UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cell-intrinsic Transforming Growth Factor-β signaling mediates virus-specific $CD8^+$ T cell deletion and lymphocytic choriomeningitis virus persistence *in vivo*

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

By

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The Dissertation of Roberto Tinoco is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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DEDICATION

To my lovely wife Erika Patricia Tinoco and my beautiful children Anahi Alyssa, Yazmin Cecilia and Erik Roberto Tinoco. To my Mother, Maria Del Carmen Morales Dueñas who I love dearly, and my Father, Roberto Tinoco Tello who lives daily in my heart.

Your unconditional love and support were the reasons why I was able to complete my Ph.D. I am excited for a bright future ahead of us. I love you with all my heart.

TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE DISSERTATION

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Professor Elina I. Zuñiga, Chair

Chronic viral infections lead to a delicate long-term equilibrium between pathogen and host and represent a major biomedical problem for human populations worldwide. Although, in both humans and mice, viral persistence has been associated with insufficient numbers and/or dysfunction of effector $CD8^+$ T cells, the underlying mechanisms involved in these pathogen evasion strategies are not completely understood. By using lymphocytic choriomeningitis virus (LCMV) infection in its natural murine host, we found that enhanced and sustained Transforming Growth Factor-β (TGF-β) expression and

phosphorylation of its signaling mediator, Smad-2, were distinctive features of virus-specific $CD8^+$ T cells during chronic *versus* acute viral infections *in vivo*. Selective attenuation of TGF-β pathway in T cells decreased the expression of the pro-apoptotic protein Bim and increased survival and numbers of virus-specific $CD8^+$ T cells. Under these conditions, virus-specific $CD8^+$ T cells showed enhanced cytotoxicity, increased production of anti-viral cytokines, and down-regulation of the inhibitory molecules programmed-death-1 (PD-1) and interleukin-10 (IL-10). The presence of these enhanced numbers of functional virus-specific $CD8^+$ T cells resulted in rapid virus eradication and generation of an effective memory T cell response that protected the host upon subsequent challenge. Notably, we found that cell-intrinsic TGF-β signaling was responsible for virus-specific-$CD8^+$ T cell apoptosis but was not necessary for their functional exhaustion. Our findings demonstrate that sustained TGF-β-Smad signaling is as a hallmark and biologically meaningful regulator of $CD8^+$ T cell responses during chronic viral infection *in vivo*.

CHAPTER 1 INTRODUCTION

Immune response to pathogens

Pathogens have evolved complex mechanisms against the host immune system to avoid detection and promote their survival. In response, vertebrates have also co-evolved highly coordinated innate and adaptive immune response mechanisms to defend against these foreign pathogens to maintain health. The vertebrate immune system is divided into innate and adaptive arms, each having a specific temporal and cellular component. Innate immune cells express receptors that recognize classes of pathogens and represent the first line of defense against infection. In contrast, cells of the adaptive immune system use highly specific rearranged receptors to eliminate pathogens from the host and generate an effective memory cell pool to protect from re-infection.

The immune system has the challenging task of maintaining homeostasis not only under steady-state conditions but also during and after infection. Under steady-state conditions, this is achieved by having cellular and molecular mechanisms that ensure tolerance for self-antigens and commensal bacteria to prevent autoimmunty. During infection, an immune response against foreign antigens needs to be elicited to eliminate pathogens, while assuring minimal immunopathology from activated lymphocytes. Once the pathogen is cleared, immune effector cells and tissues must return to a homeostatic state. Cytokines have been described as being important mediators of lymphocyte homeostatis under steady-state and infectious conditions (1).

Human chronic viral infections represent a global biomedical challenge

Viruses such as Hepatitis B (HBV) and C (HCV), and Human Immunodeficiency Virus (HIV) have evolved immunosuppressive mechanisms to exploit the host immune system to ensure their survival by establishing persistent infections. As a consequence, chronic viral infections are a major cause of death and morbidity worldwide (2, 3). There are more than 40 million people infected with HIV and 3 million deaths annually. Greater than 0.5 billion people are chronically infected with HBV and HCV, these individuals are not only at risk of death from developing liver disease and cancer but they are also a source for many new host infections (4, 5). It is important to study and dissect the molecular mechanisms by which these persistent viruses are inducing immune suppression to develop new or improve therapies to treat these persistently infected individuals.

LCMV as a model for chronic viral infections

Important immunological questions regarding T cell responses have been discovered using LCMV infections in mice. Antigen-specific expansion, contraction and the development of memory T cells that protect from re-infection have been illuminated using this model system (6-10). Using the Cl 13 variant of the parental LCMV ARM strain, mechanisms of persistent infections have also been extensively described and have led to very important discoveries. Mice can be infected with two genetically related LCMV isolates that induce either acute or chronic viral infections, but share identical T cell epitopes. The parental strain, Armstrong 53b (ARM), induces a strong $CD8^+$ T cell response, resulting in acute

viral clearance after 8-10 days post infection (p.i.) (11). In contrast, LCMV clone 13 (Cl 13) initiates an abortive $CD8^+$ T cell program that results in a persistent infection for 60-90 days in blood and most tissues (9, 12-14). Cl 13 infection induces $CD8^+$ T cell deletion and exhaustion characterized by a hierarchical loss of effector functions (9, 13-15). Specifically, chronic viral infections cannot be controlled by the host's immune system. Viruses that result in high viral burdens characterize these types of infections and clear examples include LCMV in mice and HIV, HBV, and HCV in humans. These highly productive viruses induce the impairment of adaptive immune responses, which translate into significant dysfunctional T cell responses. Significant effort and resources have aimed to develop vaccination strategies to boost immune responses against HIV (16-18), HBV (19, 20), and HCV (21-23). Vaccinations strategies have not worked because during chronic infections, immunosuppressive mechanisms are in place preventing viral clearance.

T cells have unique dysfunctional characteristic during chronic infection

Chronic viral infections lead to continuous T cell stimulation by viral antigens. This results in the constrained size of the responding T cell population and lead to the continual deterioration of T cell effector function. In addition to T cell exhaustion described in mice infected with persistent LCMV (13) other studies have shown this phenomenon to occur in human infections including, HCV (24), HIV (25-29), and simian immunodeficiency virus (SIV) in rhesus macaques (30). As T cell dysfunction ensues, there is a hierarchical loss in the ability of these cells to produce effector cytokines. Initially these virus-specific T

cells lose the ability to produce IL-2, then TNF-α, and finally IFN-γ (9, 31, 32). Remarkably, these cells are not only deficient in cytokine production, but they have diminished proliferative potential, are unresponsive to IL-7 and IL-15 cytokines (33), and in the most severe cases of exhaustion, they are almost completely deleted (13, 14). Virus-specific $CD4^+$ T cells on the other hand, also loose the ability to make IL-2 and TNF-α early after infection with chronic LCMV and are unresponsive when re-stimulated (34). Anti-viral $CD4^+$ T cells are not necessarily deleted during chronic LCMV infection, they exist rather inactivated and have the potential to be restored to provide helper function to $CD8^+$ T cells (34). $CD4^+$ T cells have been shown to produce interleukin 10 (IL-10), which is a potent immunosuppressive cytokine, during chronic LCMV infection (34, 35). In addition, T regulatory cells (Tregs) from the $CD4^+$ T cell compartment can function to minimize tissue damage during chronic infection, but at the same time help establish the chronic state in the host (36).

Exhausted virus-specific $CD8^+$ T cells express high levels of inhibitory receptors

Important contributions to a better understanding of the physiological state of exhausted $CD8^+$ T cells was elucidated from a genome-wide microarray that compared the transcriptional program from exhausted virus-specific $CD8^+$ T cells in chronic infection vs effector and memory $CD8^+$ T cells during acute LCMV infection (37). These studies uncovered that virus-specific $CD8^+$ T cells over-expressed inhibitory receptors on their surface. One of the most highly expressed receptors was programmed-death 1 (PD-1) and it was shown to

directly regulate $CD8^+$ T cell exhaustion (38). The significance of these inhibitory molecules was also shown with other highly expressed inhibitory receptors including 2B4, CTLA-4, and LAG-3. In vivo blockade of CTLA-4 or LAG-3 showed improved T cell responses during chronic LCMV infection (39). Furthermore, LAG-3 but not CTLA-4 blockade was shown to synergize with PD-1 (39). In comparison to memory $CD8^+$ T cells, exhausted $CD8^+$ T cells expressed decreased levels of cytokine receptors IL-4Rα, IL-7Rα, IL-2Rβ, and were unresponsive to IL-7 and IL-15 (33). Interestingly, this study also found that exhausted $CD8^+$ T cells expressed distinct set of transcription factors, had altered expression of genes involved in chemotaxis, migration and adhesion, as well as severe energy and metabolic deficiencies (37). Furthermore, exhausted $CD8^+$ T cells have been thought to exist in an anergic state, however, angergy-associated genes which include Egr-2, Egr-3 and grail were not selectively expressed in exhausted $CD8^+$ T cells, indicating that anergy and exhaustion were distinct processes in chronic LCMV infection (37).

In addition to expression of inhibitory receptors, suppressive cytokines like IL-10 and its role in T cell exhaustion during persistent LCMV has been extensively studied. IL-10 protein and mRNA levels were higher in Cl 13 compared to ARM infected mice (35, 40, 41). Blocking the IL-10 receptor using antibodies soon after infection or after the infection was fully established, resulted in decreased PD-1 levels on virus-specific T cells, improved T cell function and reduced viral loads (40, 41). Neutralizing IL-10 with anti-IL-10 antibodies resulted

in early functional restoration, however, the infection was not resolved and exhaustion occurred at later time points. Furthermore, blocking IL-10 with anti-IL-10 receptor antibodies and using therapeutic DNA vaccination resulted in enhanced T cell responses and viral containment (42). These findings highlight that along with inhibitory receptor expression on virus-specific T cells, immunosuppressive cytokines can also negatively impact T cell exhaustion during chronic LCMV infection.

Immunosuppression through T cell exhaustion also occurs in human chronic infections

In addition to functional T cell exhaustion observed in chronic LCMV infection, pathogens leading to human chronic infections also exploit these immunosuppressive mechanisms. The presence of high antigen in HIV for example, leads to the generation of multiple inhibitory cytokines and upregulation of PD-1 on HIV-specific $CD8^+$ T cells (43-45). As PD-1 blockade restored T cell responses in chronic LCVM infection, in vitro blockade of the PD-1/PD-L1 pathway improves functional responses in HCV-specific (46-48), HBV-specific (49), and HIV-specific (43-45) $CD8^+$ T cells which have up-regulated PD-1 levels *in vivo*. HIV-specific $CD4^+$ T cell function was improved upon CTLA-4 blockade (50) and this enhanced function could synergize with PD-1 blockade. Blocking CTLA-4 and PD-1 could also restore intra-hepatic HCV-specific $CD8^+$ T cell exhaustion (51). Interestingly, in LCMV chronic infection, anti-CTLA-4 treatment had no effect on T cell function and viral control in vivo, whereas PD-1 blockade rescued exhausted T cells and reduced viral loads (38). Furthermore, CTLA-4

deficient mice infected with LCMV Cl 13 had no significant alteration and improvement in viral-specific T cells (52). Interestingly, in the SIV macaque model for human HIV, CTLA-4 blockade did not improve viral-specific T cell responses but it induced an increase in viral replication at mucosal sites (53). Blocking PD-1 in SIV infected macaques however, enhanced SIV-specific T cell immunity and reduced viral loads, indicating that as in chronic LCMV, PD-1 is a common immunosuppressive molecule. Understanding immunosuppressive mechanisms in the mouse chronic LCMV model led to discovering PD-1 as a marker of exhaustion, these findings were then observed in CD8+ T cells in SIV infected macaques, and finally translated to in HIV infected patients. Increased IL-10 levels were shown to be detrimental to $CD8^+$ T cell responses and was an important contributor to persistence of chronic LCMV (40). Increased IL-10 production has also been observed in HIV (54, 55), HCV (56-58), and HBV (59) infections indicating that like in LCMV, in these human persistent infections, the IL-10 and IL-10R pathways play a negative role during chronic infection. The Tim-3 receptor, which is a member of the T cell Ig and mucin family of proteins, with galectin-9 as its ligand, was shown to be upregulated in $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals (60). Furthemore, the higher number of $CD8^+$ T cells with upregulated Tim-3, the higher the viral load and lower number of $CD4^+$ T cells observed in these patients (60). Interestingly, blocking Tim-3 restores $CD8^+$ T cell effector function and Tim-3 also regulated T cell exhaustion in HCV (61) and HBV (62) infections. These findings show that similar

immunosuppressive pathways leading to functional T cell exhaustion are shared and conserved within different species infected with different persistent viruses.

Therapeutic potential to combat persistent infections

One of the main goals in studying immunosuppressive mechanisms leading to viral persistence in mice is to develop therapies to cure or alleviate persistent infections in humans. The goals of developing these therapies are aimed to reverse or prevent the negative effects of the immunosuppressive environment and high antigen load observed in these types of chronic infections. Significant work has been achieved to boost T cell responses during chronic infection through vaccination; these vaccines have worked to modulate immune responses against pathogens by providing better stimulus to virus-specific T cells. The problem in these strategies though, has been the fact that they have not been effective as a therapy for HIV, HBV, and HCV. If vaccines are to be used for therapeutic purposes, they need to work in combination with a modulation of the immunosuppressive environment and synergize to improve T cell responses and clear persistent viruses. In LCMV studies for example, a combination of LCMV-GP_{33-41}-encoding vaccinia vaccine and anti PD-L1 blocking antibody improved the function of virus-specific $CD8^+$ T cells and decreased viral load in chronic LCMV infection, this response was better than either of these strategies alone (63). In addition, improved antiviral T cell responses were observed when IL-10 was neutralized and followed by administration of DNA vaccine encoding LCMV antigens (42). These findings show that by understanding immunosuppressive mechanisms in mice, these findings can

potentially be translated to human chronic infections and therapies to alleviate these infections can be developed. If therapeutic intervention will be developed to combat chronic viral infections, careful consideration must be taken to develop strategies that modulate the immunosuppressive environment to restore T cell responses and reduce viral burden. Furthermore, while inhibitory molecules like PD-1 are commonly shared mechanisms of suppression in mice, primates, and humans, others like CTLA-4 can be species and infection-specific and this difference needs to be considered to develop the most effective therapy.

Production of TGF-β is tightly regulated

TGF-β has been described as having an essential role in immune system homeostasis. It has been implicated in mechanisms of T cell development, tolerance and differentiation. As TGF-β has such an important regulatory role, production and activation of this molecule is tightly regulated. TGF-β is a member of a family of pleiotropic cytokines, it can signal on many cell types that regulate a diversity of physiological processes such as embryogenesis, cancer, and immunity (64-67). Three isoforms of TGF-β have been described in mammals, these include TGF-β_1, TGF-β_2, and TGF-β_3, with TGF-β_1 being the main form expressed in the immune system (68). A specific three-dimensional fold characterizes members of the TGF-β family; these proteins also have a conserved number and spacing of cysteine residues in the C-terminus (69, 70). TGF-β is produced as a precursor peptide; it is then processed by furin-like peptidases in the Golgi to produce the mature form of TGF-β. A homodimer of

latency-associated protein (LAP) associates non-covalently with a homodimer of mature TGF-β. This complex can now be secreted from the cell or it can be further bound to the latent-TGF-β-binding protein (LTBP), which sequesters TGF-β to the extracellular matrix. The LTBP binds to extracellular matrix proteins including but not limited to fibronecting and fibrillin 1 (71, 72). LAP-associated TGF-β is unable to bind and signal through its receptor. TGF-β biological activity is rendered through disassociation from LAP through the action of TGF-β activators, which include but is not limited to BMP-1, plasmin, urokinase-type and tissue-type plasminogen activators, thrombin, elastase, cathepsin, matrix metalloproteinases, and glycosidases (73-75).

Bioactive TGF-β signaling is a highly regulated process

Once bioactive TGF-β is released after being caged by LAP, active TGF-β can now achieve signaling by binding to TGF-βR type II (TGFBRII) which undergoes a comformational change recruiting TGFBR type I, both receptors have an intracellular serine/threonine kinase domain (76, 77). Once TGF-β is bound, a tetrameric complex of TGFBRI and TGBRII homodimers is formed. TGFBRII phosphorylates TGFBRI, as TGFBRI is activated, it can now propagate the signal by phosphorylation of Smad proteins. Smad proteins consist of eight members divided into three functional classes. The receptor-regulated Smads (R-Smads), the co-mediator Smads (Co-Smad) and the inhibitory Smads (I-Smads). Smad proteins have three domains, an N-terminal Mad-homolgy 1 (MH1) domain which has a nuclear localization signal and DNA binding domain.

They have a middle linker domain allowing interaction with isomerases and ubiquitin ligases, this region is enriched with prolines and serines or threonines that can be phosphorylated. Finally, the C-terminal has an MH2 allowing it to bind to type I receptors as well as other proteins. This domain allows for homo or hetero-oligomerization of Smads (77-79). R-Smads include 1,2,3,5, and 8, these are directly phosphorylated by TGFBRI. Phosphorylated R-Smads can now associate with common-mediator (Co) Smad 4. The resulting complex consists of a trimer of two R-Smads and a single SMAD4. These complexes can associate in the form of a SMAD2-SMAD2-SMAD4, a SMAD3-SMAD3-SMAD4 or a SMAD2-SMAD3-SMAD4 complex (80, 81). Associated Smads can now shuttle to the nucleus where binding with other nuclear co-factors can occur to regulate transcription of target genes. In fact, association with a diversity of transcription factor partners achieves high affinity DNA binding of Smad complexes (77, 82). I-Smads include Smad 6 and Smad 7, these proteins negatively regulate TGFBR signaling by competing with R-Smads for receptor and Co-Smad interaction. In fact, TGF-β Smad complexes can induce the expression of Smad 6 and Smad7, providing a negative feedback loop to regulate the strength and duration of signaling (83). In addition to TGF-β signaling propagated through Smad proteins, Smad-independent signaling pathways can also occur in a cell-type specific manner. These pathways are mediated by mitogen-activated protein kinase (MAPK), PI3K kinase, PP2A phosphatase, Rho family proteins, and Par6 (70, 84).

Complex regulation of TGF-β signaling from receptor to Smads

TGF-β signaling can be positively or negatively regulated at the level of its receptor. Sumoylation of TGFBRI enhances TGF-β signaling by facilitating recruitment of SMAD3 to the receptor for phosphorylation (85). Internalization of activated TGF-β receptors via clathrin-coated pits to early endosomes can also occur. Once in the endosome, the receptors encounter Smad anchor for receptor activation (SARA). SARA recruits SMAD2 and SMAD3 to TGFBRI to induce their subsequent phosphorylation. Furthermore, SARA can cooperate with cytoplasmic promyelocytic leukemia (cPML) tumor suppressor protein to stabilize the SARA-Smad complex (86). Negative regulation of TGF-β signaling can involve receptor dephosphorylation by PP2A phosphatases (87), which inactivates the receptor. The flow of signaling can be regulated by receptor endocytosis, this controls TGF-β availability on the cell surface. A recent in vitro study, demonstrated that excess TGF-β ligand availability can be depleted by constitutive endocytosis of the TGFBRII (88). This mode of regulation can potentially regulate and fine-tune the strength and duration of signaling on the surface of the cell. Receptor-ligand complex can also be internalized into lysosomes, where this complex is degraded. The internalization process involves trafficking via calveolae and complex association with I-Smads and SMURF1 or SMURF2 ubiquitin ligases (89). I-Smads directly bind to TGFBRI and competitively inhibit R-Smad phosphorylation, they can also recruit phosphatases in addition to ubiquitin ligases to inhibit receptor signaling by decreasing TGFB receptor levels (83). Interestingly, HSP90 chaperone proteins

can bind to TGFBRII and TGFBRI to protect from SMURF2 ubiquitylation (90). TGF-β receptor downregulation can also occur through the actions of AMP-regulated kinase member salt-inducible kinase (SIK) which is induced by TGF-β concomitantly with SMAD7 and SMURF2. SIK binds to SMAD7 and TGFBRI, this binding induces the downregulation of this receptor (91).

TGF-β effect is highly pleiotropic and can also be modulated at the level of transcriptional regulation by Smad proteins. Nuclear Smad complexes will bind to Smad-binding elements (SBEs) on DNA with weak affinity (81, 92). Smad2 is not able to bind to SBEs because it has an insertion in its MH1 binding domain while SMAD3 will recognize its SBE and achieve binding (93). Recruitment of Smad complexes to chromatin depends on direct interaction with other transcription factor-binding partners that aid in binding to DNA with high affinity (94, 95). Examples of SMAD binding partners include Zinc finger 198 (ZNF198) with unknown function, Peroxisomal biogenesis factor 6 (Pex6) involved in peroxisome assembly, Eucaryotic translation initiation factor 4E nuclear import factor 1 (4-ET) involved in nucleocytoplasmic shuttling of eIF4E, Splicing factor 3b subunit 2 (SF3b2) involved in RNA processing (96). Other binding partners include FoxH1 (97) (98), Jun, Atf2, TFe3, vitamin D receptor, OAZ, CBP/p300, MSG, and SMIF (99) (100).

TGF-β family members signal through Smad proteins

The TGF-β family is conserved throughout metazoan development and in addition to the TGF-β isoforms, the family includes bone morphogenetic proteins

(BMPs), growth differentiation factors (GDFs), activins and nodal. Signaling by these family members can lead to opposing cellular functions. For instance, in certain cell types TGF-β can inhibit proliferation while in other cells it will promote it (101-105). In stem cells Nodal can inhibit stem cell differentiation and BMP can have the opposite effect by promoting differentiation (106-108). All TGF-β family members signal by binding to type I and type II receptors. TGF-β differs from other members because the type II receptors it binds are specific for TGF-β while other family members can use multiple type II receptors. For instance, TGF-β will exclusively bind to TGFBRII, whereas BMP, GDF and Activin can all bind to BMPRII, ActRIIA, and ActRIIB type II receptors. Signaling is achieved by recruitment of type 1 receptors, also known as activin-receptor-like kinases (ALKs) and TGF-β family members can use different ALK receptors. TGF-β for example, recruits TGFBRI (ALK5), BMP can recruit ALK3 or ALK6, Activin will recruit ALK4 or ALK7 (109). The TGF-β pathway leads specifically to phosphorylation of Smad-2/3 transcription factors, however other TGF-β family members can also induce the phosphorylation of these Smad proteins. Activins for example, will signal through ActRIIA or ActRIIB type II receptors and either use ALK4 or ALK7 type I receptors to induce phosphorylation of Smad2/3 transcription factors (110). These findings have indicated that while TGF-β signaling through its receptors (e.i. TGFBRI and TGFBRII) leads to phosphorylation of Smad transcription factors, activation by other family

members through their respective receptors can also lead to phosphorylation of these same transcription factors.

TGF-β suppresses T cell activation

During immune responses against pathogens, naïve T cells are activated and differentiate to acquire effector functions. Naïve $CD8^+$ T cells differentiate into effectors and gain the ability to kill target cells while $CD4^+$ T cells gain the ability to provide helper function to other immune cells such as B cells, macrophages and $CD8^+$ T cells. T cell differentiation occurs from T cell receptor (TCR) stimulation, co-stimulation, and cytokine signaling. Once T cells differentiate, they express key transcription factors. T-bet and EOMES expression specifies naïve $CD8^+$ T cells to differentiate into cytotoxic T lymphocytes (CTLs). CD4 T cell differentiation into several T helper subsets depends on expression of specific transcription factors which are activated by contextual signaling. T-bet expression specifies Th1 cells, GATA-3 specifies Th2 cells, and RORγT generates Th17 cells (111). Since TGF-β is highly expressed in the immune system and can regulate multiple cellular events, it is not surprising that TGF-β plays such an important role in regulating T cell differentiation. These observations were first observed in germline TGF-β_1 deficient mice, these mice die early in life due to immunopathology as a result of hyperactivated T cells that infiltrate multiple organs (112, 113). If these mice are depleted of CD4 and CD8 T cells, the immunopathology is alleviated, pointing to T cells as the mediators of the observed pathology (114, 115). Further studies

showed that disease in TGF-β_1 deficient mice is not prevented in the absence of foreign antigens, that is, if these mice are raised in germ-free conditions, immunopathology is observed, likely driven by self-antigens (116).

TGF-β prevents T cell differentiation

Intact TGF-β signaling is required to prevent spontaneous T cell activation and effector cell differentiation (117, 118). Disrupting TGF-β signaling specifically in T cells resulted in complete absence of T cell homeostasis. These findings were shown in mice that express a dominant-negative mutant form of the TGF-β RII (dnTGFBRII) under the control of a CD4 promoter that lacks the CD8 silencer, expression of this receptor attenuates (but does not completely ablate) signaling in both $CD4^+$ and $CD8^+$ T cells. T cells from these mice are spontaneously activated and dnTGFBRII mice develop inflammatory disease, although symptoms of disease appear much later than those seen in TGF-β_1 deficient mice (101). T cell-specific TGFBRI or TGFBRII deficient mice develop early inflammatory disease, similar to the complete TGF-β_1 deficient mice (117-119). These findings show that T cells are directly regulated by TGF-β signaling in vivo. Further evidence showed that intrinsic TGF-β signaling prevented T cell differentiation (117, 118). In 10-week-old dnTGBRII mice, $CD4^+$ T cells differentiated into Th1 and Th2 cells (101). Interestingly, in mice with a complete T cell-specific deficiency in the TGFBRII, this $CD4^+$ T cell differentiation was more pronounced such that $CD4^+$ T cells polarized exclusively toward the Th1 subset, produced IFN-γ, and had high levels of T-bet expression (117, 118).

Only when the receptor was leaky, as in the case of dnTGFBRII mice, $CD4^+$ T cells were able to polarize towards both Th1 and Th2 cells. TGFBRII T cell-specific deficient mice that also lack T-bet expression, had reduced Th1 cells and reduced IFN-γ production, interestingly, these mice had increased Th2 cells and IL-4 production. Furthermore, these double knockout mice had an overall reduction of $CD4^+$ T cells. In fact, T-bet regulates the expression of CD122, the shared receptor beta chain for IL-15 and IL-2 (117). These studies highlight the pro-survival effect of T-bet expression in T cells. Interestingly, TGFBRII deficiency in T cells showed that naïve $CD4^+$ T cells will not survive unless they differentiated into Th1, T-bet expressing cells.

As in $CD4^+$ T cells, TGF-β is also a potent inhibitor of $CD8^+$ T cell activation and differentiation. T cell-specific TGFBRII deficient mice have activated $CD8^+$ T cells that produce high amounts of IFN-γ and effector pro-apoptotic molecules including FasL, perforin and granzymes (117, 118). A tumor model further showed that when TGF-β was neutralized by treatment with a soluble TGFBRII, $CD8^+$ T cell responses against tumor cells were potentiated. Tumor-specific effector $CD8^+$ T cells expanded and these had enhanced cytolytic activity (120). Interestingly, gene expression profile of tumor-specific CTLs indicated that TGF-β can inhibit granzyme B expression by recruitment of Smad2 and Smad3 to the Gzmb promoter, these in conjunction with CREB and ATF1 transcription factors inhibit transcription (120). These findings showed that TGF-β was an important regulator of $CD4^+$ and $CD8^+$ T cell differentiation.

TGF-β can regulate NKT and natural T reg cell differentiation

Invariant natural killer T cells are derived from $CD4^+CD8^+$ T lymphocytes, they have been shown to have an important role in immune responses against viral and bacterial pathogens, and in autoimmunity (121) and (122). NKT cells recognize lipid antigens presented on CD1d molecules expressed on antigen presenting cells (123). Studies in TGFBRII deficient mice showed marked reduction in thymic and peripheral NKT cell numbers (117, 118). NKT cells have a tissue distribution similar to T cells and can make up to 30% of these lymphocytes in liver. In lymph nodes, NKT cells can exceed the number of antigen-specific T cells by as much as 500- to 5000-fold (124).

T regulatory cells develop from $CD4^+CD8^+$ thymocytes and express their hallmark transcription factor Foxp3 (122). Treg development is regulated by TCR affinity for self-peptides presented on MHC II molecules and by co-stimulatory and cytokine signaling pathways (125). Regulatory T cells that develop in the thymus are termed natural regulatory T cells (nTregs) while those that are induced in the periphery from $CD4^+Foxp3^-$ naïve T cells are termed induced T regs (iTregs). Central and peripheral tolerance mechanisms have evolved to prevent T cell-mediated autoimmunity. High affinity self-reactive T cells are deleted in the thymus (126), while peripheral mechanisms are in place to maintain escaped self-reactive T cells in check (127). Tregs maintain peripheral tolerance by actively suppressing these self-reactive T cells. Mice lacking TGF-β_1 or Foxp3 develop early fatal inflammatory disease. Immune suppression by

TGF-β_1 or $CD4^+Foxp3^+$ Tregs are essential mechanisms in maintaining peripheral tolerance (68, 128).

Studies where TGF-β signaling was disrupted in T cells showed that in 12 to 16 day old mice, nTreg cell thymic numbers were normal, however Tregs were reduced in the spleen (117-119). Further analysis showed that TGFBRII deficient Tregs proliferated at a higher rate than wild-type T regs (117) suggesting that the failed maintenance of peripheral Tregs was due to decreased survival. In addition to thymic derived Tregs, iTregs are naïve CD4 T cells that acquire the ability to express Foxp3. $CD4^+CD25^-$ or $CD4^+Foxp3^-$ T cells activated in the presence of TGF-β in vitro are induced to express Foxp3 and gain suppressor function (129-132). Interestingly, TGF-β-induced Foxp3 expression occurred by recruitment of Smad3 molecules to a Foxp3 enhancer element (133). Two studies showed that iTreg differentiation was enhanced in the presence of T-cell derived IL-2 (134, 135), IL-2 induces Foxp3 expression by activation of STAT5. It appears that Smad3 and STAT5 can synergize to induce Foxp3 expression in T cells. The gut-associated lymphoid tissue (GALT) is a potent site for the neoconversion of iTregs. When $CD4^+Foxp3^-$ T cells were transferred into lymphopenic hosts and challenged with oral antigens, iTregs were generated and accumulated in the GALT (136). The preferential induction of iTregs at mucosal sites was attributed to the activity of GALT dendritic cells, especially $CD103^+$ DCs. GALT dendritic cells expressing $CD103^+$ have high expression of retinal dehydrogenase which is the enzyme that converts vitamin A

to retinoic acid (RA) (136). RA induces the upregulation of gut-homing receptors on T cells (137). Generation of iTregs can be attenuated upon addition of TGF-β netralizing antibodies or RA receptor inhibitors, whereas excess RA potentiates TGF-β-induced iTreg differentiation (136, 138-140). These studies showed the GALT as an important site for iTreg generation and this was dependent on TGF-β and RA and $CD103^+$ dendritic cells. TGF-β is very important in immune tolerance by direct regulation of iTregs and nTregs generation and maintenance.

Th17 cell differentiation involves TGF-β

In addition to Th1 and Th2 differentiation, $CD4^+$ T cells can also differentiate into Th17 cells. Th17 are induced upon certain bacterial and fungal infections, these cells have also been implicated in models of experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis (CIA). Th1 and Th2 cytokines inhibit Th17 differentiation. Th17-producing cells depend on RORγT expression (141, 142). Recent evidence has implicated TGF-β as an important regulator of Th17 differentiation from naïve T cells. In one study, naïve $CD4^+$ T cells activated in the presence of Tregs produced high levels of IL-17A (143). In vitro cultures containing naïve T cells, Tregs and LPS-activated dendritic cells also promoted Th17 differentiation, however if TGF-β was neutralized in these cultures, Th17 differentiation was inhibited. The study found that in addition to TGF-β, IL-6 produced from LPS-activated dendritic cells was required for Th17 induction (143). A separate study showed that while TGF-β alone can induce Foxp3 expression of naïve $CD4^+$ T cells, addition of IL-6 in

culture inhibited iTregs and diverted these T cells towards Th17 cells (144). In vivo studies showed that while TGF-β and IL-6 together initiate Th17 commitment of naïve $CD4^+$ T cells, they are not sufficient to fully differentiate these cells into full effector Th17 cells. An EAE model showed that if myelin reactive T cells were expanded in the presence of IL-23 and then transferred, mice developed pathology. In contrast if mice received TGF-β and IL-6 expanded Th17 cells, they did not develop disease, these Th17 cells were associated with increased production of IL-10, a known immunosuppressive cytokine (145). It is possible that TGF-β and IL-6 expanded Th17 cells acquire a regulatory function and only when they receive signals by IL-23 in the absence of TGF-β, can these cells obtain a full effector function where they can now produce inflammatory cytokines as well as pathogenic chemokines. These studies highlight the importance of TGF-β signaling on T cells in regulating the differentiation of both $CD4^+$ and $CD8^+$ T cells.

TGF-β role in peripheral tolerance

Of the three TGF-β isoforms, TGF-β_1 is the most critical in regulating T cell responses in vivo. Studies attempted to understand whether blood TGF-β levels were sufficient to control T cell responses in vivo. In this study, TGF-β_1 was expressed in the liver and this restored normal TGF-β_1 blood levels in TGF-β_1 deficient mice. Even though, TGF-β_1 circulating levels were restored, TGF-β_1 deficient mice developed and succumbed to disease (146). These findings show that TGF-β signaling and regulation of T cells responses occurs locally, this can

be in an autocrine or paracrine fashion. Studies showed that T cells are important TGF-β sources in vivo. Mice with a T-cell specific deletion of TGF-β_1 developed colitis similar to the dnTGFBRII mice (147), indicating that T cells are critical TGF-β sources important maintaining peripheral tolerance. TGF-β_1 deficient T cells had increased activation, proliferation, and effector differentiation phenotypes. Interestingly, TGF-β_1 T cell specific deficient mice had enhanced Treg proliferation and numbers in the periphery (147), in contrast to complete TGF-β_1 deficient mice or T cell-specific TGFBRII deficient mice that fail to maintain peripheral Tregs (117, 118, 148). This finding showed that other TGF-β_1 sources can compensate for the lack of TGF-β_1 on T cells, and this source is sufficient for their maintenance. It is also possible that other TGF-β isoforms or family members can compensate for the lack of TGF-β_1 on T cells. Colonic Tregs from T cell-specific TGF-β_1 deficient mice had reduced Foxp3 expression when compared to Tregs in spleen and lymph nodes, indicating that TGF-β_1 from T cells was required for optimal Foxp3 induction in the colon (147). A study using a model of colitis showed the relevance of TGF-β_1 derived from T cells in regulation of T cell responses. When naïve $CD4^+$ T cells are transferred into lymphopenic hosts, mice will develop colitis. If these naïve $CD4^+$ T cells are co-transferred with wildtype T regulatory cells, colitis is prevented. When naïve CD4+ T cells were transferred with TGF-β_1 deficient Tregs, mice developed colitis (147). These findings indicated that TGF-β_1 produced by Tregs was critical in protecting against developing colitis by preventing differentiation of naïve $CD4^+$

T cells towards a Th1 phenotype or that TGF-β is necessary for the development of fully functional T regulatory cells even if TGF-β is not the effector T regulatory cytokine (147).

Important cell-to-cell contact is necessary for efficient TGF-β signaling in T cells

TGF-β signaling on T cells occurs in an autocrine or paracrine fashion, the activation of latent TGF-β by integrins αvβ6 or αvβ8 are important regulatory mechanism. TGF-β_1 activation can occur either by LAP degradation or a conformational change releasing the mature TGF-β_1 homodimer. Integrins activate TGF-β by binding to the RGD (arginyl-glycyl-aspartic acid) motif on LAP from the latent TGF-β complex, these integrins have a different mechanism of action that allows spatial regulation of TGF-β. The αvβ6 integrin activates TGF-β by a conformational change induced as it binds to LAP, not by releasing TGF-β from the latent complex (149). For TGF-β to bind to its receptor and signal, a very close cell interaction is required for this to happen. The αvβ8 integrin releases TGF-β from the latent complex when it binds to LAP, degradation of LAP then occurs through the action of a metalloproteinase (150). TGF-β is then released to the extracellular matrix, activation through this mechanism allows TGF-β to signal at longer distances. Mutations in a single residue in the integrin binding site on LAP is sufficient in preventing TGF-β activation, but TGF-β production and secretion is unaffected. Surprisingly, these mutant mice develop a lethal immune phenotype identical to TGF-β_1 deficient mice, indicating an

essential role of TGF-β activation by integrins (151). Of these integrins, αvβ6 seems to be dispensable for T cell activation as mice with a deficiency in αvβ6 develop a mild inflammatory disease compared to the TGF-β1 deficient mice (151). In contrast, αvβ8 which is expressed in several immune cells, T cells, and myeloid dendritic cells seems to have a more important role in preventing inflammatory disease. When αvβ8 is deleted in dendritic cells but not in T cells, mice develop colitis and have reduced numbers of Tregs (152). These findings indicate that integrins play a critical function in regulating T cells by direct activation of latent TGF-β.

The role of TGF-β biology has been extensively studied under steady state conditions. TGF-β plays a critical role in regulating diverse cellular processes including survival, proliferation, and differentiation of T cells. Whether TGF-β is important during infectious conditions has not been examined. To investigate the role of TGF-β in T cell suppression and viral persistence, we used chronic infection with LCMV in mice as a model system (see section titled "LCMV as a model system for chornic viral infection," for more detail).

Chapter 1 contains portions of the material as it appears in the journal Immunity, "Cell-intrinsic Transforming Growth Factor-β signaling mediates virus-specific CD8+ T cell deletion and viral persistence *in vivo*." Tinoco, Roberto, Alcalde Victor, Yang, Yating, Sauer, Karsten, Zuñiga, Elina I., July 2009 Jul 17;31(1):145-57. The dissertation author is the first author of this paper.

CHAPTER 2 RESULTS

Enhanced TGF-β expression in $CD8^+$ T cells during chronic LCMV infection

In our first studies, we wanted to determine which cell types could be a potential relevant source of TGF-β production during acute vs chronic infection. To examine the source of TGF-β during infection, T cells ($CD4^+$ and $CD8^+$), B ($CD19^+$) cells, dendritic cells (DCs) ($CD11c^+CD19^-Thy1.2^-NK1.1^-$), and macrophages (Macs) ($CD11b^+CD11c^-CD19^-Thy1.2^-NK1.1^-$) were FACS-sorted at day 9-pi from ARM and Cl 13 infected mice, or from uninfected mice and analyzed for TGF-β_1 levels by immunoblot (Figure 1A and 1B). All cells examined produced TGF-β_1 under steady state conditions, however, during ARM and Cl 13 infection, B cells, DCs, and Macs had lower levels of TGF-β_1 when compared to uninfected mice (Figure 1A and 1B). Interestingly, both total $CD4^+$ and $CD8^+$ T cells had increased TGF-β_1 production during acute and chronic infection compared to uninfected mice, but the highest levels (albeit slightly) were observed in $CD8^+$ T cells from Cl 13 infected mice (Figure 1A).

Since we observed a slight increase in TGF-β levels in total $CD8^+$ T cells from Cl 13 infected mice (Figure 1A), we next wanted to understand whether TGF-β levels were differentially regulated in virus-specific $CD8^+$ T cells in acute vs chronic infection. To evaluate TGF-β protein levels in virus-specific $CD8^+$ T cells, we adoptively transferred naïve $CD45.2^+$ P14 TCR transgenic $CD8^+$ T cells that are specific for the GP_{33-41} epitope, into $CD45.1^+$ WT mice 1 day before infection with LCMV ARM or Cl 13. We then FACS sorted these P14 TCR

transgenic $CD8^+$ T cells and analyzed TGF-β levels by immunoblot (Figure 1C). At day 5 pi we observed that TGF-β_1 was produced in virus-specific $CD8^+$ T cells during both acute and chronic infection (Figure 1C). By days 8-10 pi virus-specific $CD8^+$ T cells from ARM infected mice had very low levels of TGF-β_1 compared to virus-specific $CD8^+$ T cells from Cl 13 infected mice, which had sustained production of TGF-β_1. These findings indicate that many immune cell types are able to produce TGF-β_1 during both acute and chronic infection, but while most downregulate production strong upregulation is observed in virus-specific $CD8^+$ T cells.

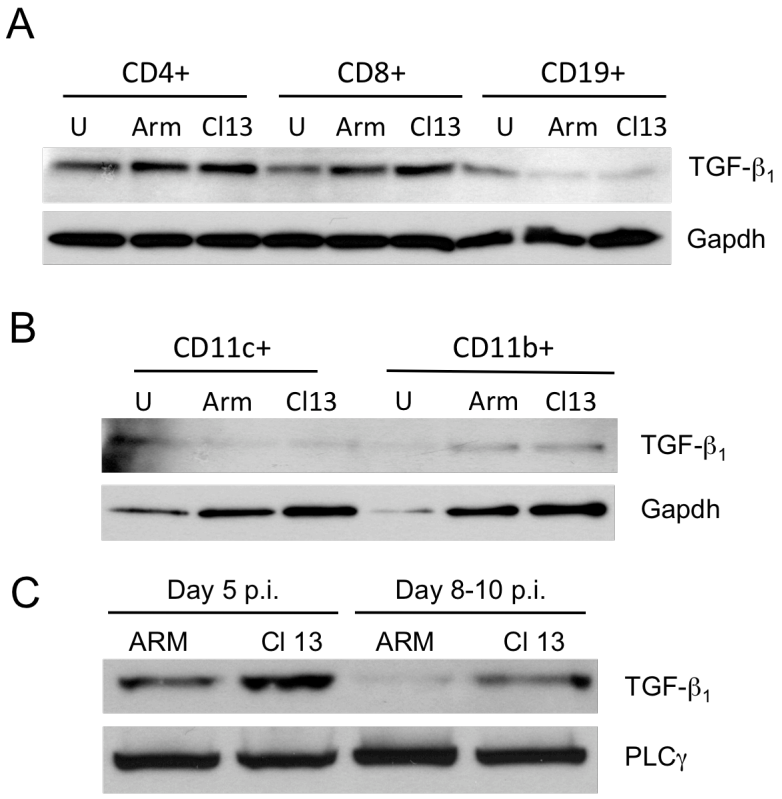

Figure 1. TGF-β_1 expression in different cell types during LCMV infection. Indicated cell populations were FACS purified from uninfected, ARM, or Cl 13 infected animals at day 9 pi (A-B) or day 5 and 8-10 pi (C). Gapdh or PLC-γ were used as loading controls as indicated. In (C) donor CD45.2$^+$ P14 TCR transgenic CD8$^+$ T cells were transferred into WT CD45.1$^+$ recipients, hosts were infected with LCMV ARM or Cl 13, and pooled P14 cells were FACS purified and processed for immunoblot. All results are representative of two or three independent experiments with three to five mice per group each.

Sustained and enhanced Smad-2 phosphorylation in $CD8^+$ T cells during chronic LCMV infection

TGF-β canonical signaling is achieved by its binding to TGF-β receptors type I and II (153-155) and is mediated by phosphorylation of Smad proteins. To evaluate TGF-β-Smad signaling on virus-specific $CD8^+$ T cells, we again adoptively transferred naïve $CD45.2^+$ P14 $CD8^+$ T cells into $CD45.1^+$ WT mice 1 day before infection with LCMV ARM or Cl 13. Immunoblot analysis of P14 cells isolated at day 8-10 p.i. showed enhanced Smad-2 phosphorylation during chronic compared to acute LCMV infection (Figure 2). In addition, a slight increase in Smad-2 phosphorylation was already evident by day 5 after Cl 13 infection. Minimal or no difference in total Smad-2 levels after ARM vs Cl 13 were observed (Figure 2).

Differential phoshorylation of Smad-2 proteins in virus-specific $CD8^+$ T cells during acute or chronic infection could result from altered expression of TGF-β receptors. To examine this possibility, we determined the level of TGFBRII expression on virus-specific D^b-$GP_{33-41}{}^+CD8^+$ and D^b-$GP_{276-286}{}^+CD8^+$ T cells by FACS. We observed no differences in the expression of TGFBRII during acute or chronic infection (Figure 3). Together, these findings indicated that the TGF-β-Smad signaling pathway was active in antiviral $CD8^+$ T cells during both acute and chronic LCMV infection but was only enhanced and sustained during persistent infection and it was not a result of differences in TGFBRII expression.

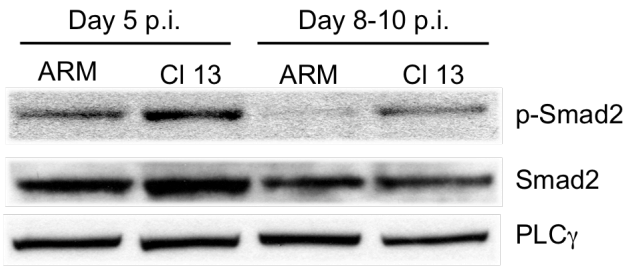

Figure 2. TGF-β-Smad-2 phosphorylation in virus-specific CD8$^+$ T cells during LCMV infection. Donor CD45.2$^+$P14 TCR transgenic CD8+ T cells were transferred into WT CD45.1$^+$ recipients. Hosts were infected with ARM or Cl 13, and P14 cells FACS purified at day 5 or 8-10 p.i. Cells were processed for immunoblot for p-Smad-2 and Smad-2, PLCγ was used as a loading control. All results are representative of two or three independent experiments with three to five mice per group.

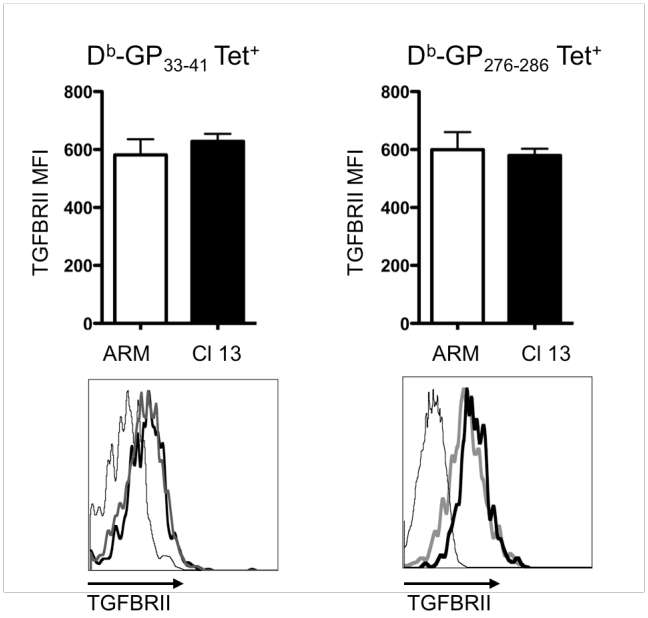

Figure 3. TGFBRII expression on virus-specific T cells during LCMV infection. C57BL/6 mice were infected with LCMV ARM (white bars and grey histograms) or Cl 13 (black bars and black histograms) and splenocytes obtained at day 9 p.i. TGFBRII expression was quantified by FACS in D^b-GP$_{33-41}$$^+$CD8$^+$ and D^b-GP$_{276-286}$$^+$CD8$^+$ cells. Bar graphs indicate the average TGFBRII MFI ± sd. Histograms depict a representative mouse per group. Thin line, Isotype control. Data are representative of two independent experiments with three to six mice per group.

Attenuation of TGF-β signaling in T cells enabled accumulation of virus-specific $CD8^+$ T cells

To investigate the biological significance of TGF-β signaling in T cells during chronic viral infection, we used dnTGFBRII transgenic mice. As described in section titled, "TGF-β prevents T cell differentiation," these mice express a dominant negative form of the TGFBRII under the control of a modified CD4 promoter lacking the CD8 silencer, as a consequence, TGF-β signaling is attenuated exclusively in T cells (101). We infected dnTGFBRII mice with LCMV Cl 13 and analyzed T cell responses in these animals. Numbers of peripheral blood $CD8^+$ T cells specific for three different MHC class I-restricted LCMV epitopes were monitored by tetramer staining throughout the course of infection (Figure 4A). Cl 13-infected dnTGFBRII mice showed considerably higher frequencies of GP_{33-41} and $GP_{276-286}$-specific T cells than WT controls. More impressive, the proportion of $NP_{396-404}$-specific $CD8^+$ T cells, which are normally deleted in WT mice during Cl 13 infection (15), was also enhanced in dnTGFBRII mice. The increased proportions of peripheral blood tetramer-positive $CD8^+$ T cells were maintained until the last time-point examined at 45 days p.i. Analysis of splenic T cells at day 9 p.i. also revealed elevated frequencies of $NP_{396-404}$, GP_{33-41}, $GP_{276-286}$-specific $CD8^+$ T cells in dnTGFBRII mice (Figure 4B). Thus, mice with attenuated TGF-β receptor II signaling in T cells have increased frequencies of virus-specific $CD8^+$ T cells in blood and spleen after Cl 13 infection.

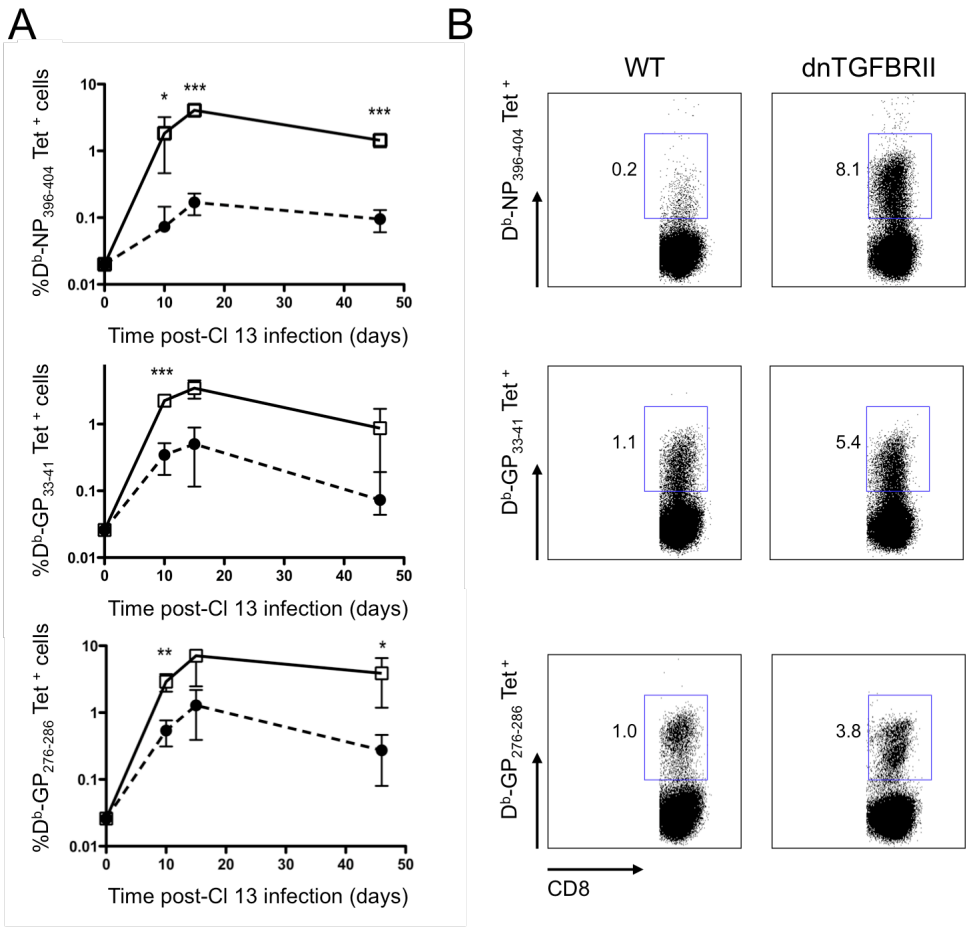

Figure 4. Percent of virus-specific CD8$^+$ T cells in blood and spleen during LCMV infection. WT (black circles) or dnTGFBRII (white squares) mice were infected with LCMV Cl 13. Virus-specific CD8$^+$ T cells from blood (A) or spleen (B) were stained with H2D^b-NP$_{396-404}$, D^b-GP$_{33-41}$ and D^b-GP$_{276-286}$ tetramers. (A) Log-scaled plots showing average frequencies ± SD of tetramer$^+$CD8$^+$ blood cells at the indicated time pi. (B) Dot plots displaying tetramer staining profiles in splenocytes from a representative mouse per group at day 9 p.i. Numbers indicate percent tetramer$^+$CD8$^+$ cells in the respective gate. All results are representative of two or three independent experiments with three or five mice per group each. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

We next examined the number of splenic $NP_{396-404}$, GP_{33-41}, $GP_{276-286}$-specific $CD8^+$ T cells in dnTGFBRII mice and observed a dramatic increase compared to WT mice. In fact, the number of virus-specific $CD8^+$ T cells was similar to WT mice infected with ARM, but more impressively, the dnTGFBRII mice had up to 300 time higher numbers than Cl 13 infected WT mice (Figure 5A). We next wanted to understand whether differences in the early expansion of virus-specific $CD8^+$ T cells in WT vs dnTGFBRII mice accounted for the increased cell numbers observed in dnTGFBRII mice at later time points. We examined the number of $NP_{396-404}$ and GP_{33-41} virus-specific $CD8^+$ T cells at days 3, 5, and 9 p.i. and observed that the initial $CD8^+$ T cell expansion was similar in WT and dnTGFBRII mice (Figure 5B). Interestingly, the difference in cell numbers was only observed after day 5 p.i. suggesting that the negative impact of TGF-β receptor signaling becomes effective after this time post infection. These findings show that diminished TGF-β receptor signaling in dnTGFBRII T cells results in the accumulation of virus-specific $CD8^+$ T cells during chronic LCMV infection, and this accumulation does not occur before day 5 p.i.

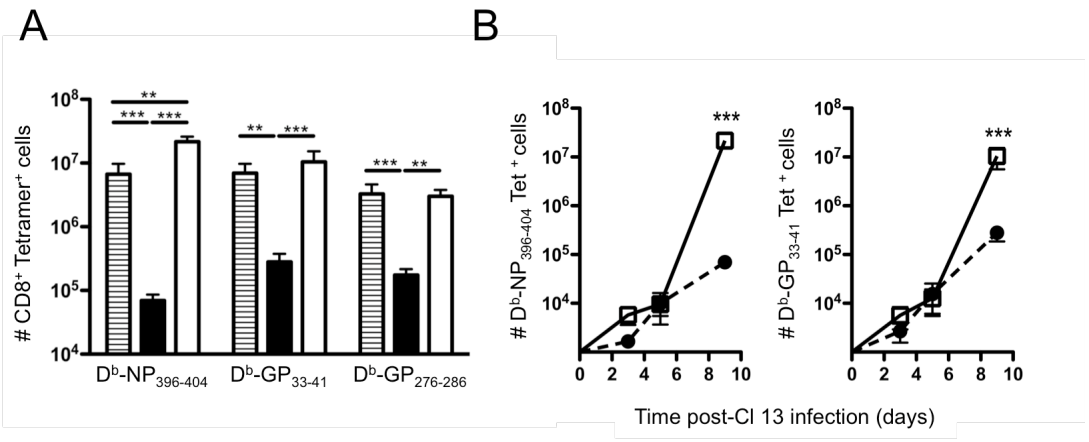

Figure 5. Accumulation of virus-specific CD8$^+$ T cells during LCMV infection. WT (black circles or bars) or dnTGFBRII (white squares or bars) mice were infected with LCMV CL 13. WT-ARM infected mice were processed as controls (striped bars). Virus-specific CD8$^+$ T cells from spleen were stained with H2D^b-NP$_{396-404}$, D^b-GP$_{33-41}$ and D^b-GP$_{276-286}$ tetramers. (A and B) Total numbers of tetramer$^+$CD8$^+$ cells per spleen at day 9 (A) or days 1 through 9 (B) p.i. Note that numbers of tetramer+ T cells were similar to background levels at day 1 and 3 p.i. All results are representative of two or three independent experiments with three or five mice per group each. **p < 0.005, ***p < 0.0005.

Virus-specific $CD8^+$ T cell proliferation is not increased in dnTGFBRII mice after Cl 13 infection

To investigate whether the increased number of virus-specific $CD8^+$ T cells observed in dnTGFBRII mice after Cl 13 infection was a result of increased proliferation, we infected WT and dnTGFBRII mice and monitored proliferation in vivo by analyzing BrdU incorporation. We isolated splenocytes at day 7 and 9 p.i. and examined proliferation in GP_{33-41} and $GP_{276-286}$ $CD8^+$ T cells. We did not detect differences in WT or dnTGFBRII virus-specific $CD8^+$ T cells at day 7 p.i. (Figure 6A and Figure 7A). In fact, by day 9 p.i. virus-specific $CD8^+$ T cells from dnTGFBRII mice had decreased proliferation, while cells from WT mice continued dividing, likely because of continuous antigen presentation.

Accumulation of virus-specific $CD8^+$ T cells in dnTGFBRII mice is a result of enhanced survival

Having determined that the increase number of virus-specific CD8+ T cells in dnTGFBRII mice was not due to increased proliferation. It remained possible this increase could be the result of altered survival of WT vs dnTGFBRII virus-specific $CD8^+$ T cells. To examine this possibility, we monitored apoptosis by Annexin V staining of GP_{33-41} and $GP_{276-286}$ $CD8^+$ T cells after Cl 13 infection. We detected that at day 7 p.i. GP_{33-41} $CD8^+$ T cells dnTGFBRII cells had reduced Annexin V staining, indicative of reduced apoptosis (Figure 6B). Furthermore, by day 9 p.i. virus-specific $CD8^+$ T cells in dnTGFBRII mice continued to have enhanced survival compared to WT mice (Figure 6B and 7B). In fact, dnTGFBRII virus-specific $CD8^+$ T cells had less Annexin V binding at later rather than earlier time points. These findings show that virus-specific $CD8^+$ T cells with

attenuated TGF-β signaling have a survival advantage compared to WT mice during Cl 13 infection, and the accumulation of these cells is a result of this enhanced survival.

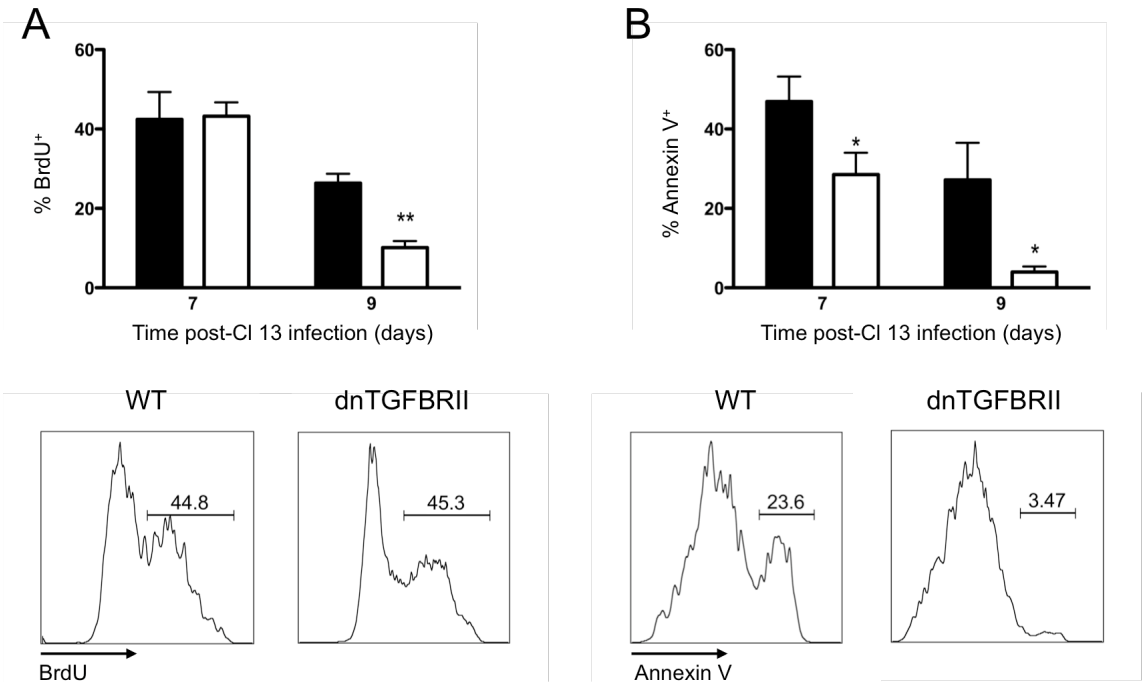

Figure 6. Proliferation and survival of virus-specific H2D^{b}-GP$_{33-41}$-specific CD8^{+} T cells during LCMV infection. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV Cl 13 and splenocytes obtained at day 7 and 9 p.i. BrdU incorporation (A) and Annexin V staining (B) of H2D^{b}-GP$_{33-41}$ tetramer^{+} CD8^{+} T cells were determined. Bar graphs depict the average frequency of positive cells ± sd. Histograms display a representative mouse per group and numbers indicate the frequency of cells within regions. Results are representative of two independent experiments with three or four mice per group. (WT *vs* dnTGFBRII, *$p < 0.05$, **$p < 0.005$).

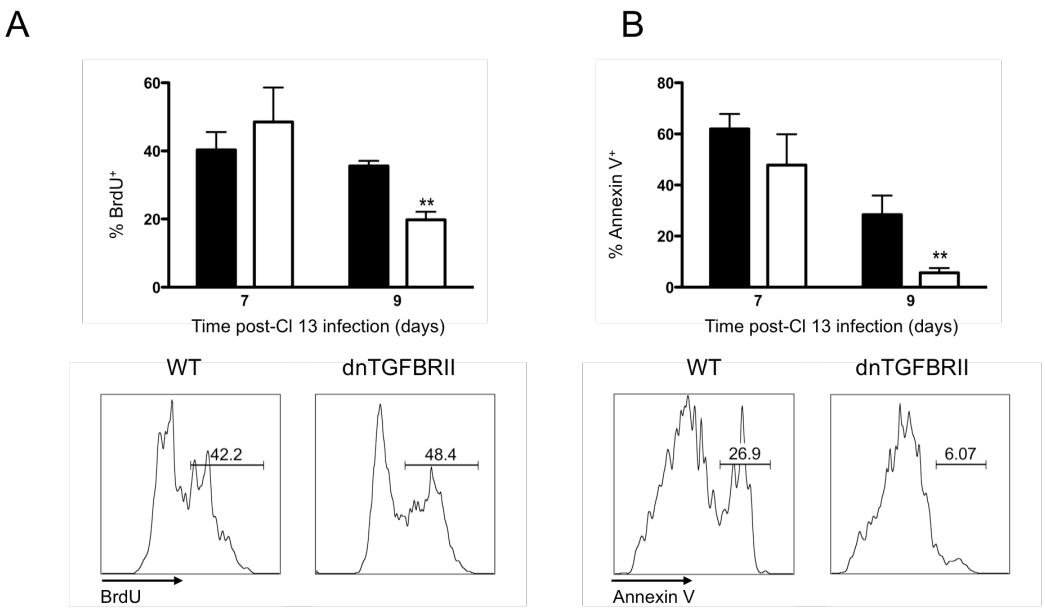

Figure 7. Proliferation and survival of LCMV $GP_{276-286}$-specific $CD8^+$ T cells during LCMV infection. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV Cl 13 and splenocytes obtained at day 7 and 9 p.i. BrdU incorporation (A) and Annexin V staining (B) of $H2D^b$-$GP_{276-286}$ $tetramer^+$ $CD8^+$ T cells were determined. Bar graphs depict the average frequency of positive cells ± sd. Histograms display a representative mouse per group and numbers indicate the frequency of cells within regions. Results are representative of two independent experiments with three or four mice per group. (WT *vs* dnTGFBRII, $^{**}p<0.005$).

Bim levels are reduced in virus-specific CD8$^+$ T cells with attenuated TGF-β signaling

We next wanted to understand the molecules involved in virus-specific CD8$^+$ T cell death during chronic infection. The BH3-only pro-apoptotic protein Bim has previously been implicated in the killing of virus-specific CD8$^+$ T cells during chronic infection with LCMV Cl 13 and murine γ-herpesvirus (156, 157). We first examined Bim levels in virus-specific CD8$^+$ T cells sorted from mice infected with ARM or Cl 13 at day 5.5 and 8-10 p.i. and observed a consistent increase in Bim EL and Bim L (Figure 8A) isoforms in virus-specific CD8+ T cells from Cl 13 infected mice. In fact, this increase in Bim expression was evident as early as day 5.5 p.i. and was greatly elevated at later time points compared to virus-specific CD8$^+$ T cells from ARM infected mice. Notably, upregulation of Bim coincided with enhanced Smad-2 phosphorylation in virus-specific CD8$^+$ T cells (Figure 2), suggesting that TGF-β signaling could potentially regulate Bim levels. We therefore examined whether Bim protein levels in virus-specific CD8$^+$ T cells were also being differentially regulated in dnTGFBRII Cl 13 infected mice and found that indeed, Bim levels were decreased in virus-specific CD8$^+$ T cells from dnTGFBRII mice compared to WT controls (Figure 8B). These findings indicated that TGF-β signaling somehow (directly or indirectly) upregulated Bim levels in virus-specific CD8$^+$ T cells during Cl 13 infection and this could contribute to the TGF-β-induced apoptosis of virus-specific CD8$^+$ T cells observed during chronic infection.

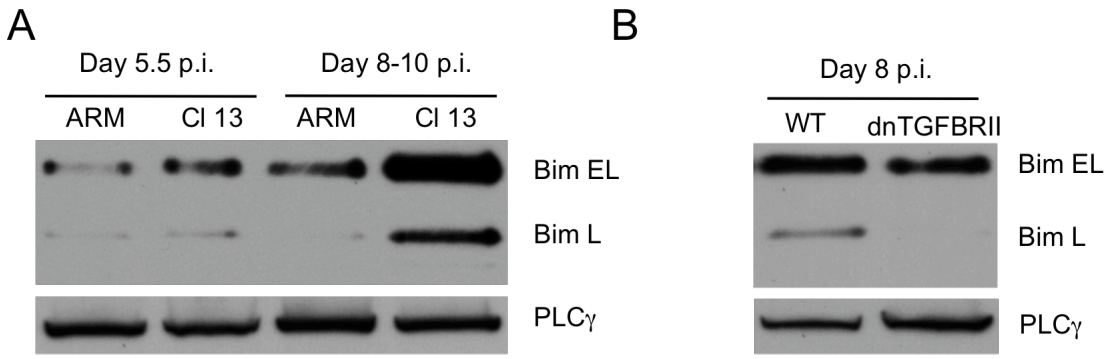

Figure 8. dnTGFBRII mice have reduced Bim expression in virus-specific CD8$^+$ T cells. (A and B) Virus-specific CD8$^+$ T cells were FACS purified from WT mice infected with LCMV ARM or Cl 13 (A) or dnTGFBRII mice infected with Cl 13 (B) at days 8-10 pi. Cells were processed by immunoblot and the amounts of Bim EL and Bim L isoforms are shown. PLCγ was used as loading control. Results are representative of 2 independent experiments with 3-5 mice per group.

dnTGFBRII mice generate a superior virus-specific CD8$^+$ T cell effector response than WT mice after Cl 13 infection

We investigated whether attenuated TGF-β receptor signaling in T cells enabled the acquisition and maintenance of effector functions after Cl 13 infection. First we examined the production of IFN-γ, TNF-α, and IL-2 in CD8$^+$ T cells from day 9 Cl 13-infected WT and dnTGFBRII mice by ex vivo peptide stimulation. As previously reported, WT Cl 13 infected mice had severe impairment in production of antiviral cytokines (9, 14). In contrast, dnTGFBRII mice had dramatic elevated frequencies of epitope-specific CD8$^+$ T cells producing IFN-γ, TNF-α, and IL-2 when re-stimulated with $NP_{396-404}$, GP_{33-41}, or $GP_{276-286}$ LCMV peptides (Figure 9B). Furthermore, we normalized the number of cytokine-producing CD8$^+$ T cells to the corresponding number of CD8$^+$ tetramer$^+$ cells in the same spleen and found that on a per cell basis, dnTGFBRII mice had more functional virus-specific CD8$^+$ T cells compared to WT (Figure 9A). To investigate the killing capacity of CD8$^+$ effectors T cells, we isolated splenocytes from WT and dnTGFBRII mice at day 9 p.i. and incubated these with $NP_{396-404}$ or GP_{33-41} loaded target cells and quantified lysis of target cells by ^{51}Cr release assay (Figure 10). We found that effectors from dnTGFBRII mice were significantly more efficient at lysing these LCMV peptide-loaded target cells than WT. These findings indicate that diminished TGF-β receptor signaling in T cells during Cl 13 infection resulted in full functional development of virus-specific CD8$^+$ T cell effectors able to produce anti-viral cytokines and kill target cells.

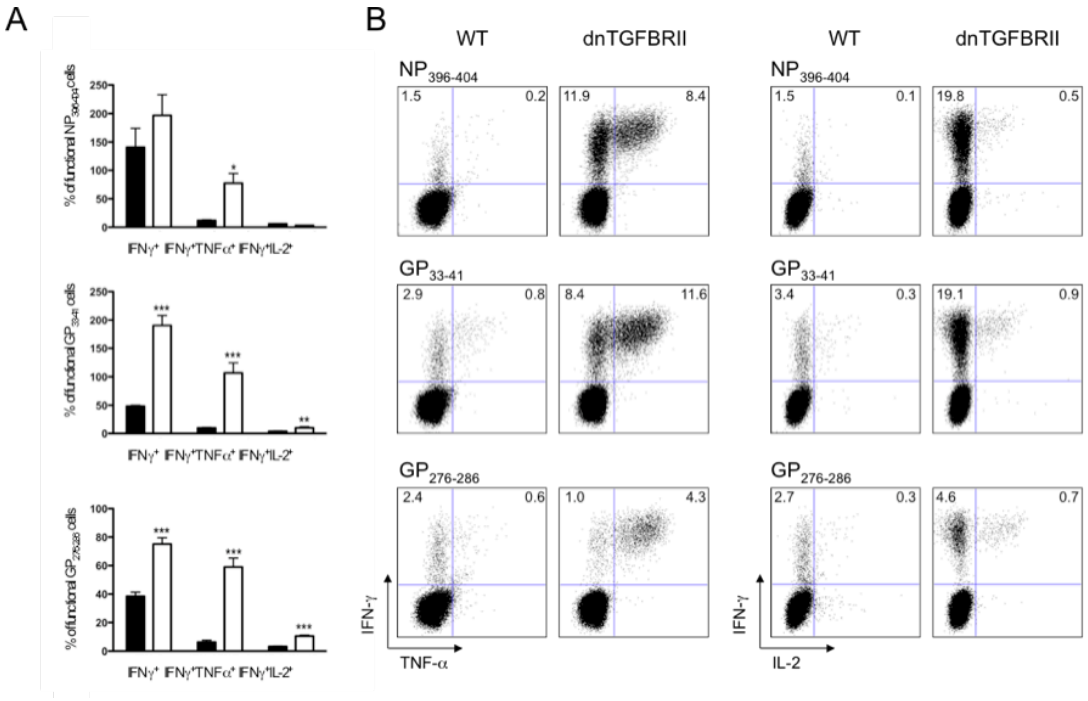

Figure 9. Effector function of virus-specific CD8$^+$ T cells after LCMV infection. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV Cl 13 and splenocytes obtained at day 9 p.i. (A) Cells were stimulated with $NP_{396-404}$, GP_{33-41}, and $GP_{276-286}$ LCMV peptides and production of IFN-γ, TNF-α, and IL-2 by CD8$^+$ T cells was analyzed. Bar graphs depict the average percent ±SD of cytokine-producing CD8$^+$ T cells normalized to the number of tetramer$^+$ cells in the same spleen. (B) Dot plots display a representative mouse for each peptide. Numbers indicate the percent of cells within the indicated gates. Results representative of two or three independent experiments with three to four mice each. WT vs dnTGFBRII; *$p < 0.05$ and **$p < 0.005$.

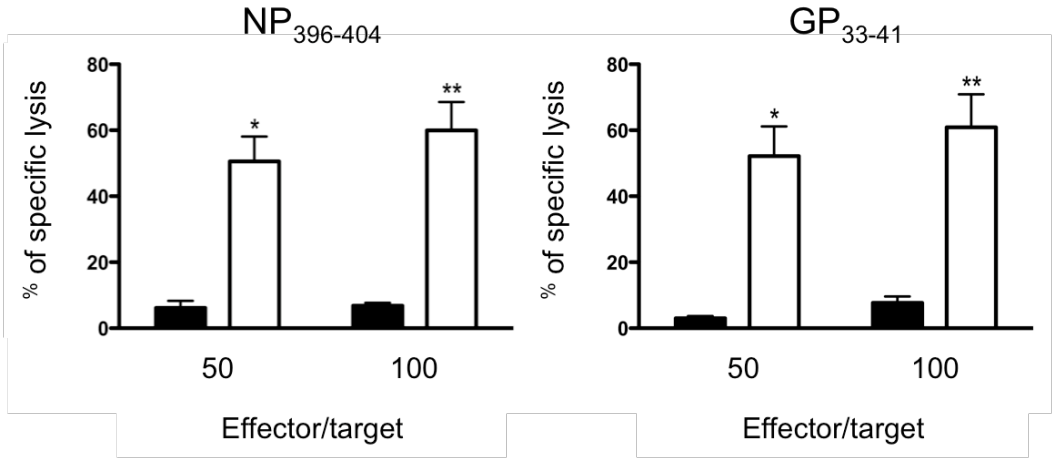

Figure 10. Cytotoxicity of virus-specific CD8$^+$ T cells in WT and dnTGFBRII mice. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV CL 13 and splenocytes obtained at day 9 p.i. The cytotoxic capacity of virus-specific CD8$^+$ T cells was quantified by ^{51}Cr release assay against target cells loaded with LCMV peptides at the indicated ratios. The graphs show average percent of specific lysis ± SD. Results are representative of two or three independent experiments with three to four mice each. WT vs dnTGFBRII; *$p < 0.05$, **$p < 0.005$.

Virus- specific $CD8^+$ T cell responses in dnTGFBRII Cl 13 infected mice is similar to WT ARM infected mice

We next investigated whether $CD8^+$ T cell responses in dnTGFBRII Cl 13 infected resembled to WT acutely infected mice. We isolated splenocytes from WT ARM, WT Cl 13, and dnTGFBRII Cl 13 infected mice at day 9 p.i. We re-stimulated these cells ex vivo with $NP_{396-404}$, GP_{33-41}, and $GP_{276-286}$ LCMV peptides and monitored IFN-γ, TNF-α, and IL-2 cytokine production (Figure 11). As previously shown, WT Cl 13 infected animals have a compromised ability to secrete IL-2, TNF-α, and IFN-γ. In contrast, dnTGFBRII mice infected with the same chronic virus mount a potent $CD8^+$ T cell response and produce copius amounts of these three antiviral cytokines (Figure 11). In comparison to WTARM infected mice, $CD8^+$ T cells from dnTGFBRII-Cl 13 infected mice produce similar levels of IL-2, TNF-α, and IFN-γ. These findings show that while $CD8^+$ T cells from WT Cl 13 infected mice fail to produce sufficient effector cytokines, $CD8^+$ T cells from mice with attenuated TGF-β signaling produce these cytokines to a similar proportion as mice infected with ARM.

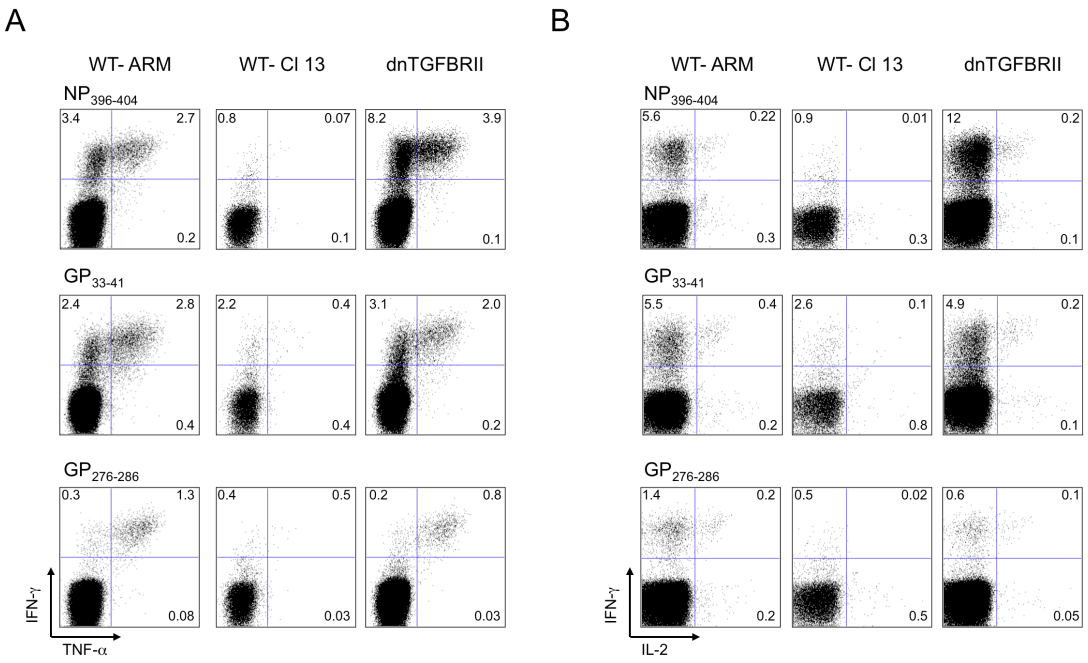

Figure 11. Cytokine production in WT and dnTGFBRII mice during LCMV ARM and Cl 13 infection. WT and dnTGFBRII mice were infected with LCMV ARM or Cl 13 as indicated and splenocytes obtained at day 9 p.i. Cells were stimulated with $NP_{396-404}$, GP_{33-41} and $GP_{276-286}$ LCMV peptides and production of IFN-γ, TNF-α and IL-2 by $CD8^+$ T cells was analyzed. Dot plots display a representative mouse per group for each peptide. Numbers indicate the percent cells within the indicted gates. Results are representative of one experiment with four mice per group.

dnTGFBRII mice have reduced PD-1 and IL-10 levels after Cl 13 infection

Production of inhibitory cytokines or expression of inhibitory receptors on virus-specific $CD8^+$ T cells is a hallmark of chronic LCMV infections. As mentioned in section "Exhausted viru-specific CD8+ T cells express high levels of inhibitory receptors," one such receptor, PD-1 is continuously upregulated in exhausted T cells, while only transiently upregulated in T cells after ARM infection, and absent on functional memory $CD8^+$ T cells (38). PD-L1 blockade during Cl 13 infection restores the proliferative capacity of virus-specific $CD8^+$ T cells and reduces viral levels (38). To investigate whether these PD-1 inhibitory receptor levels were being differentially regulated in the absence of TGF-β signaling, we examined PD-1 expression in D^b-GP_{33-41} and D^b-$_{GP276-286}$ virus-specific $CD8^+$ T cells after Cl 13 infection. At day 5 p.i. PD-1 levels on virus-specific $CD8^+$ T cells were identical in WT and dnTGFBRII Cl 13 infected mice, indicating that TGF-β signaling is not required for initial PD-1 upregulation during chronic LCMV infection. However, by day 9 p.i. virus-specific $CD8^+$ T cells in WT mice continued to sustain higher PD-1 levels while dnTGFBRII mice had significantly lower PD-1 levels similar to virus-specific $CD8^+$ T cells obtained from WT ARM infected mice at day 9 p.i. (Figure 12A and 12B). Furthermore, PD-1 levels on WT D^b-GP_{33-41} $CD8^+$ T cells remained high at day 10, 15, and 32 p.i. in blood, while virus-specific $CD8^+$ T cells in dnTGFBRII mice continued to express very low PD-1 levels (Figure 13).

IL-10 levels have been linked to T cell exhaustion in chronic LCMV infections such that both IL-10 protein and mRNA levels were increased in Cl 13 infected mice (35, 42). Blocking IL-10 levels by treatment with IL-10 receptor resulted in decreased viral loads and improved function of virus-specific T cells (35, 42). We examined IL-10 mRNA levels in spleens isolated at day 5 and 9 p.i. from WT and dnTGFBRII mice

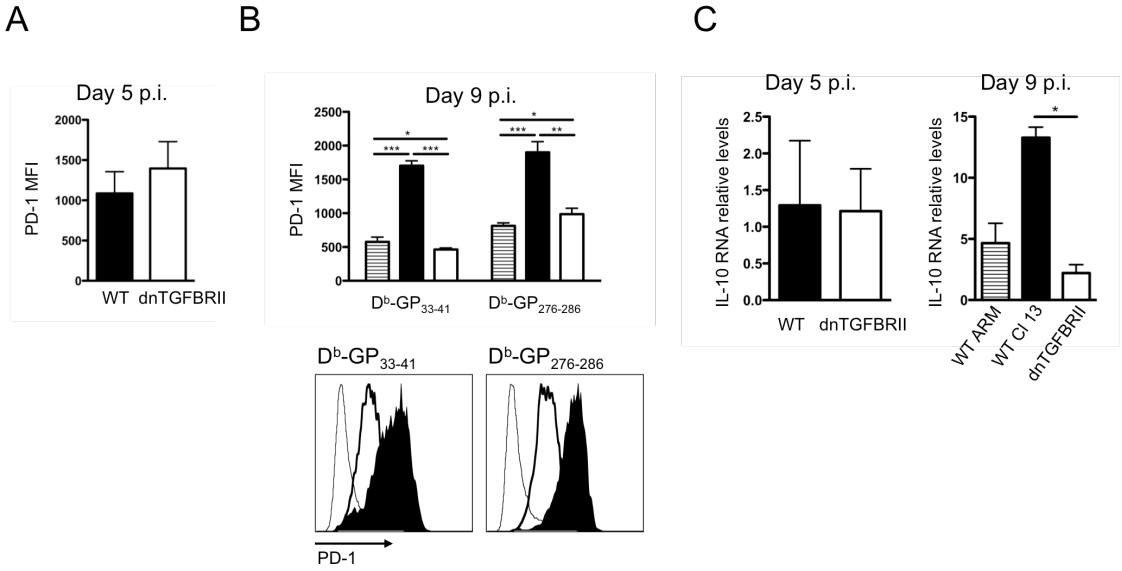

Figure 12. PD-1 and IL-10 levels during LCMV infection. WT (black bars or histograms) or dnTGFBRII (white bars or histograms) mice were infected with LCMV Cl 13 and splenocytes obtained at day 9 p.i. unless otherwise stated. (A-B) PD-1 expression was quantified in D^b-$GP_{33-41}{}^+CD8^+$ and D^b-$GP_{276-286}{}^+CD8^+$ splenocytes. Bar graphs indicate the average PD-1 MFI ±SD. The histograms show representative data from one mouse per group. Shown are PD-1 expression on total $CD8^+$ cells from uninfected controls (thin line) or on the indicated tetramer-positive dnTGFBRII (thick line) or WT (filled histogram) $CD8^+$ T cells from Cl 13-infected mice. (C) IL-10 mRNA expression was quantified by real-time PCR and are shown as average ±SD normalized to GAPDH mRNA. WT-ARM-infected mice at day 9 p.i. were processed as controls (striped bars). Results are representative of two or three independent experiments with three to four mice each. *$p < 0.05$, *$p < 0.05$, ***$p < 0.005$.

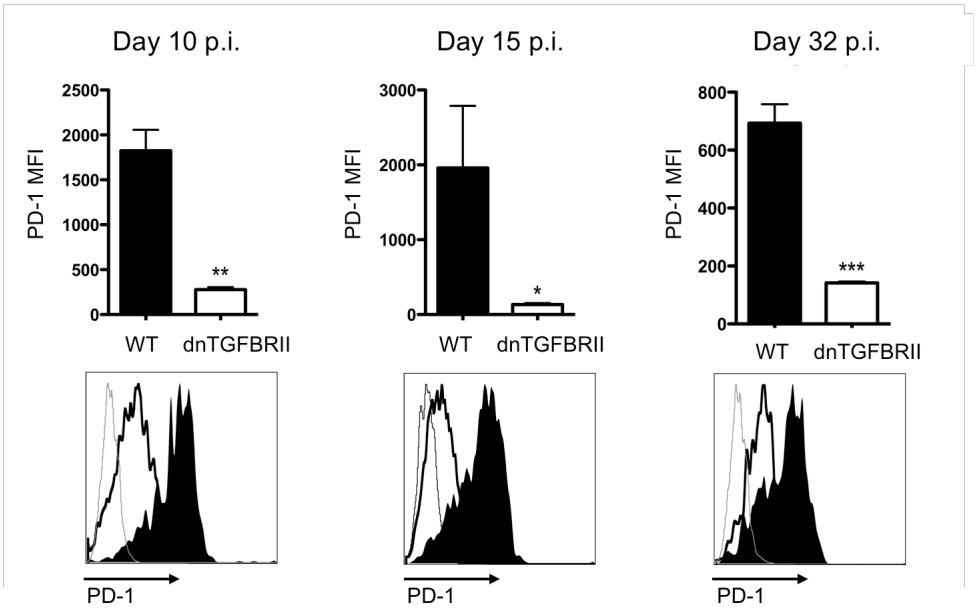

Figure 13. PD-1 expression in virus specific CD8$^+$ T cells from dnTGFBRII mice. WT (black bars or histograms) or dnTGFBRII (white bars or histograms) mice were infected with LCMV Cl 13. PD-1 expression was determined in D^b-$GP_{33-41}{}^+CD8^+$ blood cells at the indicated time p.i. Bar graphs indicate the average ± sd of PD-1 MFI. Histograms depict a representative mouse per group. Dashed line, unstained controls. Results are representative of two independent experiments with three to four mice per group. (WT *vs* dnTGFBRII, *p<0.05, **p<0.005 and ***p<0.0005)

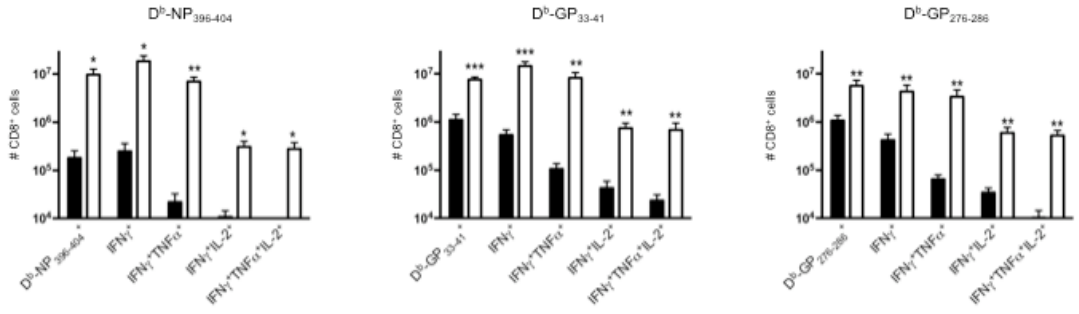

Figure 14. Numbers of epitope-specific and cytokine producing CD8$^+$ T cells in dnTGFBRII mice. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV Cl 13 and splenocytes obtained at day 9 p.i. Virus specific CD8$^+$ T cells were stained with D^b-$NP_{396-404}$, D^b-GP_{33-41} and D^b-$GP_{276-286}$ tetramers or stimulated with the corresponding LCMV peptides to assess IFN-g, TNF-a and IL-2 production. Numbers ± sd of total tetramer$^+$ or cytokine producing CD8$^+$ T cells per spleen are depicted. Results are representative of three independent experiments with three or five mice per group. (WT *vs* dnTGFBRII, *p<0.05, **p<0.005 and ***p<0.0005)

infected with Cl 13 (Figure 12C). At day 5 p.i. IL-10 mRNA levels were similar in WT and dnTGFBRII mice, however by day 9 p.i. IL-10 mRNA levels in dnTGFBRII and WT ARM infected mice were lower compared to higher levels in WT-Cl 13 infected animals (Figure 12C). These findings indicated that attenuation of TGF-β signaling during Cl 13 infection prevented the sustained upregulation of PD-1 on T cells and limited IL-10 production in spleen. Furthermore, similar expression of PD-1 and IL-10 at day 5 p.i. in dnTGFBRII and WT mice suggest that the differences observed between WT and dnTGFBRII mice at later time points after Cl 13 infection were not a direct consequence of attenuated TGF-β signaling in T cells, but instead may result from emerging differences in the infectious environment.

Attenuating TGF-β in T cells results in an increase number of multiple cytokine-producing virus-specific $CD8^+$ T cells

The presence of antigen specific T cells that can produce more than one cytokine correlates with improved viral control and prognosis in chronically infected patients and mice (158-166). To investigate the presence of multi-cytokine production by T cells, we enumerated the number of virus-specific $CD8^+$ T cells that were IFN-γ^+, IFN-γ^+TNF-α^+, IFN-γ^+IL-2^+, and IFN-γ^+TNF-α^+IL-2^+ in WT and dnTGFBRII mice at day 9 p.i. after ex vivo re-stimulation with $NP_{396-404}$, GP_{33-41}, and $GP_{276-286}$ LCMV peptides. Notably, dnTGFBRII mice had a greater number of virus-specific $CD8^+$ T cells that were capable of not only producing one or two, but three cytokines when compared to WT mice (Figure 14). Importantly, dnTGFBRII Cl 13 infected mice had multi-cytokine producing $CD8^+$ T

cells specific for the $NP_{396-404}$ epitope, which were completely eliminated in WT Cl 13 infected mice (Figure 14). These findings indicated that attenuation of TGF-β signaling in T cells during Cl 13 infection resulted in the generation of increased numbers of $CD8^+$ T cells that were multi-producers of antiviral cytokines.

Effective $CD4^+$ T cell responses in dnTGFBRII mice infected with Cl 13

$CD4^+$ T cells provide important helper function to $CD8^+$ T cells during both acute and chronic LCMV infections. Since we observed improved $CD8^+$ T cell functional responses in mice with attenuated TGF-β signaling during Cl 13 infection, we hypothesized that CD4+ T cells could also be benefiting from decreased TGF-β signals. Indeed, when we examined the frequencies and numbers of $CD4^+$ T cells specific for the IA^b-GP_{66-77} LCMV epitope at day 9 p.i. we observed a 2 fold increase in proportion and a 2.5 fold increase in numbers in dnTGFBRII compared to WT Cl 13 infected mice (Figure 15A and 15B). We next compared the ability of $CD4^+$ T cells to produce IFN-γ, TNF-α, and IL-2 upon *ex vivo* stimulation with LCMV GP_{66-77} peptides. dnTGFBRII mice had an increased proportion of $CD4^+$ T cells producing IFN-γ, TNF-α, and IL-2 (Figure 15D). Interestingly, when we enumerated the number of $CD4^+$ T cells producing more than one cytokine, the dnTGFBRII mice had a greater number of these multi-cytokine producers (Figure 15A). Furthermore, when cytokine-producing CD4+ T cells were normalized to the number of IA^b-GP_{66-77} $tetramer^+$ cells in the same spleen, $CD4^+$ T cells from dnTGFBRII mice were more functional on a per cell basis compared to WT Cl 13 infected mice (Figure 15C). These findings

indicated that like in $CD8^+$ T cells with diminished TGF-β signaling, virus-specific $CD4^+$ T cell numbers and functional responses are enhanced in dnTGFBRII mice after Cl 13 infection.

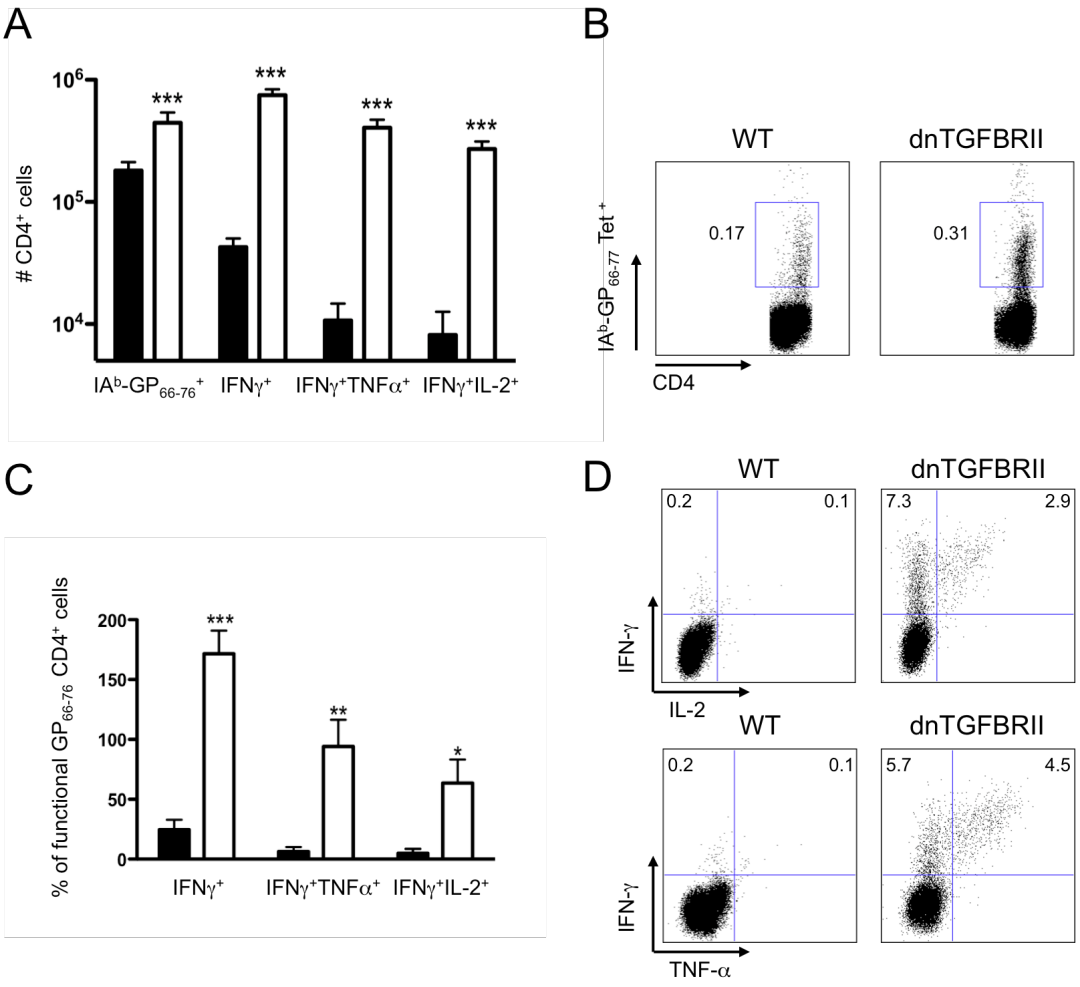

Figure 15. Accumulation and effector function of virus-specific CD4$^+$ T cells during LCMV infection. WT (black bars or black circles) or dnTGFBRII (white bars or white squares) mice were injected with LCMV Cl 13 and splenocytes obtained at day 9 p.i. Virus-specific CD4$^+$ T cells were stained with IA^b-GP$_{66\text{-}77}$ tetramers and B) or stimulated with GP$_{66\text{-}77}$ LCMV peptide to analyze the production of IFN-γ, TNF-α, and IL-2. (A) Average number ± SD of total IA^b-GP$_{66\text{-}77}^+$ or cytokine-producing CD4$^+$ cells per spleen. (B and D) Dot plots with one representative mouse per group; numbers denote the percent cells within each gate. (C) Mean percentages ± SD of cytokine-producing CD4$^+$ T cells normalized to the number of IA^b-GP$_{66\text{-}77}$ tetramer$^+$ cells in the same spleen. Results representative of three independent experiments with three to four mice per group. *p < 0.05, **P < 0.005, and ***p < 0.0005.

Functional T cell responses in dnTGBRII results in accelerated clearance of persistent LCMV Cl 13

We next investigated whether the enhanced T cell responses observed in dnTFBRII mice were sufficient to control Cl 13 replication. We determined titers in the blood of WT or dnTGFBRII mice at days 8, 15, 45 and 49 p.i. In contrast to WT Cl 13 infected mice that had higher sustained viremia at all points examined and continued to have virus until day 49 p.i., dnTGFBRII mice controlled the infection and had no detectable virus by day 15 p.i. (Figure 16A). Furthermore, we monitored viral loads in liver, brain, and lung at day 15 p.i. (Figure 16B). WT Cl 13 infected mice had very high viral burden, ~10^7 PFU/g whereas dnTGFBRII mice had completed cleared the virus in liver and brain and either cleared or were in the process of clearing the virus in lung (Figure 16B). These findings show that by attenuating TGF-β signaling on virus-specific T cells in vivo, a previously persistent virus is now controlled and cleared in these animals.

It may be possible that in dnTGFBRII mice, the early Cl 13 viral replication does not reach the levels observed in WT Cl 13 infected mice, thereby facilitating viral clearance in dnTGFBRII mice. To examine this possibility, we quantified viral titers in spleens of WT and dnTGFBRII mice at early time points, of days 1 and, 3 p.i., along with days 5 and day 9 p.i. Interestingly, early during infection, WT and dnTGFBRII mice had viral titers that were indistinguishable up until day 5 p.i. (Figure 17A). By day 9 p.i. however, spleens in dnTGFBRII mice had significantly reduced viral titers, whereas spleens from WT mice continued to

have higher viral loads, and these levels were even higher than those observed at day 5 p.i. (Figure 17A).

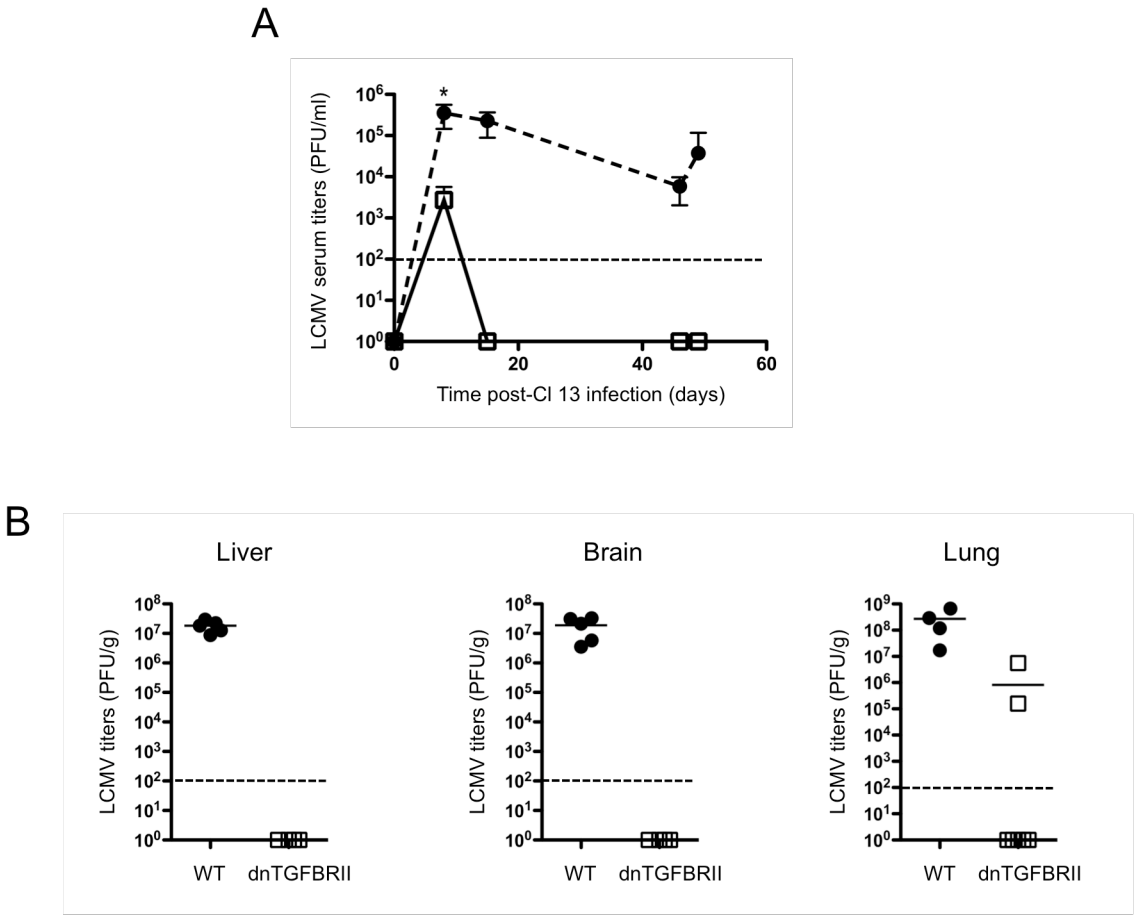

Figure 16. Viral titers in WT and dnTGFBRII mice. WT (black circles or bars) or dnTGFBRII (white squares or bars) mice were infected with LCMV Cl 13. (A) Virus titers were determined by plaque assay in blood at the inicated time points. (B) The average viral titers in liver, brain, and lung at day 15 p.i. are indicated by the lines and individual mice represented by each symbol. WT vs dnTGFBRII as indicated; *$p < 0.05$.

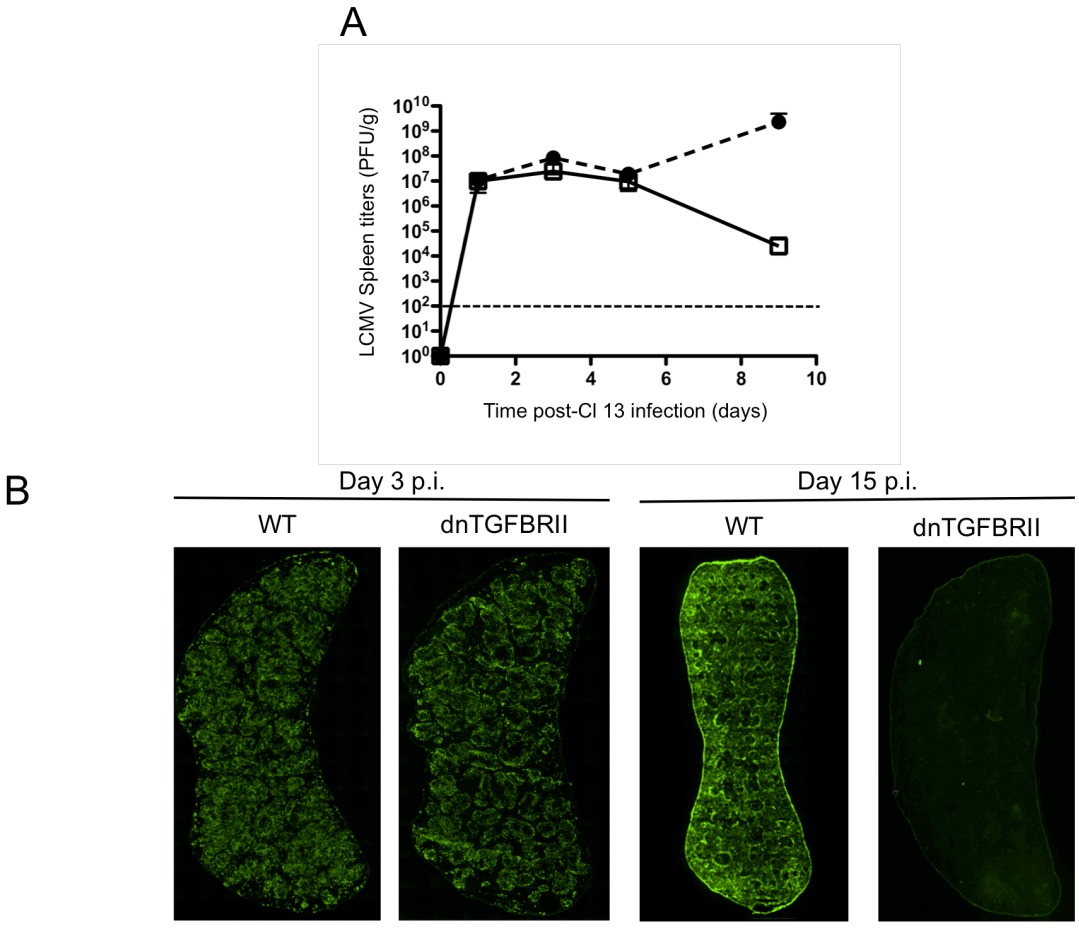

Figure 17. Viral titers in spleens of WT and dnTGFBRII mice. WT (black circles) or dnTGFBRII (white squares) mice were infected with LCMV Cl 13. (A) Virus titers were determined in spleen at the indicated time points and average LCMV titers ± SD are shown. (B) Spleens sections from WT or dnTGFBRII at day 3 or 15 p.i. were stained with anti-LCMV Ab. Panoramic spleen images are 10X magnified.

Furthermore, spleens were isolated from WT and dnTGFBRII Cl 13 infected mice at days 3 and 15 p.i. and sectioned for immunohisto-chemistry using polyclonal anti-LCMV IgG. We observed that at day 3 p.i. virus was clearly detected in both WT and dnTGFBRII spleens, however by day 15 p.i. staining for LCMV was only detected in spleens isolated from WT mice (Figure 17B). These findings show that the environment in dnTGFBRII mice did not pre-dispose these mice to control Cl 13 replication early after infection.

$CD8^+$ T cells are essential while $CD4^+$ T cells are dispensable for viral clearance in dnTGFBRII mice

$CD8^+$ T cells are required for clearance of ARM virus in WT mice, while $CD4^+$ T cells are not, but in the absence of helper function, optimal $CD8^+$ T cell memory is not attained after acute infection (167). In chronic LCMV infection, $CD8^+$ T cells are not able to control Cl 13 infection, but eventually by day 60-90 WT Cl 13 infected mice control viremia. In the absence of $CD4^+$ T cells however, $CD8^+$ T cells are more dysfunctional and Cl 13 persists indefinitely, indicating an important role of $CD4^+$ T cell help during Cl 13 infection (14, 168-175). Since dnTGFBRII mice are able to control Cl 13 infection (Figure 16), we wanted to investigate the contribution of $CD4^+$ and $CD8^+$ T cells in controlling viral replication in these animals. We infected WT and dnTGFBRII mice with Cl 13 and treated dnTGFBRII mice with CD8 depleting antibody or isotype control (Figure 18A), or CD4 depleting antibody or isotype control (Figure 18B) and examined viremia at day 12 p.i. In the absence of $CD8^+$ T cells, dnTGFBRII mice were unable to reduce Cl 13 serum levels and viremia is similar to WT Cl 13

infected mice (Figure 18A). In contrast, when dnTGFBRII mice were depleted of $CD4^+$ T cells, viremia is reduced or cleared compared to WT Cl 13 infected animals (Figure 18B). These findings indicate that while $CD8^+$ T cells are essential and required to eliminate Cl 13 in dnTGFBRII mice, $CD4^+$ T cells are dispensable. In the absence of $CD4^+$ T cell help, dnTGFBRII Cl 13 infected mice are able to prevent an otherwise persistent infection, albeit with a delay compared to CD4 T cell sufficient mice.

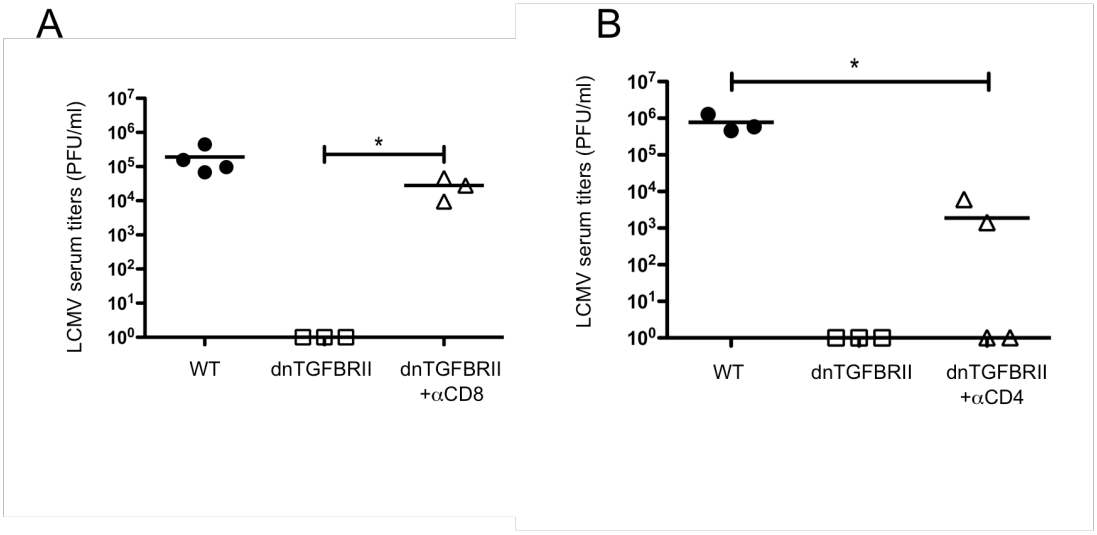

Figure 18. Serum viral titers in WT and dnTGFBRII mice after $CD8^+$ or $CD4^+$ T cell depletion. WT (black circles), dnTGFBRII (white squares) and dnTGFBRII T cell depleted (white triangles) mice were infected with LCMV Cl 13. (A) dnTGFBRII mice were injected with depleting anti-CD8 at day -2, -1, 0, 5 p.i. and virus titers in blood determined at day 12 p.i. (B) dnTGFBRII mice were injected with depleting anti-CD4 at day 0, 2.5, 9 p.i. and virus titers in blood determined at day 12 p.i. Data are representative of two or three independent experiments with three to four mice each. dnTGFBRII vs dn-TGFBRII anti-CD8, or WT vs dnTGFBRII anti-CD4; *$p < 0.05$.

dnTGFBRII mice develop functional memory $CD8^+$ T cells after Cl 13 infection

The hallmark of adaptive immunity is the development of an effective memory pool of T and B cells that protect the host from re-infection with the same pathogen. We detected LCMV-specific $CD8^+$ T cells in dnTGFBRII mice long after viral clearance (Figure 4A) suggesting that early control of LCMV Cl 13 enabled the generation of long-lived virus-specific $CD8^+$ T cells. To examine the presence and function of these long-lived virus-specific $CD8^+$ T cells, we obtained splenocytes from WT and dnTGFBRII mice at day 49 p.i. (long after virus have been eradicated from dnTGFBRII mice). Cells were re-stimulated with $NP_{396-404}$, GP_{33-41}, and $GP_{276-286}$ peptides and examined $CD8^+$ T cells for production of IFN-γ and TNF-α (Figure 19). The proportion of $CD8^+$ T cells producing IFN-γ and TNF-α was substantially elevated in dnTGFBRII compared to WT mice (Figure 19B). Furthermore, dnTGFBRII mice had an increased percentage of $CD8^+$ T cells IFN-γ^+TNF-α^+ double producers (Figure 19A and 19B). We normalized the number of $NP_{396-404}$, GP_{33-41}, and $GP_{276-286}$ $tetramer^+$ cells to $CD8^+$ T cells producing IFN-γ^+, TNF-α^+, and IFN-γ^+TNF-α^+ and found that dnTGFBRII mice had a higher percentage of functional virus-specific $CD8^+$ T cells than WT mice (Figure 19A). These findings indicated that long-lived CD8+ T cells generated in dnTGFBRII mice have functional effector cytokine production upon re-stimulation with LCMV peptides.

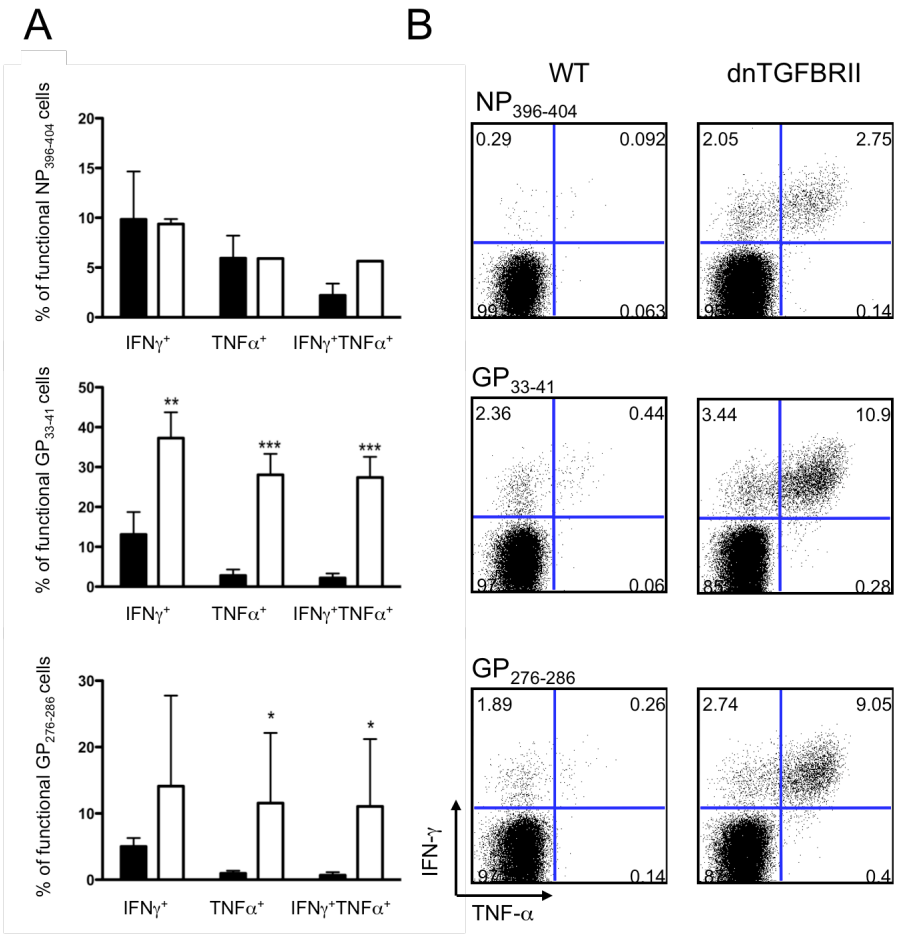

Figure 19. Memory CD8$^+$ T cell response in CI 13-infected dnTGFBRII mice. WT (black bars) or dnTGFBRII (white bars) (A) Splenocytes were obtained at day 49 p.i. and stimulated with $NP_{396-404}$, GP_{33-41}, and/or $GP_{276-286}$ LCMV peptides, and production of IFN-γ and TNF-α by CD8$^+$ T cells was analyzed. Bar graphs depict average percent ± SD of cytokine-producing CD8$^+$ T cells normalized to the number of corresponding tetramer$^+$ cells in the same spleen. (B) Dot plots display one representative mouse per group; numbers indicate the percent of cells within the respective gate. WT vs dnTGFBRII; *p < 0.05, **p < 0.005, ***p < 0.0005.

$CD8^+$ T cells undergo homeostatic proliferation and their survival is dependent on IL-7 signaling. Memory $CD8^+$ T cells in the periphery express high levels of CD127, or the IL-7 receptor (7). We investigated the level of IL-7R expression on GP_{33-41} and $GP_{276-286}$ $CD8^+$ T cells in blood from WT Cl 13, dnTGFBRII Cl 13, and WT ARM infected mice 2 months p.i. (Figure 20A and 20B). GP_{33-41} and $GP_{276-286}$ $CD8^+$ T cells from dnTGFBRII mice had significantly higher levels of CD127 expression compared to WT mice; in fact CD127 levels were similar to WT ARM infected mice (Figure 20A and 20B). Furthemore, virus-specific $CD8^+$ T cells in dnTGFBRII mice also expressed higher levels of CD122 (IL-2Rβ), and Ly6C memory markers (Figure 21A and 21B). Long-lived memory virus-specific $CD8^+$ T cells in dnTGFBRII mice expressed similar levels of memory markers as virus-specific CD8+ T cells from acutely infected mice.

We next examined a primary and secondary (re-challenge) Cl 13 infection in dnTGFBRII mice. dnTGFBRII mice were infected with LCMV Cl 13 and viral titers determined in blood and liver at day 5 p.i. Primary infection led to significant viral load in the blood (~10^5 PFU/mL) and liver (~10^7 PFU/g). In contrast, dnTGFBRII mice receiving the secondary infection had no viremia and viral levels in the liver were reduced to near the limit of detection at day 5 p.i. (Figure 20C). Together, these findings indicated that an effective anti-viral immunity developed in dnTGFBRII mice after Cl 13 infection. Furthermore, virus-specific $CD8^+$ T cells express high levels of $CD8^+$ T cell memory markers and these mice are protected from a secondary infection with the same virus. It is

also possible that antibodies could be effectively generated and it would be interesting to examine their contribution by analyzing neutralizing antibodies in serum of WT and dnTGFBRII mice at later time points (i.e. day 60 p.i.) when both WT and dnTGFBRII have cleared viremia.

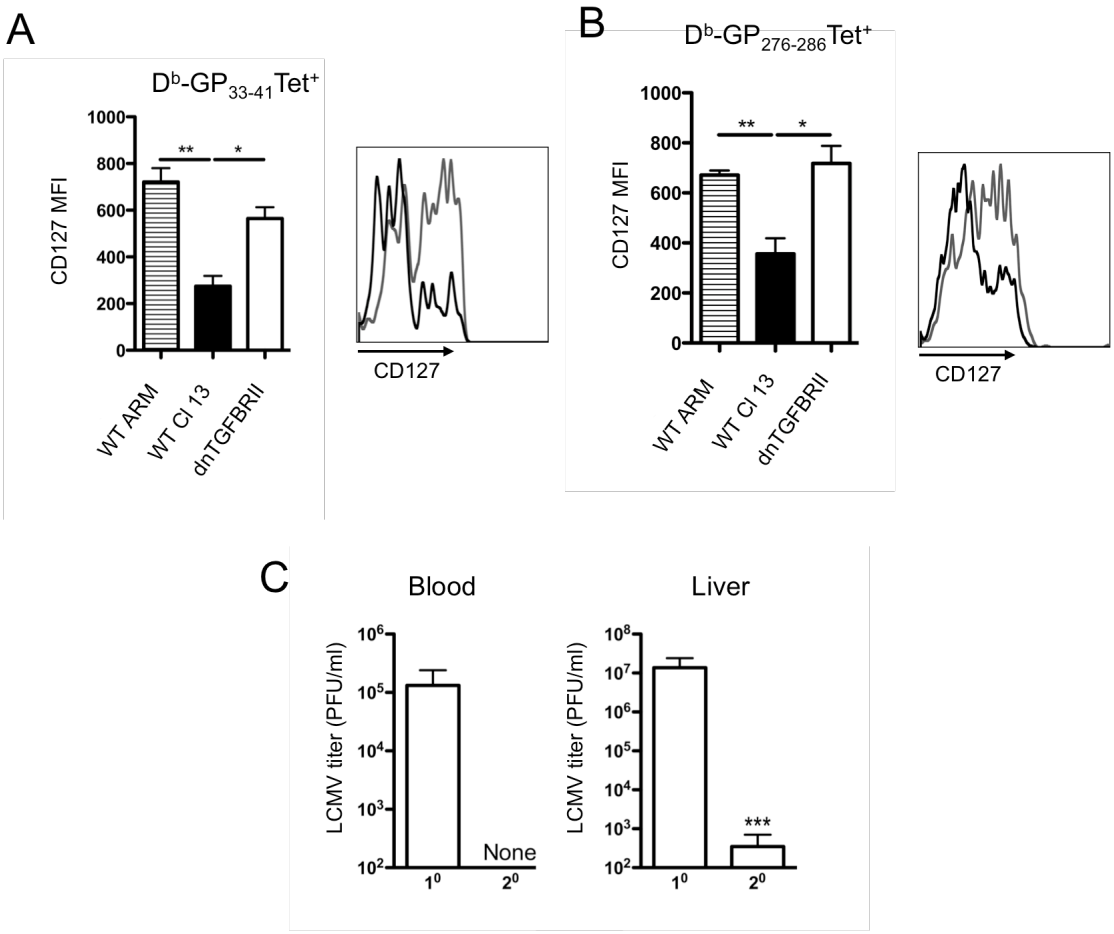

Figure 20. Memory CD8$^+$ T cell re-challenge response in Cl 13-infected dnTGFBRII mice. WT (black bars or histograms) or dnTGFBRII (white bars or histograms). CD127 expression was quantified in (A) D^b-$GP_{33-41}{}^+CD8^+$ and (B) D^b-$GP_{276-286}{}^+CD8^+$ blood cells 2 months p.i. Bar graphs indicate the average CD127 MFI ± SD. ARM-infected mice were processed as controls (striped bars). Histograms depict one representative mouse per group. (C) dnTGFBRII mice were rechallenged with LCMV Cl 13 (secondary response) and processed in parallel to primary-infected dnTGFBRII mice. Average viral titers in blood and liver at day 5 p.i. ± SD are shown. Results are representative of two experiments with three to five mice per group. WT vs dnTGFBRII; *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

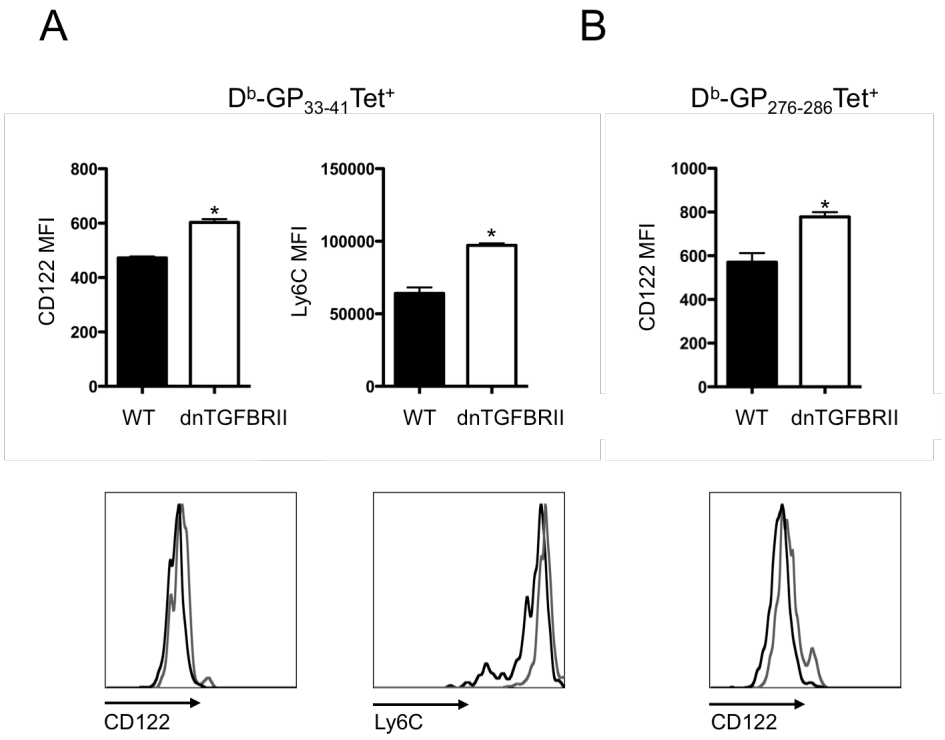

Figure 21. Memory markers in virus specific CD8$^+$ T cells from dnTGFBRII mice. WT (black bars or histograms) or dnTGFBRII (white bars or grey histograms) mice were infected with LCMV Cl 13. CD122 and Ly6C expression were determined where indicated in D^b-GP$_{33-41}$$^+$CD8$^+$ (A) and D^b-GP$_{276-286}$$^+$CD8$^+$ (B) blood cells after 2 months p.i. Bar graphs indicate the average MFI ±sd. Histograms depict a representative mouse per group. Results are representative of two experiments with three to four mice per group. (WT vs dnTGFBRII, *p<0.05)

Immunopathology was not enhanced in dnTGFBRII mice during Cl 13 infection

Exuberant immune responses can be beneficial for pathogen clearance but detrimental to vital self-tissues and may eventually kill the host. To investigate whether TGF-β attenuation in T cells could increase or accelerate tissue damage during Cl 13 infection, we compared histological sections of liver, lung, and stomach of WT and dnTGFBRII mice at day 10 p.i. (Figure 22). We observed that after Cl 13 infection, mononuclear cell infiltration was increased in all organs of both WT and dnTGFBRII mice compared to tissues from uninfected mice (Figure 22). In agreement with the more potent T cell responses in dnTGFBRII mice, the cell infiltrate in these organs was larger than in WT infected mice. Despite this increased infiltration, there was no enhanced tissue damage observed in liver, lung, or stomach of infected dnTGFBRII mice compared to WT. Fewer vacuolated hepatocytes were observed in Cl 13-infected dnTGFBRII than WT mice, likely due to the reduced viral burden in dnTGFBRII mice. Furthermore, there was a 100% survival rate of dnTGFBRII mice after Cl 13 infection up to 66 days p.i. We performed a biochemical analysis of plasma from WT and dnTGFBRII mice at day 10 p.i. and found that levels of plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), glucose, and blood urea nitrogen (BUN) were similar (Figure 23). These results indicate that despite the enhanced T cell responses observed in dnTGFBRII mice, there is no increased tissue damage and in addition, these mice survived long after clearing the infection.

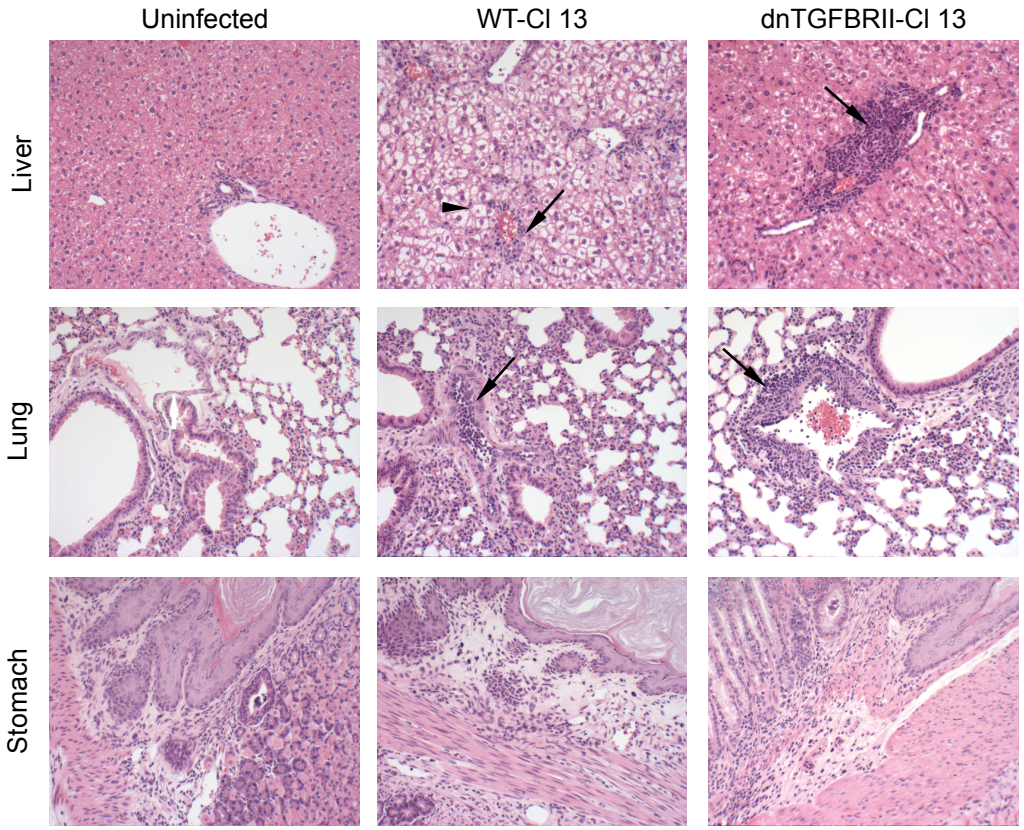

Figure 22. Histology of Liver, Lung and Stomach during virus infection of dnTGFBRII mice. Mice at ~7 weeks of age were infected with LCMV Cl 13 and tissues obtained at day 10 p.i. Organs were processed and stained for histopathological analysis. Long arrows point towards mononuclear cell infiltrates, short arrows point to vacuolated hepatocytes. 200x magnification is shown.

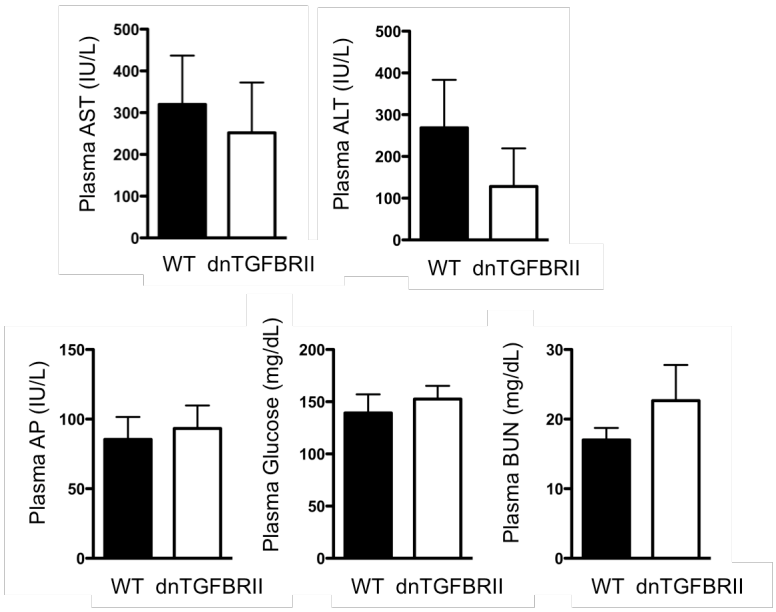

Figure 23. Biochemical parameters in plasma after LCMV infection. WT (black bars) or dnTGFBRII (white bars) mice were infected with LCMV Cl 13. Plasma amounts of the indicated molecules were determined at day 10 p.i. Results are representative of two or three independent experiments with three or five mice per group.

Cell-intrinsic TGF-β signaling was required for virus-specific CD8$^+$ T cell deletion

We observed an increased survival of virus-specific CD8$^+$ T cells with attenuated TGF-β signaling. We next wanted to understand whether this survival effect was due to the environment or whether this TGF-β-mediated effect was directly within T cells. To discriminate between cell-intrinsic versus extrinsic TGF-β effects on virus-specific CD8$^+$ T cells, we undertook two approaches. We first co-transferred WT (CD45.1$^+$CD45.2$^+$) or dnTGFBRII (CD45.2$^+$) P14-TCR transgenic CD8$^+$ T cells into CD45.1$^+$ WT hosts, infected these mice with Cl 13 one day after transfer, and examined proliferation by BrdU incorporation (Figure 24A) and survival by Annexin V staining (Figure 24B). Interestingly, we detected similar extents of proliferation, but lower Annexin V binding in P14-dnTGFBRII versus P14-WT CD8$^+$ T cells. One caveat of transferring P14-dnTGFBRII cells into WT hosts is that the dnTGFBRII transgene is of human origin, suggesting the possibility of rejection of P14 cells expressing the dnTGFBRII transgene. To control for this possibility, we generated WT and dnTGFBRII mixed bone marrow chimeric mice that are tolerant towards the presence of the dnTGFBRII transgene. We first examined the CD8$^+$ T cell chimerism in the WT and dnTGFBRII compartment in blood before infection and selected mice with a near 1:1 ratio for infection studies (Figure 25A). We infected these mice and examined the expansion of D^b-$NP_{396-404}$, D^b-GP_{33-41}, and D^b-$GP_{276-286}$ in each compartment at day 8 p.i. (Figure 25A), and observed that virus-specific CD8$^+$ T cells were significantly enriched in the dnTGFBRII compartment. As in the co-

transfer experiments, we observed this increase to be a result of enhanced survival and not proliferation. Moreover, the number of virus-specific $CD8^+$ T cells in chimeric mice were about 10 times lower than non-irradiated dnTGFBRII mice after Cl 13 infection (Figure 25B). These findings indicated that intrinsic TGF-β signaling in virus-specific $CD8^+$ T cells actively induced death of these cells while not altering their proliferation.

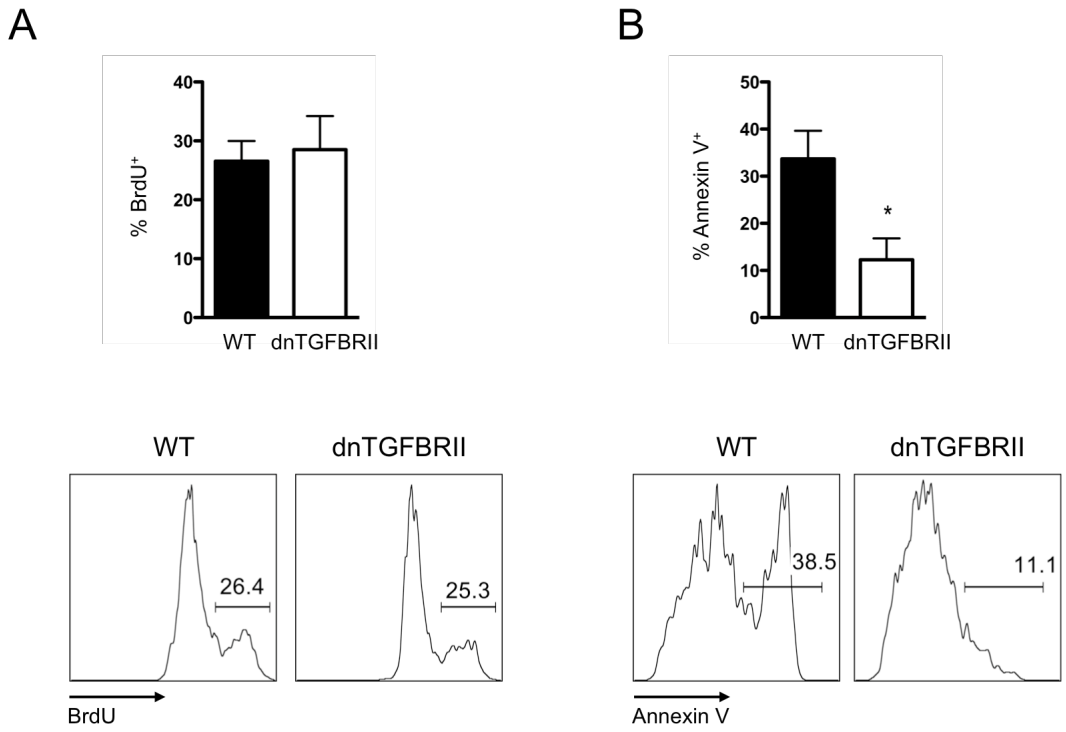

Figure 24. Intrinsic and extrinsic TGF-β effect on virus-specific CD8$^+$ T cells in transferred cells. (A and B) P14-WT (black bars) or P14-dnTGFBRII (white bars) CD8$^+$ T cells were co-transferred into WT mice 1 day before LCMV Cl 13 infection. BrdU incorporation (A) and Annexin V staining (B) of P14 cells were determined at day 8 p.i. Bar graphs depict the average frequence of positive cells ± SD. Histograms display a representative mouse and numbers indicate the frequency of cells within regions. Results are representative of two or three independent experiments with three or five mice per group with 4-8 mice per group. *$p < 0.05$.

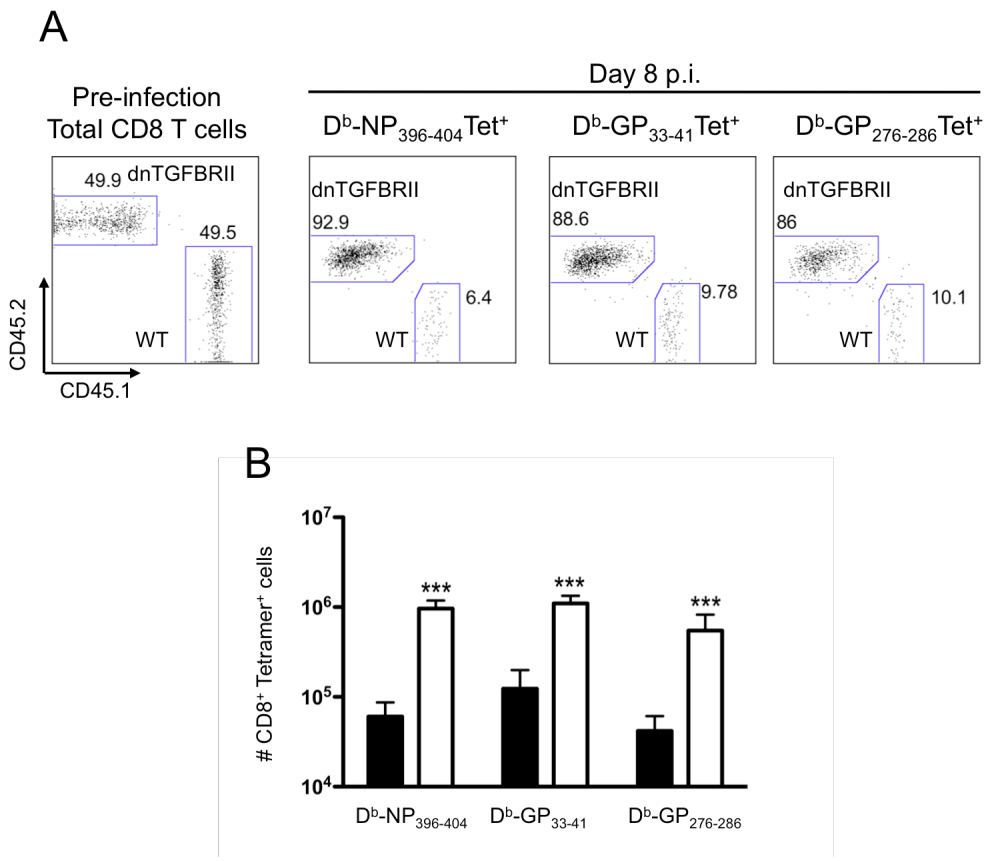

Figure 25. Intrinsic and extrinsic TGF-β effect on virus-specific CD8$^+$ T cells in chimeras. WT-dnTGFBRII mixed bone marrow chimeras were processed to analyze CD45.1$^+$ WT and CD45.2$^+$ dnTGFBRII CD8$^+$ T cells before (pre-infection) and at day 8 after Cl 13 infection. (A) Total CD8$^+$ T cells in blood before infection and within spleen D^b-NP$_{396-404}$ and D^b-GP$_{33-41}$ tetramer$^+$ cells at day 8 p.i. (B) Total numbers of tetramer$^+$CD8$^+$ cells per spleen at day 8 p.i. Results are representative of two or three independent experiments with three or five mice per group with 4-8 mice per group. *$p < 0.05$ ***$p < 0.0005$.

Virus-specific $CD8^+$ T cell functional exhaustion during Cl 13 infection is not dependent on intrinsic TGF-β signaling

Intrinsic TGF-β signaling induced apoptosis of virus-specific $CD8^+$ T cells during Cl 13 infection, we next asked whether intrinsic TGF-β signals were contributing to exhaustion of virus-specific $CD8^+$ T cells. We examined the ability of $CD8^+$ T cells in WT and dnTGFBRII mixed bone marrow chimeras to produce effector cytokines IFN-γ and TNF-α after ex vivo GP_{33-41} and $GP_{276-286}$ peptide stimulation at day 8 p.i. (Figure 26A). We observed an identically compromised production IFN-γ in WT and dnTGFBRII compartments as well as an inability of $CD8^+$ T cells to produce TNF-α. Furthermore, PD-1 levels on D^b-GP_{33-41} $tetramer^+$ cells in WT and dnTGFBRII mixed bone marrow chimeras was similar (Figure 26B). Consistent with the lower number of dysfunctional virus-specific $CD8^+$ T cells in chimeras compared to non-irradiated dnTGFBRII mice, viremia at day 9 p.i. ($7.2 \times 10^5 \pm 2.0 \times 10^5$ PFU/mL; n= 8 mice) was identical to non-irradiated WT mice. Our findings indicated that while intrinsic TGF-β signaling was responsible for virus-specific $CD8^+$ T cell apoptosis, it was dispensable for virus-specific $CD8^+$ T cell functional exhaustion and sustained PD-1 upregulation.

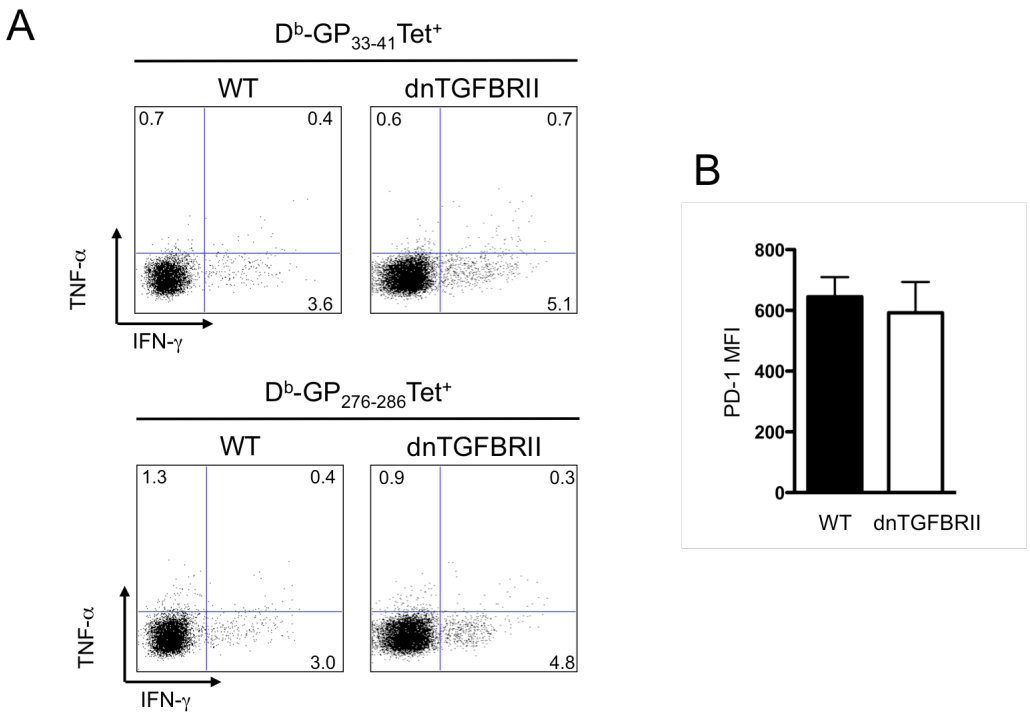

Figure 26. Intrinsic and extrinsic virus-specific CD8$^+$ T cell effector function. WT-dnTGFBRII mixed bone marrow chimeras were processed to analyze CD45.1$^+$ WT and CD45.2$^+$ dnTGFBRII CD8$^+$ T cells at day 8 after Cl 13 infection. (A) Production of IFN-γ and TNF-α after GP_{33-41} and $GP_{276-286}$ LCMV peptide stimulation at day 8 p.i. (B) PD-1 expression within D^b-GP_{33-41} tetramer$^+$ cells at day 8 p.i. Dot plots display a representative mouse and numbers indicate the frequencey of cells within each region. Results are representative of two or three independent experiments with three or five mice per group with 4-8 mice per group.

Activation status in dnTGFBRII mice does not impact the enrichment observed in dnTGFBRII after Cl 13 infection

T cells are spontaneously activated in dnTGFBRII mice (101). In addition, P14-dnTGFBRII also displayed an activated phenotype (Figure 29E) and most mixed bone marrow chimeras also had an activated phenotype in the dnTGFBRII T cells compared to WT counterparts. It is possible that this difference in activation in WT and dnTGFBRII $CD8^+$ T cells can result in a different baseline between these cells and impact the enrichment observed in the dnTGFBRII compartment in mixed bone marrow chimeras after infection. While most WT:dnTGFBRII mixed chimeras showed enhanced activation in the dnTGFBRII T cells compared to their WT counterparts, a few of these mice showed that T cells from WT and dnTGFBRII compartments in the same chimera had a similar level of activation (Figure 27A). We examined the chimerism in $CD8^+$ T cells in these groups of chimeras and observed more WT than dnTGFBRII $CD8^+$ T cells in blood before infection (Figure 27B). Interestingly, when we infected these mice and examined virus-specific $CD8^+$ T cell WT:dnTGFBRII ratio at day 8 p.i. by gating in D^b-GP_{33-41} and D^b-$GP_{276-286}$ $tetramer^+$ cells, we again observed a preferential enrichment of virus-specific $CD8^+$ T cells from the dnTGFBRII compartment (Figure 27C). These findings indicated that in the absence of disparate activation of dnTGFBRII $CD8^+$ T cells compared to WT before infection, virus-specific $CD8^+$ T cells in the dnTGFBRII compartment continue to be enriched after Cl 13 infection compared to WT.

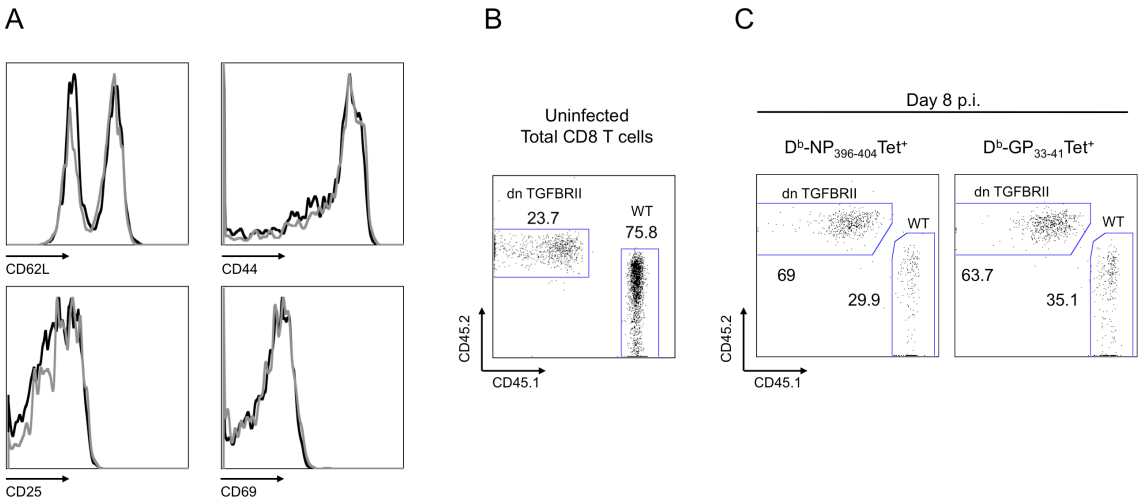

Figure 27. Dissociation among pre-infection activation status and post-infection enrichment of dnTGFBRII CD8$^+$ T cells. Blood samples from 6-week mixed-bone marrow chimeras were processed to analyze expression of CD62L, CD44, CD25 and CD69 activation markers (A) as well as CD45.1 and CD45.2 (B) in total CD8 T cells from CD45.1 WT (black histogram) or CD45.2 dnTGFBRII (grey histogram). C) Chimeras were infected with LCMV Cl 13 and spleens processed to determine the percentages of WT and dnTGFBRII cells within D^b-NP $_{396-404}$ and D^b-GP_{33-41} tetramer$^+$ cells at day 8 p.i. Histograms and dot plots display a representative mouse and numbers indicate the frequency of cells within regions. Results are representative of two mice per group.

$CD8^+$ T cells in dnTGFBRII mice display increased proliferation and death before Cl 13 infection

As dnTGFBRII mice have a more activated phenotype, we next investigated the effect of this environment on resting $CD8^+$ T cells in uninfected mice. We first examined proliferation by BrdU incorporation and survival by Annexin V staining of total $CD8^+$ T cells in WT and dnTGFBRII mice (Figure 28A). We observed that $CD8^+$ T cells in dnTGFBRII mice had increased proliferation compared to WT; this is in agreement with previous reports in uninfected mice (101, 176). Interestingly, Annexin V levels were also higher in $CD8^+$ T cells in dnTGFBRII mice indicating enhanced apoptosis in these cells (Figure 28B). We then stimulated splenocytes with PMA and ionomycin and examined production of IFN-γ, TNF-α and IL-2 (Figure 28 C and D). We observed that $CD8^+$ T cells from dnTGFBRII mice produced significantly higher levels of IFN-γ compared to WT, however, TNF-α and IL-2 levels were similar between these two groups. We next examined PD-1 levels on total $CD8^+$ T cells and observed higher PD-1 levels in dnTGFBRII compared to WT (Figure 28E). Consistent with previous studies, $CD8^+$ T cells from dnTGFBRII mice had a more activated phenotype; expressing higher levels of CD44 and CD25, lower levels of CD62L, and slight increase in CD69 levels (Figure 28F). These findings show that before infection, TGF-β signaling in T cells prevented proliferation while it enhanced the survival of $CD8^+$ T cells. Furthermore, TGF-β signaling prevented $CD8^+$ T cells activation markers, upregulation of PD-1 expression and increased IFN-γ production after T cell stimulation.

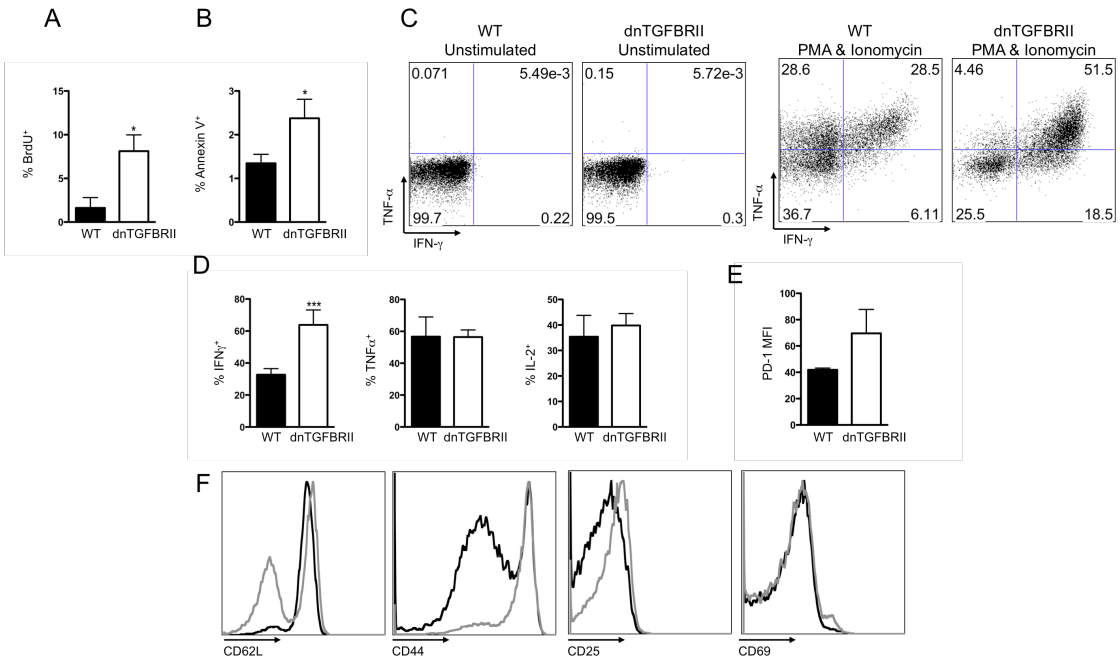

Figure 28. Pre-infection profile of total CD8$^+$ T cells from dnTGFBRII. Splenocytes were obtained from WT (black bars and black histograms) or dnTGFBRII (white bars and grey histograms) mice at ~7 weeks of age. Total CD8$^+$ T cells were analyzed by FACS to determine BrdU incorporation (A), Annexin V staining (B) Cytokine production with and without PMA-ionomycin stimulation (C and D), expression of PD-1 (E) and CD62L, CD44, CD25 and CD69 (F). Bar graphs depict the average frequency of mice per group ± sd. Histograms and dot plots display a representative mouse per group and numbers indicate the frequency of cells within regions. Results are representative of two independent experiments with four mice per group. (WT *vs* dnTGFBRII, *p<0.05 and **p<0.005)

We next investigated whether P14-dnTGFBRII $CD8^+$ T cells in uninfected mice displayed a similar phenotype as that observed in uninfected dnTGFBRII mice. We isolated splenocytes from P14-dnTGFBRII or P14-WT 7 week-old mice and stained $CD8^+$ T cells with D^b-GP_{33-41} tetramers and examined proliferation by BrdU incoporation (Figure 29A) and survival by Annexin V staining (Figure 29B). We observed that P14-dnTGFBRII tetramer$^+$ cells had increased proliferation and death compared to P14-WT cells. We next stimulated splenocytes from these mice with GP_{33-41} peptides and examined cytokine production (Figure 29 C and D). P14-dnTGFBRII $CD8^+$ T cells produced more IFN-γ, slightly less TNF-α and similar IL-2 levels compared to P14-WT. In addition, P14-dnTGFBRII tetramer$^+$ cells had higher levels of CD44 and lower CD62L levels compared to P14-WT tetramer$^+$ cells (Figure 29E), indicating a more activated state in P14-dnTGFBRII cells. These findings show that even in conditions where the T cell receptor is exclusively expressed to recognize a non-self antigen, P14 $CD8^+$ T cells expressing the dnTGFBRII transgene had increased proliferation and death, had an activated phenotype and produced higher levels of IFN-γ after peptide stimulation.

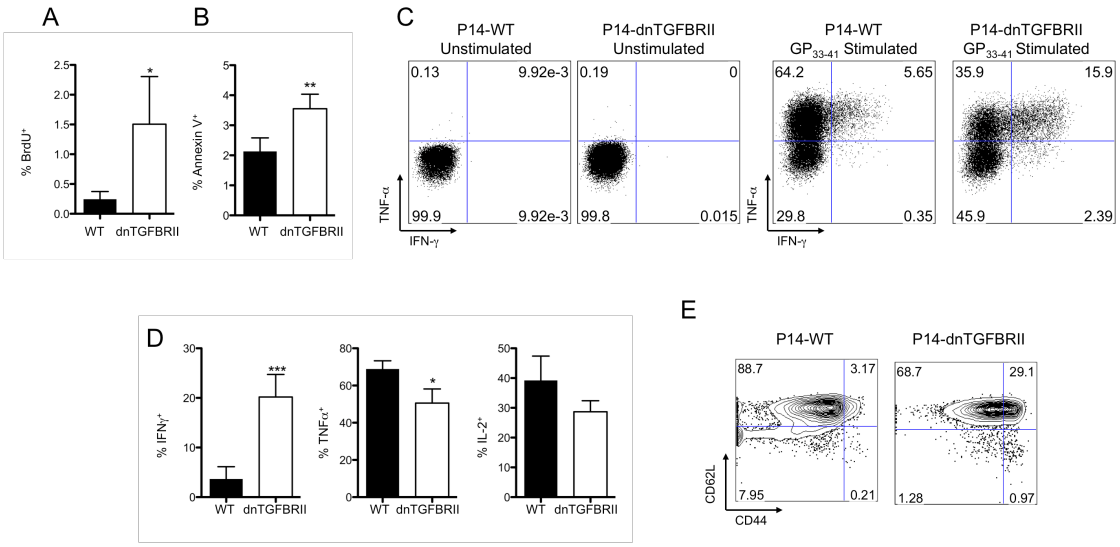

Figure 29. Pre-infection profile of P14$^+$ CD8$^+$ T cells from WT and dnTGFBRII. Splenocytes were obtained from P14 Tg WT (black bars) or P14 Tg dnTGFBRII (white bars) mice at ~7 weeks of age. Splenocytes were stained with H2-D^b-GP$_{33-41}$ tetramers, CD8$^+$ T cells were gated on tetramer$^+$ cells and analyzed by FACS to determine BrdU incorporation (A), Annexin V staining (B) Cytokine production after GP$_{33-41}$ peptide stimulation (C and D), expression CD62L and CD44 (E). Bar graphs depict the average frequency of mice per group ± sd. Dot plots display a representative mouse per group and numbers indicate the frequency of cells within regions. Results are representative of two independent experiments with four mice per group. (WT *vs* dnTGFBRII, *p < 0.05, **p < 0.005 and ***p<0.0005)

Virus-specific CD8$^+$ T cell responses in the absence of T cell-derived TGF-β

Our data indicated that total CD4$^+$ and CD8$^+$ T cells were important sources of TGF-β during Cl 13 infection (Figure 1A and C). In addition, a study observed a correlation with plasma TGF-β levels and the expansion of OT-I CD8$^+$ T cells during *Listeria monocytogenes*-OVA infection (177). We hypothesized that T cell-derived TGF-β could potentially be critical in suppressing CD8$^+$ T cell responses during chronic infection. To test this hypothesis, we bred mice that have one copy of the TGF-β gene flanked by loxp sites (147) to CD4Cre mice and generated TGF-β$^{fl/n}$ x CD4Cre$^+$ mice. These mice have one copy of the TGF-β gene deleted in CD4$^+$ and CD8$^+$ T cells, while the other copy of the TGF-β gene has a GFP insertion and renders this copy non-functional, TGF-β$^{fl/fl}$ x CD4Cre$^-$ were used as WT controls. We infected these mice with Cl 13 and analyzed proportions of D^b-GP$_{33-41}$ and D^b-NP$_{396-404}$ in blood at day 7 p.i. (Figure 30 A and B). We observed an increase percentage of virus-specific CD8$^+$ T cells in blood of mice lacking T cell-derived TGF-β at this time point. To determine whether this increased proportion of virus-specific T cells was also observed at later time points, we isolated splenocytes from these mice at day 9 p.i. and stained with D^b-GP$_{33-41}$, D^b-NP$_{396-404}$, and IA^b-GP$_{66-77}$ tetramers (Figure 31A-C). We observed that the number of CD8$^+$ and CD4$^+$ virus-specific T cells were identical in both TGF-β$^{fl/n}$ x CD4Cre$^+$ and WT mice at this time point. Since TGF-β is involved in Treg development and maintenance (119), we examined the number of CD4$^+$Foxp3$^+$ cells at day 9 p.i. (Figure 31D). We did not observe a difference in Treg numbers in TGF-β$^{fl/n}$ x CD4Cre$^+$ and WT mice. Consistent with

similar numbers of virus-specific $CD8^+$ T cells in these mice, proliferation of D^b-GP_{33-41} $tetramer^+$ $CD8^+$ T cells was similar (Figure 32A) as well as the survival of these cells (Figure 32B).

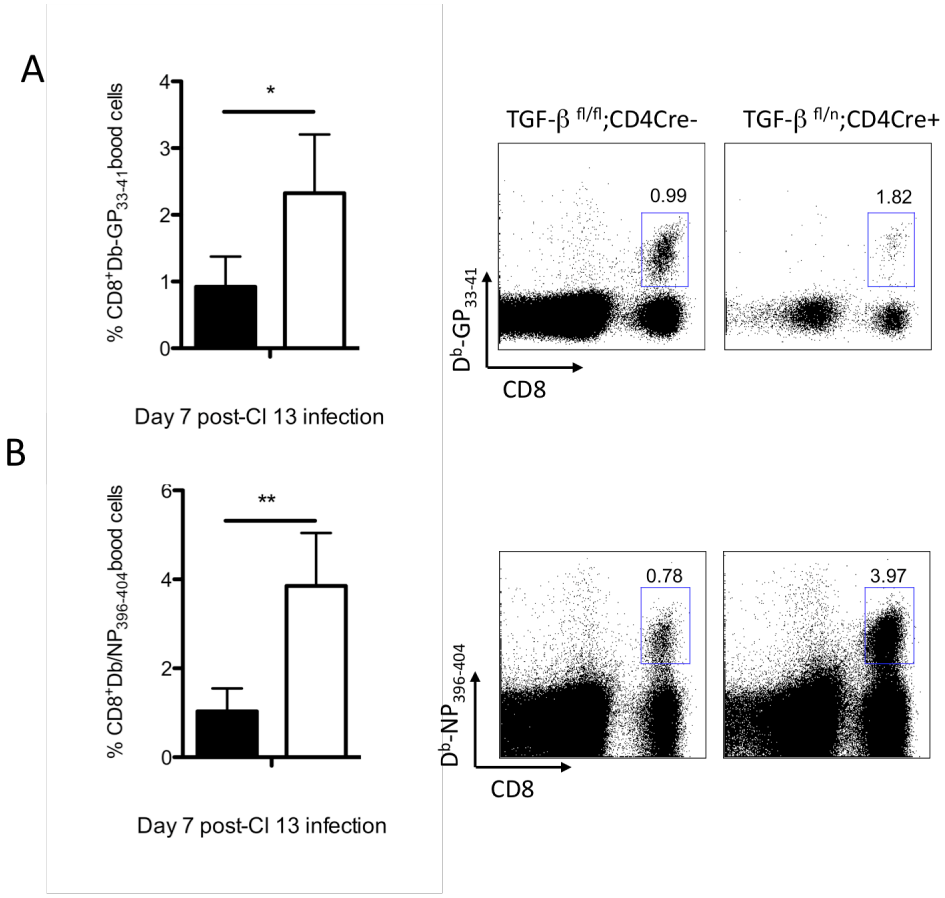

Figure 30. Virus-specific CD8$^+$ T cells in T cell-specific TGF-β deficient mice. WT (black bars) or TGF-β $^{fl/n}$ x CD4Cre$^+$ (white bars) mice were infected with LCMV Cl 13 and blood isolated at day 7 p.i. and stained with H2-D^b-GP$_{33-41}$ (A) and H-2-D^b-NP$_{396-404}$ tetramers (B). Bar graphs depict the average frequency of mice per group ± sd. Dot plots display a representative mouse per group and numbers indicate the frequency of cells within regions. Results are representative of two independent experiments with four mice per group. (WT vs TGF-β $^{fl/fl}$;CD4Cre+, *p < 0.05, **p < 0.005)

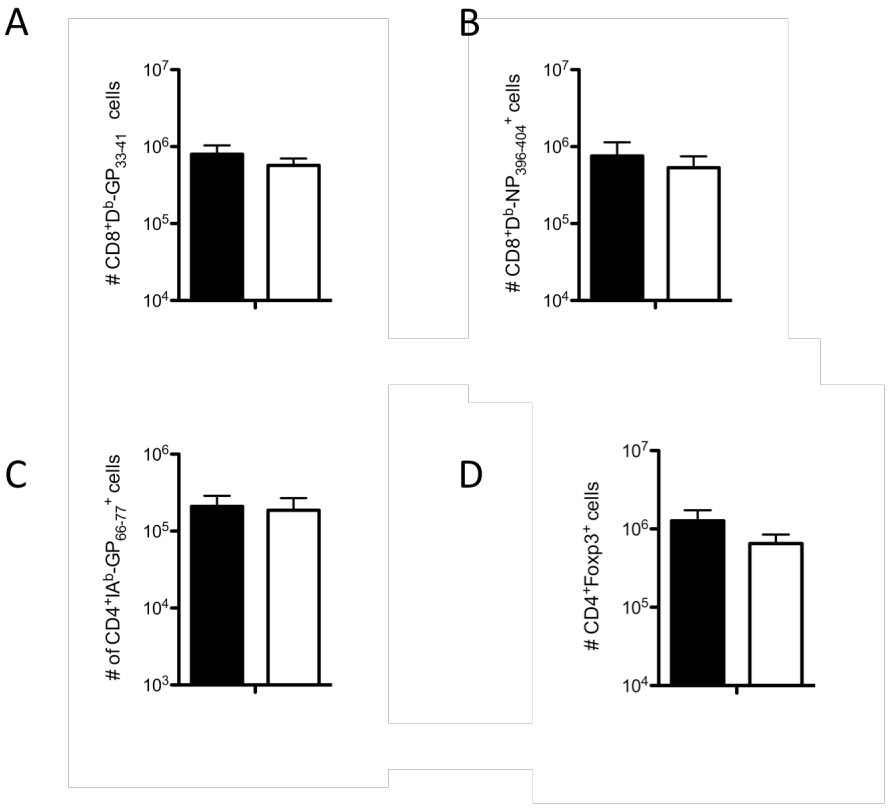

Figure 31. Virus-specific CD8$^+$ T cells and Tregs in T cell-specific TGF-β deficient mice. WT (black bars) or TGF-β $^{fl/n}$ x CD4Cre$^+$ (white bars) mice were infected with LCMV Cl 13 and splenocytes isolated at day 9 p.i. and stained with H2-D^b-GP$_{33-41}$ (A), H-2-D^b-NP$_{396-404}$ (B), IA^b-GP$_{66-77}$ tetramers, or Foxp3 (D). Bar graphs depict the average frequency of mice per group ± sd. Results are representative of two independent experiments with four mice per group.

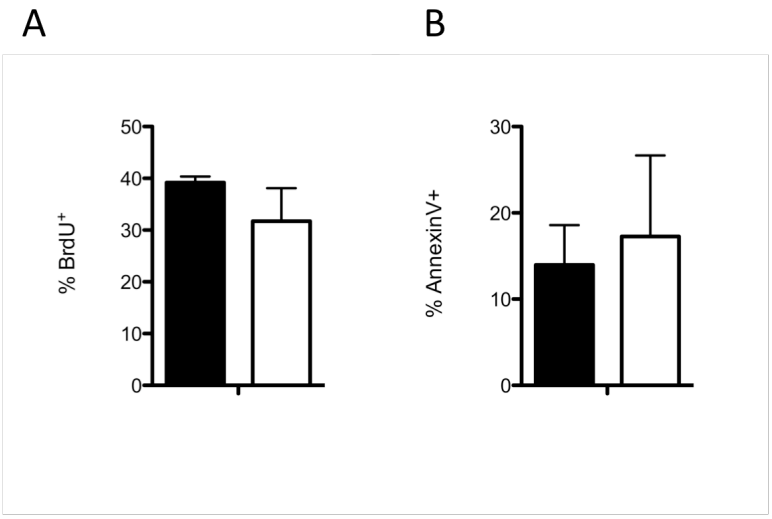

Figure 32. Proliferation and survival of virus-specific H2D^{b}-GP$_{33-41}$-specific CD8^{+} T cells during LCMV infection. WT (black bars) or TGF-$\beta^{fl/n}$ x CD4Cre^{+} (white bars) mice were infected with LCMV Cl 13 and splenocytes obtained at day 9 p.i. BrdU incorporation (A) and Annexin V staining (B) of H2D^{b}-GP$_{33-41}$ tetramer^{+} CD8^{+} T cells were determined. Bar graphs depict the average frequency of positive cells ± sd. Results are representative of two independent experiments with three or four mice per group.

We next examined the functional state of $CD8^+$ T cells in TGF-$\beta^{fl/n}$ x $CD4Cre^+$ and WT mice by ex vivo stimulation with GP_{33-41} peptides and analyzed cytokine production (Figure 33A). Both TGF-$\beta^{fl/n}$ x $CD4Cre^+$ and WT $CD8^+$ T cells produced similar levels of IFN-γ and TNF-α, and these levels were significantly reduced compared to ARM controls (Figure 33A). In addition, PD-1 expression in D^b-GP_{33-41} $tetramer^+$ $CD8^+$ T cells was indistinguishable in both groups of animals (Figure 33B). We next examined degranulation in $CD8^+$ T cells after GP_{33-41} peptide stimulation in the presence of CD107a-b antibodies and found similar proportion of $CD8^+$ T cells that were IFN-γ^+CD107a-b^+ (Figure 33C). Finally, we examined TGF-β protein levels by immunoblot in sorted $CD4^+$ and $CD8^+$ T cells from TGF-$\beta^{fl/n}$ x $CD4Cre^+$ and WT mice at day 9 p.i. and confirmed TGF-β protein in WT $CD4^+$ and $CD8^+$ T cells and complete deletion of TGF-β protein in TGF-$\beta^{fl/n}$ x $CD4Cre^+$ T cells (Figure 33D). Furthermore, we examined TGF-β-Smad signaling in these purified $CD4^+$ and $CD8^+$ T cells by examining Smad-2 phosphorylation and observed that T cells sorted from TGF-$\beta^{fl/n}$ x $CD4Cre^+$ continued to have phosphorylation of Smad-2 proteins to a similar extent as WT T cells (Figure 33D). These findings indicated that in the absence of T cell-derived TGF-β, virus-specific $CD8^+$ T cells are increased in proportion in blood at day 7 p.i., however the number of virus specific $CD8^+$ T cells in the spleen at day 9 p.i. are not increased compared to WT. One possible reason for these differences observed at day 7 and 9 p.i. may be that WT T cells in blood are exposed to sufficient T cell-derived TGF-β and this signal is sufficient to

decrease their proportion, the opposite is observed in T cells lacking TGF-β protein. In contrast, many cell types in the spleen can generate and may activate TGF-β to signal in T cells consequently affecting cell numbers and highlighting the fact that T cell-derived TGF-β is not the only cellular source during infection. Furthermore, we did not detect differences in proliferation, survival, and effector function between both groups. Finally, even though $CD4^+$ and $CD8^+$ T cells have complete absence of TGF-β protein, they continue to receive TGF-β signals as indicated by the phosphorylation of Smad-2 proteins. These findings suggest that there are other relevant sources of TGF-β_1 or molecules leading to Smad-2 phosphorylation other than TGF-β_1, which are operating during Cl 13 infection.

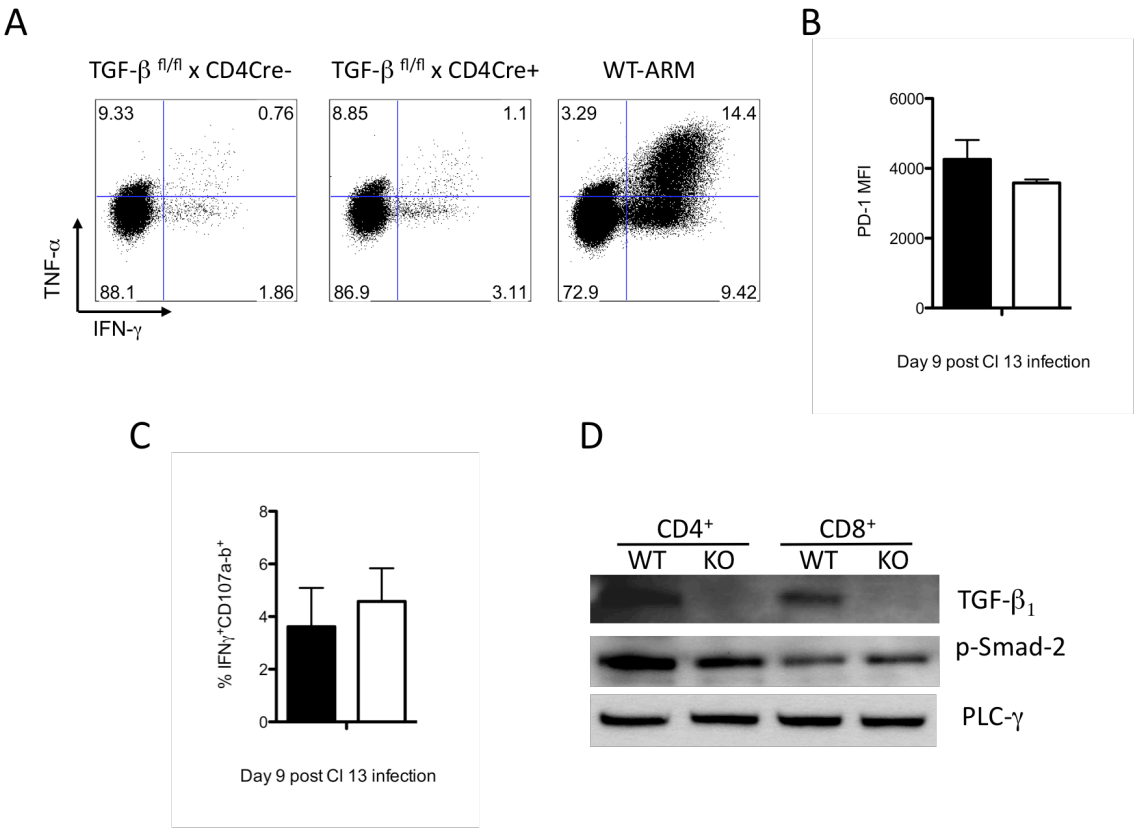

Figure 33. Virus-specific H2D^b-GP$_{33-41}$-specific CD8$^+$ T cell function and TGF-β$_1$ levels in T cells. WT (black bars) or TGF-β$^{fl/n}$ x CD4Cre$^+$ (white bars) mice were infected with LCMV CL 13 and splenocytes isolated at day 9 p.i. Splenocytes were ex vivo stimulated with GP$_{33-41}$ LCMV peptides and total CD8$^+$ T cells analyzed for IFN-γ and TNF-α production (A), WT-ARM was used as control. Splenocytes were stained with H2-D^b-GP$_{33-41}$ tetramer and Tet$^+$ cells analyzed for PD-1 levels (B). Cells were stimulated with GP$_{33-41}$ peptides in the presence of FITC-labeled anti-CD107a-b (C). CD4$^+$ and CD8$^+$ T cells were FACS sorted and analyzed for TGF-β$_1$ and p-Smad-2 levels by immunoblot. PLC-γ was used as a loading control. Dot plots represent one representative mouse per group.

Complete TGF-β deletion in mice did not enhance virus-specific $CD8^+$ T cell responses during Cl 13 infection

We next investigated whether complete deletion of TGF-β_1 before infection could rescue virus-specific $CD8^+$ T cell responses during infection. To examine this possibility, mice that have TGF-β flanked by loxp sites(TGF-β $^{f/n}$) were bred with estrogen receptor cre mice (ERCre) that expressed 4OH-tamoxifen-inducible cre gene under an ubiquitous Rosa26 promoter. TGF-β $^{f/n}$ x $ERCre^+$ and TGF-β $^{f/n}$ x $ERCre^-$ (WT) mice were treated with 4-OH-tamoxifen (2 mg/mouse) daily for 5 days, rested 1 day, and infected with Cl 13. Since TGF-β signaling in T cells prevents spontaneous activation, we bled WT and TGF-β $^{f/n}$ x $ERCre^-$ mice before infection and examined CD44 and CD62L expression on $CD4^+$ and $CD8^+$ T cells (Figure 34A and B). We observed that both $CD4^+$ and $CD8^+$ T cells expressed similar levels of CD44 and CD62L indicating a similar activation state in both TGF-β $^{f/n}$ x $ERCre^+$ and WT at the time of infection.

We examined viral levels in TGF-β $^{f/n}$ x $ERCre^+$ and WT mice at day 5 and 8 p.i. (Figure 35A). We found no differences in viremia between these two groups of mice at both time points examined. Finally, we examined TGF-β protein levels in enriched total $CD8^+$ T cells from TGF-β $^{f/n}$ x $ERCre^+$ and WT mice, as well as in splenocytes obtained from each individual mouse within each group (Figure 35B). We observed that while WT $CD8^+$ T cells had high levels of TGF-β protein, TGF-β $^{f/n}$ x $ERCre^+$ $CD8^+$ T cells had complete deletion of TGF-β protein. Furthermore, splenocytes from each WT individual mouse had TGF-β protein while splenocytes from each TGF-β $^{f/n}$ x $ERCre^+$ mouse had complete

TGF-β deletion. We then examined phosphorylation of Smad-2 proteins in the same cells and observed that $CD8^+$ T cells from TGF-$\beta^{f/n}$ x $ERCre^+$ mice had continued Smad-2 phosphorylation (Figure 35C).

We next examined the number of virus-specific $CD8^+$ T cells in spleen at day 8 p.i. by staining with D^b-GP_{33-41}, D^b-$NP_{396-404}$, and IA^b-GP_{61-67} tetramers (Figure 36A). We found no difference in the number of virus-specific $CD8^+$ and $CD4^+$ T cells in TGF-$\beta^{f/n}$ x $ERCre^+$ and WT mice. To examine the function state of these cells we ex-vivo stimulated splenocytes with GP_{33-41} peptides and analyzed $CD8^+$ T cells for the ability to secrete IFN-γ and TNF-α (Figure 36B). We found that $CD8^+$ T cells in both groups produced similar levels of IFN-γ and had similar proportions of IFN-γ^+TNF-α^+ $CD8^+$ T cells (WT ~24%, TGF-$\beta^{f/n}$ x $ERCre^+$ ~28%; n=3 each). In addition, GP_{33-41} $CD8^+$ T cells expressed similar PD-1 levels (Figure 36C). Since TGF-β is necessary for Treg induction, we enumerated $CD4^+Foxp3^+$ cells in these animals (Figure 36D) and observed no difference in Treg cells.

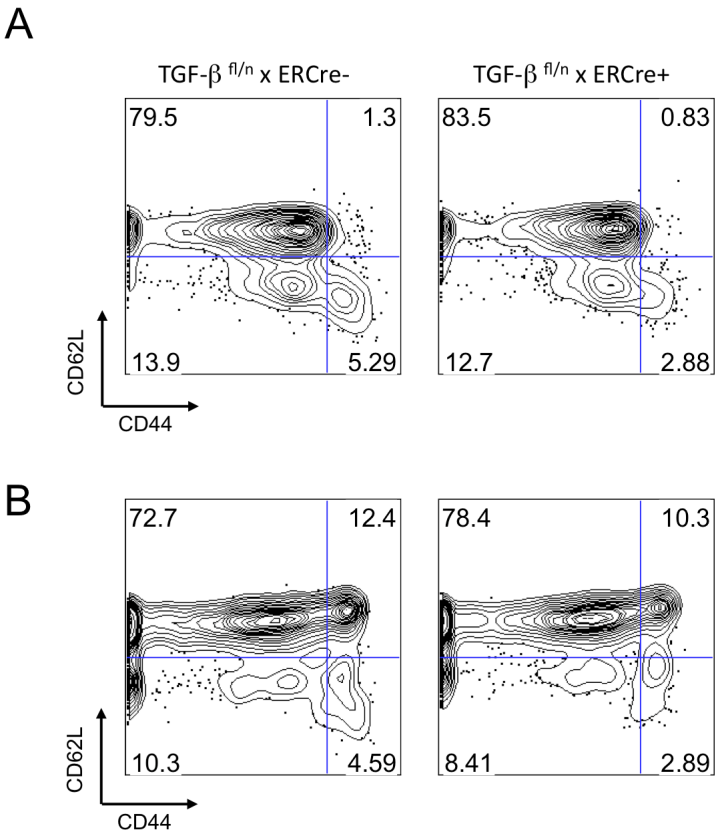

Figure 34. Activation markers in CD4$^+$ and CD8$^+$ T cells from tamoxifen treated TGF-β$^{f/n}$ x ERCre- and TGF-β$^{f/n}$ x ERCre$^+$ mice. TGF-β$^{f/n}$ x ERCre$^-$ and TGFb$^{f/n}$ x ERCre+ mice were ip injected 2 mg 4-hydroxytamoxifen for 5 consecutive days, rested for one day, bled and analyzed for CD44 and CD62L expression in CD4$^+$ T cells (A) and CD8$^+$ T cells (B). Dot plots representative of one mouse per group.

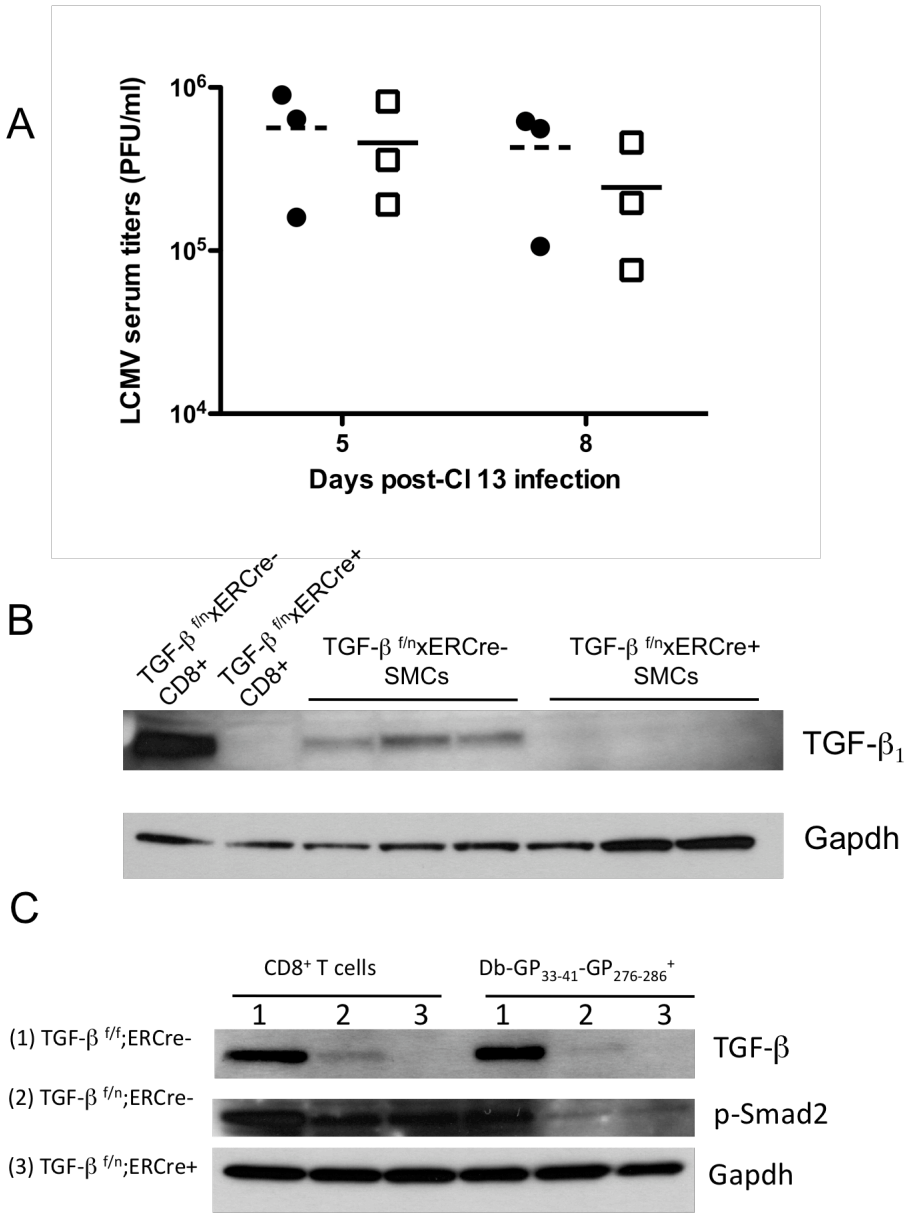

Figure 35. Serum viral titers and TGF-β levels in tamoxifen treated TGF-$\beta^{f/n}$;ERCre^{-} and TGF-$\beta^{f/n}$;ERCre^{+} mice after LCMV Cl 13 infection. TGF-$\beta^{f/n}$;ERCre^{-} (black circles) and TGF-$\beta^{f/n}$;ERCre^{+} (white squares) mice were ip injected 2 mg 4-hydroxytamoxifen for 5 consecutive days, rested for one day, infected with LCMV Cl 13 and serum obtained at day 5 and 8 p.i. to examine serum viral titers (A). Splenocytes and enriched CD8^{+} T cells were examined for TGF-β_1 levels by immunoblot (B). Sorted CD8^{+} T cells and D^{b}-GP$_{33-41}$/GP$_{276-286}$ Tet^{+} cells were examined for TGF-β_1 and p-Smad2 levels. Gapdh was used as a loading control (C).

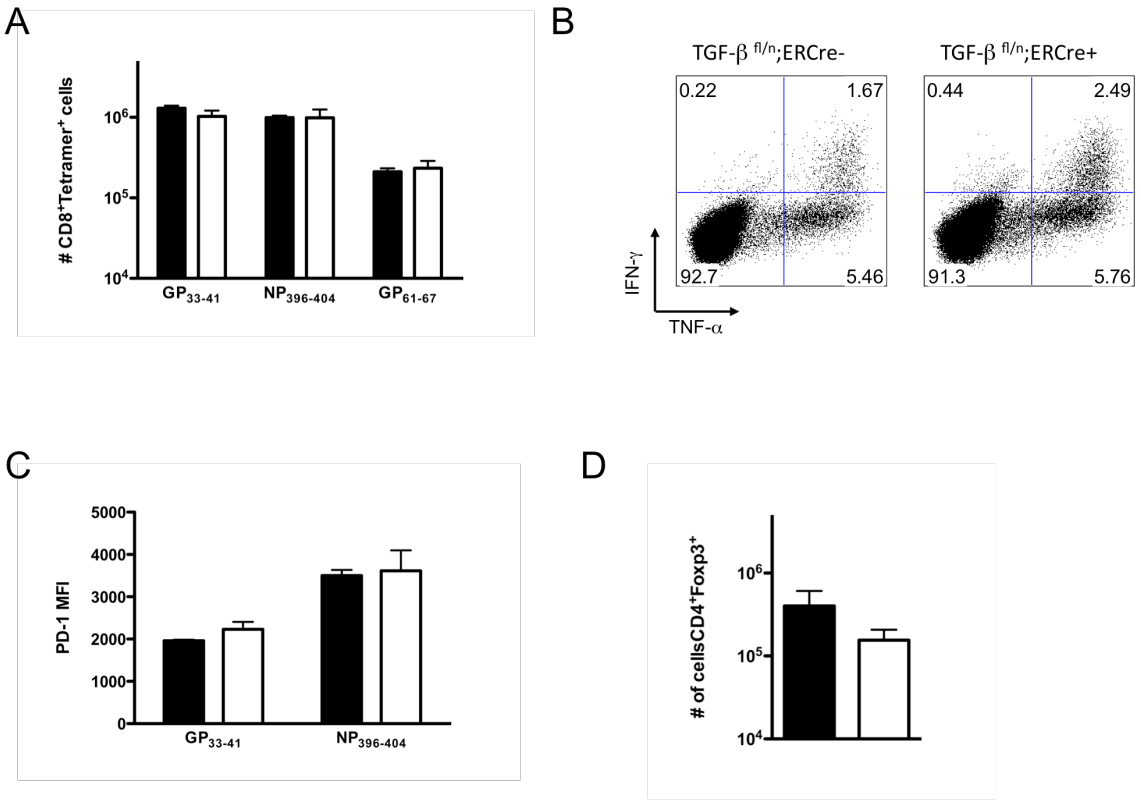

Figure 36. Virus-specific T cell and function in tamoxifen treated TGF-$\beta^{f/n}$;ERCre^{-} and TGF-$\beta^{f/n}$;ERCre^{+} mice after LCMV Cl 13 infection. TGF-$\beta^{f/n}$;ERCre^{-} and TGF-$\beta^{f/n}$;ERCre^{+} mice were ip injected 2 mg 4-hydroxytamoxifen for 5 consecutive days, rested for one day, infected with LCMV CL 13 and splenocytes isolated at day 8 p.i. Splenocytes were stained with H2-D^{b}-GP$_{33-41}$, H2-D^{b}-GP$_{33-41}$, and IA-^{b}GP$_{61-67}$ tetramers (A). Splenocytes were stimulated with GP$_{33-41}$ peptides and CD8^{+} T cells examined for IFN-γ and TNF-α production (B). PD-1 levels on tetramer^{+} cells (C). CD4^{+} T cells were stained with Foxp3 (D). Bar graphs depict the average frequency of positive cells ± sd. Dot plots representative of one mouse per group.

These findings show that complete TGF-β deletion before infection does not induce exuberant T cell activation. During infection however, virus-specific $CD8^+$ T cell numbers are not enhanced, virus-specific $CD8^+$ T cell function and PD-1 expression, and viremia in TGF-β $^{f/n}$ x $ERCre^+$ and WT are not affected. Interestingly, even though $CD8^+$ T cells from TGF-β $^{f/n}$ x $ERCre^+$ and spleens in these animals have no detectable TGF-β protein, these $CD8^+$ T cells have continued phosphorylation of Smad-2 proteins indicating that other molecules leading to Smad-2 phosphorylation are operating during Cl 13 infection.

Therapeutic potential of anti-TGF-β administration during chronic LCMV infection

Since the TGF-β signaling pathway is actively deleting virus-specific $CD8^+$ T cell and contributing to the persistence of LCMV, and the fact that complete genetic deletion of TGF-β_1 does not result in improved T cell responses we wondered whether signaling via the other two TGF-β isoforms, TGF-β_2 or TGF-β_3, could be limiting T cell responses. To examine this possibility we administered neutralizing antibodies against TGF-β (clone 2G7) during Cl 13 infection (178). This antibody has been shown to effectively block TGF-β signaling in T cells in vivo (179) as well as blocking the activity of all three TGF-β isoforms (178). We infected two groups of WT mice with LCMV Cl 13 and injected one group with 1 mg anti-TGF-β at days 4 through 7 p.i. and left the other group untreated. Mice infected with LCMV ARM strain (which causes an acute infection) were used as controls. We first monitored the number $CD8^+$ D^b-$GP_{276-286}^+$ blood cells at days 7, 9, 15 and 32 p.i. (Figure 37A). We observed that

the numbers of virus specific $CD8^+$ T cells in WT anti-TGF-β treated or WT untreated mice were indistinguishable, indicating that the antibody treatment had no effect on the number of virus-specific $CD8^+$ T cells in these mice (Figure 37A). We next monitored the level of viremia at days 5, 15, and 32 p.i. (Figure 37B). Viremia was similar in antibody-treated or untreated WT mice at day 5, 15 and 32 p.i. (Figure 37B). We analyzed IFN-γ and TNF-α antiviral cytokine production in $CD8^+$ T cells after ex vivo LCMV $GP_{276-286}$ peptide stimulation. Production of IFN-γ in WT antibody-treated or WT untreated control was similar and both groups had a compromised ability to secrete TNF-α (Figure 37C). Finally, we monitored inhibitory receptor programmed death-1 (PD-1) levels on $CD8^+$ D^b-$GP_{276-286}^+$ blood cells at days 7, 9, 15 and 32 p.i. and found that PD-1 expression in WT anti-TGF-β treated or WT untreated controls were statistically indistinguishable, while virus-specific $CD8^+$ T cells from ARM infected mice had significantly reduced PD-1 levels at all time points examined (Figure 37D). Together, these findings indicate that administration of anti-TGF-β (clone 2G7) during LCMV Cl 13 infection did not affect the numbers of virus-specific $CD8^+$ T cells, IFN-γ and TNF-α production, PD-1 expression, or viremia.

Chapter 2 contains portions of the material as it appears in the journal Immunity, “Cell-intrinsic Transforming Growth Factor-β signaling mediates virus-specific CD8+ T cell deletion and viral persistence *in vivo*.” Tinoco, Roberto, Alcalde Victor, Yang, Yating, Sauer, Karsten, Zuñiga, Elina I., July 2009 Jul 17;31(1):145-57. The dissertation author is the first author of this paper.

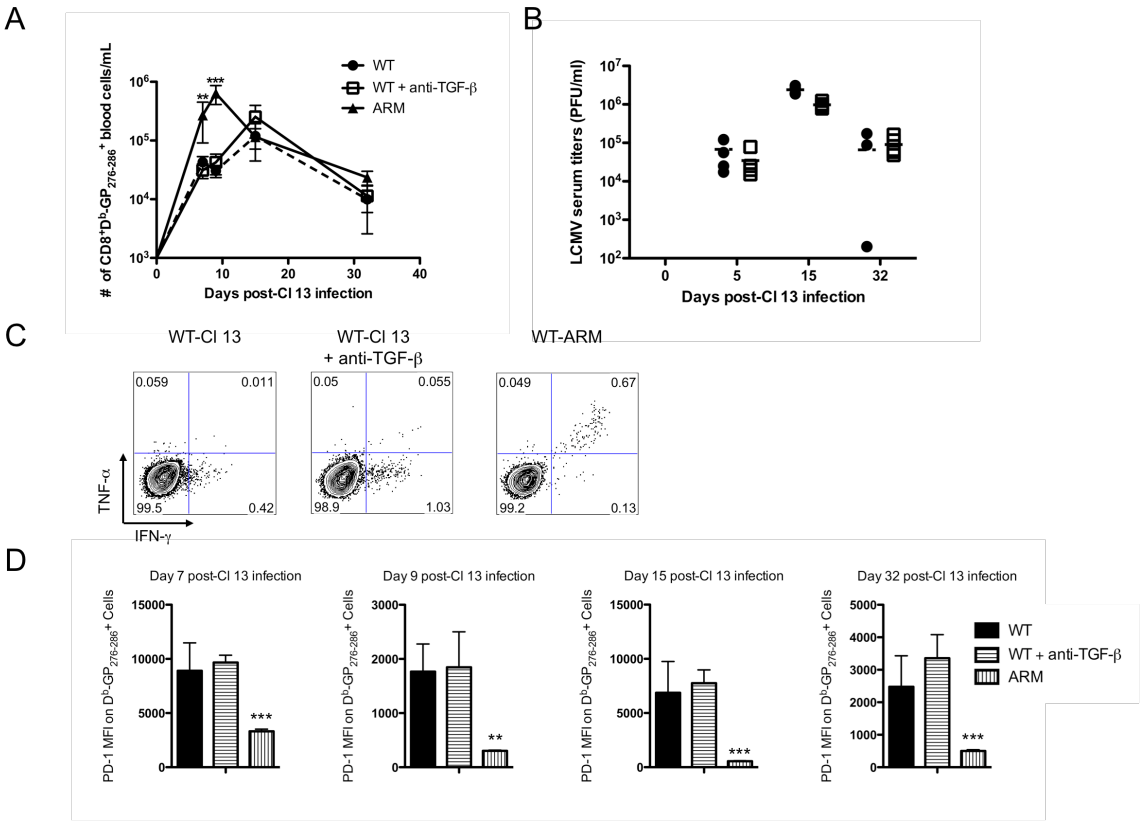

Figure 37. Anti-TGF-β treatment of WT mice during LCMV Cl 13 infection. WT mice were infected with Cl 13 and left untreated (black circles or black bars) or treated during day 4 through 7 with i.p. injections of anti-TGF-β mAb (clone 2G7) 1mg/mouse in PBS (white squares or bar with horizontal stripes). WT mice were infected with ARM as controls (black triangles or bars with vertical stripes). Blood cells were stained with H2-$D^b$$GP_{276-286}$ tetramers at day 7, 9, 15, and 32 p.i. and analyzed by FACS (A). Serum viral titers at day 5, 15, and 32 p.i. were determined by plaque assay (B). PD-1 levels on H2-$D^b$$GP_{276-286}$ tet^+ cells at different times post infection. Results representative of 5 mice per group. (WT vs Arm; **p < 0.005, ***p < 0.0005)

CHAPTER 3 DISCUSSION

Chronic viral infections represent a major health concern and afflict the lives of millions of individuals globally. Chronic viral infections in humans are examples of an ongoing battle between the immune system, which tries to control viral spread and the pathogen, which aims to continue replicating. Under conditions where viral replication is not controlled, persistent antigen induces the suppression of immune responses preventing further damage to the host from immunopathology while facilitating the persistence of the virus. Using the chronic LCMV infection model in mice, we identified TGF-β as an immunosuppressive cytokine contributing to viral persistence.

TGF-β production and signaling is altered during infection

We investigated TGF-β production during acute and chronic infection by examining the levels of TGF-β protein in different cell types. We found that under steady state conditions, TGF-β was produced by all cell types examined (Figure1) however, during chronic infection all cell types had lower TGF-β levels compared to uninfected or acutely infected mice, T cells on the other hand, showed an increase expression of this protein. Furthermore, we found that virus-specific $CD8^+$ T cells produced high levels of TGF-β during early stages of acute and chronic infection, but these TGF-β levels were sustained for longer periods only during chronic infection. It is important to note that we only examined TGF-β levels in B cells, T cells, dendritic cells and macrophages and found TGF-β production in these cell types. It is still possible that many other cell types such

as fibroreticular cells or non-lymphoid cells can actively be making TGF-β, contributing to the overall systemic circulating levels of this cytokine or to the overall levels that are deposited on the extracellular matrix. These findings indicated that there is not one specific source of TGF-β under steady state conditions or during infection. Since we knew that TGF-β levels in virus-specific $CD8^+$ T cells were upregulated during chronic viral infection, we investigated the level of signaling in these cells by examining phosphorylation of Smad-2 proteins, which are downstream effectors of TGF-β receptor signaling (Figure 2). We observed that Smad-2 phosphorylation was occuring in virus-specific $CD8^+$ T cells during both acute and chronic infection but this phosphorylation was enhanced and sustained in virus-specific $CD8^+$ T cells from chronically infected mice. It is interesting that virus-specific $CD8^+$ T cells during Cl 13 infection not only produce more TGF-β_1, but also receive more TGF-β stimulation as indicated by modulation of downstream TGF-β mediators. It is still unclear how other molecules that are known to be regulated by TGF-β signaling are being modulated during acute vs chronic infection. For instance, TGF-β can also affect the levels of Smad-3 phosphorylation or affect Smad-independent pathways such as MAPK, PI3K, PP2A, Rho, Par6 (84, 87) which can certainly affect cellular processes located downstream of the TGF-β receptors. The levels of inhibitory Smads in $CD8^+$ T cells can also be differentially regulated during steady state conditions or during infection as well as the activity of proteases and phosphatases that can be activated or repressed to dampen or strengthen

downstream TGF-β receptor signaling, and eventually affect expression of infection-specific genes. TGF-β provides a critical immune homeostatic role during steady state conditions and many immune cell types are actively secreting it. During infectious conditions however, immune system homeostasis is dysregulated and TGF-β production in different cell types is altered in response to infection. In addition to TGF-β production during infection, the activation mechanisms also need to be considered. TGF-β can be produced at normal levels but if it is not activated it will not signal its specific cell target (75, 149, 150, 152). We did not examine possible activation mechanisms during our LCMV studies; future work is needed to determine whether activation mechanisms are dysregulated during chronic infection.

TGF-β signaling in T cells decreased the numbers of virus-specific T cells

TGF-β production and signaling in virus-specific $CD8^+$ T cells was enhanced during chronic vs acute infections. When we examined the biological significance of TGF-β signaling in T cells during chronic viral infection we found that by attenuating the TGF-β receptor-signaling pathway in T cells, both virus-specific $CD4^+$ and $CD8^+$ T cell numbers were higher compared to WT controls (Figure 4 and 5). This finding indicated that during persistent LCMV infection, TGF-β signaling is an active signaling pathway in virus-specific T cells and it is activated to limit T cell responses by decreasing the numbers of these T cell effectors. T cells in dnTGFBRII mice are spontaneously activated and mice harboring this transgene develop autoimmune disease after 3-4 months of age

(101). A possible effect of enhanced T cell activation in dnTGFBRII mice could mean that T cell responses in dnTGFBRII mice already start from a different baseline, such that upon T cell priming, virus-specific $CD8^+$ T cells have an advantage over WT T cells, and this leads to their early expansion and accumulation after Cl 13 infection. Interestingly, when virus-specific $CD8^+$ T cells were enumerated throughout the course of Cl 13 infection in spleen, virus-specific $CD8^+$ T cell expansion was identical in WT and dnTGFBRII mice up until day 5 p.i. (Figure 5). The accumulation was not observed early after infection, indicating that despite the increased activation state observed in dnTGFBRII mice, this did not pre-dispose these cells to have an expansion advantage early during infection. Future studies will aim to determine the TGF-β contribution in limiting T cell responses to eventually manipulate TGF-β signaling in antigen-specific T cells during HIV, HBV, and HCV infections and restore the number of antigen-specific effector T cells *in vivo*. The TGF-β pathway is an important avenue to pursue as it has already been implicated in the aforementioned chronic infections. A study using genomewide association studies (GWASs) of HIV infected patients aimed to understand the genetic factors that predispose individuals to rapid progression to AIDS found the TGF-β pathway as being important in the pathogenesis of HIV-1 disease (180). Hepatitis B virus-specific $CD4^+$ T cells generated ex vivo had enhanced apoptosis when exposed to TGF-β (181). Furthermore, TGF-β_1 inhibited T cell cytotoxicity from peripheral blood mononuclear cells (PBMC) of patients with chronic hepatitis B (182). In HCV infection, HCV-specific T cell responses were weak against recall antigens.

Blocking antibodies against all three TGF-β isoforms and IL-10 increased HCV-specific T cell responses in patients infected with HCV alone or co-infected with HCV and HIV, although less functional restoration occurred in blood from patients co-infected with HCV-HIV (183). Furthermore, IFN-γ enahancement was observed in both $CD4^+$ and $CD8^+$ T cells and was mediated primarily by TGF-β neutralization (183). Our findings show that by attenuating the TGF-β pathway in T cells during Cl 13 infection resulted in enhanced virus-specific T cell response and numbers, highlighting its important biological role. The fact that the TGF-β pathway has also been implicated in human chronic viral infections indicates that this signaling pathway has therapeutic potential and should be further explored.

The magnitude of the $CD8^+$ T cell response is limited by direct TGF-β induced death of virus-specific $CD8^+$ T cells

A recent study showed that in simian immunodeficiency viral infections in macaques and LCMV infections in mice, the early timing and magnitude of excess effectors to infected cells correlated with control and clearance vs partial or poor control and viral persistence (184). Our findings show that TGF-β signaling in virus-specific $CD8^+$ T cells induced death, diminishing the magnitude of effectors able to control viral replication. When this pathway is attenuated, virus-specific $CD8^+$ T cells survived and these critical numbers of effectors were restored to similar numbers as observed during acute LCMV infection, preventing the establishment of persistent infection (Figure 16 and 17). Acute vs chronic infection outcomes depends on the race between viral replication and T cell responses generated to contain the virus, our findings support the notion that

during the early stages of chronic LCMV infection, TGF-β signaling limits the $CD8^+$ T cell response by direct killing of these cells allowing viral replication to continue. The accumulation of virus-specific $CD8^+$ T cells after Cl 13 infection can depend on altered proliferation and death or a combination of these two cellular processes. We investigated whether the accumulation of virus-specific $CD8^+$ T cells observed in dnTGFBRII mice was a result of proliferation after Cl 13 infection and observed no enhancement compared to WT (Figure 6A and 7A). Upon examining survival however, virus-specific $CD8^+$ T cells from dnTGFBRII mice survived better than WT cells (Figure 6B and 7B). Studies using chronic LCMV infection have shown that antigen load and T cell exhaustion are intricately linked processes such that if any of these two is altered, it will inevitably affect the other in a positive or negative manner. More specifically, sustained antigen presentation drives T cell exhaustion and negatively impacts virus-specific T cell responses during chronic infection (185). Furthermore, a study showed that during LCMV Cl 13 infection, the emergence of a LCMV variant that abrogates presentation of the GP_{33-41} epitope resulted in the preservation of function of D^b-GP_{33-41} CD8+ T cells, these cells also had decreased PD-1 levels, and re-expressed memory marker CD127 (186). During the early stages of Cl 13 infection, up until about day 5 p.i., T cell responses are similar to ARM infected mice even though the virus is actively replicating. In contrast to acutely infected mice, which begin controlling the virus, chronically infected mice fail to control viral replication and eventually inhibitory mechanisms become effective to induce immunosuppression allowing the virus to persist (14,

31, 32, 37, 38). If viral loads are not controlled, then a chronic infection becomes fully established. If immunosuppressive mechanisms are alleviated such that T cell exhaustion does not occur or if these cells are exhausted and functionally restored to reduce viral burden, then all the conditions are in place to control viral persistence. In the case of PD-1 for example, blockade of this inhibitory pathway in mice restored $CD8^+$ T cell responses and this resulted in decrease in viral loads (38). In addition, blocking the receptor for IL-10 in mice led to improved function of virus-specific $CD8^+$ T cells and resolution of chronic LCMV infection (35, 40).

The proapoptotic molecule, Bim has been implicated in the killing of virus-specific $CD8^+$ T cells during chronic LCMV infection and murine herpes virus (156, 157). We confirmed elevated Bim levels in virus-specific $CD8^+$ T cells of chronically vs acutely infected mice (Figure 8). Interestingly, in virus-specific $CD8^+$ T cells from Cl 13 infected dnTGFBRII mice, Bim levels were also reduced (Figure 8). A study using OT-I WT and OT-I dnTGFBRII $CD8^+$ T cells also observed a survival of OT-I dnTGFBRII cells and they observed elevation in pro-survival Bcl-2 levels (177). Our findings indicated that Bim was important in mediating the survival phenotype in dnTGFBRII mice. It is possible that by eliminating Bim in virus-specific $CD8^+$ T cells, death of these cells will be prevented. This is not the case however as Bim deficient mice infected with Cl 13 had only a slight rescue of D^b-$NP_{396-404}$ specific $CD8^+$ T cells and not the other T cell clones specific for other epitopes. These findings indicate that Bim is not

the only apoptotic molecule involved in death of virus-specific $CD8^+$ T cells (156). It is not surprising that survival of all virus-specific $CD8^+$ T cells in Bim deficient mice is not observed, as apoptosis is induced or prevented by the balance between anti and pro-apoptotic molecules (177). It is important to note that our findings do not elucidate between TGF-β direct or indirect induction of Bim. It is possible that TGF-β increased $CD8^+$ T cell survival through a different pathway leading to reduced viral loads which in turn result in reduced Bim expression in dnTGFBRII mice. Further work is needed to determine whether Bim and other apoptotic molecules are directly regulated by TGF-β or whether the balance between these molecules is simply a result of reduced viral burden in dnTGFBRII mice.

It has been reported that under steady-state conditions, TGF-β signaling prevents proliferation and death of naïve $CD8^+$ T cells (101, 102, 117, 187). Indeed, we examined TGF-β effects on proliferation and death of total $CD8^+$ T cells (Figure 28A and B) and P14 Tg $CD8^+$ T cells (Figure 29A and B). We observed that in uninfected mice and in the absence of TGF-β signaling, $CD8^+$ T cells proliferated and had decreased survival compared to WT. These findings show opposing effects of TGF-β signaling in $CD8^+$ T cells before infection, where it functions to maintain survival and prevent proliferation, whereas during infection TGF-β signaling promotes death of virus-specific $CD8^+$ T cells and has no effect on proliferation. These findings are important because they show that the role that TGF-β is playing in $CD8^+$ T cells is very different before and during

infection. Interestingly, we found the survival advantage of virus-specific $CD8^+$ T cells during chronic infection to be an intrinsic property of cells with diminished TGF-β signaling (Figure 24 and 25). In terms of therapeutic potential, it would be interesting to determine the TGF-β-dependent transcriptional program before and during infection to explore which genes are specifically regulated to induce death of virus-specific $CD8^+$ T cells. The goal would be to evaluate whether these genes and their products could be specifically regulated during infection to rescue the survival of virus-specific T cells and control or prevent a persistent infection while not affecting autoreactive T cells. These future therapies could be useful in rescuing T cell numbers in chronic infections; the challenge is targeting these therapies exactly to virus-specific T cells. Caution however, needs to be carefully considered as these potential therapies can also result in autoimmunity and perhaps have more detrimental consequences than the actual infection.

TGF-β was shown to induce death of activated OT-I WT $CD8^+$ T cells by overriding IL-15 signals. Furthermore, this TGF-β-induced death was prevented by IL-2 and IL-7 signaling (177). This group and others have shown that short-lived effector cells (SLECs) during the expansion and contraction phase depend on IL-15 signals for survival and not IL-7 since they fail to express the IL-7 receptor (8, 177, 188, 189). SLECs are terminally differentiated $CD8^+$ T cell effectors that express high KLRG1 and low IL-7 receptor. These cells are terminally differentiated, have low proliferative potential, and maintain effector molecule expression. In contrast, memory precursor effector cells (MPECs)

express low KLRG1 and high levels of IL-7 receptor. These MPECs are characterized as being long-lived, proliferate more rapidly and re-acquire effector function upon re-stimulation with antigen, maintain a stable memory $CD8^+$ T cell population via self-renewal, and maintain multipotency (8, 190-193). It is possible that TGF-β signaling regulates the magnitude of the immune response by overriding these IL-15 signals during chronic infection. It would be interesting to understand whether virus-specific $CD8^+$ T cell subsets, such as short-lived effector cells, are also differentially enriched in dnTGFBRII mice after Cl 13 infection. In terms of therapeutic potential, it is important to understand TGF-β-dependent SLECs vs MPECs regulation. Understanding the molecular regulation in these subsets will be important to manipulate the survival of SLECs that will continuously secrete effector cytokines and kill infected cells as well as the survival of MPECs that will protect the host from re-infection. For example, strategies may involve delivering molecules that block TGF-β-dependent genes and cytokines like IL-2 or IL-7 in nanoparticles to target virus-specific $CD8^+$ T cells and promote T cell subset survival.

TGF-β is a biological meaningful signaling pathway limiting T cell responses and promoting viral persistence

We evaluated the impact of TGF-β signaling in virus-specific $CD4^+$ and $CD8^+$ T cell function and observed that in mice with attenuated TGF-β signaling, T cell responses were dramatically improved compared to WT (Figure 9, 11, 15). After LCMV peptide stimulation, T cells produced greater levels of IFN-γ, TNF-α, and IL-2. In contrast to WT mice, dnTGFBRII virus-specific T cells were more

functional on a per cell basis (Figure 9A and 15C). Furthermore, we enumerated the number of multi-cytokine producers and observed that dnTGFBRII mice had a higher number of these multi-producers (Figure 14). In addition, $CD8^+$ T cell effectors from dnTGFBII mice were better at killing LCMV peptide-loaded target cells (Figure 10). These findings indicated that attenuation of TGF-β signaling in T cells led to the effective development of functional T cells that produced anti-viral cytokines and were able to kill infected cells.

Dysfunctional virus-specific T cells express high levels of the PD-1 (38) inhibitory receptor. We found that virus-specific $CD8^+$ T cells in dnTGFBRII had high PD-1 levels early after infection, however, these levels were reduced to those observed in acutely infected mice (Figure 12A and B). Furthermore, PD-1 levels on virus-specific $CD8^+$ T cells remained low up until day 32 p.i. while WT cells continued to express high levels of this receptor (Figure 13). Interestingly, when we examined whether PD-1 levels were differentially expressed in virus-specific $CD8^+$ T cells in mixed bone marrow chimeras, we observed that PD-1 levels were identical in both compartments (Figure 26B). Furthermore, $CD8^+$ T cells in WT and dnTGFBRII mixed chimeras were equally dysfunctional as both had a compromised ability to secrete effector cytokines (Figure 26A). These findings indicated that while intact dnTGFBRII mice have reduced PD-1 levels in virus-specific $CD8^+$ T cells later during the infection, virus-specific $CD8^+$ T cells in WT-dnTGFBRII mixed chimeras expressed the same level of this inhibitory receptor. These findings show that functional exhaustion is not a direct

consequence of TGF-β signaling in T cells. We have dissociated that TGF-β signaling is mediating death of virus-specific $CD8^+$ T cells but not directly affecting their functional exhaustion. High IL-10 levels were observed in chronically infected mice (35, 40). Similar levels of IL-10 were observed in WT and dnTGFBRII mice at day 5 p.i. however, these levels were reduced in dnTGFBRII mice at day 9 p.i. and sustained in WT mice (Figure 12C). It is interesting that in dnTGFBRII mice, likely because we shift the effector to target ratio over the critical point where LCMV Cl 13 can be controlled, all the positive effects of a successful viral resolution are observed, such as decreased PD-1 levels on virus-specific $CD8^+$ T cells as well as diminished IL-10 levels. More interesting is the fact that if WT and dnTGFBRII virus-specific $CD8^+$ T cells are exposed to the same infectious environment, functional restoration does not occur even though the survival of dnTGFBRII virus-specific $CD8^+$ T cells is preserved. It is possible that other cell types, such as regulatory T cells, are actively suppressing dnTGFBRII virus-specific $CD8^+$ T cells. In fact, the number of WT regulatory T cells is not reduced during Cl 13 infection, whereas the number of regulatory T cells in dnTGFBRII mice is decreased. It would be interesting in future studies to evaluate whether established chronic viral infections can be fully controlled by blocking pathways like PD-1 to obtain functional restoration as well as enhancing the survival of virus-specific $CD8^+$ T cells by manipulation of the TGF-β pathway.

We investigated whether the enhanced T cell response in dnTGFBRII mice was sufficient to control replication of LCMV Cl 13. We observed that dnTGFBRII mice were indeed able to control this infection while WT mice continued to have high levels of virus in blood and several organs (Figure 16 and 17). The superior numbers and functional capacity of virus-specific $CD8^+$ T cells in dnTGFBRII mice were sufficient to prevent the establishment of persistent LCMV infection. In addition, this clearance depended on the presence of $CD8^+$ T cells while $CD4^+$ T cells were dispensable (Figure 18), indicating that $CD8^+$ T cells were central in viral control and help by CD4 T cells was not essential to clear, albeit maximized viral control. One possibility to consider when examining viral control in dnTGFBRII mice is that T cells in these animals display an activated phenotype (101) (Figure 28F and 29D). When stimulated, $CD8^+$ T cells from dnTGFBRII mice also produced higher levels of IFN-γ (Figure 28 C-D and Figure 29 C-D) compared to WT mice. It is possible that because T cells in dnTGFBRII mice were more activated, they already started from a different baseline and T cell expansion and accumulation in these mice is altered to limit initial infection in dnTGFBRII mice. We show however, that in terms of virus-specific $CD8^+$ T cell expansion, up until day 5 p.i. we observed no differences in cell numbers between both groups (Figure 5B), indicating that even though these cells were more activated, they did not preferentially expand early during the infection. Furthermore, we quantified viral levels in spleen in a kinetic study to determine whether in fact viral levels could be different in dnTGFBRII mice early during infection (Figure 17). We observed that up until day 5 p.i., viral titers in

WT and dnTGFBRII spleens, which is also the secondary lymphoid organ where we have examined T cell responses, viral titers were indistinguishable, in fact whereas viral titers are significantly reduced by day 9 p.i., WT viral titers in spleen continued increasing and titers were higher at day 9 than day 5 p.i. These findings indicated that while dnTGFBRII mice had a more activated phenotype, LCMV Cl 13 titers were identical as WT early during infection, but controlled later during the infection. It is interesting that viral titers in spleen were identical in both WT and dnTGFBRII mice from days 1-5 p.i., but by day 9 p.i. viral titers in WT mice increased while dnTGFBRII mice were controlling the virus (Figure 17A). It is possible that the initial phase of the infection days 1-5 p.i. represents the control elicited by the innate and adaptive immune system and by day 5 p.i. the adaptive immune system must fully "kick in" to control the infection. In the case of WT mice this does not occur because T cell responses are compromised and thus LCMV Cl 13 continues replicating. In the case of dnTGFBRII mice, full T cell restoration controls this virus; it would be interesting to examine the contribution of innate immune cells in dnTGFBRII and whether these cells contribute or not to enhance T cell responses and help control viral replication. It would also be interesting to determine whether global TGF-β levels in dnTGFBRII are altered during infection such that they may be having a positive effect on other cell types and again, indirectly helping the adaptive immune response.

Our studies have focused in understanding the early phases of chronic viral infection, by attenuating TGF-β signaling in T cells, chronic LCMV is controlled and a persistent infection does not occur. We know the magnitude and the early timing of the $CD8^+$ T cell response can determine whether a virus will be cleared or whether it will persist. By manipulating TGF-β signaling, we shifted the equilibrium towards increasing the number of effectors that can now reduce viral levels. As the virus titer is reduced, a positive feedback mechanism is in place that benefits the host. Reduced antigen allows T cells to retain full functional capacities; T cells are exposed to decreased inhibitory signals, which further amplifies the positive T cell response to eliminate the otherwise persistent virus. We show that manipulation of the TGF-β pathway in T cells can rescue the number of virus-specific $CD8^+$ T cells than can help fight off the infection, in terms of potential therapies in humans, this approach would be useful when the host has recently been exposed to a persistent pathogen such as an accidental needle prick with contaminated blood. TGF-β would have to be attenuated during this initial phase to allow T cell responses to prevent the establishment of a chronic infection. We have no evidence to show whether TGF-β signaling in T cells is important during the later phase of a chronic viral infection. It is possible that at later stages during infection, TGF-β continues signaling, but whether it contributes to cell death or not needs to be determined. This is an important point to consider because most chronic infections in humans are diagnosed after they have reached equilibrium with the host immune system and have been fully established. If TGF-β is to be used as a potential therapy, will it be able to

rescue sufficient numbers of virus-specific $CD8^+$ T cells in HIV, HBV, or HCV infections to have an impact on viral control or whether it can be used in addition to other antiviral therapies needs to be determined. It will also be important to understand whether using these potential therapies to restore the number of other antigen-specific T cells in the absence of virus-specific T cell clones such as the LCMV $NP_{396-404}$ specific $CD8^+$ T cells in chronic LCMV infections or $CD4^+$ T cells in HIV are sufficient to promote a positive disease outcome. In the case of $NP_{396-404}$ –specific $CD8^+$ T cells for example; there is a correlation of this virus-specific $CD8^+$ T cells and viral outcome, such that the presence of these cells protected mice from persistent infection (194). Further work is needed to determine the relevance of TGF-β signaling during the later stages of chronic viral infection and whether this pathway can result in successful restoration of T cell numbers to decrease or eliminate high viral loads. In fact, Trabedersen, an antisense molecule that targets TGF-β_2 mRNA from Antisense Pharma GmbH is in Phase III for brain cancer and Phase I/II for colorectal, pancreatic, and skin cancer. Other companies are also using several inhibitors against the TGF-β pathway and are in clinical trials for cancer, autoimmune, and dermatological diseases (SciBX 2(29); doi:10.1038/scibx.2009.1152).

Effective memory responses develop in mice with attenuated TGF-β signaling

After effectively eliminating the persistent virus, dnTGFBRII mice develop long-lived virus-specific $CD8^+$ T cells expressing high levels of “memory” markers (Figure 4A, 20, and 21). These long-lived $CD8^+$ T cells were able to respond to

re-stimulation with LCMV peptides by producing high levels of effector cytokines (Figure 19). When dnTGFBRII mice were re-challenged with a secondary infection with the same chronic virus, the virus was completely eliminated as early as day 5 p.i., indicating that effective memory responses were generated in these animals. The significance of these findings is that by attenuating TGF-β signaling in T cells, vaccine strategies can be designed to target T cell responses and generate long-lived antigen-specific $CD8^+$ T cells that can protect from re-infection. If such strategies are found to be effective, vaccines can be developed not only to combat chronic infections but can also be used to combat pathogens that elicit acute but dangerous infections. One factor to consider is whether antigen-specific T cells can be specifically targeted and whether blocking TGF-β signals can affect autoreactive cells. In addition, caution needs to be considered as to whether keeping these activated T cells alive for longer periods can potentially have negative consequences for the host. At least in dnTGFBRII mice, we observed no significant immunopathology at day 10 p.i. (Figure 22 and 23), and dnTGFBRII continued living long after the infection, however dnTGFBRII mice develop autoimmunity later in life. This does not mean however, that these dnTGFBRII mice that resolved the infection did not develop more signs of autoimmunity than uninfected dnTGFBRII aged-matched mice. We examined immunopathology at a relatively early time after infection, it would be important to determine whether more or less immunopathology exists in dnTGFBRII mice that resolved the infection vs uninfected dnTGFBRII mice.

Which is the relevant TGF-β source during LCMV Cl 13 infection?

We found that TGF-β production during Cl 13 infection occurred in B and T cells, as well as in dendritic cells and macrophages (Figure 1A and B). More importantly, we found that during Cl 13 infection, virus-specific $CD8^+$ T cells were an important source of TGF-β production (Figure 1C). A separate study found a correlation of increased TGF-β serum levels with antigen-specific $CD8^+$ T cell expansion during *Listeria monocytogenes* infection (177). We evaluated whether T-cell derived TGF-β was the critical source inducing T cell deletion during persistent LCMV infection. We found that in the absence of T cell produced TGF-β, the proportion of virus-specific $CD8^+$ T cells in blood is increased at day 7 p.i. (Figure 30). At later time points however, virus-specific $CD8^+$ T cell numbers in spleens of mice that lack T cell-derived TGF-β are identical to WT (Figure 31) and no differences in survival and proliferation were observed (Figure 32). Furthermore, $CD8^+$ T cells in both groups of animals were equally dysfunctional, had similar PD-1 surface expression (Figure 33). More importantly was the fact that TGF-β signaling was detected in mice that lacked T cell-derived TGF-β (Figure 33D). These findings showed that while virus-specific $CD8^+$ T cells are an important relevant source of TGF-β early during infection, there are many other potential sources of TGF-β that can continue signaling on T cells to maintain similar numbers of virus-specific T cells as in WT mice after Cl 13 infection. From these studies it seems that there is not one specific TGF-β cellular source during Cl 13 infection that is important in limiting T cell responses in vivo. We examined TGF-β in this restricted population of immune cells and

detected the presence of this protein. It is possible that other immune cells are producing TGF-β during Cl 13 infection. During development and under steady-state conditions, many cells are able to produce TGF-β (195, 196) and it would not be surprising to discover TGF-β production in other cell types during Cl 13 infection. In addition, the activation mechanisms leading to release of bioactive TGF-β that can now signal in virus-specific $CD8^+$ T cells must be further examined. TGF-β production and activation mechanisms are intricately linked, whether these mechanisms are dysregulated during Cl 13 infection need to be further evaluated. It is also possible that in specific cell types, TGF-β can have opposing effects during uninfected vs infected state, but future studies are needed to examine whether this difference (survival vs proliferation) can be beneficial or detrimental to immune responses during chronic infections in mice and humans.

Complete TGF-β deletion does not prevent virus-specific T cell deletion and viral persistence

We evaluated whether complete temporal deletion of TGF-β could prevent death of virus-specific T cells and reduce viral levels (Figure 35 and 36). Since TGF-β germline deficient mice die from multi-focal inflammation (112), we used TGF-β $^{f/n}$ x $ERCre^+$ mice to temporally delete this gene. We obtained complete deletion in processed spleen mononuclear cells and enriched $CD8^+$ T cells before infection (Figure 36A). We observed no differences in the numbers of virus-specific $CD4^+$ and $CD8^+$ T cells in WT and TGF-β deficient mice (Figure 35A). Furthermore, virus-specific $CD8^+$ T cells produced similar levels of effector

cytokines and had similar levels of PD-1 (Figure 35B and C). We observed no differences in viral titers at day 8 p.i. in WT vs TGF-β deficient mice (Figure 36A). We examined TGF-β signaling in enriched $CD8^+$ T cells and observed phosphorylated Smad-2 proteins, indicating continued signaling through this pathway. These findings indicated that in the absence of detectable TGF-β_1 levels during infection, virus-specific $CD8^+$ T cell numbers are not enhanced, PD-1 levels and function are similar, and enriched $CD8^+$ T cells in the absence of TGF-β_1 continue to have phosphorylation of Smad-2 proteins. This result was unexpected, as we believed that in the absence of TGF-β_1, smad-2 phosphorylation would be completely abrogated (101). These findings raise the possibility that other compensatory mechanisms can be effective in the absence of TGF-β_1. It is possible that TGF-β_2 or TGF-β_3 levels are elevated in these animals to compensate for the lack of the TGF-β_1 isoform, as a consequence, these other isoforms continue signaling through TGF-β receptors and induce phosphorylation of Smad-2. Another possibility is that other TGF-β family members, such as activins which can signal through Smad-2 phosphorylation (197), can be activated in the absence of TGF-β_1 and compensate and perhaps induce a negative signal in T cells. It is also possible that while TGF-β_1 is completely eliminated from processed splenocytes, latent TGF-β can be found to the latent TGF-β binding protein which localizes this complex in the extracellular matrix, this “reserves” of TGF-β can be enough to signal in virus-specific $CD8^+$ T cells to limit their expansion during Cl 13 infection. These findings highlight the

complexity of the TGF-β signaling pathway during Cl 13 infection and indicate that a further understanding of the intrinsic TGF-β -dependent gene regulation program is required to understand whether there are potential gene and protein candidates that can be further evaluated for their therapeutic potential.

Therapeutic potential of blocking TGF-β in vivo during Cl 13 infection

In vivo Inhibitory molecule blockade has been shown to successfully restore T cell responses and reduce viral loads (35, 39, 42, 63). We examined whether the administration of TGF-β neutralizing antibody, which blocks activity of all three TGF-β isoforms in vivo (198), could rescue virus-specific $CD8^+$ T cell numbers and reduced viral titers during chronic LCMV infection (Figure 37). We monitored the $CD8^+$ T cell response in blood and did not observe enhanced numbers or function of virus-specific $CD8^+$ T cells (Figure 37A and C). In addition, virus-specific $CD8^+$ T cells from untreated or antibody treated mice had identical PD-1 expression throughout the course of the infection (Figure 37D). Not surprisingly, viremia in these antibody-treated animals was identical to untreated-mice (Figure 37B). Our findings indicated that in vivo TGF-β blockade did not rescue T cell numbers, as we observe in dnTGFBRII mice. This could be due to several possibilities, because TGF-β signaling occurs in the very local environment and requires cell-to-cell contact (75, 151, 152, 198), perhaps as TGF-β is being produced by T cells, it is rapidly activated and binds to TGF-β receptors on T cells, this rapid activation and signaling prevents neutralizing antibodies from binding to TGF-β and having an effect. A study showed that T

regulatory cells express glycoprotein A repetitions predominant (GARP) on their surface, which can bind latent TGF-β (199, 200). Latent TGF-β retention by GARP on regulatory T cells can therefore be a mechanism by which TGF-β signaling is achieved on virus-specific $CD8^+$ T cells during infection. The antibody can potentially block circulating TGF-β levels, but these TGF-β "reservoirs" on T regulatory cells are sufficient to induce signaling in T cells during cell-to-cell contact. Another possibility is that perhaps TGF-β serum circulating levels are highly elevated during Cl 13 infection, the antibody neutralizes this circulating TGF-β, but fails to neutralize the very locally-produced TGF-β that signals in T cells while these are in tissues. We did not determine whether TGF-β signals as mediated by Smad-2 phosphorylation were reduced in T cells from antibody-treated mice. It is possible that as TGF-β is neutralized, again other compensatory mechanisms continue signaling in these T cells preventing the expansion we observe when these cells express the dnTGFBRII. These findings however, show that manipulating the TGF-β pathway for therapeutic purposes not only poses the challenge of leaving autoreactive T cells unaffected, but also shows the difficulty of targeting this TGF-β pathway specifically in virus-specific $CD8^+$ T cells. Nevertheless, the TGF-β -dependent gene and proteins that are being differentially regulated in virus-specific $CD8^+$ T cells to induce their death need to be explored as these can be potential candidates to specifically target these cells during infection.

SUMMARY

As with many highly tuned biological processes, alteration of TGF-β–Smad signaling has been associated with tumorigenesis, atherosclerosis, and autoimmunity, but its role in regulating T cells responses and viral persistence during *in vivo* chronic infection remained unknown. Here, we report that virus-specific $CD8^+$ T cells exhibited increased Smad-2 phosphorylation and TGF-β expression during chronic compared to acute viral infection *in vivo*. Selective attenuation of TGF-β pathway in T cells decreased the expression of the pro-apoptotic protein Bim and increased survival and numbers of virus-specific $CD8^+$ T cells. Under these conditions, $CD8^+$ T cells exhibited enhanced cytotoxicity, increased production of anti-viral cytokines, and down-regulation of the inhibitory molecules, PD-1 and IL-10. Notably, while direct TGF-β signaling in virus-specific $CD8^+$ T cells was required for their increased apoptosis and reduced numbers, it was not essential for their functional exhaustion and PD-1 up-regulation. The end result was rapid virus eradication and generation of an effective memory T cell response that protected the host upon subsequent challenge. The potent T cell responses and rapid clearance of a persistence-prone virus that we described upon genetic attenuation of TGF-β signaling in T cells not only brings to light a novel molecular mechanism by which persistent viruses curtail immune responses but also offers promising novel opportunities for the much-needed improvement of treatment for chronic viral diseases.

Chapter 3 contains portions of the material as it appears in the journal Immunity, “Cell-intrinsic Transforming Growth Factor-β signaling mediates virus-specific CD8+ T cell deletion and viral persistence *in vivo*.” Tinoco, Roberto, Alcalde Victor, Yang, Yating, Sauer, Karsten, Zuñiga, Elina I., July 2009 Jul 17;31(1):145-57. The dissertation author is the first author of this paper.

CHAPTER 4 MATERIALS AND METHODS

Mice and viruses

dnTGFBRII mice (101) were purchased at The Jackson laboratory or generously provided by Dr. Richard Flavell (School of Medicine, Yale University). C57BL/6 mice or control littermates were used as WT controls. C57BL/6 CD45.1$^+$ and D^bGP_{33-41} TCR-tg (P14) mice were a generous gift from Dr. Stephen Hedrick (University of California San Diego-UCSD). Mice were bred and maintained in a closed breeding facility and mouse handling conformed to the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of UCSD. Unless otherwise stated, mice (6-8 weeks) were infected intravenously (iv) with 2 x 10^6 PFU of LCMV ARM or Cl 13. Re-challenge with LCMV Cl 13 was performed with the same dose and route of infection as primary infections. Viruses were grown, identified and quantified as described (11, 201).

To delete TGF-β in T cells, mice that have TGF-β flanked by loxp sites (TGF-β $^{f/n}$) were bred with mice expressing Cre under a CD4 promoter. Mice used in these studies were between 6-8 weeks of age. Mice were bled before infection to examine CD44 and CD62L expression on CD4$^+$ and CD8$^+$ T cells. Mice were infected with Cl 13 and T cell responses examined at day 7 p.i. in blood and day 9 p.i. in spleen. Total CD4$^+$ and CD8$^+$ T cells were FACS sorted from both groups to examine TGF-β and phospho-Smad-2 levels by immunoblot.

Mice that have TGF-β flanked by loxp sites (TGF-β $^{f/n}$) were also bred with estrogen receptor cre mice (ERCre) that expressed 4OH-tamoxifen-inducible cre gene under a ubiquitous Rosa26 promoter. TGF-β $^{f/n}$ x ERCre^{+}(KO), TGF-β $^{f/n}$ x ERCre^{-} (WT heterozygous) and TGF-β $^{f/f}$ x ERCre^{-} (WT) mice were treated with 4-OH-tamoxifen (2 mg/mouse) daily for 5 days, rested and infected with Cl 13. Mice were bled on the same day before infection and blood cells were stained with CD44 and CD62L on CD4^{+} and CD8^{+} T cells to examine activation state. Splenocytes from individual animals from each group were used to examine TGF-β levels by immunoblot. Splenocytes were pooled from each group of infected mice at day 8 p.i. and virus-specific and total CD8+ T cells were FACS sorted and examined for TGF-β and phospho-Smad-2 levels by immunoblot.

Adoptive transfer and CD4^{+} T cell depletion

Where indicated CD8^{+} T cells were purified from the spleens of naive P14xWT (CD45.1^{+}CD45.2^{+}) and/or P14xdnTGFBRII (CD45.2^{+}) mice by negative selection (StemCell Technologies), and equal number (1-10 x 10^{3}) Vβ8^{+} cells from each population were adoptively transferred iv into C57BL/6 CD45.1^{+} recipient mice 1-2 days before LCMV Cl 13 infection. Where indicated, P14 cells were purified after the infection by FACs sort as described before (202). For CD4^{+} T cell depletion, dnTGFBRII mice were intraperitoneally injected with control rat IgG or anti-CD4 (clone GK1.5; 300 μg/mouse) at day 0, 2.5 and 9 p.i. This treatment resulted in >95% reduction in the numbers of CD4^{+} T cells at day

5 and 10 p.i. For $CD8^+$ depletion mice were injected with anti-CD8+ (clone 53-6.72; 200 μg/mouse) at day -2, -1, 0, and 5 after infection.

Generation of mixed BM chimeras

To obtain mixed BM chimeras, WT $CD45.1^+$ C57BL/6 recipient mice were lethally irradiated with 1000 rads and reconstituted one day after with a mixture of BM cells from $CD45.1^+$ WT mice and $CD45.2^+$ dnTGFBRII mice. Bone-marrow cells were isolated from femurs and tibia of donor mice and 10 million total cells were i.v. transferred into the irradiated recipient mice. Recipient mice were treated with antibiotics (Trimethoprim 8 mg/ml and Sulfamethoxazole 40 mg/ml supplied in the drinking water) for three weeks to prevent infection and allow immune reconstitution. Reconstitution was analyzed 6-8 weeks after bone-marrow transfer and the ratio of WT-dnTGFBRII cells was determined to be ≤1 for $CD8^+$ T cells. At this point mice were infected with LCMV Cl 13 as indicated above.

$CD8^+$ T cell proliferation and survival in uninfected mice

Splenocytes were obtained from WT or dnTGFBRII mice or from P14 Tg WT or P14 Tg dnTGFBRII mice at ~7 weeks of age. Mice were IP injected with 2 mg BrdU dissolved in 200uL of PBS 16 hours prior to sacrificing. Proliferation was examined on stained $CD8^+$ T cells or in H2-D^b-$GP_{33-41}{}^+$ $CD8^+$ T cells by staining using BrdU Flow kit (BD Biosciences) following the manufacturer instructions. Survival in these subsets was examined by Annexin V staining (BD Biosciences). As indicated below in Ex-vivo T cell stimulation methods, CD8+ T

cells were stimulated with PMA and ionomycin or GP33-41 LCMV peptides (P14 Tg cells) for 5 hours and intracellularly stained for production of IFN-γ, TNF-α, and IL-2. Activation state was examined by staining with CD44, CD62L, CD25, and CD69 antibodies.

TGF-β levels in different cell types

WT mice were infected with LCMV ARM or Cl 13. Splenocytes were isolated at day 9 p.i. from a total of 5 mice in each group of infected animals as well as uninfected mice. Splenocytes were treated with collagenase, pooled and processed in PBS with 0.1% BSA to control for any TGF-β protein that is found in FBS. Single-cell suspensions were generated and cells stained with appropriate antibodies to FACS sort CD19+, $CD4^+$, $CD8^+$, $CD11c^+$, and $CD11b^+$ cells. Purity of sorted cells was confirmed in a separate LSRII flow cytometry machine (BD, San Jose), cells were centrifuged and supernatant removed. Dry pellets were stored at -80°C until these were used for immunoblot analysis.

TGF-β blockade using neutralizing antibodies

WT mice were infected with LCMV Cl 13 and divided into two groups (5 mice per group). One group was IP injected with 1 mg of anti-TGF-b antibody (clone 2G7 provided by Genentech, San Francisco) dissolved in 200 uL of PBS at days 4 through 7 p.i. while the other group was untreated. T cell responses and viral titers were determined in blood at days 7, 9, 15, and 32 p.i.

Flow cytometry

The following antibodies purchased from E-bioscience or BD-bioscience were used to stain blood or spleen cells: anti-IFN-γ-APC, anti-TNF-α-FITC, anti-IL-2-PE, anti-PD-1-PE, anti-Annexin-V-PE, anti CD45.1-PECy7, anti-CD45.2-APC-Alexa-750, anti-CD44-PECy7, anti-CD62L-FITC, anti-CD4-Alexa-700, anti-CD127-PE, Anti-CD122-FITC, anti-TGFBR-PE, anti-Ly6C-biotin, Streptavidin-PercP-Cy5.5 and isotype-control-IgG-PE. Anti CD8-pacific blue was purchased from Caltag. Anti p-Smad-2-3 (Ser 423-425) was purchased from Santa Cruz Biotechnology and its control goat IgG from Jackson Immunoresearch. For staining with IA^b-GP_{66-77}, D^b-$NP_{396–404}$, D^b-$GP_{276–286}$ (provided by NIH Tetramer Core Facility; Atlanta, GA) or D^b-$GP_{33–41}$ (Beckman Coulter; Fullerton, CA) tetramers, cells were incubated for 1 h and 15 min at room temperature. To quantify incorporation of BrdU by tetramer$^+$ $CD8^+$ T cells, mice were injected with 2 mg of BrdU (Sigma-Aldrich) 16 h before analysis and splenocytes stained with BrdU Flow kit (BD Biosciences) following the manufacturer instructions. Cells were acquired using the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed with FlowJo software.

Ex vivo-T cell stimulation

Splenocytes were stimulated with 2 μg/ml of the MHC class-I-restricted LCMV $NP_{396–404}$, $GP_{33–41}$ or $GP_{276–286}$ peptides (all >99% pure; Synpep) in the presence of 50 U/ml recombinant murine IL-2 (R&D Systems) or PMA (10 ng/ml) and ionomicyn (0.5 μg/ml). Cells were cultured for 5 h in the presence of brefeldin A (1 μg/ml; Sigma) and stained for surface expression of CD8, fixed,

permeabilized and stained with Abs to IFN-γ, TNF-α and IL-2. To evaluate cell degranulation splenocytes were incubated in the presence of anti-CD107a-FITC and anti-CD107b-FITC (BD-Biosciences). All cultures without peptide performed in parallel show no production of cytokines or degranulation.

^{51}Cr release assays

LCMV-specific CTL activity was evaluated in splenocytes isolated at day 9 p.i. by standard ^{51}Cr release assay. MHC-matched MC57 (H-2^{b}) cells either unloaded or loaded with 1 μg/ml of LCMV $NP_{396–404}$ or $GP_{33–41}$ were used as targets. Cells were mixed at a 50:1 and 100:1 effector/target ratio. Samples were performed in triplicates and ^{51}Cr release was measured in the supernatant after 5 h as previously described (201).

Immunofluorescence Microscopy

Spleens were removed, frozen in OCT, and cut in 6-μm sections with a Leica CM3050 S Cryostat. Slides were fixed with 4% PFA for 4 min, washed in PBS, and then incubated for 1 h in 5% Normal Donkey Serum flowed by 1h incubation at RT with a guinea pig anti-LCMV Ab (1:1,500). Tissues were washed and incubated for 1 h at RT with a FITC labeled anti-guinea pig Ab (Jackson Immuno; 1:200). Fluorescence was captured at 10x using an Olympus DSU Disk Scanning Confocal Microscope, and a montage was created using SlideBook software.

SDS-PAGE and Immuno-blotting

Cells were lysed in a Ripa buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Calbiochem). Protein homogenates were run on 4-12% SDS-PAGE gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore) using a semidry transfer cell (Bio-Rad). Blots were blocked in blocking buffer (Phosphate buffer saline (PBS) containing 0.1 % tween-20 and 5% non-fat milk) and incubated with primary anti-pSmad-2, anti-Smad-2-3, anti-TGF-β_1, anti-Bim or anti-phospholipase C γ (PLC-γ) mAbs (All from Cell Signaling; 1/1000 in blocking buffer) at 4°C overnight, or at room temperature for 2 h. HRP-conjugated anti-rabbit IgG (Cell Signaling; 1/5000 in blocking buffer) was then added for 45 min at room temperature and ECL (GE Healthcare) was used to visualize the proteins.

Histology and biochemical blood tests

Liver, lung and stomach were obtained and fixed in 10% formalin, stained with hematoxylin-eosin and processed for histopathological analysis at the Histology and Immunohistochemistry Shared Resource (UCSD). For evaluation of AST, ALT, AP, Glucose, BUN and creatinine, plasma samples were processed at the UCSD Chemical and Coagulation Core Laboratory using a Beckman CX-7 analyzer.

Real-time RT-PCR

Total RNA was extracted from splenocytes using RNeasy kits (Qiagen), digested with DNase I (RNase-free DNase set; Qiagen) and reverse transcribed

into cDNA. cDNA quantification was performed using SYBR Green PCR kits (Applied Byosistems) and a Real-Time PCR Detection System (ABI). The RNA levels of the IL-10 gene (Forward: GGT TGC CAA GCC TTA TCG GA; Reverse: ACC TGC TCC ACT GCC TTG CT) were normalized to cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA levels.

Statistical analysis

Unpaired student's t-tests or ANOVA tests were performed using the InStat 3.0 software (GraphPad, CA.).

Chapter 4 contains portions of the material as it appears in the journal Immunity, “Cell-intrinsic Transforming Growth Factor-β signaling mediates virus-specific CD8+ T cell deletion and viral persistence *in vivo*.” Tinoco, Roberto, Alcalde Victor, Yang, Yating, Sauer, Karsten, Zuñiga, Elina I., July 2009 Jul 17;31(1):145-57. The dissertation author is the first author of this paper.

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