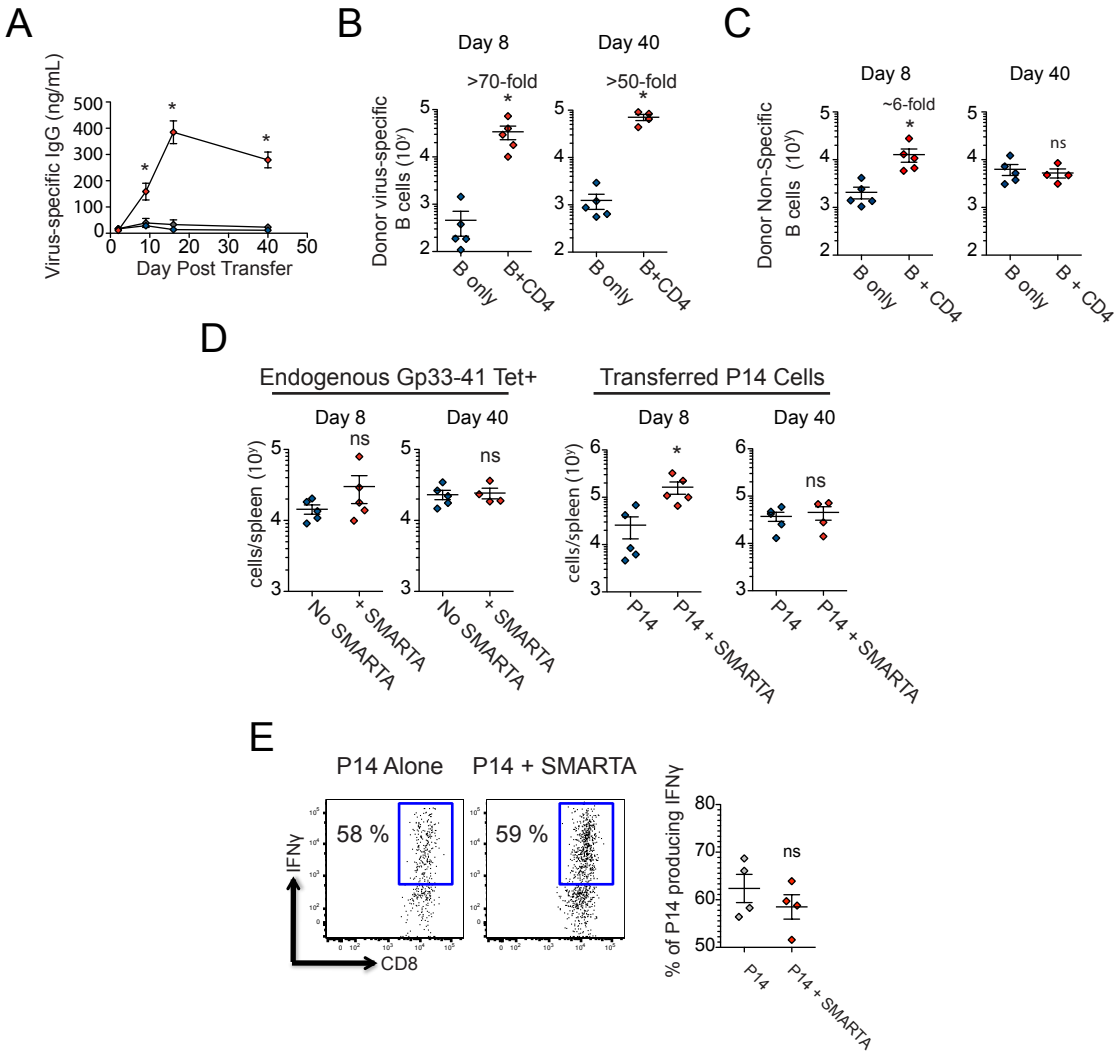

A
Virus-specific IgG (ng/mL)
500
400
300
200
100
0
0 10 20 30 40 50
Day Post Transfer
B
Day 8
Day 40
>70-fold
>50-fold
Donor virus-specific B cells (10^)
B only
B+CD4
C
Day 8
Day 40
~6-fold
ns
Donor Non-Specific B cells (10^)
B only
B + CD4
D
Endogenous Gp33-41 Tet+
Transferred P14 Cells
Day 8
Day 40
cells/spleen (10^)
ns
No SMARTA
+ SMARTA
P14
P14 + SMARTA
E
P14 Alone
P14 + SMARTA
58 %
59 %
IFNγ
CD8
% of P14 producing IFNγ
80
70
60
50
ns
P14
P14 + SMARTA

Figure 3.9. ***In vivo*** **boosting of costimulatory signaling does not enhance late primed virus-specific CD4 Th1 differentiation.**

(A) CD62L, Granzyme B, IL-2Rα, Tbet, and Bcl6 expression on early primed (white histograms), late primed (red histograms), and anti-CD28 treated late primed (blue histograms) SMARTA cells in the spleen 60 hours after transfer. Gates show percentage of SMARTA cells positive for marker or expression level based on geometric mean fluorescence intensity (MFI).

(B) Flow plots illustrate Th1 ($SLAM^{hi}$, CXCR5-) and Tfh ($SLAM^{lo}$, CXCR5+) differentiation by splenic SMARTA cells 60 hours after transfer.

(C) Flow plots and graphs illustrate Th1 ($SLAM^{hi}$, CXCR5-) and Tfh ($SLAM^{lo}$, CXCR5+) differentiation by splenic SMARTA cells 8 days after transfer.

*=$p<0.05$; ns= not significant. Data is representative of 2 independent experiments with 4 mice per group.

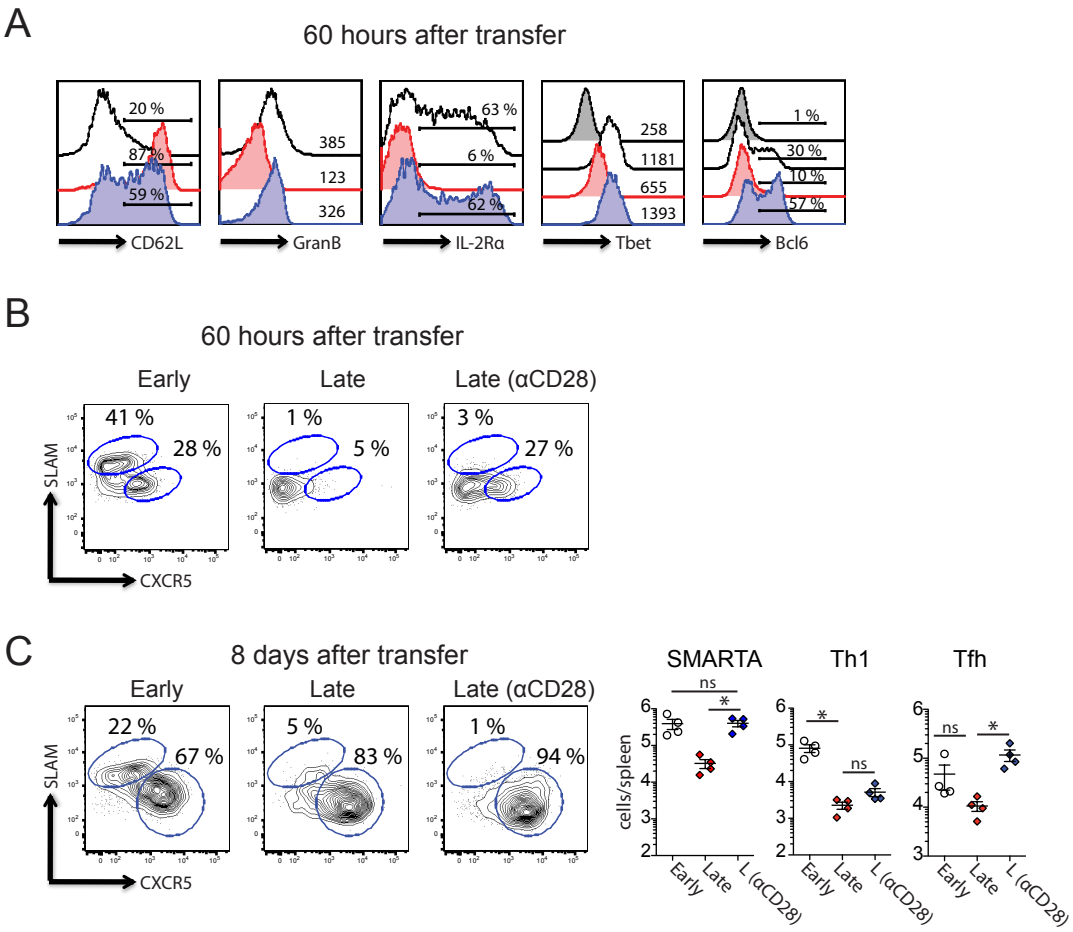

A
60 hours after transfer
20 %
87 %
59 %
CD62L
385
123
326
GranB
63 %
6 %
62 %
IL-2Rα
258
1181
655
1393
Tbet
1 %
30 %
10 %
57 %
Bcl6
B
60 hours after transfer
Early
Late
Late (αCD28)
41 %
28 %
1 %
5 %
3 %
27 %
SLAM
CXCR5
C
8 days after transfer
Early
Late
Late (αCD28)
22 %
67 %
5 %
83 %
1 %
94 %
SLAM
CXCR5
SMARTA
Th1
Tfh
cells/spleen
ns
*
Early
Late
L (αCD28)

Figure 3.10. IFNR blockade restores Th1 differentiation during late priming.

(A-D) Early and late primed conditions were treated with isotype antibody or with anti-IFNR blocking antibody. Antibody treatment was initiated 2 days prior to SMARTA cell transfer and then every 2 days through day 6 after transfer.

(A) Flow plots illustrate the frequency and number of Th1 and Tfh SMARTA cells in the mesenteric lymph node and spleen 8 days after priming in the presence of the indicated antibody treatment. Graphs quantify total number of SMARTA cells and number of Th1 and Tfh SMARTA cells within the organs.

(B) Flow plots illustrate Th1 ($SLAM^{hi}$, $IL\text{-}2R\beta^{hi}$) formation in liver SMARTA cells 8 days after early or late priming and isotype treatment (iso) or anti-INFR blocking antibody treatment. Graphs quantify total SMARTA cell number and number of Th1 SMARTA cells within the liver.

(C) Graphs quantify the total SMARTA cell number within the GI tract after indicated antibody treatment.

(D) IFNγ and TNFα production following ex vivo peptide stimulation by brachial/axillary lymph node-derived early and late primed SMARTA cells 8 days after transfer.

*=$p<0.05$, ns=not significant. Data is representative of 4 independent experiments with 3-4 mice per group.

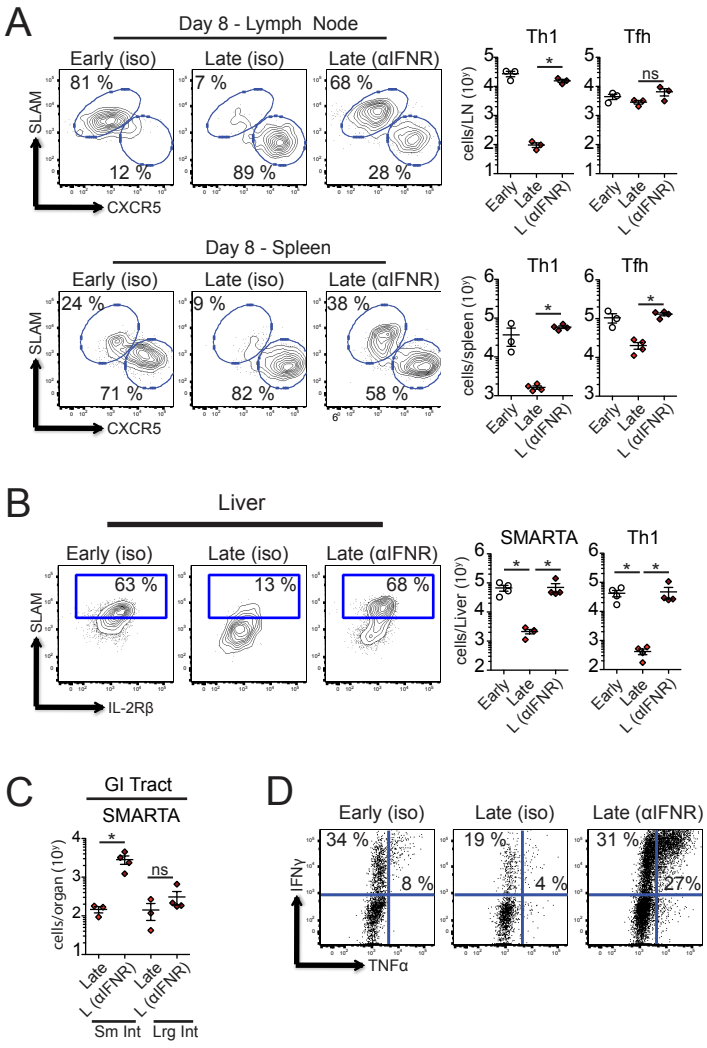

A
Day 8 - Lymph Node
Early (iso)
Late (iso)
Late (αIFNR)
81 %
12 %
7 %
89 %
68 %
28 %
SLAM
CXCR5
Th1
Tfh
cells/LN (10⁶)
Early
Late
L (αIFNR)
Day 8 - Spleen
24 %
71 %
9 %
82 %
38 %
58 %
cells/spleen (10⁶)
B
Liver
63 %
13 %
68 %
IL-2Rβ
SMARTA
cells/Liver (10⁶)
C
GI Tract
SMARTA
cells/organ (10⁶)
Sm Int
Lrg Int
D
34 %
8 %
19 %
4 %
31 %
27%
IFNγ
TNFα

Figure 3.11. IFNR blockade at the onset of persistent infection leads to enhanced Tfh but not Th1 formation.

Flow plots and graphs illustrate Th1 and Tfh formation by splenic SMARTA cells 9 days after isotype or anti-IFNR blocking antibody treatment at the onset of infection.

*=$p<0.05$, ns=not significant. Data is representative of 4 independent experiments with 3-4 mice per group.

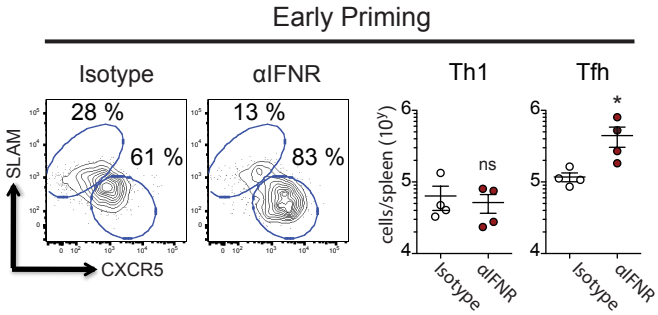

Early Priming
Isotype
αIFNR
28 %
61 %
13 %
83 %
SLAM
CXCR5
Th1
Tfh
cells/spleen (10^7)
6
5
4
ns
*
Isotype
αIFNR

Figure 3.12. Indirect IFN-I signals suppress CD4 Th1 differentiation at the time of priming.

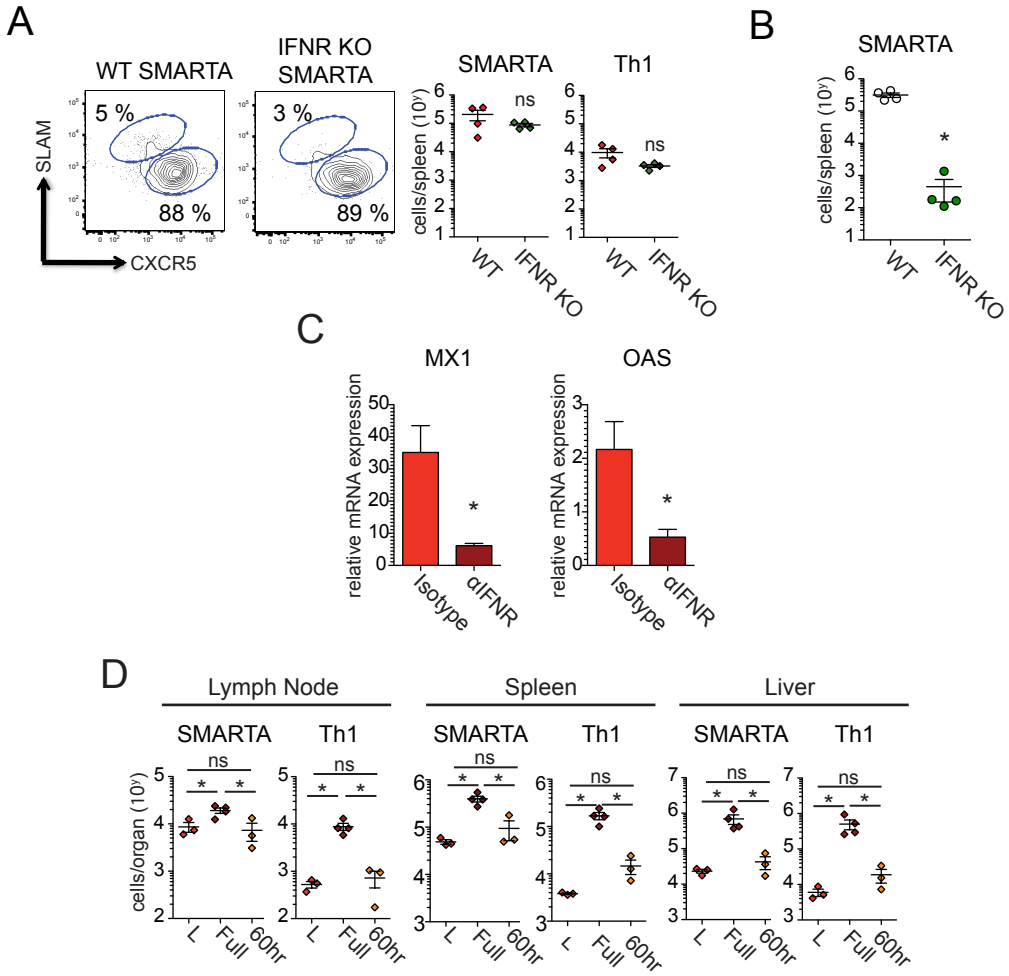
(A) Flow plots and graphs demonstrate Th1 and Tfh differentiation by late primed wild type and IFNR-/- SMARTA cells 8 days after priming. WT, wild-type SMARTA cells; IFNR KO, IFNR-/- SMARTA cells. Cells were transferred into animals infected 21 days prior with LCMV Cl13.

(B) Wild type or IFNR-/-SMARTA cells were transferred into mice immediately prior to LCMV-Cl13 infection. The graph shows the number of splenic SMARTA cells 8 days after infection.

(C) On day 21 after LCMV-Cl13 infection mice were treated with isotype or anti-IFNR blocking antibody. Graphs display mRNA expression of interferon-inducible genes MX1 and OAS in peripheral blood mononuclear cells 24 hours after treatment.

(D) Starting one day before SMARTA transfer, animals were treated with either isotype antibody, anti-IFNR blocking antibody or isotype antibody followed by anti-IFNR blocking antibody at 60 hours post transfer. Cells were transferred into animals infected 21 days prior with LCMV Cl13. Graphs illustrate number of Th1 and Tfh SMARTA cells in the mesenteric lymph nodes, spleen, and liver 8 days after late priming.

*=$p<0.05$, ns=not significant. Data is representative of 2 independent experiments with 3-4 mice per group.


A
WT SMARTA
IFNR KO SMARTA
5 %
88 %
3 %
89 %
SLAM
CXCR5
SMARTA
Th1
cells/spleen (10⁷)
ns
WT
IFNR KO
B
SMARTA
cells/spleen (10⁷)
*
C
MX1
OAS
relative mRNA expression
Isotype
αIFNR
D
Lymph Node
Spleen
Liver
SMARTA
Th1
cells/organ (10⁷)
L
Full
60hr

CHAPTER FOUR

Type I interferon promotes terminal effector differentiation and suppresses effector memory potential in CD8 T cells primed in the midst of persistent infection

ABSTRACT

Virus-specific CD8 T cells recruited at the onset of a persistent viral infection initially form robust short-lived effector cell (SLEC) and memory precursor effector cell (MPEC) populations. However, prolonged antigen exposure induces progressive virus-specific CD8 T cell dysfunction and abrogates their ability to form antigen-independent memory after prolonged antigen exposure. Virus-specific CD8 T cells recruited in the midst of low-level persistent infections develop superior effector and memory responses compared to the dysfunctional virus-specific CD8 T cells recruited at the onset of infection, but it is unclear whether this is true for highly-replicating persistent viral infections. Herein we demonstrate that virus-specific CD8 T cells primed in the midst of a highly-replicating persistent viral infection are driven towards terminal effector differentiation, develop few MPECs, and form poor effector memory responses, despite displaying a transcriptional program associated more closely with memory formation. These alterations in effector differentiation are dependent upon type I interferon signaling, and IFN-I signaling blockade reduced the frequency of terminally differentiated SLECs and restored MPEC formation. Our findings highlight unique a mechanism of control over CD8 T cell effector programming during persistent viral infection.

INTRODUCTION

CD8 T cells are a critical subset of adaptive immunity that control infections by viral pathogens by directly killing infected cells and secreting proinflammatory cytokines such as IFNγ and TNFα[5]. Upon encounter with viral antigen and the appropriate costimulatory signals, a CD8 T cell undergoes a program of rapid expansion and differentiation into potent short-lived effector cytotoxic lymphocytes (SLECs) and memory precursor effector cells (MPECs)[77,137]. After elimination of infection, the majority of the virus-specific SLECs undergo apoptosis. However, MPECs preferentially survive this contraction to form antigen-independent memory cells, becoming dependent on the cytokines IL-7 and IL-15 for survival[78]. Broadly, memory virus-specific CD8 T cells can be subdivided into two different classes. CD62L- effector memory cells circulate within the blood and perform surveillance of non-lymphoid organs, whereas CD62L+ central memory cells are primarily retained in the lymphoid organs and give rise to a more robust recall response in the case of antigen re-encounter[138,139]. Both central and effector memory cells persist potentially for the lifetime of the host, and serve to rapidly and efficiently eliminate their target pathogen in the case of re-infection.

Although most viral infections trigger a potent CTL response that serves to control and purge infection, a number of viral pathogens are capable of overcoming this control and establishing a persistent infection. Escape mutations within the viral genome generate viral variants that cannot be killed by the most potent CTL effectors, and prolonged antigen stimulation and inhibitory cell surface and secreted cytokine signals lead to CTL exhaustion or physical deletion[1,140,141]. Nevertheless, even exhausted CD8 T cells critical to maintain a limited degree of control over viral replication, and the outcome in multiple persistent viral infections correlates well with the robustness of the CTL response[142,143,144]. Interestingly, during some persistent infections, the exhausted CD8 T cell responses primed at the onset of infection are supported and continuously

replenished by new virus-specific CD8 T cells emerging from the thymus[109]. These "latecomer" CD8 T cells could also help control viral variants that have escaped from the original effector CTL response. Furthermore emerging techniques in hematopoietic stem cell (HSC) engineering may provide the opportunity to introduce virus-specific T cell receptors into HSCs to boost the antiviral immune response and control persistent infection[99]. Both natural and therapeutic supplementation of existing antiviral immunity would rely on the activation of new virus-specific CD8 T cells within a persistently infected immune environment.

The type and quality of signals a CD8 T cell received during priming can substantially alter the quality of its effector response, the degree of terminal differentiation it undergoes, and its potential to establish protective memory responses[145,146]. Thus, the immediate exposure to high levels of antigen and inflammatory and suppressive factors, changes in antigen presenting cell dynamics, and disrupted lymphoid organ architecture[21,49] may affect the function of naïve CD8 T cells primed during persistent infection. Given the potential biological and therapeutic utility of generating new antiviral CD8 T cell responses in the midst of persistent infection, we sought to determine how priming in the midst of persistent infection would affect the cellular and effector functions of virus-specific CD8 T cells. In this chapter, we demonstrate that CD8 T cells primed during persistent infection are characterized by high levels of terminal differentiation, expression of memory-associated markers and transcriptional regulators, and reduced peripheral presence. This altered differentiation state is dependent on type I and II interferon, and blockade of type I or II interferon signaling restores normal CD8 T cell effector differentiation during late priming. Our findings indicate that manipulation of the immune environment and type I interferon-dependent pathways may help engineer a more optimal CD8 T cell effector response in the midst of persistent infection.

MATERIALS AND METHODS

Mice and virus

C57BL/6 (WT) mice were purchased from The Jackson Laboratory or the rodent breeding colony at University of California, Los Angeles. B cell deficient μMT, Hen-egg lysozyme transgenic (Hel-tg), and CD11c-DTR mice were purchased from The Jackson Laboratory. LCMV-GP_{61-80} specific CD4 TCR transgenic (SMARTA) and LCMV-GP_{33}-specific CD8 TCR transgenic (P14) mice have been described previously[100,116]. All mice were housed under specific pathogen–free conditions. Mouse handling conformed to the experimental protocols approved by the University of California, Los Angeles Animal Research Committee (ARC). In all experiments the mice were infected i.v. via the retroorbital sinus with 2×10^6 PFU of LCMV-Armstrong or LCMV-Clone 13. Virus stocks were prepared and viral titers were quantified as described previously [100].

Isolation and adoptive transfer of virus-specific T cells

LCMV-specific P14 cells were isolated from the spleens of respective transgenic mice by negative selection (StemCell Technologies). All cell transfers were performed i.v. in the retroorbital sinus. To assess priming and differentiation of virus-specific CD4 T cells in the midst of persistent infection, we transferred 1,000 P14 cells into either naive mice that were infected with LCMV-Cl13 or LCMV-Armstrong 1 hour later (early priming) or into mice that had been infected with LCMV-Cl13 21 days earlier (late priming). For experiments in which the mice were sacrificed at 60 hours after transfer, 250,000 P14 cells were transferred to enable detection at this early time point. For late priming during acute infection, 1,000 P14 cells were transferred into mice infected for 4 days with LCMV-Armstrong.

Assessment of virus-specific T cell memory formation

To determine the capacity of early and late primed virus-specific CD8 T cells to form antigen independent memory, 10,000 P14 cells were transferred into naïve mice infected with LCMV-CL13 1 hour later, or mice that had been infected with LCMV-Cl13 21 days earlier. Sixty hours after transfer, splenocytes were isolated, pooled by cohort, and depleted of B cells by negative selection on an autoMACS (Miltenyi Biotec). Total number of P14 cells in each cohort was determined, and 700 early or late primed P14 cells were injected into LCMV immune mice. LCMV immune mice were generated by infection with low dose (2×10^5 PFU) of LCMV-Armstrong.

***In vivo* Type I Interferon Receptor (IFNR1) blockade**

To block IFN-I signaling in vivo during persistent infection, mice were treated i.v. with 500μg anti-IFNR1 blocking antibody (clone MAR1-5A3; Leinco Technologies) or isotype control antibody 1 or 2 days before P14 cell transfer (on day 19 or 20 of infection), and every 48 hours subsequently through day 27 or 28 after infection.

Isolation of peripheral organ and intestinal lymphocytes

Lymphocytes from the spleen were isolated by mashing the organ through a 100 micron filter. For experiments in which intrahepatic or intestinal lymphocytes were analyzed, mice were perfused with 25 ml sterile PBS by intracardiac injection to remove blood from peripheral tissues. Intrahepatic lymphocytes were isolated by mashing the organ through a 100 micron filter and by subsequent centrifugation in 35% Percoll (GE healthcare).

***In vivo* dendritic cell depletion**

To deplete dendritic cells, CD11c-DTR mice were treated i.v. with 100ng diphtheria toxin in PBS (List Biological Laboratories) one day prior to SMARTA cell transfer. A second

treatment was given one day after cell transfer. PBS vehicle was injected into undepleted controls.

quantitative RT-PCR

1000 P13 cells were transferred into naïve mice that were immediately infected with LCMV-C13 (early priming) or mice infected with LCMV-Cl13 21 days earlier (late priming). Samples contained cells isolated from the spleens of 6 pooled mice per group. Sixty hours after P14 transfer, early and late primed P14 cells were isolated by FACSorting using an Aria II (Beckton Dickinson). Post-sort purity was verified as >97% on the FacsVerse (Beckton Dickinson). RNA was isolated from the sorted cells using the RNeasy extraction kit (Qiagen). Three to four biological replicates were obtained per group. RNA was normalized for input and amplified directly using the One-Step RT-PCR kit (Qiagen). PRDM1 and HPRT were amplified using Applied Biosystems Assays-on-Demand TaqMan pre-made expression assays. mRNA amplification was detected on the iCycler (Bio-Rad Laboratories). RNA expression was normalized to HPRT.

Flow Cytometry

Analysis of immune subsets was performed by staining lymphocytes obtained from spleen, lymph nodes, liver, and intestinal fractions ex vivo for the expression of CD4, CD8, KLRG1, CD127 (IL-7R), CD25 (IL-2Rα), CD62L, IFNγ, TNFα, CD138, CD11c, MHCII (I-A/I-E), Tbet, EOMES (BioLegend); CD45.1, Granzyme B (eBioscience); Thy1.1, Bcl6 (BD Biosciences). Intracellular cytokine staining was performed after six hour ex-vivo stimulations with GP_{33} peptide. CFSE dilution analysis was performed by incubating naïve P14 cells with 2.5 μM CFSE (Life Technologies). Flow cytometric data was collected on the FacsVerse (Beckton Dickinson).

Statistical analysis

Student's *t* tests (two-tailed, unpaired) were performed using GraphPad Prism 5 software (GraphPad Software, Inc.).

RESULTS

Virus-specific CD8 T cells primed in an established persistent infection undergo an alternative pathway of effector differentiation.

To investigate the activation and effector differentiation of CD8 T cells primed in the midst of persistent infection, we employed an experimental approach similar to what we described in the previous chapter (see figure 3.1). Briefly, we transferred transgenic virus-specific CD8 (P14) T cells into mice that had been infected 21 days earlier with LCMV-Cl13 or into naïve mice immediately infected with LCMV Cl-13. As with our CD4 T cell model, this allowed a direct comparison of CD8 T cell priming at the onset and in the midst of persistent infection. Importantly, transgenic P14 CD8 T cells mimic the host-derived immunodominant (Gp_{33} tetramer+) CD8 T cell response to LCMV-Cl13[100]. Herein, we continue the nomenclature we established in Chapter 3, and refer to CD8 T cell responses primed at the onset of infection as “early priming,” and CD8 T cell responses primed in the midst of persistent infection as “late priming.”

Sixty hours after transfer, we observe that early and late primed CD8 T cells proliferate, upregulate the activation marker CD44, and expand to similar levels (Figure 4.1A). Similar to late priming of virus-specific CD4 T cells, efficient expansion of virus-specific CD8 T cells during late priming required DCs, and depletion of DCs prior to late priming potently inhibited CD8 T cell proliferation and expansion (Figure 4.1B). Despite similar expansion and activation early after transfer, late primed virus-specific CD8 T cells exhibit few characteristics of CTL effector differentiation. Notably, early primed CD8 T cells downregulate the lymph node retention molecule CD62L, and upregulate the IL-2 receptor alpha chain (IL-2Rα), the cytotoxic effector protein Granzyme B, as well as Tbet and Blimp1, the transcriptional regulators responsible for programming CTL effector differentiation, whereas these changes were not observed in late primed virus-specific CD8 T cells (Figure 4.1C). The T-box transcriptional regulator Eomesodermin (Eomes)

can serve a redundant role with Tbet in regulating CD8 T cell effector functions, as well as to promote CD8 T cell memory formation. Interestingly late primed CD8 T cells express higher amounts Eomes sixty hours after priming (Figure 4.1C), suggesting they may be undergoing an alternate effector differentiation pathway. In support of this, we observe that late primed virus-specific CD8 T cells produce similar levels of the effector cytokine IFNγ, notable, albeit slightly reduced levels of TNFα, and increased levels of IL-2 (Figure 4.1D). This stands in stark contrast with our findings regarding late priming in virus-specific CD4 T cells, where no effector cytokines apart from IL-2 were produced by late primed CD4 T cells (Figure 3.2). Ultimately, our data demonstrate that unlike late primed virus-specific C4 T cells, late primed virus-specific CD8 T cells assume some effector functions by sixty hours after priming, and suggest that these functions are programmed by Eomes rather than Tbet and Blimp1.

Virus-specific CD8 T cells primed during an established persistent infection demonstrate a higher degree of terminal differentiation but may retain the potential to form memory.

To further determine how priming in the midst of persistent infection affects virus-specific CD8 T cell differentiation, we assessed early and late primed CD8 T cell responses eight days after priming. We noted that late primed CD8 T cell responses expand to lower peak numbers within the spleen at this time point (Figure 4.2A). Additionally, we continued to observe a reduced expression of Tbet and increased expression of Eomes (Figure 4.2B). Furthermore, these changes were not observed when virus-specific CD8 T cells were transferred into an established acute LCMV-Arm infection, indicating that the suppression of Tbet and induction of Eomes is specific to priming in the midst of a persistent infection (Figure 4.2B). High levels of Tbet and Blimp1 drive CD8 T cell terminal effector differentiation and formation of short-lived

effector cells (SLECs), whereas Eomes expression is associated with CD8 T cell memory formation[77,147,148]. Because late primed virus-specific T cells expressed low levels of Tbet and high levels of Eomes, we sought to determine whether this would lead to preferential formation of MPEC over SLECs. Surprisingly, we observed that an increased frequency of virus-specific cells primed in the midst of persistent infection express the SLEC terminal differentiation marker KLRG1 (Figure 4.2C). Additionally, IL-7 receptor (IL-7R) expression and MPEC formation was reduced in late primed virus-specific CD8 T cells (Figure 4.2D). Functionally both early and late primed virus-specific CD8 T cells continued to produce similar amounts of IFNγ (Figure 4.2E), as well as TNFα and IL-2 (not shown). Together, these data indicate that late priming accelerates terminal differentiation in virus-specific CD8 T cells and reduces memory potential.

We next sought to determine how priming in the midst of persistent infection affects the long-term differentiation of virus-specific CD8 T cells. Fifty days after priming, we noted that similar numbers of early and late primed virus-specific CD8 T cells were present within the spleen (Figure 4.3A). Nevertheless, late primed virus-specific CD8 T cells displayed a greater frequency and number of KLRG1+ SLECs at this time (Figure 4.3B). Interestingly, we observed a notable population of KLRG1-, IL7R+ MPEC cells, similar to the memory compartment that develops fifty days after acute LCMV-ARM infection, but which does not develop in virus-specific CD8 T cells primed at the onset of persistent LCMV-Cl13 infection (Figure 4.3B). Furthermore, we observe that late primed virus-specific CD8 T cells demonstrate an increased frequency of polyfunctional IFNγ and TNFα producers (Figure 4.3C), although this data may represent a rebound of function secondary to viral clearance. It has been established that cells primed at the onset of persistent infection lose their capacity to form antigen-independent memory and respond to homeostatic signals by IL-7 and IL15[80]. Our data suggest that late primed

virus-specific CD8 T may be capable of establishing antigen-independent memory after infection has been cleared.

Virus-specific CD8 T cells primed during an established persistent infection have a reduced per-cell capacity to form peripheral effector memory

Increased SLEC and reduced MPEC frequency in late primed virus-specific CD8 T cells eight days after priming indicate that the memory potential of late primed virus-specific CD8 T cells may be impaired. However, the appearance of an MPEC population in late but not early-primed virus-specific CD8 T cells suggests that late primed virus-specific CD8 T cells may have superior memory-forming capacity in the setting of low or absent persisting antigen. To resolve this question, we isolated early and late primed virus-specific CD8 T cells sixty hours after priming and transferred equal numbers LCMV-immune mice. We chose this early time point to analyze the impact of priming without the confounding factor of prolonged persisting antigen, which is known to impair memory potential in early primed virus-specific CD8 T cells[80]. Tracking the memory virus-specific CD8 T cell response within the peripheral blood over a one-month period, we observed that early primed virus-specific CD8 T cells were present at increased levels within the peripheral blood (Figure 4.4A). Furthermore, although the number of memory of early and late primed CD8 T cells was not significantly different within the spleen 30 days after transfer, late primed memory CD8 T cells were not detectable in the liver (Figure 4.4B). Collectively, these data indicate that late primed virus-specific CD8 T cells exhibit a profound defect in the formation of a peripheral effector memory response, while central memory formation appears to be intact.

Type I interferon signaling promotes Eomes expression and SLEC formation during an established persistent infection.

As we have demonstrated in the previous chapter, type I interferon (IFN-I) signaling is also capable of modulating *de novo* immunity during persistent viral infection. Furthermore, IFN-I signaling has been demonstrated to induce Eomes expression and effector differentiation in bystander naïve non-virus-specific CD8 T cells during viral infection, and to limit CD8 T cell proliferative responses[146,149]. To determine whether IFN-I similarly modulates virus-specific CD8 T cells primed during persistent infection, we blocked IFN-I signaling *in vivo* with an antibody against the type I interferon receptor (IFNR)[11]. 8 days after priming, virus-specific CD8 T cells in anti-IFNR treated animals expanded to greater levels and downregulated Eomes expression, although virus-specific CD8 T cell expression Tbet expression remained the same in anti-IFNR and isotype control treated animals (Figure 4.5A-B). This data demonstrates that IFN-I signaling is responsible for limiting the overall expansion of late primed virus-specific CD8 T cell responses and for instructing a program of effector differentiation characterized by high levels of Eomes.

To further determine how IFN-I signaling affects the effector differentiation of late primed virus-specific CD8 T cell responses, we assessed SLEC and MPEC formation 8 days after priming. We observed that IFNR blockade increased the frequency of IL-7R+ MPECs and reduced the frequency of KLRG1+ SLECs (Figure 4.5C). SLEC and MPEC frequency in anti-IFNR treated mice is similar to what we observed during early priming, suggesting that IFN-I signaling is primarily responsible for altering the effector differentiation of virus-specific CD8 T cell primed during viral persistence. Interestingly, we did not observe significant change in IFNγ and TNFα secretion in late primed virus-specific CD8 T cells after IFNR blockade (Figure 4.5D), indicating that IFN-I signals primarily affects MPEC and SLEC differentiation potential rather than CD8 T cell effector function.

DISCUSSION

The environment a CD8 T cell encounters during priming plays a significant part in its ultimate fate. Inflammatory signals, cytokine levels, antigen load, and the timing of CD8 T cell priming can influence both antiviral effector functions, as well as the rate of exhaustion as well as the ability to form central and effector memory responses[146,150,151]. Previous studies have indicated that CD8 T cell recruitment several days into an acute infection or in the midst of a low-level persistent mouse polyomavirus infection results in preferential programming of long-lived CD8 T cell memory responses, reduced exhaustion, and superior effector function[151,152]. Surprisingly, our data demonstrate the opposite effect. CD8 T cells primed in the midst of a persistent LCMV infection were driven toward an SLEC phenotype, while the formation of MPECs was repressed. LCMV-Cl13 replicates at a high level within the mouse, while polyomavirus generates a "smoldering" low-level infection and acute LCMV-Arm infection is cleared rapidly within several days. It is therefore likely that discrepancies in viral replication and the inflammatory milieu between these types of viral infections help determine the outcome of late primed CD8 T cell responses.

CD8 T cell priming late during an infection, or the recruitment of non-virus-specific bystander CD8 T cells by an inflammatory environment is frequently associated with the expression of memory-associated markers and transcriptional regulators[146,151]. Our findings that late primed virus-specific CD8 T cells express higher levels of the transcriptional regulator Eomes and memory-associated lymphoid retention molecule CD62L and lower levels of Tbet and Blimp1 appears to corroborate these findings. Nevertheless, despite an apparent memory-like transcriptional and phenotypic program, late primed virus-specific CD8 T cells formed SLECs at a higher frequency and demonstrated a pronounced defect in the generation of memory when transferred into an antigen-free environment. Furthermore, our data suggest that phenotypic

resemblance of early effector cells to memory cells may not always precisely indicate memory potential. Interestingly, the memory formation defect we observed was primarily the result of the absence of peripheral effector memory responses, while the capacity to form lymphoid resident central memory was equal in early and late primed cells. Importantly, in our experiment cells were transferred into an antigen-free environment very early after priming, thus avoiding potentially confounding effects of reprogramming through prolonged antigen contact. Our results therefore indicate that the deficiency in memory formation in virus-specific CD8 T cells primed during viral persistence is programmed during the initial priming events. It may be that this failure to form effector memory is a parallel mechanism to the defective formation of Th1 responses by late primed virus-specific CD4 T cells. If so, this would indicate a common mechanism that specifically downregulates effector responses functioning within the periphery during persistent viral infection. Although a previous report indicated that virus-specific CD8 T cells primed several days into an acute LCMV infection phenotypically resemble central memory cells and are retained at higher levels within the spleen over a long-term period, formation of peripheral effector memory was not assessed[152]. Additionally, we did not observe the same transcriptional reliance on Eomes by virus-specific CD8 T cells primed late during an acute infection. It would therefore be interesting to determine the effector memory formation defect we observed is specific to priming during a persistent LCMV infection, or whether it translates to late priming in other infectious scenarios.

IFN-I is a potent regulator of the immune response. During LCMV infection, IFN-I is necessary for the proper formation of virus-specific CD8 T cell responses; however, sustained IFN-I signaling is ultimately detrimental to antiviral immunity and promotes viral persistence[11,153]. Curiously, our findings regarding the role of IFN-I during late priming stand in stark contrast to the role of IFN-I during early priming in inducing Blimp1 expression and a high rate of CD8 T cell proliferation[153]. Whereas blockade of IFN-I

signaling at the onset of LCMV and other viral infections induces deficient CD8 T cell responses[154], we observed that blockade of IFN-I during late priming induced greater levels of expansion in virus-specific CD8 T cells eight days after priming. This suggests that the timing and context of IFN-I signaling are important for determining the effects it exerts during T cell programming.

As seen in the previous chapter, IFN-I signaling is capable of modulating the effector differentiation of *de novo* antiviral CD4 T cell immunity during persistent infection. In this chapter, we demonstrate that IFN-I induced Eomes-driven effector differentiation and suppression of MPEC formation in late primed virus-specific CD8 T cells. Interestingly, Marshall et al. had previously reported that IFN-I induces cytokine secretion and Eomes expression in bystander non-virus specific CD8 T cells[146]. The similarity between these findings and ours suggest a common INF-I mediated mechanism that sensitizes CD8 T cells toward an Eomes-driven acquisition of effector function. Interestingly, Marshall et al. show that IFN-I does not need to signal directly to CD8 T cells to promote this effect. Although additional experiments need to be performed to determine whether this is the case with late priming of virus-specific CD8 T cells, our findings regarding virus-specific CD4 T cell late priming in the previous chapter also suggest a T-cell extrinsic mechanism. Although it is still unclear how IFN-I mediates its effects, the necessity of APCs for late priming of virus-specific CD8 T cells indicates that many of the instructive signals late primed CD8 T cells receive may ultimately be dependent on the APC. Ultimately, modulation of IFN-I signaling and downstream mechanisms could help appropriately tailor antiviral immunity during persistent infection.

Figure 4.1. Late primed virus-specific CD8 T cells demonstrate Eomesodermin-driven effector function.

(A) CFSE-labeled virus-specific CD8 P14 T cells were transferred into recipient mice and spleens isolated 60 hours after transfer. Early priming (E; white histogram); Late Priming (L; red histogram); Gray histogram (CFSE labeled P14 cells injected into naïve recipients that were not infected with LCMV).

(B) On day 20 after infection, CD11c-DTR transgenic mice were treated with PBS or diphtheria toxin (DT) to deplete dendritic cells, and CFSE-labeled naïve P14 cells were transferred 24 hours later. 24 hours after transfer, mice were again given DT or PBS. Mice were sacrificed 60 hours post transfer. Flow plot demonstrates CFSE dilution and the graph indicates the number of P14 cells 60 hours after transfer in the spleen in PBS (red) or DT (brown) treated mice.

(C) Expression of the indicated protein and Blimp1 mRNA on early (E; white) and late primed (L; red) virus-specific CD4 SMARTA T cells 60 hours after priming. Numbers on plots quantify percent of cells within gate (where applicable) or geometric fluorescence intensity of the population.

(D) Flow plots display IFNγ, TNFα, and IL-2 secretion by P14 cells sixty hours after priming. Percentages indicate the frequency of cells within the appropriate gate.

*=$p<0.05$, ns=not significant. Data is representative of 3 independent experiments with 3-5 mice per group.

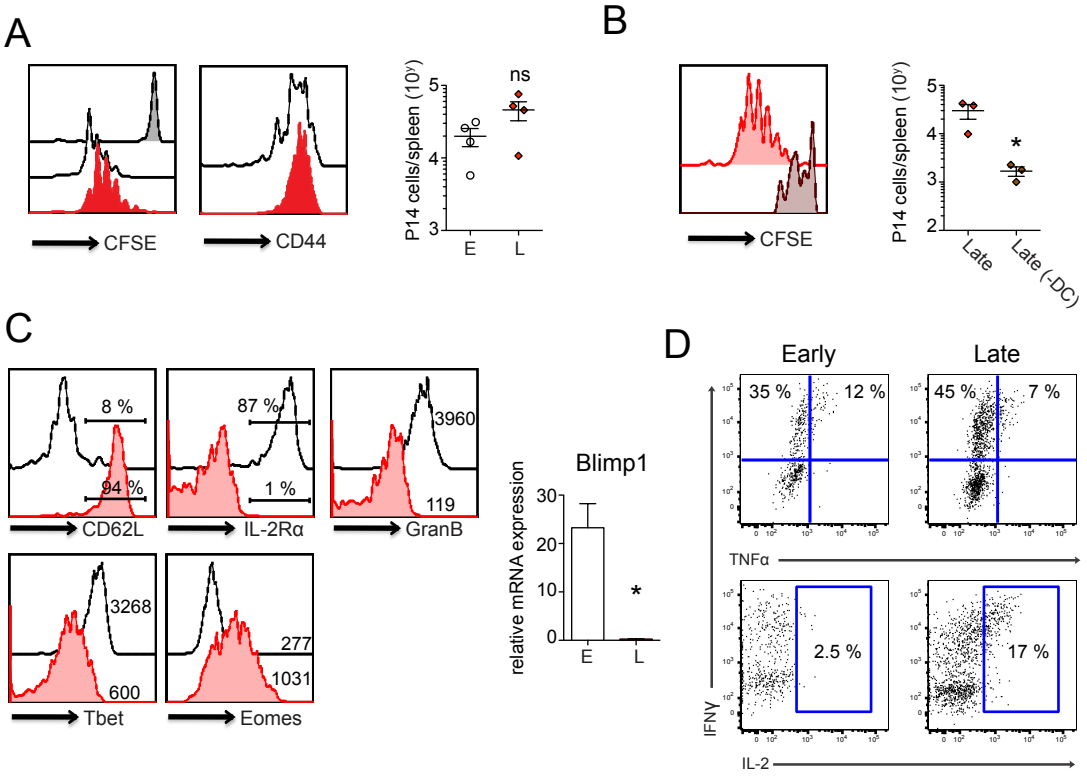

A
CFSE
CD44
P14 cells/spleen (10⁵)
ns
E
L
B
CFSE
P14 cells/spleen (10⁵)
*
Late
Late (-DC)
C
8 %
94 %
CD62L
87 %
1 %
IL-2Rα
3960
119
GranB
3268
600
Tbet
277
1031
Eomes
Blimp1
relative mRNA expression
*
E
L
D
Early
Late
35 %
12 %
45 %
7 %
TNFα
2.5 %
17 %
IFNγ
IL-2

Figure 4.2. Virus-specific CD8 T cells primed during persistent infection demonstrate a prolonged upregulation of Eomes, enhanced frequency of SLECs, and reduced MPEC formation.

(A) Graph illustrates the total number of P14 cells within the spleen eight days after early or late priming.

(B) Flow plots and graphs illustrate Tbet and Eomesodermin levels within P14 cells primed at the onset of LCMV-Armstrong infection (Arm-Early; blue), four days into LCMV-Armstrong infection (Arm-Late; brown), at the onset of LCMV-Cl13 infection (Cl13-Early; white), or 21 days into LCMV-Cl13 infection (Cl13-Late; red).

(C) Flow plots illustrate KLRG1 expression by P14 cells primed at the onset of acute LCMV infection (Armstrong), or at the onset (Early) or in the midst (Late) of persistent LCMV infection.

(D) Graph illustrates the frequency of IL-7R+ P14 MPECs eight days after priming at the onset of acute or persistent LCMV infection, and in the midst of persistent infection.

(E) Flow plots illustrate IFNγ secretion by P14 cells eight days after priming at the onset of LCMV-Arm infection, at the onset of LCMV-Cl13 infection, or 21 days into LCMV-Cl13 infection.

*=$p<0.05$, ns=not significant. Data is representative of 3 independent experiments with 3-5 mice per group.

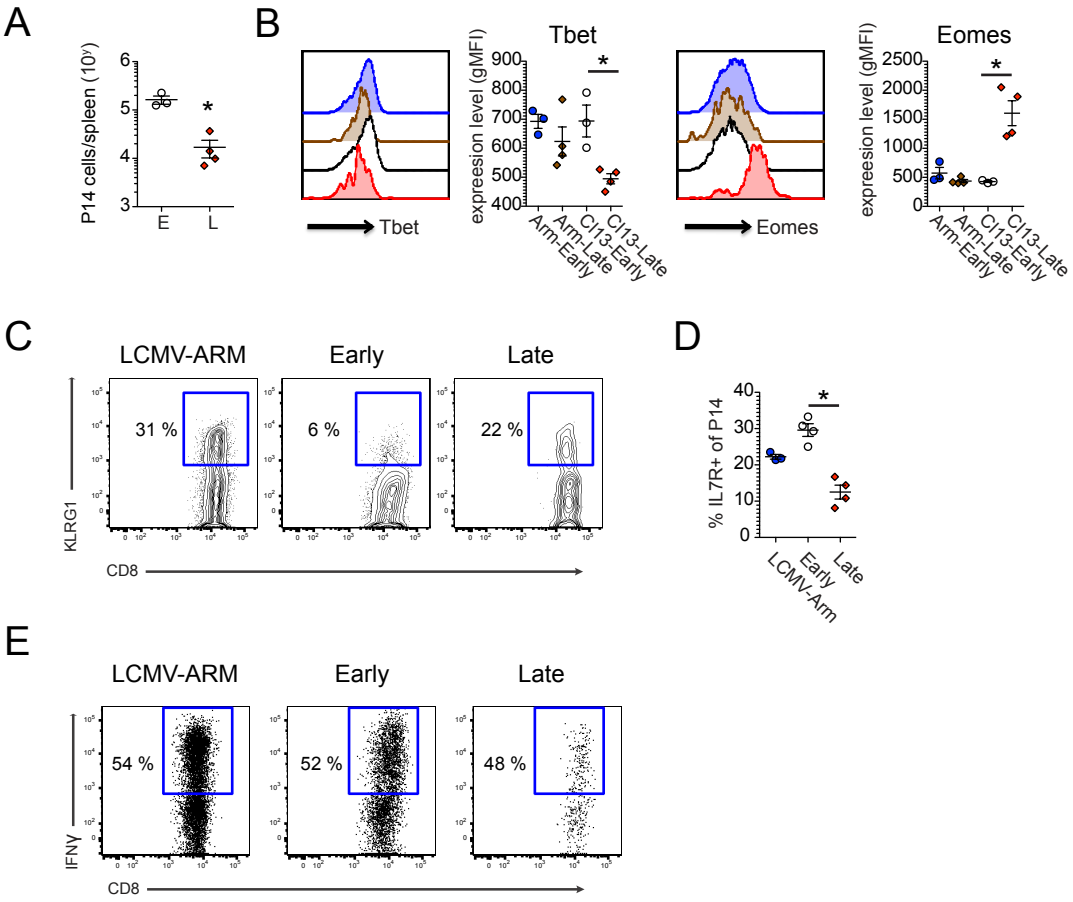

A
P14 cells/spleen (10⁵)
E
L
B
Tbet
expression level (gMFI)
Arm-Early
Arm-Late
Cl13-Early
Cl13-Late
Eomes
C
LCMV-ARM
Early
Late
31 %
6 %
22 %
KLRG1
CD8
D
% IL7R+ of P14
LCMV-Arm
E
54 %
52 %
48 %
IFNγ

Figure 4.3. Increased SLEC formation is maintained long-term in virus-specific CD8 T cells primed in the midst of persistent infection.

(A) Graph illustrates the total number of P14 cells within the spleen fifty days after priming at the onset of acute LCMV-Arm infection, or early or late priming during persistent LCMV-Cl13 infection.

(B) Flow plots and graphs illustrate KLRG1 and IL-7R expression and, correspondingly, MPEC and SLEC formation within P14 cells fifty days after priming at the onset of LCMV-Armstrong infection, at the onset of LCMV-Cl13 infection, or 21 days into LCMV-Cl13 infection.

(C) Flow plots and graphs illustrate IFNγ and TNF secretion by P14 cells fifty days after priming at the onset of LCMV-Arm infection, at the onset of LCMV-Cl13 infection, or 21 days into LCMV-Cl13 infection.

*=$p<0.05$, ns=not significant. Data is representative of two experiments with 3-4 mice per group.

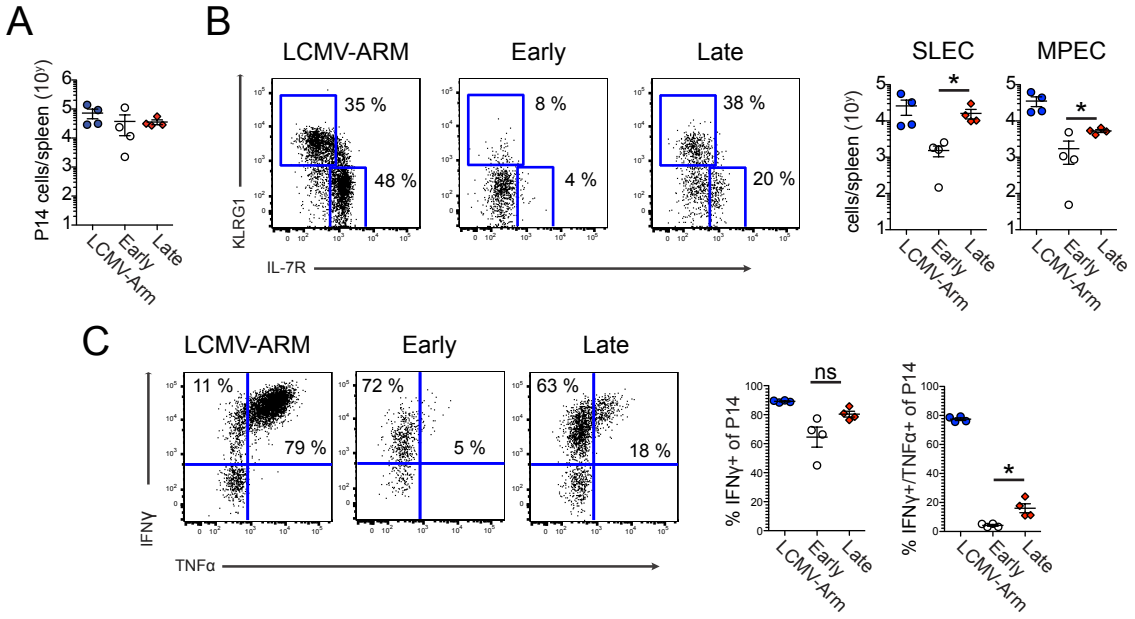

A
P14 cells/spleen (10⁶)
LCMV-Arm
Early
Late
B
LCMV-ARM
Early
Late
35 %
48 %
8 %
4 %
38 %
20 %
KLRG1
IL-7R
SLEC
MPEC
cells/spleen (10⁶)
*
*
C
LCMV-ARM
Early
Late
11 %
79 %
72 %
5 %
63 %
18 %
IFNγ
TNFα
ns
% IFNγ+ of P14
% IFNγ+/TNFα+ of P14
*

Figure 4.4. Virus-specific CD8 T cell priming in the midst of persistent infection results in impaired effector memory potential.

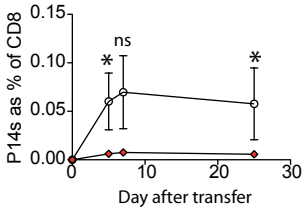
(A-B) Early or late primed P14 cells were isolated sixty hours after priming and transferred at equal numbers into LCMV-immune mice.

(A) Graph illustrates the frequency of early (white circles) or late (red diamonds) primed P14 cells within the peripheral blood at multiple time points after transfer into LCMV-immune mice.

(B) Graphs illustrate the number of early or late primed P14 cells within the spleen and the liver 30 days after transfer into LCMV-immune mice.

*=$p<0.05$, ns=not significant. Data is representative of one experiment with 6 mice per group.

A


P14s in Peripheral Blood
P14s as % of CD8
0.15
0.10
0.05
0.00
ns
*
*
0
10
20
30
Day after transfer

B

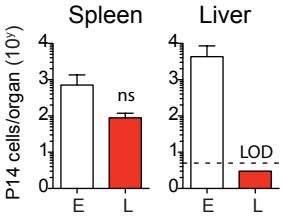

Spleen
Liver
P14 cells/organ (10⁵)
4
3
2
1
0
ns
LOD
E
L
E
L

Figure 4.5. Type I interferon signaling in the midst of persistent infection drives Eomes and SLEC formation, and suppresses MPEC formation.

(A-D) Mice were treated with isotype control antibody (iso) or an antibody blocking the type I interferon receptor (αIFNR) starting on day 20 of LCMV-Cl13 infection and every 48 hours subsequently for eight days. P14 cells were transferred 21 days after LCMV-Cl13 infection, one day after the initiation of treatment.

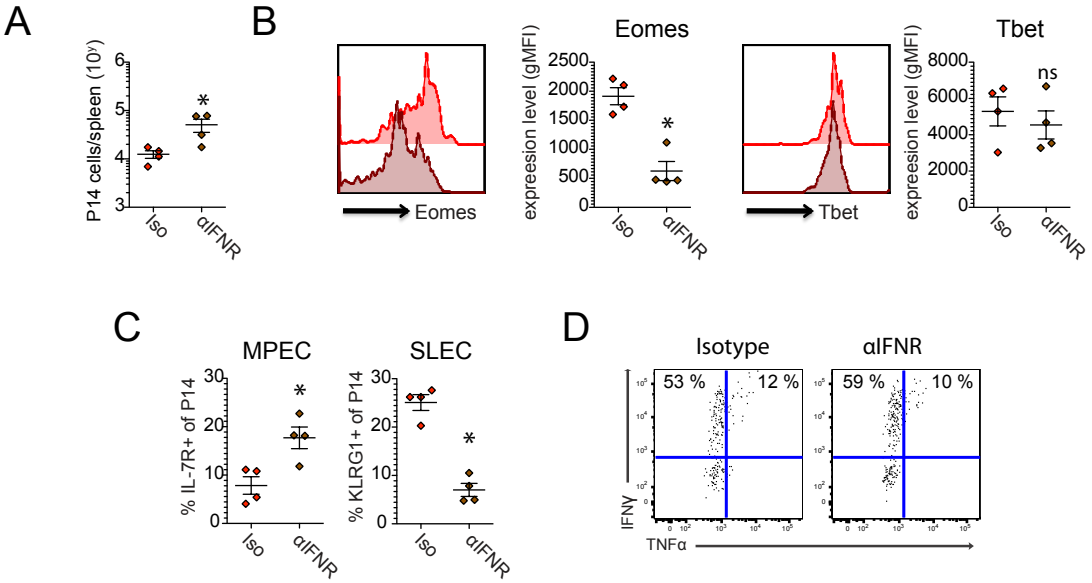
(A) Graph illustrates the number of P14 cells within the spleen after late priming in isotype or anti-IFNR treated mice.

(B) Flow plots and graphs illustrate Eomes and Tbet levels in P14 cells eight days after late priming in isotype or anti-IFNR treated mice.

(C) Graphs illustrate the frequency of MPECs and SLECs in P14 cells eight days after late priming in isotype or anti-IFNR treated mice.

(D) Flow plots illustrate IFNγ and TNFα secretion by in P14 cells eight days after late priming in isotype or anti-IFNR treated mice.

*=$p<0.05$, ns=not significant. Data is representative of one experiment with 4 mice per group.


A
P14 cells/spleen (10⁵)
Iso
αIFNR
B
Eomes
expression level (gMFI)
Tbet
ns
C
MPEC
SLEC
% IL-7R+ of P14
% KLRG1+ of P14
D
Isotype
αIFNR
53 %
12 %
59 %
10 %
IFNγ
TNFα

CHAPTER FIVE

Conclusions

GENERATING AND REGULATING IMMUNITY DURING PERSISTENT INFECTION

The last several decades have presented medical science with a unique challenge; namely, two persistent viral infections that cannot, as of yet, be vaccinated against or effectively treated into drug-free remission in the majority of cases. Although there are rumors of a drug that achieves high rates of remission for HCV infection, HIV remains a medical and scientific challenge. More and more, the scientific community has begun to investigate how persistent viral pathogens and the host immune system interact in the hopes of harnessing the immune system to eliminate persistent viral infection. Within this dissertation, we present findings that extend and integrate some of the current research regarding the regulation of immunity during persistent infection. It is our hope that the work demonstrated here will ultimately lead to significant advances in the search for a cure for infectious diseases of the present and future.

Modulation of immunity is a potential key to combating persistent infections, and several approaches have emerged in an attempt to put that theory to practice. One potential strategy is to revitalize existing immune responses against the virus by blocking immunosuppressive pathways in a persistently infected individual[9]. However, some strategies in development aim to achieve control over viral replication by introducing transgenes such as antiviral TCRs into hematopoietic stem cells and allowing new and hopefully more effective antiviral immune responses to develop naturally in an infected host[99]. The collective data within this dissertation suggests that there may be multiple additional problems that need to be overcome before the latter approach can be fully effective. The broad infection-induced disruption of thymopoiesis with an infected host may delay the emergence of new antiviral immunity within an infected host for a prolonged period, and possibly for the lifetime of the host if the virus targets thymic stromal cells or antigen presenting cells within the thymus. Because thymic atrophy during infection is mediated by virus-specific CD8 T cells and critical early secretion of

IFN-I, preventing thymic atrophy without negatively affecting viral control may be difficult. Introduction of new antiviral immunity after thymic recovery, or in the case of HIV, after rigorous antiretroviral therapy, may be the best way to achieve an optimal response.

Our results indicate that T cells that make it through the developmental stage and are primed in the periphery may not form optimal antiviral responses. Most striking is our finding that late primed virus-specific CD4 T cells fail to generate Th1 immunity. Although late primed CD4 T cells still form Tfh effectors, which are capable of assisting B cells with maturation and the production of antibody, strong Th1 responses are the best correlate for viral control[132,134,155]. Furthermore, our data indicate that both virus-specific CD4 and CD8 T cell responses primed during persistent infection are restricted in their level of expansion, thus generating far fewer virus-specific effector cells at the peak of their immunological response. However, these cells do not appear to contract to any great degree either, unlike T cells primed at the onset of infection. Their capability for long-term survival may thus prove beneficial for control of persistent infection.

Many persistent viral, as well as parasitic and bacterial infections increase the risk of secondary infection[156]. In addition to the inhibition of *de novo* virus-specific Th1 differentiation, *de novo* CD4 Th1 development against co-infection pathogens during persistent infection might also be inhibited, which could have profound effects on pathogens that require Th1 responses for control. For example, both the incidence and severity of *Mycobacterium tuberculosis* infection are increased in HIV-infected individuals[157,158,159,160]. Effective control of *M. tuberculosis* infection requires a strong Th1 response[160]; thus, it is interesting to speculate that Mtb-specific CD4 T cells primed in the midst of a persistent HIV infection would exhibit diminished Th1 development and may in part underlie the increased susceptibility to *M. tuberculosis*. Further, the failure of late primed CD4 T cells to efficiently accumulate in peripheral tissues suggests that persistently infected individuals may become more susceptible to secondary infections

and re-infection in those organs. Furthermore, the failure of late primed virus-specific CD8 T cell responses to form peripheral effector memory responses may contribute to secondary infections and reduced efficacy of vaccination in individuals suffering from an infection. Fortunately, the data presented in our dissertation reveals that IFN-I is the primary mechanism behind the defects observed in generating *de novo* T cell responses during persistent infection, raising the possibility that modulating this pathway could help enhance antiviral immunity.

TYPE I INTERFERON – COMPLEX REGULATOR OF ADAPTIVE AND INNATE IMMUNITY

Increasingly, IFN-I is coming to be regarded as not simply a cytokine that directly suppresses viral replication, but one that has numerous effects on all branches of host immunity. IFN-I can activate, potentiate, or inhibit a large number of intracellular signaling pathways[81,83], and regulate the activation and differentiation of innate immune cells, as well as T cells and B cells[153,154,161,162,163,164]. In this dissertation, we discovered that IFN-I plays a critical role regulating T cell development, differentiation, and function during persistent viral infection. Given the pantropic effect that IFN-I exerts on nearly every aspect of the immune system examined so far, this was not entirely surprising. It did surprise us however, that the effects of IFN-I on developing immunity during persistent viral infection were almost entirely inhibitory. Canonically, IFN-I is considered to be immunostimulatory, and is even used as a moderately effective antiviral therapy in human HCV infection[95]. However, IFN-I is used therapeutically to inhibit the immune response in certain autoimmune scenarios[96], and recent data suggests that it may play an immune inhibitory role during persistent viral infection[11]. Early blockade of IFN-I can inhibit the generation of virus-specific T cells, whereas later blockade can bypass this effect or even stimulate T cell responses[11,154]. Collectively, our data support a model

where long-term chronic IFN-I signaling, or IFN-I signaling in the context of a chronically infected environment may downregulate immune responses and repress the generation of *de novo* immunity, possibly as a means to limit damaging immunopathology in the infected host.

The immune system requires an effective off switch and uncontrolled immune activation can lead to dangerous levels of inflammation and death to immune-mediated shock[165]. Our findings suggest that IFN-I can serve to restrict the potency of T cell responses primed during a persistent infection. Virus-specific CD4 and CD8 T cells primed during viral persistence expanded to 10-50-fold lower levels than T cells primed at the onset of infection, and blockade of IFN-I led to increased expansion to levels resembling early priming. In the case of CD4 T cells, it could be argued that IFN-I blockade increases peak T cell numbers by restoring Th1 differentiation, but we observed a numerical increase in the Tfh population as well, thus suggesting that the effect of IFN-I on limiting T cell expansion is independent of its effect on controlling CD4 T cell fate. However, the ability of IFN-I to repress Th1 cell formation may also be a mechanism to limit inflammation, as Th1 cells are potent producers of proinflammatory cytokines such as IFNγ, TNFα, and IL-2[49,50]. Th1 cells are also specialized for helping other immune responses at non-lymphoid sites such as the liver, lung, or gut epithelium, so our findings thus suggest that IFN-I specifically serves to limit responses capable of acting within the periphery while leaving more centrally-acting responses intact. The inability of late primed virus-specific CD8 T cells to form peripherally circulating effector memory may thus be a CD8 T cell-specific parallel to the defect in late primed CD4 T cell Th1 formation.

The mechanism of IFN-I-mediated regulation of *de novo* CD4 and CD8 T cell immunity has some interesting parallels and differences. We see similar transcriptional and phenotypic changes in both late primed virus-specific CD4 and CD8 T cells. Late

priming in both T cell subsets leads to the preferential retention of lymphoid homing molecule CD62L expression, which perhaps underlies the defect in forming peripheral responses. Additionally, the effector programming transcriptional regulators Tbet and Blimp1 are downregulated during late priming, as is the IL-2 receptor, which plays a critical role in coupling IL-2 signaling to Blimp1 expression[166]. During early priming, IL-2 signals, Tbet, and Blimp1 play a crucial role in the expansion and formation of a large pool of antiviral CD4 and CD8 T cell effectors[166], and the reduced numbers of late primed virus-specific CD4 and CD8 T cells, as well as the lack of CD4 Th1 formation during late priming is likely related to these transcriptional changes. Interestingly, late primed T cell responses come to rely on alternate transcriptional regulators for programming their effector function – Bcl6 for CD4 T cells and Eomesodermin for CD8 T cells. As IFN-I signaling blockade both increases Tbet and IL-2R expression and downregulates Eomes expression in CD8 T cells, we cannot be sure which is the primary effect of IFN-I signaling and which is the secondary. It is likewise interesting that although IFN-I represses Th1 formation in late primed CD4 T cells, the formation of SLECS in late primed CD8 T cells, a population that in many ways is the parallel of Th1 effectors, is maintained and even enhanced. This may be due to the fact that Tbet and Bcl6 regulate vastly different transcriptional programs, whereas Tbet and Eomes are closely related homologues with many overlapping functions[167]. This is likely why the secretion of the canonical CD8 T cell and Th1 effector cytokine IFNγ is greatly reduced in late primed CD4 T cells, whereas late primed CD8 T cell maintain the capacity to secrete IFNγ at a similar frequency to early primed cells. Ultimately, our data suggests that IFN-I impairs certain common effector differentiation mechanisms in CD4 and CD8 T cells, and that intrinsic differences between CD4 and CD8 T cells control their ultimate fate.

Interestingly, while the suppression of late primed T cell responses is an effect of chronic IFN-I signaling, the atrophy of the thymus caused by persistent LCMV infection is caused by IFN-I signaling at the onset of infection. Although IFN-I may impair thymic development by acting directly on thymocytes or thymic stroma during retroviral infection[106,107], our findings indicate that IFN-I alone is not sufficient to mediate thymic atrophy. Both acute and persistent LCMV infections stimulate high levels of IFN-I early after infection, but thymic atrophy is seen only in persistent LCMV infection. Furthermore, mice that had CD8 T cells depleted before infection, or mice that are unable to mount a virus-specific CD8 T cell response to LCMV did not experience thymic atrophy despite IFN-I production. Together, this suggests that IFN-I signaling blockade reduced the level of thymic atrophy by inhibiting the immunostimulatory effect of IFN-I on virus-specific CD8 T cells, thus reducing the damage to the thymic microenvironment. In contrast to our findings regarding the role of IFN-I in late T cell priming, our data on thymic atrophy highlights a potential proinflammatory role of IFN-I leads to immune dysfunction.

Our line of research has left us with a number of open questions regarding the function of IFN-I during persistent infection. This leaves some room for speculation about the function and mechanisms of IFN-I signaling and, excitingly, provides us with future avenues of research to further explore the effect of IFN-I on immunity. One of the questions that remain unanswered is how IFN-I exerts such disparate effects on virus-specific T cell programming early and late during persistent infection. One potential answer comes from the ability of IFN-I to integrate and modulate a number of biological pathways[81]. Thus the downstream effects of a burst of IFN-I in the setting of the onset of inflammation may be fundamentally different than chronic low-level IFN-I signaling in a chronically inflamed immune environment. This is supported by the finding that DCs from patients persistently infected with HCV, particularly those unresponsive to IFN-I therapy,

are not response to IFN-I levels that stimulate DCs from uninfected individuals[164], thus highlighting how IFN-I no longer exerts the same immunostimulatory effects in the setting of persistent infection. Likewise, blockade of IFN-I in the midst of persistent infection restored Th1 development to virus-specific CD4 T cells, whereas blockade early during infection generated increased levels of Tfh. Indeed, early IFN-I signaling may actually promote Th1 development through repression of STAT3 signals[162]. Thus, IFN-I mediated changes to cellular programming during persistent infection, or other factors of the immune environment fundamentally alter how IFN-I modulates T cell responses. Furthermore, it remains unclear what cell type IFN-I is acting on to modulate T cell programming. We demonstrated that INF-I is not acting directly on late primed CD4 T cells, and recent studies suggest this may also be the case for CD8 T cells as well[146]. The most likely culprit for the integration of IFN-I signaling and T cell programming is the DC, which we determined is necessary for late priming, is generally sensitive to the presence (and lack of) IFN-I, and conveys signals that strongly influence T cell differentiation[163,164]. Our group has previously shown that blockade of IFN-I signaling during persistent infection enhances expression of costimulatory molecules on DCs[11], but it remains to be determined whether the DC is the key in IFN-I-mediated repression of late primed T cell responses and how IFN-I signals during persistent infection and blockade of IFN-I alter DC programming. Ultimately, this line of research could lead to the discovery of pathways that can be selectively modulated to alter immunity during persistent infection without losing the beneficial antiviral effects of IFN-I.

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