University of California, San Diego

Transforming Growth Factor-beta mediated suppression of cellular response to chronic viral infection

A Dissertation in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Gavin M. Lewis is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

Thanks MOM!!

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LIST OF ABBREVIATIONS

Dendritic cells (DC)

- Conventional (cDC)
- Plasmacytoid (pDC)

Natural Killer cells (NK)

Pattern Recognition Receptors (PRRs)

Toll-Like Receptors (TLR)

Retinoic acid Inducible gene-I-like Receptors (RLR)

Type I Interferons (IFN-I)

Major histo-compatibility (MHC)

Human Immunodeficiency virus (HIV)

Hepatitis C virus (HCV)

Hepatitis B virus (HBV)

Lymphocytic choriomeningitis virus (LCMV)

- Armstrong 53b (ARM)
- Clone 13 (CL13)

Human Cytomegalovirus (HCMV)

Murine Cytomegalovirus (MCMV)

Latency Associated Protein (LAP)

Transforming Growth Factor-Beta (TGFß)

Transforming Growth Factor-Beta Receptor 2 (TGFßRII)

dominant negative TGFβ-receptor transgene (CD11c-dnTGFβRII)

intraperitoneal (i.p.)

intravenously(i.v)

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PUBLICATIONS

Lewis GM, Macal M, Hesser CR, Zuniga EI (2015) Constitutive but not inducible attenuation of transforming growth factor beta signaling increases natural killer cell responses without directly affecting dendritic cells early after persistent viral infection. *Journal of virology 89: 3343-3355*.

Macal M, **Lewis GM**, Kunz S, Flavell R, Harker JA, et al. (2012) Plasmacytoid dendritic cells are productively infected and activated through TLR-7 early after arenavirus infection. *Cell host & microbe 11: 617-630*.

Harker JA, **Lewis GM**, Mack L, Zuniga EI (2011) Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science 334: 825-829*.

Sohn SJ, **Lewis GM**, Winoto A (2008) Non-redundant function of the MEK5-ERK5 pathway in thymocyte apoptosis. *The EMBO journal 27: 1896-1906*.

Lipinski MJ, Amigues Y, Blasi M, Broad TE, Cherbonnel C, et al. (2007) An international parentage and identification panel for the domestic cat (Felis catus). *Animal genetics 38: 371-377.*

FIELDS OF STUDY

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Studies in Immune response to pathogens
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ABSTRACT OF THE DISSERTATION

Transforming Growth Factor-beta mediated suppression of cellular response to chronic viral infection

Gavin M. Lewis

Doctor of Philosophy in Biology

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Professor Elina Zuniga, Chair

Chronic viral infections remain a global health burden. Here we study the role of immunosuppressive Transforming Growth Factor-Beta (TGFß) signaling on the immune response to different chronic infections in mice. We use genetic systems to modulate TGFßRII and SMAD4 adapter availability in a cell type specific and inducible manner similar to therapeutic approaches. We found that TGFß signaling minimally influenced Dendritic cell or Natural Killer cell responses early during infections. We further found TGFß signaling did not alter antiviral CD8 T cell number and function but did however suppress CD4 T cell differentiation during chronic viral infection. TGFß signaling on any of these cell types alone did not promote viral persistence. Importantly, we found TGFß/SMAD4 signaling did prevent hematopoietic-based pathology during chronic infection, which should be considered in therapy design to combat chronic infections.

Introduction

Chronic viral infections are a significant global health burden.

Approximately 33 million people are infected with Human Immunodeficiency virus (HIV-1 or -2), 170 million with Hepatitis C Virus (HCV) and 350 million with Hepatitis B Virus (HBV), many who progress to severe disease pathology. Herpes viruses and human cytomegalovirus (HCMV) reach up to %50 prevalence in human populations and can cause serious complications in immune-compromised individuals, such as HIV infection or cancer patients.

Rapid viral dissemination has been associated with low antiviral host responses and viral persistence in both mice and humans (1, 2). Indeed, the coordinated magnitude of both innate and adaptive immune responses can be critical to limit the establishment and maintenance of potentially chronic infections. Chronic viral infections in both mice and humans then create an immunosuppressive environment detrimental to both ongoing antiviral responses as well as secondary immune responses to unrelated pathogens or cancers (3, 4).

The innate immune response is an immediate host reaction, comprised of mainly dendritic cells (DC), Natural Killer cells (NK), and macrophages, utilizing generalized Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLR) and Retinoic acid Inducible gene-I-like Receptors (RLR) for DCs, or activating and inhibitory self-ligands for NK cells, to recognize invading pathogens (5, 6). Activation of innate cells triggers the release of antiviral cytokines including Type I Interferons (IFN-I) and pro-inflammatory cytokines to limit viral replication, and alongside antigen presentation by Dendritic cells, promotes the adaptive immune response (7-11).

The adaptive immune system responds by clonally expanding lymphocytes containing highly specific receptors against foreign pathogens to potently to eliminate viruses and microbes, all while maintaining self-tolerance and avoiding immune mediated pathology. Major histo-compatibility (MHC) haplotypes in unique DC subsets present specific antigens on either MHC-I or MHC-II molecules to activate CD8 cytotoxic or CD4 helper T cells(12). Native antigen recognized by B cells also generates many neutralizing antibody, some requiring CD4 T cell help to undergo affinity maturation(13). Indeed CD8 T cells, CD4 T cells, and B cell are necessary to control chronic infections (14-20).

Models of Chronic Infection.

Lymphocytic Choriomeningitis Virus (LCMV) is a negative sense single strand RNA virus of the Arenaviridae family and is a natural mouse pathogen and model of persistent replicating viruses such as HIV and HCV infections. In addition, members of this family of viruses are responsible for significant disease in humans(21-23). One strain of LCMV, Armstrong 53b (ARM), efficiently primes adaptive CD8 cytotoxic T cells leading to viral clearance and memory T cell development. The Clone13 (Cl13) strain of LCMV via only two amino acid substitutions has expanded cell tropism, pronounced reduction of antiviral T cells, and persists as a continuously replicating virus in the host (24-28). This replication continuously subverts both innate Dendritic cell and adaptive T cell responses (29-32), the nature of which is an active area of research.

Human cytomegalovirus (HCMV) lays dormant in a latent phase to hide from immune surveillance and maintain low level persistence(33). MCMV is a natural

mouse model to study responses to CMVs. Early during MCMV infection innate responses such as NK cells and Dendritic Cell Interferon production are critical to eliminate pathogen and survive acute infection (1, 34-37). Upon establishing latency in the salivary gland, viral re-activation is controlled by CD8 and CD4 T cell adaptive immune responses (38).

CD8 T cell response to chronic infection.

$CD8^+$ T cell responses to persistent replicating viruses are required for ultimate control of pathogens. Indeed, studies in LCMV chronically infected mice as well as SIV and HBV infected monkeys have shown that CD8 T cells are able to control viral replication without causing excess immunopathology (39-42). T cell hypo-responsiveness has been termed “exhaustion” and is a distinctive feature in numerous chronic viral infections from mice to humans(43).

Exhaustion was originally defined in LCMV Cl13 as a progressive loss of antiviral functions, with first IL-2, then TNFα and finally IFNγ secretion being lost upon cognate peptide recognition (4, 44), Exhausted T cells also express many inhibitory surface receptors, including PD-1, LAG3, TIM3, and 2B4 that negate specific functions upon antigen encounter (31, 45-48). In addition, degranulation and overall cytotoxic capability are lower in exhausted compare to effector or memory T cells (44, 49, 50). Importantly therapeutic blockade of inhibitory receptors can restore T cell function(51, 52).

Exhaustion is also defined on a transcriptional level, first in LCMV, and subsequently HIV (31, 53, 54) as transcriptionally distinct from the other forms of T cell hypo-responsiveness, senescence and anergy (55), memory (53) or continuously

stimulated effector memory CD8 T cells (56). Exhausted CD8 T cells are characterized by unique transcription factor milieu of reduced expression of Tbet and up-regulation of Eomes, Blimp1, and BATF (31, 57, 58). During an acute infection subsets of $CD8^+$ T cells with memory potential or terminally differentiation can be distinguished by CD127 and KLRG1 expression respectively, with the transcription factor Tbet driving effector differentiation (59). Chronic infection leads to a population that is devoid of the $KLRG1^+$ Short-lived effector T cells, yet the cells also fail to upregulate IL-7R (CD127) to form memory, which results in an $Eomes^{hi}$ $Tbet^{lo}$ antigen dependent cellular phenotype(4). Importantly CD4 T cell as well as innate DCs and NK cells shows signs of dysfunction during chronic infection (30, 32, 60).

CD4 T cell response to chronic infection.

Subsets of $CD4^+$ helper T cells differentiate into Th1 cells and have the capacity to sustain and enhance both $CD8^+$ T cells via IFNγ and IL-2 production. Alternatively CD4 T cells aid the humoral antibody response through T follicular helper cell expression of CXCR5, BCL6 and IL-21 production(61). $CD4^+$ T cell help is a critical component of the anti-viral immune response during chronic, but not acute LCMV infection(62, 63). Enhanced virus specific $CD4^+$ T cell responses early after HIV, HBV or HCV infection correlate with viral control (64-66). Similarly $CD4^+$ T cells promote control of LCMV in mice, as CD4 depleted mice fail to control chronic LCMV infection in mice (18, 67, 68).

$CD4^+$ T cells also exhibit altered function during chronic infection including loss of IL-2, TNF-α and IFN-γ production (60, 62, 67). Indeed comparative analysis of antigen specific $CD4^+$ and $CD8^+$ T cells described a common core exhaustion gene

signature during chronic LCMV(69). Persistent antigen also drives CD4 differentiation towards a follicular T cell helper phenotype characteristic high PD1 as well as CXCR5, ICOS, and BCL6 to drive germinal center B cell and antibody responses(70). However there remains a late emergence of antibody mediated immunity during chronic infection(17, 71).

TGFß signaling in the immune system.

In addition to antigen driven exhaustion, chronic infection drives a cellular environment with dynamic waves of inflammation(72), devoid of select stimulatory cytokines, yet contains increased inhibitory cytokines such as IL-10 and Transforming Growth Factor-Beta (TGFß) that inhibit immune responses (73-76). Indeed increased TGFß production and signaling is present in immune cells during human chronic infections with Hepatitis C, HIV, and Tuberculosis as well as mouse models of infections with Lymphocytic Choriomeningitis Virus (LCMV), Malaria, or Listeria Monocytogenes (74, 77-82). In addition, influenza Neuraminidase protein has evolved to activate latent TGFß (83). However the role of TGFß signaling on individual cells types at different times after infection in-vivo remains unclear.

TGFß is a pleiotropic cytokine with critical roles in the development of the hematopoietic system indicated by mouse genetic knockouts of the receptor subunits, RI and RII (84, 85). TGFß is normally produced as an inactive complex associated with Latency Associated Protein (LAP). Upon cleavage by a variety of proteases and integrins in-vitro TGFß is available to signal through its receptor. Downstream TGFß signaling is typical mediated by canonical phosphorylation of SMAD2/3 with adapter SMAD4 or TIF1γ as well as phosphorylation of MAP kinase pathways in different

settings (86-88). SMAD proteins have weak DNA binding affinity and associate with different transcription factors to regulate unique target genes in every cell type(89).

TGFß1-null mice or T cell specific targeting of TGFß-RII deficiency during development results in lethal multifocal inflammatory diseases by 3-4 weeks of age that is CD4 T cell dependent (90-94). In contrast deletion of TGFβRII in post-thymic T cells using dLCK-cre model or in adult CD4 T cells using an inducible CD4 specific (ER)Cre system did not lead to colitis or wasting syndrome in-vivo (95, 96). T cells are a main target but TGFβ has been shown to also suppress cells of the innate immune system that lead to autoimmunity (97-100).

TGFß mediated therapies.

Importantly, long-term treatment of mice or humans with TGFß antagonists does not lead to the severe autoimmune phenotype of TGFß deficiency during development (101, 102). On the other hand, TGFß antagonists have been used successfully in animal cancer models of melanoma and breast cancer via CD8 T cell activity and are currently in use in clinical trials to boost intra-tumor responses in humans (103-105). Furthermore, we and others have reported that T-cell-specific dominant negative TGFß receptor transgenic mice exhibit increased pathogen and tumor specific CD8 T cell responses (74, 80, 106), a result only modestly replicated by initial therapeutic attempts of TGFß antagonism during chronic infection (107, 108). Hence, the developmental abnormalities in T cells has precluded analysis of the direct effects of TGFß signaling in-vivo on individual cell types, especially during immune response to pathogens.

Here we utilized advanced genetic models that allow cell-type specific and temporal ablation of TGFß/SMAD signaling in adult mice in order to establish the role of TGFßR signaling on 3 distinct inflection points of the immune response: Early DC and NK innate responses, adaptive CD4 and CD8 T cell responses, and finally late hematopoietic resolution of inflammation during chronic viral infection. The results are presented here as three respective chapters.

First, TGF-βR deficient mice showed comparable early innate NK and DC responses to both LCMV and MCMV pathogens by inflammatory cytokine production as well as surface activation markers and underlying transcription factor networks.

Second, while TGFßR deficient animals contained similar adaptive CD8 T cell responses; we found enhanced CD4 T cell proliferation, terminal differentiation and a cytotoxic program characterized by Granzyme B and K, Perforin and transcription factor EOMES expression in the absence of cell-intrinsic TGF-βR signaling in both LCMV and MCMV infections.

Strikingly, SMAD4 deficiency gave opposing results. Importantly, TGFβ signaling was continuously necessary late during chronic LCMV infection to suppress Eomes and terminal differentiation in CD4 T cells. EOMES overexpression was sufficient to recapitulate the terminally differentiated phenotype of TGFßR deficient CD4 T cells.

Third and finally, we show TGFβ/SMAD4 signaling in hematopoietic cells is necessary at both early and late time points during chronic inflammation to prevent lethal pathology upon chronic but not acute infections, a result not dependent on TGFß signaling in T cells. Overall our data highlight several important roles for TGFß signaling during immune response to chronic viral infection.

Chapter 1: TGFβ and Innate immune responses to chronic infection

Rapid innate responses to viral encounters are crucial to shaping the outcome of infection, from viral clearance to persistence. Transforming Growth Factor-Beta (TGFβ) is a potent immune suppressor that is up-regulated early upon viral infection and maintained during chronic infections in both mice and humans. However, the role of TGFβ signaling in regulating individual cell types *in vivo* is still unclear. Using two different infections with potentially persistent viruses, Murine Cytomegalovirus (MCMV) and Lymphocytic Choriomeningitis Virus (LCMV Cl13), in their natural rodent host, we observed that TGFβ signaling on Dendritic cells (DCs) did not dampen DC maturation or cytokine production in the early stages of either chronic viral infection *in vivo*. In contrast, TGFβ signaling prior to (but not during) chronic viral infection directly restricted Natural Killer (NK) cell number and effector function. This restriction likely compromised both early control and host survival upon MCMV infection but not long-term control of LCMV infection. These data highlight the context and timing of TGFβ signaling on different innate cells that contribute to the early host response, which ultimately influences the outcome of chronic viral infection *in vivo*.

Introduction

The coordinated effort of both the innate and adaptive immune system is necessary to control invasive pathogens. Rapid viral dissemination has been associated with low antiviral host responses and viral persistence in both mice and humans (1, 2). Thus the magnitude of initial innate responses can be critical to limit the establishment of potentially chronic infections. Human chronic infections with hepatitis C virus (HCV), Hepatitis B (HBV), human immunodeficiency virus (HIV), and Tuberculosis as well as mouse infections with Lymphocytic Choriomenigitis virus (LCMV) and Malaria are associated with increased production and/or signaling of the immunosuppressive molecule TGFβ in immune cells (74, 76-79). However the precise role of TGFβ-mediated suppression on innate and adaptive immune components during infection is not fully deciphered.

Among innate cells, dendritic cells (DCs) and natural killer (NK) cells play pivotal roles in anti-viral defense. DCs are a heterogeneous population that can be broadly subdivided into conventional (c) and plasmacytoid (p) DC subsets. cDCs are required for priming antigen specific T cells, while pDCs are specialized to produce large amounts of type I interferons (IFN-I), a group of potent anti-viral mediators (109-111). On the other hand, NK cells sense high levels of activating receptor and/or decreased levels of inhibitory receptors in virally infected cells to induce cell death through the release of ready-made cytotoxic granules containing granzymes and perforin (5). DC-derived cytokines drive maturation of NK cells, including upregulating transcription factors TBET and BLIMP1 to enhance cytotoxic effector functions during inflammation (112). NK cells can also kill DCs and T cells, to limit

antiviral immune responses or pathology, establishing a fluid cross talk with other immune cell populations (34, 113, 114).

Transforming growth factor beta (TGFβ) is an important negative regulator of the immune system, as best illustrated by rapid death of TGFβ deficient mice due to multi-organ inflammatory disease at 3-4 weeks of age (92). T cells are the main mediators of this pathology (91) but TGFβ has been shown to also suppress cells of the innate immune system. For example, autocrine TGFβ is required for the development of tissue resident DC subtypes including Langerhans Cells and tolerogenic $CD103^+$ DC in the gut (97-99). TGFβ also inhibits IFN-I production by pDCs *ex vivo* (100) and the expression of co-stimulatory molecules MHCII, CD40 and CD80/CD86 in bone marrow derived DCs *in vitro* (115). Notably, DCs from mice infected with a persistent strain of Lymphocytic Choriomenigitis virus (LCMV CL13) have reduced maturation (30) and pDCs produce substantially reduced IFN-I compared to pDCs from non-infected hosts (32) similar to pDCs from HIV infected individuals (116). These studies raised the possibility that TGFβ signaling may limit DC maturation and/or cytokine production during *in vivo* viral infection, attenuating early viral containment and promoting viral persistence.

TGFβ also suppresses NK cell function ex-vivo during HBV, HIV and HCV infection (117-119). Treatment of mice with recombinant TGFβ *in vivo* can suppress early NK cell activation in response to acute infection with parental strain LCMV-Armstong53b (120). Furthermore, Laouar and colleagues used mice expressing a dominant negative TGFβ-receptor transgene in both $CD11c^+$ DCs and NK cells (hereafter referred to as CD11c-dnTGFβRII mice) to show that TGFβ suppresses NK cell maturation in neonatal mice *in vivo*, resulting in enhanced NK cell numbers both

before and after infection with murine cytomegalovirus (MCMV) (121, 122). However, whether TGFβ directly limits innate responses to chronic viral infections in adult mice after infection is unknown.

We therefore set out to determine the role that TGFβ signaling may play on DCs and NK cells during early response to *in vivo* chronic viral infections in two animal models. First, we used CD11c-dnTGFβRII mice to attenuate TGFβ signaling only on DCs and NK cells. Second, we crossed mice with temporally controlled tamoxifen responsive ERcre transgene (123, 124) to mice harboring a conditional TGFβRII flox allele (125) (hereafter referred to as ERcre-TGFβRII). We then evaluated the early innate responses in these genetically modified adult mice following infection with the actively replicating persistent RNA virus LCMV Cl13 and/or the latent DNA virus (MCMV).

We observed that while TGFβ signaling blockade in innate cells indirectly affected DC numbers, cytokine production and maturation, direct TGFβ signaling had minimal or no effects on early DC responses during either MCMV or LCMV infections. In contrast, CD11c-dnTGFβRII adult mice had increased numbers and differentiation of NK cells before and during both MCMV and LCMV infections. This enhanced NK cell response was accompanied by increased viral control and improved survival upon MCMV (but not LCMV) infection. In contrast, deletion of the TGFβRII conditional allele (ERcre-TGFβRII) in adult mice (just prior to infection) did not directly affect either DC or NK cell responses to MCMV. Taken together these data indicate that, while cell-intrinsic TGFβ signaling suppresses NK cell response before infection, its direct signaling after *in vivo* infection with either DNA or RNA persistent viruses does not play a major role in regulating DC or NK responses.

Materials and Methods

Mice and viral stocks. C57BL/6 (CD45.2), B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ (CD45.1) and ERt2-cre mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). CD11c-dnTGFβRII mice on a C57BL/6 background were a kind gift of R. Flavell (Yale University) as described previously (121). Mice were bred and maintained as transgene hemizygotes to C57BL/6 or congenic CD45.1 mice and transgene negative age- and sex-matched littermates used as WT controls. TGFβRII$^{flox/flox}$ mice (126) were generously provided by Dr. M. Li (Sloan Kettering) and crossed to hemizygous ER-cre mice, both on C57BL/6 backgrounds. 1:1 mixed chimeras were generated by transferring 2x10^{6} bone marrow cells of each indicated genotype into irradiated CD45.1 mice and allowed 8 weeks to reconstitute. 1mg/day Tamoxifen (Sigma) emulsified in Sunflower seed Oil (Sigma) was injected *intraperitoneal* (i.p.) for 5 days and mice were used for experiments 7 days after last treatment. Mice were maintained in a closed breeding facility in compliance with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego. 6-12 week old mice were infected *intravenously* (i.v.) with 2x10^{6} plaque forming units (PFU) of LCMV Cl13 or 2x10^{4} or 2x10^{5} PFU of MCMV (Smith strain) i.p. All viruses were grown, identified, and quantified as previously described (24, 127).

Cell Purification. Spleens were treated with collagenase D (1mg/mL, Roche, Indianapolis, IN) 20 min @ 37˚C and were depleted of T and B cells using Thy1.2 and CD19 antibodies and bead magnetic enrichment. Splenic NK cells and pDCs were FACS-purified using a BD ARIA (BD Biosciences, San Jose, CA) for pDCs (CD19^{-}

Thy1.2$^-$NK1.1$^-$CD11c$^+$CD11b$^-$B220$^+$PDCA$^+$) and NK cells (NK1.1$^+$CD11c$^+$ Thy1.2$^-$). For TGFβRII deletion, T cells (Thy1.2$^+$), B cells (B220$^+$), NK cells (NK1.1$^+$ CD11b$^+$), Monocytes (Thy1.2$^-$ B220$^-$ NK1.1$^-$ CD11b$^+$, Ly6C$^+$ Gr1$^-$) and Neutrophils (Thy1.2$^-$ B220$^-$ NK1.1$^-$ CD11b$^+$, Ly6c^{lo}, Gr-1$^+$) were sorted from pooled blood by congenic marker (CD45.1, CD45.2). Purity for all cell types was >95%.

Flow cytometry. Surface and intracellular cytokine staining and gating strategy for DCs was performed as previously described (128). pDCs were gated as (CD19$^-$Thy1.2$^-$NK1.1$^-$CD11c$^+$CD11b^{lo}B220$^+$PDCA1$^+$), cDCs gated as (CD19$^-$Thy1.2$^-$ NK1.1$^-$CD11c$^+$CD11b$^{hi/mid}$ B220$^-$CD8α$^{+/-}$), and NK cells as (CD3$^-$ NK1.1$^+$ DX5$^+$ Ly49H$^{+/-}$) and separated by congenic marker CD45.1 and CD45.2 where indicated. Antibodies used were purchased from Ebioscience or BD Pharmingen (San Diego, CA) to stain splenocytes except LIVE/DEAD (Aqua-Invitrogen) and TGFβRII-PE (R&D systems): CD16/CD32 Fc block, CD19 PerCPCy5.5, Thy1.2 PerCPCy5.5, NK1.1 PE or PE-TR, DX5 PE or PB, CD3 PB, GranzymeB PE, IFNγ APC or Alexa700, Ly49H APC, CD11c APC or PE, PDCA1 FITC, CD11b PECy7 or PB, CD40 PE, PDL1-PE, B220 Alexa780, CD8a Alexa700, IL-12 Alexa647, TNFα efluor450, CD86 PeCy7, IAb FITC or PB, KLRG FITC, CD69 PerCPCy5.5, CD45.1 BV605, CD45.2 BV650. LCMV specific tetramers D^b:GP$_{31-41}$ and I-A^b:GP$_{67-77}$ were provided by the NIH tetramer core facility. Samples were acquired on a BD LSR II (BD biosciences) and analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

Cytokine Measurements. IFN-I bioactivity was measured using L-929 cells transfected with an interferon-sensitive luciferase standardized with recombinant mouse IFNβ (Research Diagnostics, Concord, MA) (129). To measure NK and DC intracellular cytokines by flow cytometry, splenocytes were incubated in the presence

of 1 μg/mL Brefeldin A (BFA-Sigma, St. Louis, MO) without exogenous stimulation for 5 hrs at 37˚C before staining. Secretion of IFNγ, TNF and IL-2 in LCMV specific T cells was performed ex-vivo using GP_{31-41} or GP_{67-77} peptide stimulation and BFA.

Quantitative Real-time RT-PCR analysis. Total RNA was extracted from purified cells using RNeasy Microkit (Qiagen, Valencia, CA) digested with DNase I and reverse transcribed into cDNA (Superscript III, Invitrogen, Carlsbad, CA). cDNA quantification was performed using SYBR Green PCR and Real-Time PCR Detection System (Applied Biosystem, Carlsbad, CA), normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) RNA. Primers used were: gapdh: F 5'-CCAGTATGACTCCACTCACG-3' R 5'- GACTCCACGACATACTCAGC-3'; ifn-α4/6 (ifnα): F 5'- TATGTCCTCACAGCCAGCAG-3' R 5'- TTCTGCAATGACCTCCATCA-3', ifn-β: F 5'- CTGGCTTCCATCATGAACAA-3' R 5'-AGAGGGCTGTGGTGGAGAA-3; tbx21: F 5'-AGCAAGGACGGCGAATGTT-3' R 5'- GTGGACATATAAGCGGTTCCC-3'; prdm1: F 5'-ACATAGTGAACGACCACCCCTG-3' R 5'-CTTACCACGCCAATAACCTCTTTG-3',TGFßr2: F5'-ATGTGGAAATGGAAGCCCAGA-3' R 5'- TGCAGGACTTCTGGTTGTCG-3'.

Statistical Analysis. Statistical differences were determined by student's T-test (or paired T-test for mixed chimeras) using the InStat 3.0 software (Graphpad, La Jolla, CA).

Results

1.1 Limited DC response to MCMV infection in CD11c-dnTGFβRII mice

TGFβ signaling is rapidly activated in many tissues, especially the spleen and liver, within 6 hrs following TLR ligation *in vivo* (130). To examine the role of innate TGFβ signaling during pathogen challenge, we first infected transgenic CD11c-dnTGFβRII mice, where $CD11c^+$ cells have attenuated TGFβ signaling, or littermate controls (WT) with MCMV. Early TLR recognition of MCMV by pDCs and subsequent cytokine (i.e. IFN-I and IL-12) production are critical for pathogen control (109), thus we initially characterized splenic DC populations by flow cytometry at peak response, 36 hours post-infection (h.p.i). As reported (131), MCMV infection of WT mice resulted in decreased numbers of DCs (Fig 1-1a); this decrease, however, was significantly attenuated in CD11c-dnTGFβRII infected mice which showed increased numbers of $CD11b^+$ cDCs, $CD8\alpha^+$ cDCs and pDCs compared to WT infected mice (Fig 1-1a). We next analyzed the ability of DCs to produce cytokines upon MCMV infection. Because pDCs are a major source of IFN-I at 36 h.p.i. (132) we analyzed purified splenic pDCs from both WT and CD11c-dnTGFβRII mice and measured *ifn-α4/6 (ifnα)* and *ifn-β* mRNA transcripts by qPCR. While pDCs from WT MCMV infected mice showed high levels of *ifn-α* and *ifn-β* compared to naïve mice, pDCs from CD11c-dnTGFβRII mice had significantly reduced IFN-I transcripts (Fig 1-1b). In contrast to WT mice, which exhibited high levels of IFN-I activity in serum, CD11c-dnTGFβRII mice had substantially reduced systemic IFN-I (Fig 1-1c).

Direct viral recognition of MCMV also induces production of IL-12 and TNFα by all DC subsets. Both the proportion and total number of DCs producing IL-12 and/or TNFα was substantially reduced in CD11c-dnTGFβRII compared to WT mice

challenged with MCMV (Fig 1-1d). All DC subsets in CD11c-dnTGFβRII mice also displayed reduced levels of the prototypic activation molecules CD86 and Programmed Cell Death Ligand 1 (PDL-1) compared to DCs in littermate control mice (Fig 1-1e). Importantly, both DC number and activation were similar in CD11c-dnTGFβRII and WT mice before infection (not shown). Overall these data indicate that there is an attenuated DC response to MCMV infection in the absence of innate TGFβ signaling.

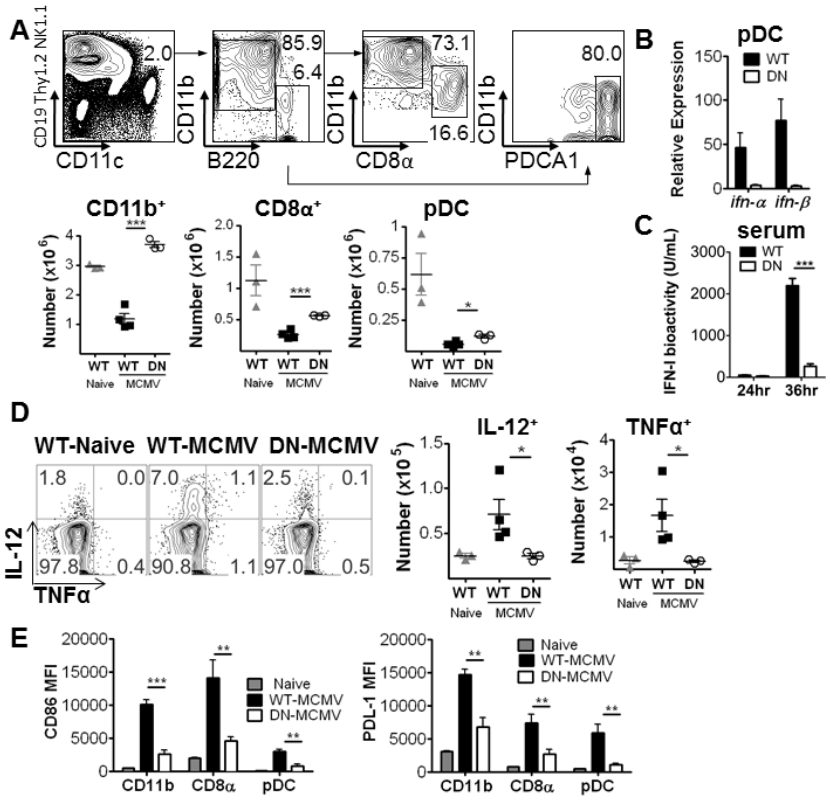

Figure 1-1. Limited DC response to MCMV infection in CD11c-dnTGFβRII mice. CD11c-dnTGFβRII (DN) mice or littermate control (WT) were infected i.p. with $2x10^4$ pfu MCMV and sacrificed 36 hr p.i. Naïve WT mice were processed as controls. **A**) Representative gating of CD11b, CD8α and pDCs and total number of DC subpopulations in splenocytes analyzed by flow cytometry **B)** RNA from pooled groups of mice sorted for pDCs and analyzed for *ifn-α* and *β* transcripts by qPCR relative to *gapdh*. **C)** IFN-I bioactivity (U/mL) from serum obtained at indicated time points p.i **D)** IL-12 and TNFα production by spleen DCs after 5hr culture in BFA. Representative FACS plot gated on total $CD11c^+$ DCs ($CD19^-$ $Thy1.2^-$ $NK1.1^-$) as in (A) (left) and total number plotted as individual mice (right). **E)** MFI of CD86 and PDL-1 expression on indicated subpopulations of DCs. Data are representative of 3 experiments of 3-5 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.2 Increased NK cell response during MCMV infection in CD11c-dnTGFβRII mice

NK cells are critical for the elimination of MCMV-infected cells. Both IFN-I and IL-12 are important activators of NK cells, with IFN-I driving proliferation and cytotoxicity (e.g. granzyme B upregulation) and IL-12 and IL-18 promoting IFNγ production (133-135). In C57BL/6 mice, MCMV resistance is dictated by direct recognition of infected cells by Ly49H+ NK cells (1, 36, 136), however early activation is cytokine-dependent and non-specific (37, 137). Given our observed changes in DC-derived IFN-I and IL-12 in CD11c-dnTGFβRII MCMV infected mice (Figure 1b-c) and given that CD11c-dnTGFβRII mice were previously reported to exhibit significantly higher numbers of NK cells before and after MCMV infection (121, 122), we next examined NK cell responses in CD11c-dnTGFβRII mice infected with MCMV.

We observed that indeed CD11c-dnTGFβRII mice had substantially higher numbers of NK cells 36 h.p.i. compared to WT controls (Fig 1-2a). A large proportion of WT NK cells produced IFNγ and GranzymeB after MCMV infection. In contrast, a smaller fraction of NK cells from CD11c-dnTGFβRII mice secreted IFNγ compared to WT NK cells, and the mean fluorescence (MFI) intensity of IFNγ staining was also reduced in CD11c-dnTGFβRII mice. Despite the reduced proportion of IFNγ-producing NK cells, the total number of IFNγ-producing NK cells was elevated in CD11c-dnTGFβRII mice compared to WT mice (Fig 1-2b). Both a greater proportion and total number of NK cells from CD11c-dnTGFβRII mice produced Granzyme B compared to WT NK cells (Fig 1-2c). Consistent with lower serum IFN-I levels, NK cells from CD11c-dnTGFβRII mice had lower levels of the early activation and IFN

sensitive marker CD69 (138), compared to WT mice (Fig 1-2d). Lastly, to examine the differentiation state of NK cells we purified NK cells 36 h.p.i. and analyzed transcript levels of T-bet (*tbx21*) and Blimp1 (*prdm1*). NK cells from CD11c-dnTGFβRII mice had increased expression of these transcription factors compared to WT mice (Fig 1-2e). Overall these data indicate that both NK cell number and differentiation towards terminal effector cells are more pronounced during MCMV infection in the absence of TGFβ signaling. These results are consistent with previous characterization of CD11c-dnTGFβRII mice where increased differentiation to mature NK cells expressing CD11b and T-bet was present even before infection (122).

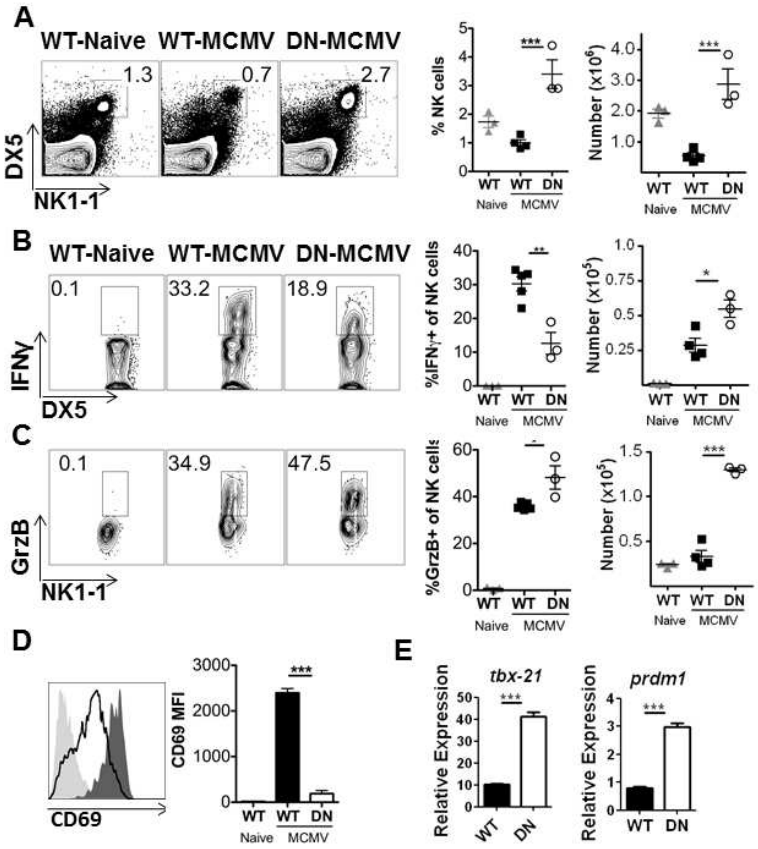

Figure 1-2. Increased NK cell response to MCMV infection in CD11c-dnTGFβRII adult mice. CD11c-dnTGFβRII (DN) mice or littermate control (WT) were infected i.p. with $2x10^4$ pfu MCMV and spleen NK cells analyzed 36 hr p.i. Naïve WT mice were processed as controls. **A)** Total percent and number of NK cells ($DX5^+NK1.1^+CD3^-$) **B-C)** IFNγ and Granzyme B production were measured after 5hr culture ex-vivo in the presence of BFA. Representative FACS plots IFNγ **(B)** or granzyme B **(C)** production in gated NK cells (left) with corresponding percentages and number plotted as individual mice (right). **D)** CD69 MFI on NK cells with representative histogram (left) depicting WT (dark fill), DN (open), Naïve (light fill) and average MFI in bar graph (right) **E)** *tbx21* (TBET) and *prdm1* (BLIMP1) mRNA expression compared to *gadph* (x100) from FACS purified splenic NK cells from infected mice. Data are representative of 3 experiments of 3-5 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.3 Direct TGFβ signaling does not modulate DC responses during MCMV infection.

To determine the intrinsic effects of TGFβ signaling on both DC and NK cell responses versus the indirect effects due to changes in the surrounding environment,

we generated mixed chimeras by co-transferring WT (CD45.2) and CD11c-dnTGFβRII (CD45.1) bone marrow into irradiated C57BL/6 WT (CD45.2) mice. Eight weeks post-reconstitution, chimeric mice were infected with MCMV and splenic DCs and NK cells were analyzed 36 h.p.i. The overall representation of WT versus CD11c-dnTGFβRII $CD11b^+$ and pDCs was unchanged compared to total spleen mononuclear cell (SMC) chimerism. However in this "competitive" setting, CD11c-dnTGFβRII donor cells developed into $CD8\alpha^+$ DCs with reduced frequency compared to their WT counterparts (Fig 1-3a).

To measure inflammatory responses by DCs both WT and CD11c-dnTGFβRII pDCs and cDCs were analyzed. In contrast to non-chimeric CD11c-dnTGFβRII mice (Fig 1b above), WT and CD11c-dnTGFβRII derived pDCs had similar levels of *ifn-α* and *ifn-β* transcripts following MCMV infection (Fig 1-3b). Similarly, both WT and DN derived DCs produced similar amounts of IL-12 (Fig 1-3c). Furthermore, expression of co-stimulatory molecules CD86 and PDL-1 were similarly low before infection and equally up regulated after infection in both WT and DN derived DCs (Fig 1-3d&e). These results suggest TGFβ signaling on DCs does not directly influence acute cytokine production or co-stimulatory molecule expression *in vivo* upon MCMV challenge and suggest that the reductions observed in non-chimeric CD11c-dnTGFβRII mice (Fig 1) were a result of changes in the DC surrounding environment, presumably increased viral control by NK cells.

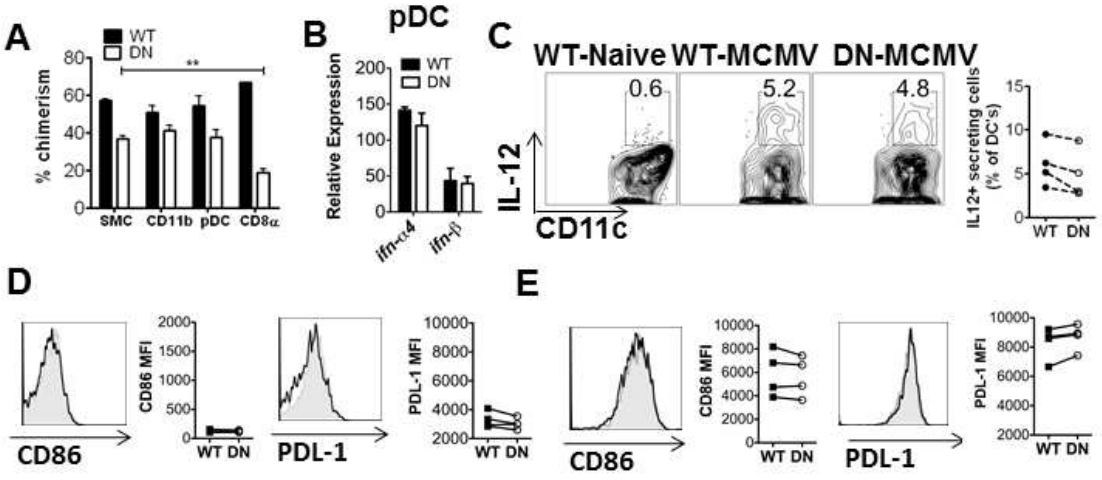

Figure 1-3. Direct TGFβ signaling does not modulate DC responses during MCMV infection. Mixed BM chimeras of C57BL/6 (WT;CD45.2) and CD11c-dnTGFβRII (DN;CD45.1) BM cells transferred into irradiated C57BL/6 mice. Eight weeks later, mice were infected i.p. with $2x10^4$ pfu MCMV and spleens were analyzed 36 hr p.i. **A)** Percent chimerism of DC cell populations from the DN compartment was compared to that of all splenocytes (SMC) as determined by flow cytometry. **B)** RNA from sorted pDCs analyzed for *ifn-α4* and *ifn-β* transcripts by qPCR and graphed relative to *gadph* expression. **C)** IL-12 production by total $CD11c^+$ ($CD19^-$ $Thy1.2^-$ $NK1.1^-$) cells after 5hr ex-vivo culture in the presence of BFA. Representative FACS plots of total $CD11c^+$ DCs (left) and total number plotted as individual mice (right). **D-E)** CD86 and PDL1 expression on Naïve (D) and MCMV infected (E) total $CD11c^+$ cells shown as representative histograms (left) with WT (grey fill) or DN (black line) compartments and MFI of individual mice (right). Data are representative of 2-3 experiments with n=4 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.4 Cell-intrinsic NK cell alterations in WT:CD11c-dnTGFβRII mixed chimeras.

In contrast to DCs, and consistent with recent work (122), NK cells from the CD11c-dnTGFβRII compartment were over-represented in proportion to WT NK cells in the blood both before and after infection compared to normal proportions of T cells from each WT or DN compartment (Fig 1-4a). Before infection both WT and CD11c-dnTGFβRII derived NK cells do not spontaneously produce IFNγ after 5hr in-vitro culture, however CD11c-dnTGFβRII derived NK cells do show enhanced Granzyme B expression (Fig 1-4b). Similar to non-chimeric CD11c-dnTGFβRII mice (Fig 2 above), NK cells derived from CD11c-dnTGFβRII donors produced slightly less IFNγ

and maintained higher Granzyme B on a per cell basis than WT NK cells after MCMV challenge (Fig 1-4c). CD11c-dnTGFβRII NK cells also had slightly increased expression of CD69 prior to infection (Fig 4d) that changed to slightly reduced levels of CD69 compared to WT NK cells after MCMV infection (Fig 1-4e). Lastly, we analyzed the levels *tbx21* & *prdm1* to test whether intrinsic TGFβ signaling altered effector differentiation of NK cells. Indeed CD11c-dnTGFβRII derived NK cells had higher levels of these transcripts compared to WT cells after MCMV infection (Fig 4f). Together the aforementioned results suggest that TGFβ signaling had a cell-intrinsic effect on NK cells, enhancing their numbers and effector functions before MCMV infection as previously described (122) while indirectly affecting DC numbers and function.

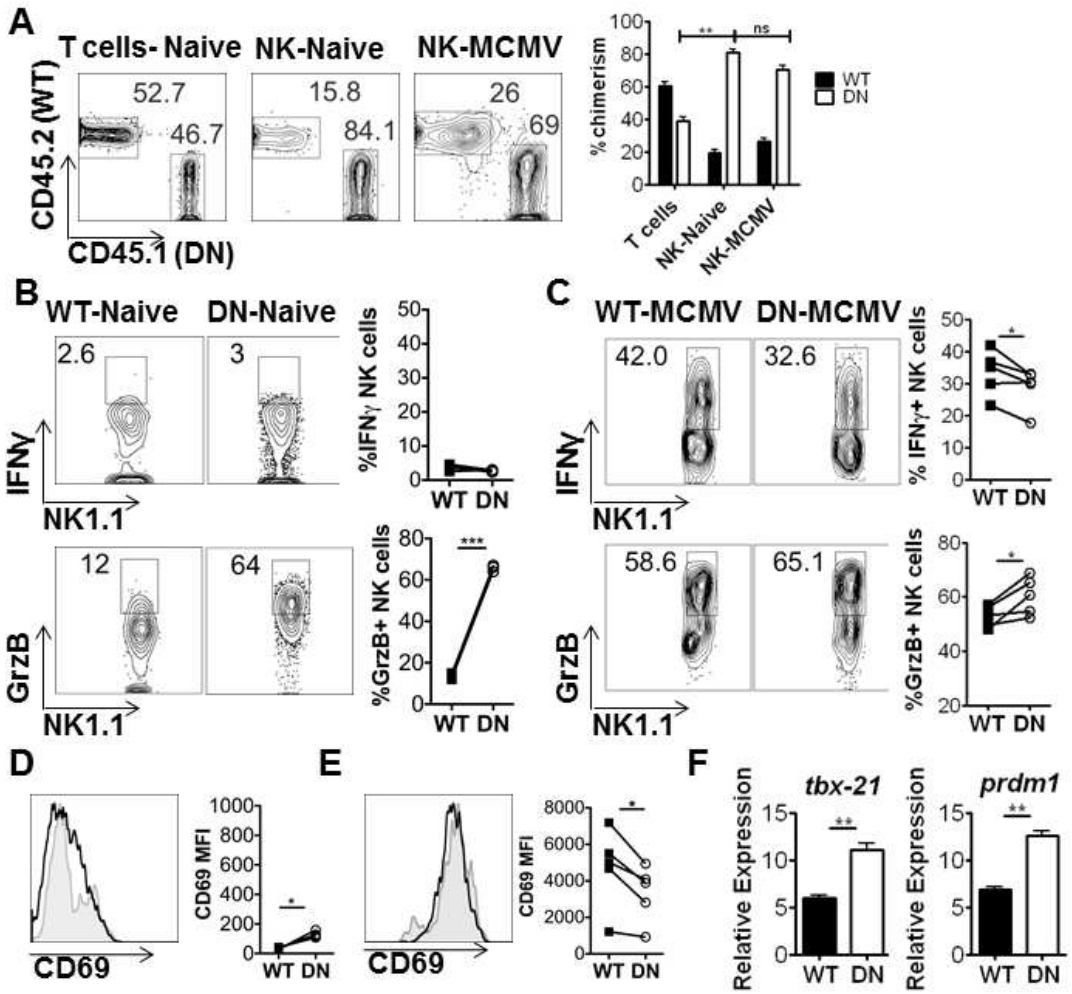

Figure 1-4. Cell-intrinsic NK cell alteration in WT:CD11c-dnTGFβRII mixed chimeras. Mixed BM chimeras of C57BL/6 (WT; CD45.2) and CD11c-dnTGFβRII (DN;CD45.1) BM cells transferred into irradiated C57BL/6 mice. Eight weeks later, mice were infected i.p. with $2x10^4$ pfu MCMV and splenic NK cells were analyzed 36 h.p.i. A) Percent chimerism of each compartment in NK cells ($DX5^+NK1.1^+CD3^-$) in the blood before and after infection were determined by flow cytometry and compared to T cells B-C) IFNγ and Granzyme B production by NK cells after 5 hr ex-vivo culture with BFA in Naïve (B) or MCMV infected (C) animals. Representative FACS plots IFNγ or Granzyme B production in gated NK cells (left) with corresponding percentages plotted as individual mice (right). D-E) CD69 expression on NK cells in Naïve (D) and MCM infected (E) mice depicted as histogram (left) of WT compartment (grey fill) to dnTGFβRII compartment (open) and MFI of individual mice (right). F) tbx21 (T-BET) and prdm1 (BLIMP1) mRNA expression compared to gapdh (x100) from FACS purified splenic NK cells ($CD3^-Nk1.1^+DX5^+$) during MCMV infection. Data are representative of 2-3 experiments with n=4 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.5 Increased NK cell (but not DC) responses during LCMV Cl13 infection in CD11c-dnTGFβRII mice

To compare and contrast the responses of DCs and NK cells from CD11c-dnTGFβRII mice upon infection with a persistent virus other than MCMV we inoculated mice with LCMV Cl13. In contrast to the rapid control of replicating MCMV by NK cells (which precedes MCMV latency), LCMV is not highly susceptible to NK cell mediated clearance and can persist in a continuously replicating state for 60-200 days post-infection (d.p.i.) in multiple tissues (24, 139). However NK cells have recently been shown to limit adaptive immune responses early during LCMV infection to promote viral persistence or protect against immune pathology (113, 114).

LCMV induces a potent IFN-I response that is dependent on many innate cells including pDCs (111, 140, 141) where pDCs ultimately contribute to late LCMV control (142, 143). We observed no difference in the number of pDCs or cDCs derived from WT or CD11c-dnTGFβRII compartment at day 1-3 p.i. (data not shown). Furthermore, we found that WT mice and CD11c-dnTGFβRII mice exhibited similarly high levels of IFN-I in the blood 24h after LCMV infection (Fig 1-5a). IFN-I is also a main contributor to cDC co-stimulatory molecule up-regulation by 3 d.p.i. (144). Accordingly, the expression of CD86 and CD40 were unchanged in DC subsets from CD11c-dnTGFβRII mice compared to WT mice (Fig 1-5b). We next measured NK cell number and activity at 1 d.p.i. and found that CD11c-dnTGFβRII mice had increased NK numbers compared to WT mice (Fig 1-5c), however the proportions of NK cells producing IFNγ and Granzyme B were similar (Fig 1-5d). Despite similar systemic IFN-I levels in the absence of innate TGFβ signaling, NK cells had slightly decreased CD69 compared to WT mice following infection (Fig 1-5e). Both CD4 and

CD8 T cells are critical to control persistent LCMV Cl13 infection, although they become deleted and functionally exhausted, producing low levels of antiviral cytokines (44, 60). To assess the potential impact of innate TGF□ signaling on anti-viral T cell responses we next examined the magnitude and function of immunodominant LCMV specific T cells in CD11c-dnTGFβRII mice at day 9 p.i. We found similar numbers of anti-viral CD8 D^b:GP_{31-41} T cells in WT versus CD11c-dnTGFβRII mice; however CD11c-dnTGFβRII mice exhibited fewer numbers of LCMV specific CD4 T cells determined by I-A^b:GP_{67-77} tetramer staining (Fig 1-5f). Secretion of IFNγ, TNF□ and IL-2 upon ex-vivo GP_{31-41} or GP_{67-77} peptide stimulation was similar in CD8 and CD4 T cells from WT versus CD11c-dnTGFβRII mice, respectively (Fig 1-5g). These results indicate that TGFβ signaling influences NK cells, but not DCs, therefore limiting NK cell early responses during chronic LCMV infection and this may in turn affect T cell responses as previously described (113, 114).

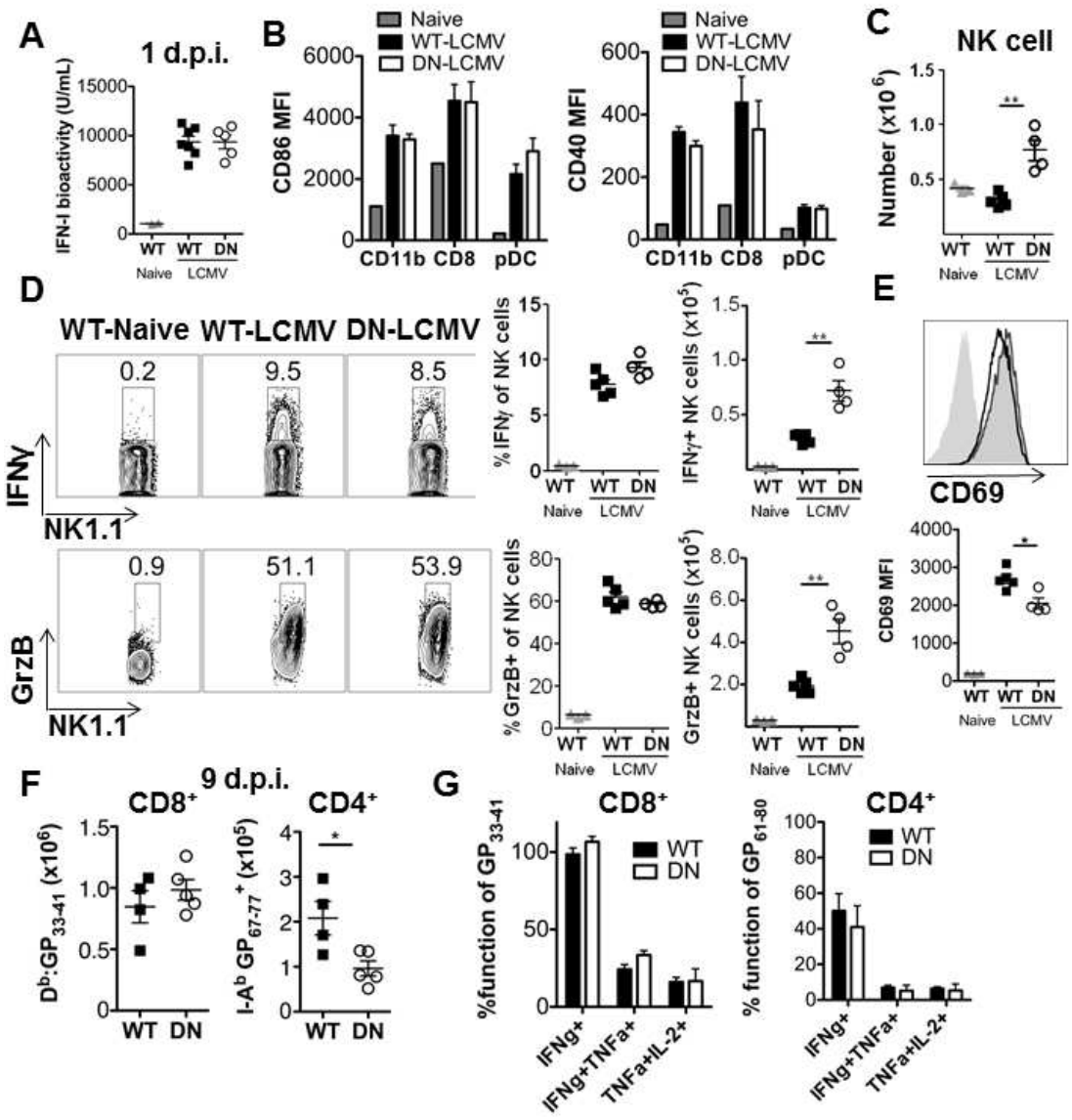

Figure 1-5. Increased NK cell (but not DC) responses in LCMV infected CD11c-dnTGFβRII mice. CD11c-dnTGFβRII mice or littermate controls (WT) were infected with $2x10^6$ pfu of LCMV Cl13. **A)** Serum IFN-β levels at 1 day post-infection (d.p.i.) **B)** CD86 and CD40 expression (MFI) in DC subsets were determined by flow cytometry 3 d.p.i. **C)** Total number of splenic NK cells 1 d.p.i. **D)** IFNγ and Granzyme B production after 5 hr culture in BFA at 1 d.p.i. Representative FACS plot gated on total NK cells (left) with percent of NK cells and total number plotted as individual mice (right). **E)** CD69 expression on NK cells at 1 d.p.i. **F)** Total number of $D^{b:}GP_{31-41}$ and $I\text{-}A^b{:}GP_{61-80}$ virus specific CD8 and CD4 T cell responses 9 days p.i. in the spleen. **G)** Cytokine production as a percentage of virus specific cells from (F) upon cognate GP_{31-41} or GP_{67-77} peptide stimulation in the presence of BFA**.** Data are representative of 2-4 experiments of 3-5 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.6 Enhanced control of MCMV but not LCMV Cl13 viral loads in CD11c-dnTGFβRII mice

Infant CD11c-dnTGFβRII mice survived otherwise lethal MCMV challenge presumably due to enhanced NK cell maturity that is present in the first week of birth (122). In the next series of experiments we tested whether this observation could be extended to adult mice. We observed that CD11c-dnTGFβRII mice are better able to control acute MCMV replication in the liver than their WT counterparts by 3 d.p.i. (Fig 1-6a). Consistently CD11c-dnTGFβRII mice were protected from an otherwise 50 percent lethal dose of MCMV in WT C57BL/6 mice (Fig 1-6b). In contrast, CD11c-dnTGFβRII mice showed similar viral loads to WT mice in the liver at day 3 post LCMV Cl13 challenge (Fig 1-6c). Moreover, comparable or slightly increased viremia persisted for 60 days in CD11c-dnTGFβRII mice compared with WT mice when challenged with LCMV Cl13 infection (Fig 1-6d). These results suggest that heightened NK cell responses due to innate TGFβ blockade likely have the capacity to limit acute viral replication and enhance survival when adult mice are challenged with MCMV, but this is insufficient to influence the course of chronic LCMV infection.

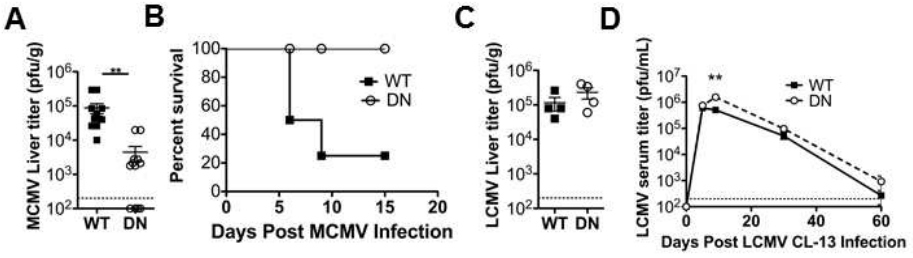

Figure 1-6. Enhanced control of MCMV but not LCMV Cl13 viral loads in CD11c-dnTGFβRII mice. CD11c-dnTGFβRII (DN) mice or littermate control (WT) were infected with $2x10^4$ **(A)** or $2x10^5$ **(B)** MCMV or with $2x10^6$ LCMV CL13 **(C-D)**. **A&C)** Viral titers in liver homogenate measured by plaque assay at day 3 p.i. **B**) Percent surviving mice at indicated times, pooled results from 2 experiments: n=12 WT and n=10 DN mice. **D)** Viremia determined by plaque assay at indicated time points. Dotted line represents the limit of assay detection, 200 plaque-forming units (PFU). Data are representative of 2-3 experiments of 3-5 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.7 Cell-intrinsic TGFβRII deletion in adult mice does not influence DC cell responses upon MCMV infection

To assess the role of TGFβ signaling during infection (independently of its developmental roles (122)) and to rule out putative dominant effects of the dnTGFβRII transgene recently reported in T cells (145), we used ERcre-TGFβRII mice in which TGFβRII is ubiquitously deleted upon tamoxifen treatment (123, 146). To analyze the cell-intrinsic effect of TGFβ signaling in DC and NK cells *in vivo* (independent of TGF□ signaling on other cells) we created 1:1 ERcre-TGFβRII:CD45.1 (WT) mixed BM chimeras. Eight weeks post-reconstitution mice were treated with Tamoxifen for 5 days and gene deletion was confirmed 5 days later in blood leukocytes (Fig 1-7a&b). While TGFβRII protein expression was readily detected by flow cytometry in WT (but not ERcre-TGFβRII) T cell and B cells, we could not detect minimal surface TGFβRII expression in NK cells, Monocytes or Neutrophils from either WT or ERcre-TGFβRII origin (Fig 1-7a). However, we did find *tgβr2* mRNA in WT NK cells and monocytes and this was dramatically reduced to the limit of detection in ERcre-TGFβRII cells (Fig 1-7b). These data indicated that five-day tamoxifen treatment effectively deleted TGFβRII from ERcre-TGFβRII leukocytes.

Therefore, we next infected the tamoxifen-treated ERcre-TGFβRII:WT mixed BM chimeras with MCMV and analyzed splenic DC responses 36 h.p.i. Unlike constitutive expression of dnTGFβRII, where CD11c-dnTGFβRII mice had reduced CD8□$^+$ DC differentiation in a competitive setting (Fig 3a) , all ERcre-TGFβRII DC subsets had similar representation to WT DCs in ERcre-TGFβRII:WT mixed chimeras (Fig 1-7c). In addition both WT and TGFβRII deficient DCs produced equivalent

amounts of IL-12p40 (Fig 1-7d) and comparable upregulation of I-A^b, CD86 and CD40 molecules after MCMV infection (Fig 1-7e). These data further support our conclusions from CD11c-dnTGFβRII mice, indicating that TGFβ signaling does not affect early DC responses to viral infections *in vivo*.

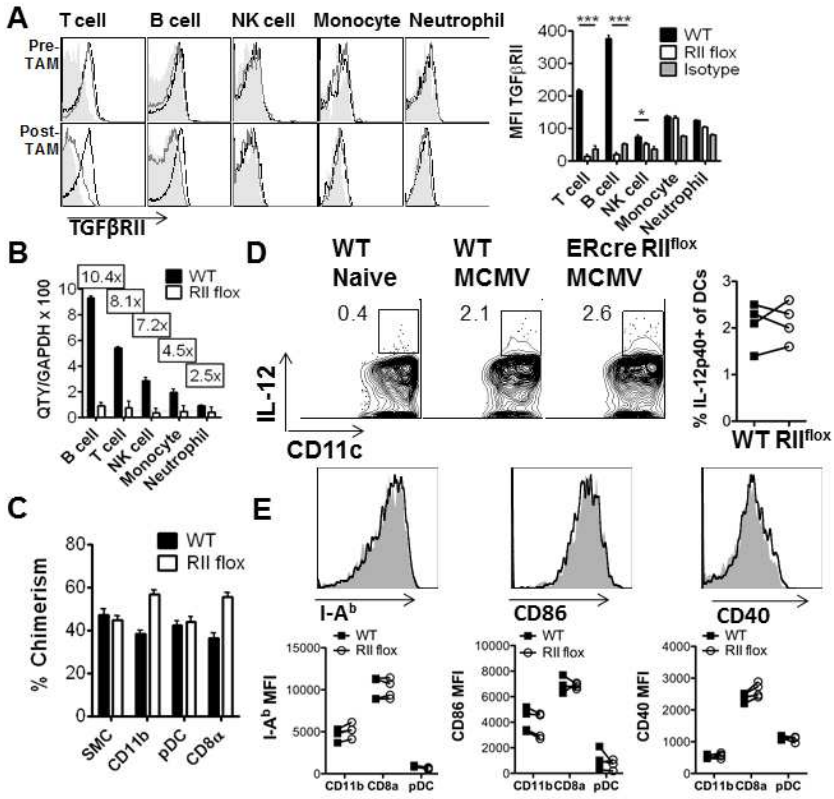

Figure 1-7. Inducible deletion of TGFβRII in adult mice does not affect DC responses to MCMV. 1:1 mix of BM from C57BL/6 (WT; CD45.1) and ERcre-TGFβRII (RII^{flox} ; CD45.2) mice was transferred into irradiated CD45.1 hosts. Eight weeks later, 1mg/day of Tamoxifen (TAM) was injected I.P. for 5 days to induce Cre activity. Five days later blood leukocytes were analyzed for receptor expression (A-C), mice infected with 2x10^4 pfu of MCMV and splenic DCs were studied by flow cytometry 36 hrs p.i. (D-F). A) TGFβRII protein expression was determined in indicated leukocytes populations by flow cytometry and MFI post-Tamoxifen is shown (right). B) *TGFßr2* relative to *gapdh* mRNA levels were quantified in FACS-purified leukocytes populations by qPCR. Numbers in box indicate fold change. C) Percent chimerism of indicated DC subsets from each compartment. D) IL-12 production by DCs after 5hr culture in BFA. Representative FACS plot gated on total CD11c$^+$ DCs (left) and percentages plotted as individual mice (right). E) I-A^b, CD86 and CD40 expression on DC subsets. Representative histograms are shown for CD11b$^+$ DCs with WT (grey fill) and ERcre-TGFβRII (black line) overlays. MFI for each molecule in indicated DC subsets is plotted for individual mice below. Data are representative of 2 experiments of 4 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

1.8 Cell-intrinsic TGFβRII deletion in adult mice does not affect NK cell responses upon MCMV infection

In the next series of experiments we measured early NK cells responses to MCMV infection in the ERcre-TGFβRII:WT mixed chimeras described above. We first observed similar proportion of WT and ERcre-TGFβRII NK cells in the blood before and after infection, indicating no preferential expansion sans TGFβ signaling (Fig 1-8a). We further found the proportion of NK cells from the ERcre-TGFβRII compartment in spleen and liver after infection was similar to the blood pre-infection (Fig 1-8b). Early activation 36 h.p.i. is cytokine dependent and independent of Ly49H response (37), however we further found that the proportion of blood Ly49H$^+$ NK cells increased during infection in both WT and ERcre-TGFβRII NK cells and that this increase is only slightly greater in ERcre-TGFβRII compared to WT NK cells at day 6 (but not day 1.5) p.i. relative to their corresponding starting points (Fig 1-8c) indicating little MCMV-specific repression of Ly49H$^+$ NK cells by TGFβ signaling. We next analyzed spleen NK cell cytokine production in WT:ERcre-TGFβRII chimeras at 36 h.p.i. We observed that NK cells from the ERcre-TGFβRII compartment had equivalent production of IFNγ and Granzyme B to WT NK cells (Fig 1-8d). Given that NK cells are recruited to the liver rapidly after MCMV infection (147, 148), we next examined NK cell function in the livers of infected WT:ERcre-TGFβRII chimeras at 36 h.p.i. Again, we found that WT and TGFβRII deficient NK cells exhibited similar levels of IFNγ and Granzyme B expression in the liver (Fig 1-8e). Furthermore, we measured expression of CD69 in splenic NK cells and found that the acute deletion of TGFßRII did not affect CD69 upregulation after infection (Fig 1-8f). Finally, T-BET expression, which was intrinsically up-regulated in CD11c-dnTGFβRII NK cells (Fig

4d above), was unaltered in splenic ERcre-TGFβRII NK cells compared to their WT counterparts (Fig 1-8g). Overall these data indicate that (in contrast to constitutive expression of dnTGFβRII) inducible deletion of TGFβRII in adult mice does not directly affect early NK cell responses upon MCMV infection.

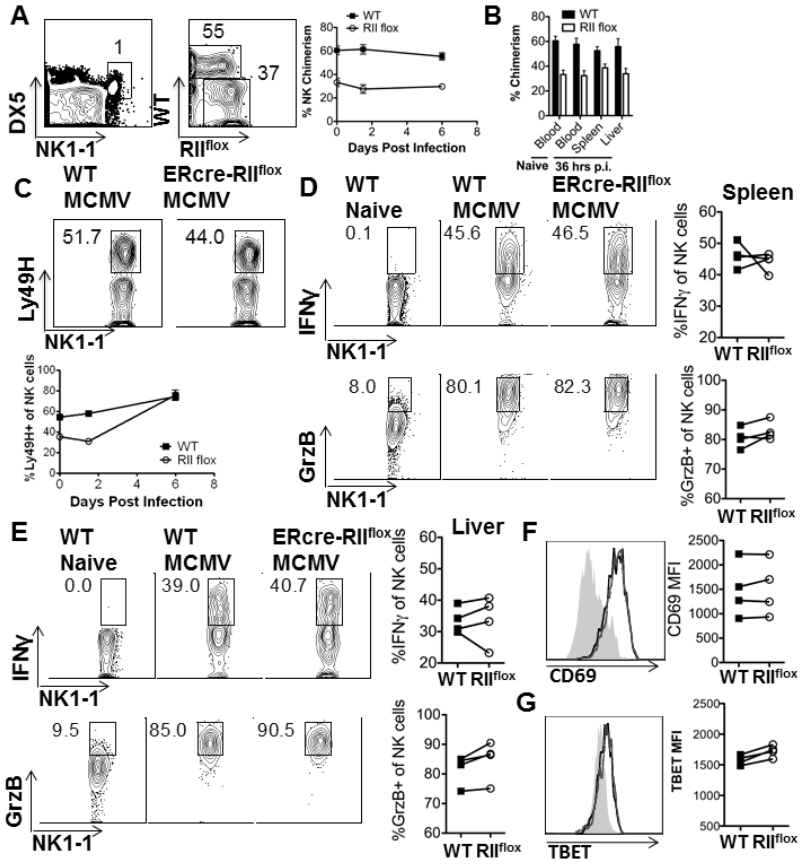

Figure 1-8. Inducible deletion of TGFβRII in adult mice does not affect NK cell responses to MCMV. 1:1 mix of BM from C57BL/6 (WT; CD45.1) and ERcre-TGFβRII (RII^{flox}; CD45.2) mice was transferred into irradiated CD45.1 hosts. Eight weeks later, 1mg/day of Tamoxifen was injected i.p. for 5 days, mice infected with 2x10^4 pfu of MCMV and NK cells studied by flow cytometry A&B) Chimerism of NK cells in the blood during infection (A) and tissues at 36 h.p.i. (B) C) Percent Ly49H+ cells within blood NK cells. Representative FACS plots (upper) and average values at indicated time points (lower). D&E) IFNγ and Granzyme B production in the spleen (D) and Liver (E) NK cells after 5hr culture with BFA. Representative FACS plot gated on total NK cells (left) and percentages plotted as individual mice (right). F&G) CD69 (F) and T-BET (G) expression on NK cells depicted as histogram (left) of WT compartment (black open) to ERcre-TGFβRII compartment (grey open) , over Naïve mice (grey fill) and MFI of individual mice (right). Data are representative of 2 experiments of 4 mice/group. *p<0.05, **p<0.005, ***p<0.0005.

Discussion

The early presence of host resistance factors can influence the balance between host and microbe to favor rapid pathogen eradication. For example, a strong innate response via Ly49H$^+$ NK cells controls MCMV in C57BL/6 mice whereas susceptible Balb/C mice, lacking a protective MCMV specific Ly49H$^+$ haplotype, demonstrate a higher mortality rate or establish more latent virus depending on inoculation dose (1, 35). Similarly rapid and robust adaptive CD8 T cell responses correlate with improved outcome of LCMV in mice and SIV in macaques (2). TGFβ is a major immunosuppressive cytokine that limits innate (DC and NK cell) and adaptive immunity *in-vitro* and *in-vivo* in different systems, with high levels of TGFβ correlating with enhanced susceptibility and viral persistence to human HCV and SIV in rhesus macaques (149, 150) as well as LCMV and Malaria in mice (74, 79).

In this study we show that while constitutive genetic attenuation of TGFβ signaling in innate cells (via expression of dnTGFβRII transgene in CD11c$^+$ cells) did not directly affect key DC functions, it resulted in increased NK cell responses before and after infection and therefore promoted early control of MCMV but not LCMV chronic infections *in vivo*. In contrast, genetic deletion of TGFβRII signaling in adult mice (just before infection) did not change early innate responses to MCMV infection. Our findings, therefore, support two conclusions. First, despite previous evidence that TGFβ suppresses DCs *in vitro* (100, 115) and in inflammatory disease models (97, 151-153), cell-intrinsic TGFβ signaling does not modulate key DC responses early after infection with potentially persistent viruses *in vivo*. Second, that cell-intrinsic

TGFβ signaling on NK cells before (but not after) viral infection limits their early responses *in vivo*.

By using either CD11c-dnTGFβRII mice or inducible TGFβRII deletion in adulthood, our results indicate that TGFβ does not directly affect early DC numbers, cytokine production or maturation in spleens after viral infection. This is true for both LCMV and MCMV infections regardless of the different route and doses of inoculation, the RNA versus DNA nature of viral genomes, and the different pathogen recognition receptors activated upon these infections (111). Our findings are unexpected given numerous previous reports supporting a role for TGFβ regulation of DCs in different settings. *In vitro* TGFβ can suppress pDC IFN-I production (132, 142) and cDC maturation and cytokine production (115). In addition, a number of studies using different models support an *in vivo* role for TGFβ on DCs. For instance, in long term chronic inflammatory models using CD11c-dnTGFβRII mice, such as EAE (153) and atherosclerosis (154), innate TGFβ signaling suppresses disease. Blockade of TGFβ on innate cells also decreases latent HSV-1 (155). Ramalingam et al recently showed Cd11c-Cre mediated DC deletion of TGFβRII causes increased IFNγ and TNFα in DCs in long-term spontaneous autoimmunity but did not affect steady state MHC-II co-stimulatory expression and had no apparent increase in NK cells (156), highlighting the differences in genetic models as well as infectious vs non –infectious contexts.

pDCs and IFN-I are essential for control of MCMV (132, 142) and IFN-I induction by pDCs is tightly linked to the presence of virus and TLR9 activation in this infection (157). Previous work by Andrews et al has shown that a protective haplotype of Ly49H in NK cell responses limits pDC cytokine response during MCMV

infection, clearly indicating feedback between NK cell and DC activation (35). This seems to be mediated both indirectly though viral control, reducing TLR9 stimulation, and also direct DC killing by NK cells. Thus, the limited DC activation and IFN-I production we see in MCMV infected CD11c-dnTGFβRII mice is most likely due to initial enhanced viral control by NK cells as mixed chimeras show that DCs with attenuated TGFβ signaling have the same activation potential and cytokine production as WT DCs when in the same environment *in vivo*. Consistently, in LCMV Cl13 infection, where viral loads are not altered in CD11c-dnTGFβRII mice, we did not detect differences in IFN-I or DC activation early after infection.

DC-derived IL-12 and IFN-I are potent inducers of IFNγ and Granzyme B in NK cells, respectively (111, 134) alongside MCMV m157 – Ly49H recognition in C57BL/6 mice (136). In this study the constitutive attenuation of intrinsic TGFβ signaling on NK cells resulted in increased Granzyme B and decreased IFNγ and CD69 expression. Consistently, Laouar and colleagues have recently shown that TGFβ signaling suppresses number and maturation towards terminally differentiated NK cells without altering Ly49H, Ly49C/I, Ly49D or NKG2D distribution and infant CD11c-dnTGFβRII mice are resistant to MCMV for this reason (122). Terminally differentiated NK cells express the most Granzyme B and Blimp1 (112). In line with this, we found that CD11c-dnTGFβRII NK cells were over-represented in competition with WT cells and had higher levels Granzyme B even before infection. CD11c-dnTGFβRII NK cells maintained higher levels of TBET, BLIMP1 and Granzyme B expression after MCMV infection, confirming the presence of a more mature phenotype. Furthermore, we also observed enhanced MCMV resistance in adult CD11c-dnTGFβRII mice likely resulting from increased NK cell response.

Interestingly, even in the presence of this heightened NK cell frequency and effector function LCMV Cl13 was able to rapidly establish infection, indicating the context specific capacity of NK cells to control viral loads. Recent reports demonstrated NK cells are capable of directly killing CD4 and CD8 T cells during LCMV Cl13 infection to limit fatal immune pathology and promote viral persistence (113, 114). In line with these studies, we did observe reduced virus specific CD4 T cells in CD11c-dnTGFβRII mice at day 9 p.i, however, the enhanced NK cell numbers in CD11c-dnTGFβRII mice appears insufficient to alter CD8 T cell responses or the ultimate outcome of chronic LCMV infection. It should be noted, however, that the dominant negative TGFβRII transgene has recently been shown to enhance T cell homeostatic proliferation and activation when a CD4 promoter drives expression even in absence of the normal TGFβRII (145). Therefore, off-target effects of the TGFβRII transgene driven by CD11c promoter in CD11c-dnTGFβRII mice cannot be completely ruled out and represent a potential limitation of this model.

To assess the role of direct TGFβ signaling during infection independently of developmental effects of TGFβ in NK cells and putative off-target effects of the dnTGFβRII transgene, we utilized ERcre-TGFβRII:WT mixed chimeras in which TGFβRII was deleted in adult mice just before MCMV infection. In this system we observed minimal differences in MCMV specific spleen or liver NK cell accumulation, IFNγ and Granzyme B secretion, CD69 or T-bet expression between WT and TGFβRII deleted cells. This is in contrast to a previous study showing that injection of recombinant TGFβ suppresses NK cell number during acute LCMV infection (120) possibly due to differences in endogenous versus inoculated TGFβ doses and target cells. Furthermore, the absence of endogenous TGFβ effect on NK cells after MCMV

infection may result, at least in part, from the low TGFβR expression that we observed in WT NK cells.

Taken together our study supports a role for TGFβ during the development of NK cells, as previously proposed (122), and contrasts this TGFβ function with no apparent direct effect on DCs. Furthermore, neither mature NK cells nor DCs in the periphery of adult mice appear susceptible to TGFβ mediated suppression as limiting cell-intrinsic TGFβ signaling did not affect their subsequent early anti-viral responses. However, TGFβ is known for its context-dependent effects and, therefore, signaling on innate cells could have different outcomes during tissue-specific infections, tumor models, or long-term chronic inflammatory environments.

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Chapter 1, in full, is a reprint of the material as it appears in Journal of Virology. Lewis, GM et al 2015. Gavin M. Lewis was the primary investigator and author of this paper. I would like to thank co-authors Dr. Monica Macal and Charles Hesser for their contributions, as well as Dr. James Harker for critical discussion.

Chapter 2: TGFβ and Adaptive immune responses to chronic infection

Persistent viruses create a profound immunosuppressive environment that is conserved between mice and humans, including increased TGFβ signaling, accompanied by large reduction in quality and quantity of the adaptive immune response, including antiviral T cells. While several pathways suppressing CD8 T cell responses have been identified, much less is known about the immune regulatory molecules directly restricting the numbers and helper function of CD4 T cells. By using lymphocytic choriomeningitis virus (LCMV) infection in mice, we observed TGFβ-R signaling did not substantially influence CD8 T cell responses; however CD4 T cell proliferation, terminal differentiation and a cytotoxic program characterized by Granzyme B and K, Perforin and EOMES expression were significantly suppressed by cell-intrinsic TGFβ-R signaling. EOMES expression was sufficient to mediate cytotoxic gene expression. Finally, TGFβ signaling also suppressed cytotoxic molecules and EOMES in CD4 T cells late during established chronic infections as well as during MCMV infection. Altogether, these data uncover a common TGFβ-dependent axis that restricts EOMES-driven differentiation in murine CD4 T cells throughout chronic viral infection.

Introduction

Chronic viral infections in both mice and humans create an immunosuppressive lymphopenic environment detrimental to both ongoing antiviral responses as well as secondary immune responses to unrelated pathogens or cancers (3, 4, 158, 159). As a result antiviral CD4 and CD8 T cells disintegrate and express many inhibitory surface receptors that negate specific functions upon antigen encounter (31, 45-47). In addition, the cellular environment is devoid of stimulatory cytokines, and contains increased inhibitory cytokines such as IL-10 and Transforming Growth Factor-Beta (TGFß) that inhibit immune responses (73-76). Indeed increased TGFß production and signaling is present in immune cells during human chronic infections with Hepatitis C, HIV, and Tuberculosis as well as mouse models of infections with Lymphocytic Choriomeningitis Virus (LCMV), Malaria, or Listeria Monocytogenes (74, 77-82). However the role of TGFß signaling on individual cells types at different times after infection in-vivo remains unclear.

TGFß is a pleiotropic cytokine with critical roles in the development of the hematopoietic system indicated by mouse genetic knockouts of the receptor subunits, RI and RII (84, 85). Downstream TGFß signaling is typical mediated by canonical phosphorylation of SMAD2/3 with adapter SMAD4 or TIF1γ as well as phosphorylation of MAP kinase pathways in different settings (87, 88). TGFß1-null mice or T cell specific targeting of TGFß-RII deficiency during development results in lethal multifocal inflammatory diseases by 3-4 weeks of age that is CD4 T cell dependent (90-92). Less complete abrogation of signal using a T cells specific dominant negative receptor transgene or conditional deletion of TGFß1 ligand in T

cells allowed early onset autoimmunity symptoms and colitis apparent at 3 months of age (93, 94). In contrast deletion of TGFβRII in post-thymic T cells did not lead to colitis or wasting syndrome in-vivo (95, 96). Consistently, long-term treatment of mice or humans with TGFß antagonists does not lead to the severe autoimmune phenotype (101, 102).

On the other hand, TGFß antagonists have been used successfully in animal cancer models of melanoma and breast cancer via CD8 T cell activity and are currently use in clinical trials to boost intra-tumor immune responses in humans (103-105). Furthermore, we and others have reported that T-cell-specific dominant negative TGFß receptor transgenic mice exhibit increased pathogen and tumor specific CD8 T cell responses (74, 80, 106, 160, 161), yet these mice have substantial autoimmunity as well as transgene dominant effects (93, 145). Indeed initial therapeutic attempts of TGFß antagonism during chronic infection only modestly replicate genetic models (107, 108). Hence, the developmental abnormalities in T cells has precluded analysis of the direct effects of TGFß signaling in-vivo on individual cell types, especially during immune response to pathogens.

We utilized advanced models that allow cell-type specific and temporal ablation of TGFß/SMAD signaling in adult mice in order to revisit the role of TGFßR signaling in T cells during chronic viral infection. While TGF-βR deficient mice showed comparable CD8 T cell responses; CD4 T cell proliferation, terminal differentiation and a cytotoxic program characterized by Granzyme B and K, Perforin and EOMES expression were significantly enhanced in the absence of cell-intrinsic TGF-βR signaling. Strikingly, SMAD4 deficiency gave opposing results. Importantly, TGFβ signaling was continuously necessary late during chronic infection to suppress

Eomes and terminal differentiation in CD4 T cells while EOMES overexpression was sufficient to recapitulate the terminally differentiated phenotype of TGFßR deficient CD4 T cells. Finally, we show the aforementioned TGFβ-Eomes signaling network was common to chronic murine cytomegalovirus (MCMV) infection and human CD4 T cells from HIV+ patients. Altogether, these data uncover a TGF-βR-dependent axis that restricts EOMES-driven terminal differentiation and potential cytotoxicity in murine and human CD4 T cells.

Materials and Methods

Mice and viral stocks. C57BL/6 (CD45.2), B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1) and ERt2-cre mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). CD8-cre;TGFβRII^{flox} (Exon2) were generously provided by Dr. M. Li (Sloan Kettering)(146). SMAD4^{flox} mice on a C57BL/6 background were provide by Dr. Frank Jirik (University of Calgary)(162). Hemizygous ER-cre mice were bred to TGFβRII$^{flox/flox}$ mice (126) or SMAD4$^{flox/flox}$ both on C57BL/6 backgrounds. CD4-ERcre knock-in mice crossed to TGFβRII$^{flox/flox}$ (Exon3/4) were generously provided by Dr. T. Buch (95). 1:1 mixed chimeras were generated by transferring 2x10^{6} bone marrow cells of each indicated genotype into irradiated CD45.1 mice and allowed 8 weeks to reconstitute. For gene deletion, 1mg/day Tamoxifen (Sigma) emulsified in Sunflower seed Oil (Sigma) was injected *intraperitoneal* (i.p.) for 5 days and mice were used for experiments 7 days after last treatment. For gene deletion after infection mice were injected for 5 days starting 12 d.p.i.. 6-12 week old mice were infected *intravenously* (i.v.) with 2x10^{6} plaque forming units (PFU) of LCMV Cl13 or 2x10^{4} PFU of MCMV (Smith strain) i.p. All viruses were grown, identified, and quantified as previously

described (24, 127). Mice were maintained in a closed breeding facility in compliance with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego.

Cell Purification and transfers. Spleens were treated with collagenase D (1mg/mL, Roche, Indianapolis, IN) 20 min @ 37˚C and were depleted B cells using CD19 antibodies and bead magnetic enrichment. Splenic $CD4^+CD8^-PD1^+CD49d^+$ T cells from mixed chimeras were FACS-purified using a BD ARIA (BD Biosciences, San Jose, CA) by congenic marker (CD45.1, CD45.2). For retroviral transduction, LCMV specific SMARTA T cells were transduced after 48 hours of anti-CD3 and anti-CD28 as previously described(163). For adoptive transfer 2500 congenic CD45.1 LCMV specific SMARTA T cells FACS-purified by retroviral marker (GFP or Thy1.1) were injected i.v. 24 hours before LCMV infection. Purity for all cell types was >95%.

In-vivo CTL. β_2M KO splenocytes were incubated 3hours ex-vivo with 2.5uM pool of LCMV peptides GP_{67-77}, $GP1_{26-140}$, and $NP_{311-326}$ or 2.5uM irrelevant peptide $OVA_{323-340}$, CFSE labeled with 500nM or 50nM CFSE and 2.5x106 splenocytes of each target transferred 7 days post-infection. 12 hours later, spleens and livers harvested and the ratio of Live MHCII+ B cells target with LCMV vs OVA targets calculated normalized to the ratio of LCMV vs OVA splenocytes in naive animals by equation: (1- Experimental ratio/ control ratio) x100.

Flow cytometry. Surface and intracellular cytokine staining was performed as previously described (128) and separated by congenic marker CD45.1 and CD45.2 where indicated. Antibodies used were purchased from Ebioscience, Biolegend or BD Pharmingen (San Diego, CA) to stain splenocytes except LIVE/DEAD (Aqua-Invitrogen) and TGFβRII-PE (R&D systems): CD16/CD32 Fc block, Eomes PE-TR,

CD8a Alexa700, CD4 BV650, GranzymeB PE, Granzyme A PeCy7, IFNγ APC, IL-12 Alexa647, TNFα efluor450, IL2 PE, I-Ab eflour450, KLRG FITC, CD127 APCeflour780, PD1 BV605, CD49d PerCPCy5.5, CD69 PerCPCy5.5, CD45.1 BV605, CD45.2 BV650. LCMV specific tetramers D^b:GP_{31-41}, D^b:$GP_{276-286}$, D^b:$NP_{396-404}$ and I-A^b:GP_{67-77} were provided by the NIH tetramer core facility. Samples were acquired on a BD LSR II (BD biosciences) and analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

Cytokine Measurements. Secretion of IFNγ, TNFα and IL-2 in LCMV specific T cells was performed ex-vivo using the following peptides: GP_{31-41} or GP_{67-77} peptide stimulation of splenocytes incubated in the presence of 1 μg/mL Brefeldin A (BFA-Sigma, St. Louis, MO) for 5 hours at 37˚C before staining.

Microarray Analysis. RNA from 3 biological replicates, each pooled Splenic T cells from n=5 mixed chimeras were purified using Qiagen microRNA kit and microarray performed by UCSD genomics core facility on Affymetrix mouse 1.0 ST arrays. Similarly 3 biological replicates of SMARTA+ T cells over-expressing Eomes or empty vector were analyzed by microarray, each from 5 mice pooled, 8 days after co-transfer into the same chronic infectious environment. Differentially regulated genes were defined by False Discovery Rate (FDR) < 0.01 or Bonferroni adjusted p-value using VAMPIRE software. Hierarchical clustering and heatmaps of expression values for individual samples were generated using GENE-E. Gene set enrichment analysis (GSEA)(164) was performed using the java implementation from the Broad Institute website (http://www.broadinstitute.org/gsea) and applied to the following available microarray data sets: virus specific CD4 and CD8 T cells from acute and chronic LCMV infection in-vivo(69), in-vitro differentiate T helper subsets Th1, Th2,

Th17, and Tregs(165), Tfh(166), Anergic T cells(167), CD4+ CD8+ T cells from ThPOK deficient spleens(168), CD4 T cells from Ezh2-/- lymph nodes(169).

Statistics. Means were compared using Student's *t*-test in control littermates to corresponding gene-modified groups. Mann–Whitney U test was applied if data sets failed the Pearson omnibus normality test (alpha = 0.05). All analyses were two-tailed and carried out using GraphPad Prism 6.0. $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.0005$. Animal groups were matched by litter, age and sex. No blinding or statistical methods were used to predetermine sample size or groups.

Results

2.1 Sustained TGFβ signaling in CD8 and CD4 T cells during chronic LCMV infection

We have previously found increased TGFß production and signaling by anti-viral CD8 T cells early during chronic LCMV infection (74). Increased TGFß production and signaling by CD4 T cells also occurs during HIV infection (170). We first measured TGFβ signaling in the murine cell types over the course of chronic LCMV infection (LCMV CL13) compared to acute LCMV–Armstrong53b (LCMV-ARM) infection by flow cytometry. Gating on activated splenic CD4 and CD8 T cells expressing exhaustion marker PD1 we found sustained levels of intracellular phosphorylated SMAD2/3 during chronic LCMV CL13 infection over acute LCMV ARM infected or T cells from naive animals (Fig 2-1a). We confirmed this finding over time by western blot for activated CD4 T cells during chronic infection (Fig 2-1b). Similarly we found sustained levels of SMAD7, a downstream target and negative regulator of TGFβ signaling in $PD1^+$ T cells during chronic infection (Fig 2-1c). Next we analyzed SMAD7 levels on after gating on virus specific $I\text{-}A^b{:}GP_{67\text{-}77}$ CD4 T cells

and D^b:$GP_{276-286}$ CD8 T cells. Again LCMV specific CD4 and CD8 T cells from chronically infected mice have increased SMAD7 compared to acutely infected mice 8 days p.i. (Fig 2-1d). Increased SMAD7 is also present in CD4 PD1 cells from multiple tissues (Fig 2-1e). Overall these data indicate sustained TGFß signaling in activated CD4 T cells during chronic infection.

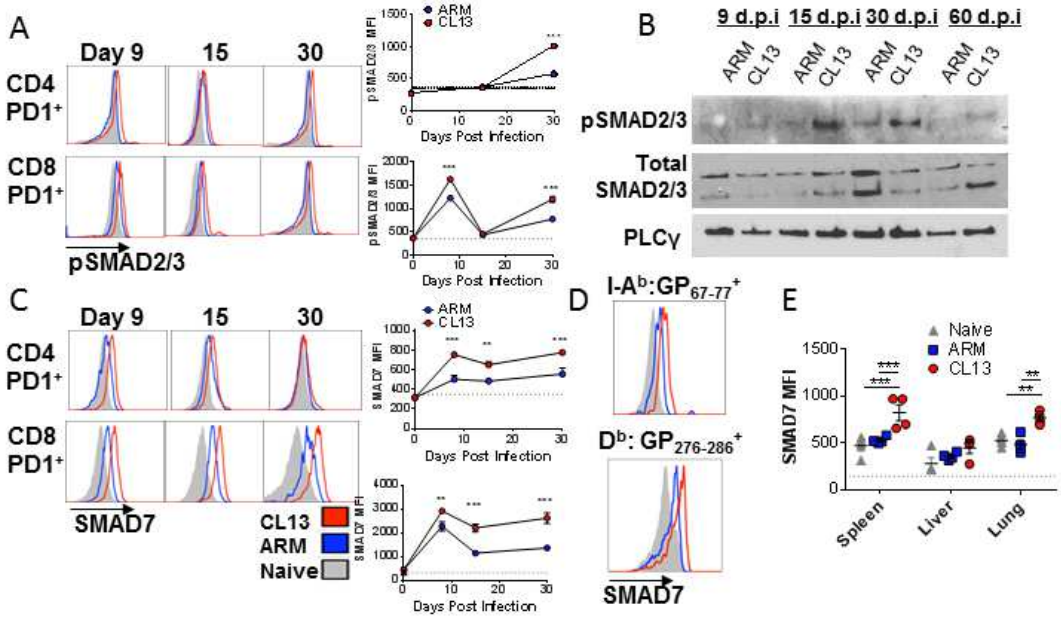

Figure 2-1. Sustained TGFβ signaling during chronic infection. C57BL/6 mice were infected with $2x10^6$ PFU of LCMV ARM or LCMV Cl13 i.v. or left uninfected. **A&C)** At indicated day post infection (d.p.i.) splenic $CD4^+$ or $CD8^+$ $PD1^+$ T cells were stained for intracellular phospho-SMAD2/3 or SMAD7 by flow cytometry. **B)** $CD4^+$ $CD44^+$ T cells were isolated from ARM and CL13 infected mice and phospho-SMAD2/3 (S465/7) measured by western blot at indicated times. **D)** LCMV specific CD4+ $I\text{-}A^b\text{-}GP_{67\text{-}77}$ or CD8+ $D^b\text{-}GP_{33\text{-}41}$ T cells were stained for SMAD7 at day 8 p.i. **E)** SMAD7 levels on CD4 $PD1^+$ T cells in indicated tissues 8 d.p.i. Representative of 2 independent experiments of 3-4 mice/group.

2.2 CD8 restricted TGFβRII deletion does not influence the course of chronic infection

To address the contribution of TGFβ signaling on individual cell types towards control of chronic infection we first used CD8(cre)xTGFβRII$^{fl/fl}$ to delete TGFβ Receptor exclusively in CD8 T cells during development and monitor T cell responses and viral titer in the blood over the course of chronic LCMV Cl13 infection. FACS analysis of surface TGFBRII expression before infection demonstrate ~90% reduction in TGFβRII expression in CD8 T cells but no reduction in CD4 T cells or B cells (Fig 2-2a). This model also has spontaneous activation of CD8 T cells expressing CD44 and KLRG1 before infection (Fig 2-2b). After LCMV Cl13 infection, CD8(cre)xTGFβRII$^{fl/fl}$ mice had similar early numbers of activated CD8 T cells in the blood 8 days p.i. compared to littermate control mice and maintained increased frequency of activated PD1^{+} CD8 T cell responses beyond day 30 post infection (Fig 2-2c). The number of immune-dominant NP396 CD8 T cells, which undergo substantial deletion (44), was similar between TGFβRII deficient and WT T cells. Whereas TGFβRII deficient LCMV GP_{33-41} specific CD8 T cells were increased in percentage and number in blood after D45 days post infection compared to littermate controls (Fig 2-2d&e). Heterozygous CD8cre^{+} TGFßRII$^{flox/+}$ mice resemble Cre^{-} WT littermate controls and are omitted for clarity.

To assess the degree of functional exhaustion we measured virus specific LCMV GP_{33-41} CD8 T cells cytokine production after cognate peptide stimulation ex-vivo. LCMV GP_{33-41} CD8 T cells produced more IFNγ in absence of TGFβRII compared to control CD8 T cells only after 30 days post infection (Fig 2-2f). These TGFβRII deficient cells also expressed more terminal differentiation marker KLRG1

late but nor early after chronic infection (Fig 2-2g). These results indicate TGFβRII signaling limits accumulation and function of some anti-viral CD8 T cells late, but not early, after chronic infection.

However despite some increase in CD8 T cell number and function, viremia in CD8(cre)xTGFβRII$^{fl/fl}$ mice persisted similar to WT controls falling below limit of detection around Day 60 p.i. (Fig 2-2h). These data suggest that deletion of TGFβ signaling on CD8 T cells is not sufficient to impact viral control. Because CD8 T cells from CD8(cre)xTGFβRII$^{fl/fl}$ mice delete the TGFβ-Receptor during development and they are more activated and express more KLRG1 (prior to infection), as well as Granzyme B and Eomes (not shown) we cannot rule out a contribution of TGFβ signaling before infection in this model.

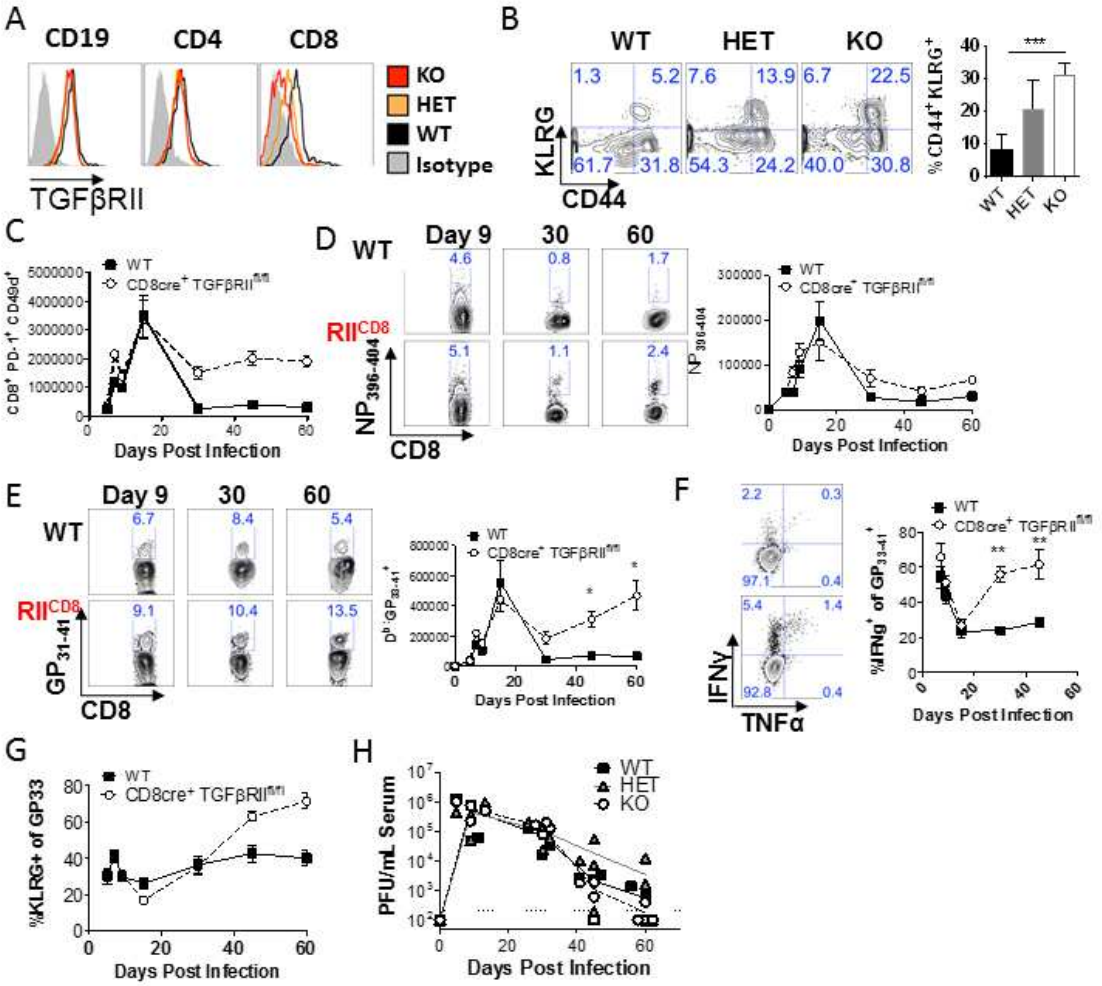

Figure 2-2. CD8 restricted TGFβRII deletion does not influence the course of chronic infection. A) CD8cre^{+} TGFβRII$^{flox/flox}$ (RII^{flox}) mice and Cre- littermate controls were infected with 2x10^{6} PFU of LCMV Cl13 . Blood was monitored for the presence of LCMV specific T cells by flow cytometry. **A**) TGFßRII expression on B cells CD4 T cell and CD8 T cells prior to infection. **B**) Percentage of activated CD8 T cells expressing KLRG prior to infection**. C)** Number of PD1+ CD49d+ CD4 T cells. **D)** Number of virus specific D^{b}:NP$_{396-404}^{+}$ cells over time post infection. **E)** Percentage and number of virus specific D^{b}:GP$_{33-41}^{+}$ cells over time post infection. **F)** IFNγ and TNFα production upon cognate peptide ex-vivo graphed as a percentage of tetramer+ cells from (c). **G)** KLRG expression on GP33 virus specific CD8 T cells. **H)** Control of viremia over time by plaque assay as PFU/mL serum (dotted line, limit of detection). Representative of 3 independent experiments of n=4-5 mice/group.

2.3 Cell-intrinsic TGFβRII signaling in adults does not influence LCMV-specific CD8 T cells

To examine the contribution of TGFß signaling toward immune regulation solely during chronic viral infection we next used a temporally controlled ERcre strain crossed to TGFßRII conditional allele ($TGFßRII^{flox/flox}$) to proximately delete the TGFßRII using injection of Tamoxifen (TAM) in adult mice(123). To further isolate direct effects of TGFß on the immune response to LCMV we generated mixed bone marrow chimeras of 50 percent WT (CD45.1) bone marrow and 50 percent ERcre+$TGFßRII^{fl/fl}$ (RII^{flox}) bone marrow into lethally irradiated CD45.1 host mice. Eight weeks post reconstitution, mice were tamoxifen injected and subsequently infected with high dose LCMV CL13 to establish chronic infection. After 5-day treatment, surface TGFßRII levels in circulating lymphocytes were reduced to 90 percent of levels in ERcre- littermate control mice (Fig 2a). We have previously shown that this treatment leads to 95% reduction in TGFβr2 mRNA in circulating leukocytes(171). Previous pan-mouse or T cell specific genetic models in which TGFß signaling is decreased exhibited T cell activation associated with TGFß deficiency during development and lymphopenia in young mice (96, 145). In contrast, we observed that after TGFßRII deletion in adult mice, CD4 and CD8 T cells had similarly low levels of activation as WT mice before infection, analyzed by CD44 and CD62L expression (Fig 2b).

To find potential cell intrinsic differences in hematopoietic cell types in absence of TGFßRII we analyzed lymphocyte responses of mixed chimeric animals in multiple tissues at 9 days post-infection. Antiviral CD8 T cell responses to the immuno-dominant epitope D^b:GP_{33-41} were similar in representation from either the

WT compartment or TGFßRII KO CD8 T cell compartment in both lymphoid and non-lymphoid organs (Fig 2-3c). Both $NP_{396-404}$ and $GP_{276--286}$ epitopes were also similar between WT and TGFßRII KO CD8 T cells (Fig 2-3d). TGFß signaling also minimally changed antiviral cytokine secretion by D^b:GP_{33-41} LCMV specific CD8 T cells upon cognate peptide stimulation, where WT exhausted T cells lose TNFα and IL-2 production early after chronic infection (Fig 2-3e).

Consistent with similar chimerism above, we do not see changes in proliferation of TGFβRII deficient CD8 T cells compared to WT T cells in the same environment (Fig 2-3f). We also found adult TGFßRII deletion only minimally suppresses KLRG or Ly6C expression in virus specific CD8 T cells (Fig 2-3g&h). In addition, total Granzyme B expression in CD8 T cells was similar between WT and TGFßRII KO compartments in both spleen and peripheral tissues early during chronic infection (Fig 2-3i). Consistent with effector function and differentiation, we found similar TBET expression and equally high levels of EOMES in antiviral CD8 T cells without TGFβRII compared to their WT counterparts during chronic LCMV infection (not shown). Overall these data suggest that inducible deletion of TGFβRII in adult mice only minimally affected CD8 T cell antiviral responses early after chronic LCMV infection, dramatically contrasting the profound cell-intrinsic CD8 T cell effects that we and others previously reported in dnTGFßRII mice challenged with LCMV Cl13, or other pathogens and tumors(74, 80, 106, 155, 160, 161).

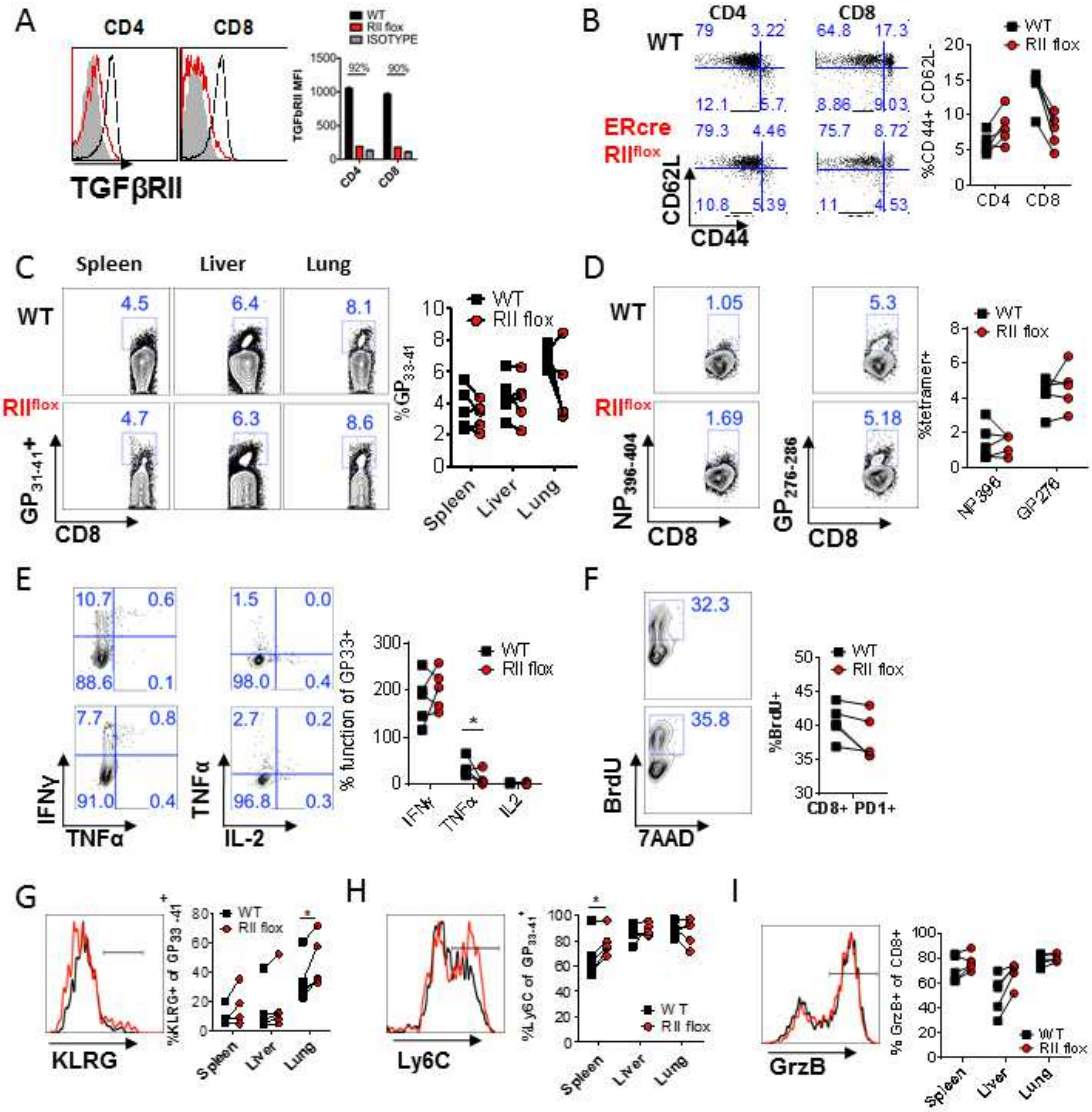

Figure 2-3. Cell-intrinsic TGFβRII signaling in adults does not influence LCMV-specific CD8 T cells. 8 weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+} TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow, mice were tamoxifen treated and infected with 2x10^{6} PFU of LCMV Cl13. Spleens and tissues were analyzed 9 days p.i. for the presence of LCMV specific T cells by flow cytometry. **A)** Prior to infection, levels of surface TGFßRII on circulating leukocytes. **B**) CD44 and CD62L activation markers on total CD4 and CD8 T cells before infection. **C)** Percentage of D^{b}:GP$_{33-41}$ CD8 T cells after gating on CD8 T cells from donor compartments in indicated tissue **D**) percentage of D^{b}:NP$_{396-404}$ and D^{b}:GP$_{276-286}$ specific LCMV T cell in the spleen. **E)** Co-production of intracellular IFNγ, TNFα, IL-2 after 5 hr stimulation with GP$_{33-41}$ cognate peptide. **F)** Incorporation of BrdU after 16 hour pulse in splenic CD8 PD1^{+} T cells from either WT compartment (upper) or ERcre+TGFßRII$^{flox/flox}$ (lower). **G&H)** Overlays and percentages of virus specific D^{b}:GP$_{33-41}$$^{+}$ cells expressing KLRG and Ly6C in WT (Black) and ERcre^{+} TGFβRII^{flox} (Red) compared to Naïve Cd4 T cells (Grey). **I)** Granzyme B expression in CD8 T cells from WT or TGFβRII^{flox} compartments. Representative of 2-3 independent experiments of n=4-5 mice/group.

2.4 Cell-intrinsic TGFßRII signaling in adults limits CD4 T cell proliferation

CD4 T cells are critical to maintain CD8 T cell and B cell responses during chronic infection. Here, TGFß signaling significantly limited the accumulation of activated PD1^{+} CD4 T cells across tissues during infection (Fig 2-4a). Accumulation of PD1^{+}CD4 T cells was accompanied with enhanced proliferation measured by Brdu incorporation in the absence of TGFßRII but not significant changes in the apoptosis marker Annexin V (Fig 2-4b). To specifically compare virus specific CD4 T cells we analyzed I-A^{b}:GP$_{61-80}$ tetramer staining in WT versus TGFßR deficient CD4 T cells. Again TGFßRII deficient LCMV specific T cells accumulated over their WT counterparts (Fig 2-4c).

We next evaluated CD4 T cell differentiation in WT versus TGFßR deficient compartments in mixed chimeric mice. Th1 responses that are typically generated in viral infection depend on TBET and IFNγ secretion. TGFβ is known to inhibit TBET and IFNγ production by CD4 T cells in-vitro and in-vivo (172, 173). Chronic infection also causes progressive decreases in cytokine production and TBET expression compared to acute infection (60, 70). We found, however, similar levels of IFNγ secretion per tetramer+ cell upon cognate peptide stimulation with GP$_{67-77}$ LCMV peptide (Fig 2-4d). Consistently we found no difference in TBET expression (Fig 2-4e). In addition, TGFßRII deficient IFNγ^{+} producing CD4 T cells produced similar TNFα and Il-2 than IFNγ+ WT CD4 T cells after stimulation on a per cell basis indicating no change in T cell exhaustion.

Th1 CD4 T cells exhibit increased SLAM expression whereas Tfh subset can be distinguished by high CXCR5 surface levels alongside increased expression of the

transcription factor BCL6 (174). We found TGFßRII KO I-A^b:GP_{61-80} CD4 T cells trended toward decreased proportion of CXCR5+ cells (Fig 2-4g). However, we found no differences levels of BCL6 expression, delineating T follicular helper T cells (Tfh), in virus specific GP_{61-80} CD4 T cells (Fig 2-4f). CD4 T regulatory cells (Treg) that express FOXP3 relies on TGFß for development in-vitro and in-vivo (175-177). However, TGFß signaling is not necessary for the maintenance of FOXP3 or Treg function in-vivo(95). While I-A^b:GP_{61-80}^+ T cells do not become Treg cells (178) we observed slight decreases in Treg representation in activated CD4 $PD1^+$ T cells between TGFßRII deficient and WT animals consistent with previous work (Fig 2-4h). Overall these data indicate TGFß signaling limits CD4 proliferation but does not substantially influence Th1 differentiation.

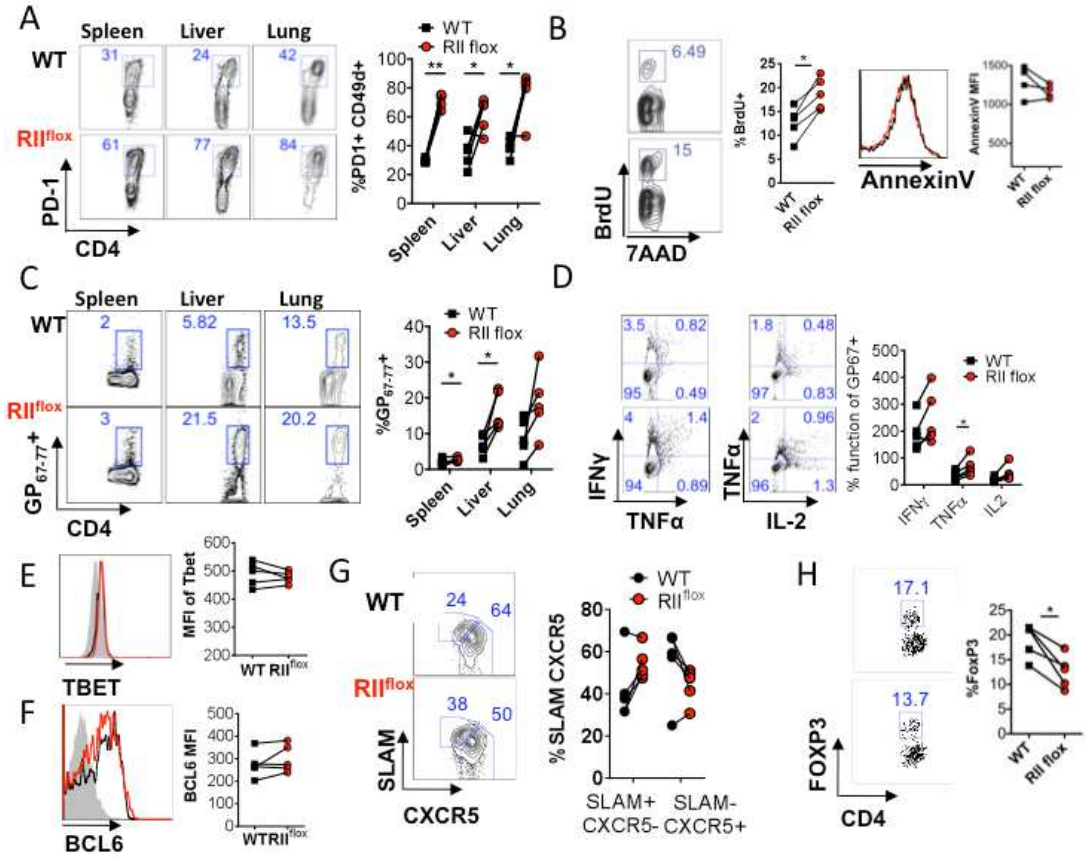

Figure 2-4. Cell-intrinsic TGFβRII signaling in adults limits CD4 proliferation. Eight weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+} TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow, mice were tamoxifen treated and infected with 2x10^{6} PFU of LCMV Cl13. Spleens and tissues were analyzed 9 days p.i. for the presence of LCMV specific T cells by flow cytometry. **A)** Percentage of PD1^{+} CD4 T cells after gating on CD4 T cells from each donor compartment in indicated tissue **B)** Incorporation of BrdU after 16 hour pulse in splenic CD4 PD1^{+} T cells from either WT (upper) or ERcre+TGFßRII$^{flox/flox}$ (lower) or Annexin V staining (right) **C)** Percentages of virus specific I-A^{b}:GP$_{67-77}$$^{+}$ cells in indicated tissue. **D)** Co-production of intracellular IFNγ, TNFα, IL-2 after 5 hr stimulation of splenocytes with GP$_{67-77}$ cognate peptide. **E&F)** Representative overlays and MFI plotted for TBET and BCL6 expression in CD4 I-A^{b}:GP$_{61-80}$ T cells in WT (Black) and Ercre^{+} TGFβRII^{flox} (Red) compared to Naïve Cd4 T cells(Grey). **G)** SLAM vs CXCR5 staining on CD4 I-A^{b}:GP$_{61-80}$ T cells. **H)** FOXP3 expression in CD4 PD1^{+} T cells from WT or TGFßRII KO compartment Representative of 2-3 independent experiments of n=4-5 mice/group.

2.5 Direct TGFβRII signaling in CD4 T cells suppressed terminal differentiation and NK/CD8 T cell cytotoxic gene program during chronic LCMV infection

We next performed microarray analysis on sorted PD1^{+} CD49d^{+} CD4^{+} (CD8^{-}) T cells from chronically infected mixed chimeric mice. We found that CD4 T cells from the TGFßRII deficient compartment expressed more Granzyme K, Granzyme B, Perforin, KLRG, Ly6C and the transcription factor EOMES than their WT counterparts (Fig 2-5a). TGFβRII deficient CD4 T cells also expressed more NK cells markers associated with cytotoxicity, NKG2C, CrTAM, and CD94. Concomitantly TGFβRII deficient cells had decreased FOXP3, BCL6, IL6r, and other Treg and Tfh associated genes. We further analyzed TGFBRII deficient microarray by GSEA analysis to published in-vitro derived helper subsets as well as T cell exhaustion signatures comparing CD4 and CD8 T cells from acute and chronic LCMV infections (31, 69). Indeed we found TGFβRII deficient CD4 T cell gene network over-represented in Th1, Th2 and Th17 specific genes in-vitro, where as Tfh and Treg genes were underrepresented (Fig 2-5b). Strikingly, TGFβRII deficient CD4 T cells gene networks more closely resembled exhausted CD8 T cells as well as effector CD8 T cell during acute LCMV infection rather than effector or exhausted CD4 T cells (Fig 2-5c).

Previous reports of responding CD4 T cells in acute LCMV infection are described as expressing both PSGL1 and Ly6C where PSGL1^{-}Ly6C^{-} are enriched for Tfh, PSGL1^{+}Ly6C^{-} are effector memory, and PSGL1^{+}Ly6C^{+} are terminally differentiated Th1 cells(179). These Ly6C^{+} Th1 CD4 T cells express more TBET and Granzyme B during acute infection, which is silenced ultimately by DNA methylation in Tfh (180). During chronic infection Ly6C effector cells are lost (181), and Tfh

predominate as a T helper cell subset (182). In absence of TGFß signaling, we found that activated CD4 T cells in the spleen and non-lymphoid tissues express more Granzyme B (Fig 2-5d). Gating on activated $PD1^+$ $CD49d^+$ CD4 T cells showed increased PSGL1 and Ly6C as well (Fig 2-5e). Importantly, gating on splenic $Ly6C^+$ cells expressed more Eomes and Granzyme protein on a per cell basis (Fig 2-5f), indicating the gene pattern by microarray cannot fully be explained by shifts in Tfh vs Th1 differentiation or proliferation of TGFßRII deficient Ly6C+ T cells and might represent a gain of function.

Given the increased expression of cytotoxicity associated genes in TGFßR deficient CD4 T cells we next evaluated if this phenotype resulted in increased CD4 T cell CTL activity during chronic LCMV infection. Cytotoxicity by CD4 T cells does occur in-vivo during peak LCMV-ARM response in intact C57BL/6 mice and can be as efficient as CD8 T cells (183, 184). CTL-like CD4s are also generated in influenza and West Nile Virus infection (185, 186). To test CTL activity during chronic infection we performed an in-vivo CTL assay with adoptively transferred 1:1 ratio of CFSE labeled β_2M-/- splenocytes coated with either NP_{311} GP_{126} GP_{67} (LCMV-Targets) or OT-II peptides (OVA-Control) followed by organ analysis. Indeed acutely infected LCMV-ARM mice have decreased proportion of LCMV coated $MHC\text{-}II^+$ B cell targets to control targets compared to proportions of $MHC\text{-}II^-$ cells in the same mouse or to proportions of $MHC\text{-}II^+$ B cell targets in naïve mice, found in both the Spleen and Liver. However at the same time after chronic LCMV Cl13 infection, there is little detectable change in LCMV target to control target proportions, indicating minimal in-vivo MHC-II dependent CTL activity during chronic LCMV infection (Fig 2-5g). These

results suggest, consistent with CD8 effector function, CD4 T cells CTL activity is also suppressed early during chronic infection.

To investigate the role of TGFß signaling on endogenous CD4 T cell cytotoxic activity during chronic infection, we transferred target cells into WT or tamoxifen-treated ERcre TGFßRII deficient mice at day 8 p.i.. Although some experiments suggested a tendency towards more CD4 T cell CTL activity in ERcre^{+} TGFßRII deficient animals compared to WT mice, we were unable to reproducibly detect increased CTL activity (Fig 2-5h), indicating ablation of TGFß signaling is not sufficient to restore CTL activity to CD4 T cells during chronic infection.

Overall, these results indicated TGFß signaling prevented terminal differentiation of CD4 T cells and suppressed aberrant expression of cytotoxic associated genes normally not expressed by CD4 T cells but rather in CD8 T cells and NK cells.

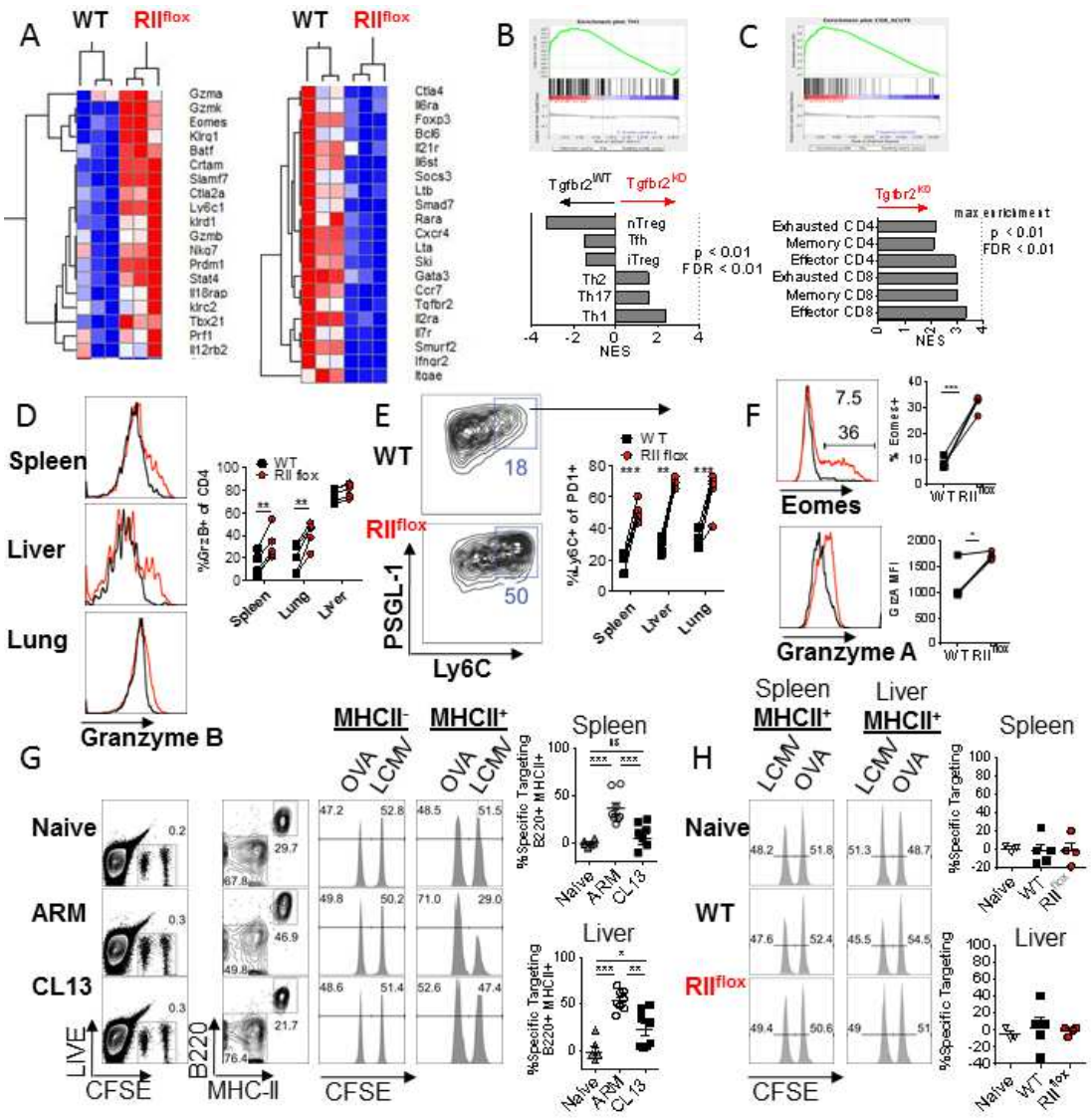

Figure 2-5. Direct TGFβRII signaling in CD4 T cells suppressed terminal differentiation and NK/CD8 T cell cytotoxic gene program during chronic LCMV infection. 8 weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+}TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow, mice were tamoxifen treated and infected with 2x10^{6} PFU of LCMV Cl13 and spleens and tissues analyzed 9 days later. **A**) FACS sorted CD4^{+}CD8^{-} PD1^{+}CD49d^{+} cells from each compartment were analyzed by microarray. Representative genes shown as a relative heat map of expression values (Blue = Min; Red = Max) from differentially regulated genes (FDR<0.05) **B)** GSEA analysis and Noramlized enrichment score (NES) score of TGFßRII CD4 T cell array signature compared to different published T cell helper subsets generated in-vitro **C)** GSEA comparison to published exhausted Cd4 and Cd8 T cells microarrays during LCMV infection. **D)** Flow cytometry analysis of Granzyme B in total CD4 T cells in tissues. **E)** PSGL1 and Ly6C gated on CD4 PD1^{+} cells **F)** Eomes and Granzyme A expression gated on Ly6C^{+} from (d). **G)** In-vivo CTL assay in spleen and liver 12 hours after transfer 2.5x10^{6} B2M-/- splenocytes coated with LCMV target or control peptides with 500nM or 50nM CFSE. **H)** In-vivo CTL in LCMV CL13 infected ER-cre^{+}TGFßRII deficient mice 8 days p.i. Representative of 2-3 independent experiments of n=5 mice/group.

2.6 SMAD4 and EOMES oppositely recapitulated TGFBRII deficient CD4 T cell phenotype during chronic LCMV infection

SMAD4 binds and suppresses Granzyme and Perforin cytotoxic loci in CD8 T cells in tumors (187). SMAD3 and SMAD4, but not SMAD2 suppresses EOMES and not TBET downstream of TGFßRI chemical inhibition to regulate cytotoxic function in CD8 T cells allowing control of B16 melanoma bearing C57LB/6 mice in-vivo(188). However in CD4 T cells Eomes expression is only mildly SMAD2/3 dependent and is functionally JNK dependent in the context of Th17 differentiation in-vivo (189, 190).

To determine if TGFßRII induced changes are SMAD dependent in activated CD4 T cells we again utilized conditional temporal knockout of SMAD4^{flox} allele with Tamoxifen responsive ERcre. Mixed chimeras of 1:1 WT and ERcre^{+} SMAD4^{flox} were allowed to reconstitute for 8 weeks followed by tamoxifen treatment and LCMV Cl13 infection. We found that in contrast to TGFβRII deficiency, SMAD4 deficiency during infection substantially limited accumulation of both anti-viral CD4 in the blood 8 days p.i. (Fig 2-6a). In addition SMAD4 KO T cells express low amounts of Ly6C, Granzyme B, Eomes, and KLRG (Fig 2-6b). These results correspond to recent work using GrzB-Cre to delete SMAD4 in CD8 T cells upon LCMV-ARM infection, which identified SMAD4 as a necessary co-factor for c-myc up-regulation to drive cell division underlying a similar cellular deficiency (191). Here we find SMAD4 deficiency compromises effector differentiation as well.

On the other hand, Eomes is sufficient to drive cytotoxicity genes directly in CD8 T cells and NK cells early during immune responses as well as in CD4 T cells in-vitro (192-194). To test the role of Eomes in modulating antiviral T cell differentiation in-vivo we retrovirally transduced SMARTA TCR-transgenic T cells specific for the

LCMVI-A^b:GP_{61-80}^+ epitope (195) to express constitutively active Eomes (EO^{VP16}) or dominant negative EOMES (EO^{DN}) (194) and adoptively transferred T cells into C57BL/6 mice prior to LCMV CL13 infection. SMARTA cells were analyzed in the blood 9 days after infection. SMARTA T cells expressing constitutive active Eomes significantly increased in number compared to empty vector controls, whereas dominant-negative Eomes suppressed T cell accumulation (Fig 2-6c). Constitutive active Eomes also expressed more PSGL1 and Ly6C compared to empty vector SMARTA T cells in vivo while dominant negative Eomes reduced Ly6C expression (Fig 2-6d). These results indicate modulating Eomes expression partially recapitulates the accumulation and terminal differentiation phenotype of TGFßRII deficient CD4 T cells during chronic LCMV infection.

To evaluate the extent of overlap we co-transferred SMARTA over-expressing Eomes (IRES-GFP) with SMARTA expressing empty vector (IRES-Thy1.1) into the same mice prior to chronic LCMV infection. Eight days post infection splenic SMARTA cells were isolated and analyzed by microarray (Fig 2-6e). Eomes induced gene expression pattern overlapped with that of TGFβRII deficiency. Nineteen of 150 genes upregulated in absence of TGFßRII overlap with the 60 genes upregulated by Eomes (Fig 2-6f left). A heatmap of these overlapping genes, all of which are NK associated or cytotoxic molecules including perforin, Granzyme A and K, klrg1, and klrc2 are modulated by EOMES in CD4 T cells (Fig 2-6f right). These changes were confirmed by protein via FACS where SMARTA-$EOMES^{VP16}$ cells were analyzed for Eomes and Granzyme A and B expression in a competitive setting (Fig 2-6g). Indeed EOMES expressing cells had higher levels of Granzyme B and Granzyme A in-vivo compared to empty vector control SMARTA T cells. These data indicate a portion of

TGFβRII induced changes, including proliferation, terminal differentiation, and cytotoxic gene expression can be replicated by constitutively active Eomes.

Recently ThPOK suppressed CD8 identity and a similar set of cytotoxic genes including CD8a in CD4 T cells during development(168). Indeed cross analysis of TGFβRII array targets here with SMARTA-Eomes above or published ThPOK deficient $CD4^+$ $CD8^+$ double-positive T cell microarrays shows significant overlap of gene networks enriched by GSEA analysis (fig 2-6h). Similarly, Ezh2 deficiency leads to Eomes up regulation in CD4 T cells(169) and Ezh2 microarrays overlap substantially with TGFßRII arrays during chronic infection (Fig 2-6h). Overall these data suggest SMAD4 and Eomes integrate TGFβ signaling to suppress both number and terminal differentiation of CD4 T cells. TGFβ might also maintain CD4 identity distinct from cytotoxic T cells during inflammatory conditions, similar to ThPOK and Ezh2.

Finally we tested whether transfer of $Eomes^{VP16}$ or $Eomes^{DN}$ SMARTA cells could impact long-term outcome of LCMV infection. For that, we monitored viremia over time after adoptive transfer into individual mice as in (a). EOMES modulation in SMARTA CD4 T cells did not change the outcome of CL13 infection (Fig 2-6i), compared to empty vector SMARTA T cell transfer. These data indicate sustained Eomes expression in CD4 T cells does not overtly influence the viral load of chronic LCMV infection.

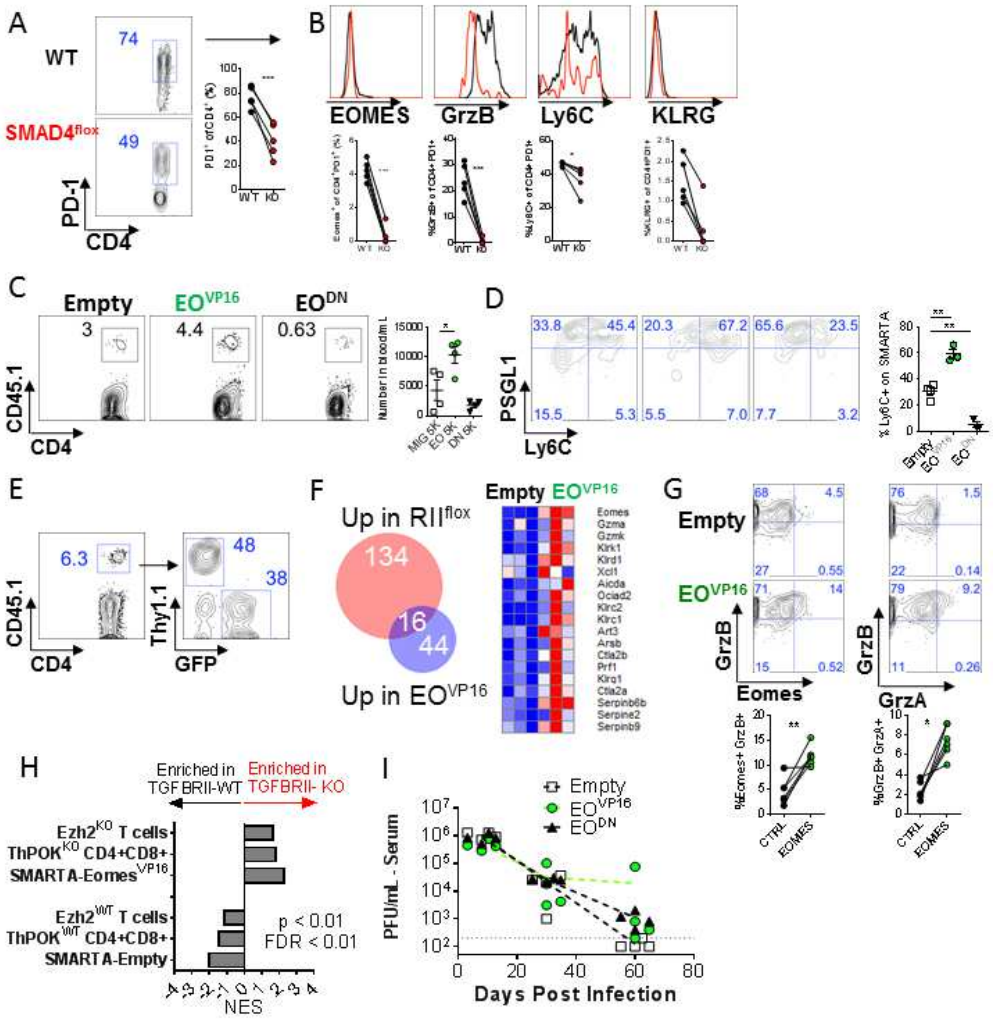

Figure 2-6. **SMAD4 and EOMES oppositely recapitulated TGFBRII deficient CD4 T cell phenotype during chronic LCMV infection. A)** 8 weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+}SMAD4$^{flox/flox}$ (SMAD4^{flox}-CD45.2) bone marrow, mice were tamoxifen treated and infected with 2x10^{6} PFU of LCMV Cl13 and spleens and tissues analyzed 9 days later for the presence of LCMV specific T cells by flow cytometry. Percent of activated PD1^{+} CD4 T cells after gating on congenic marker. **B)** Expression of indicated markers on PD1^{+} cells from (A). **C)** Retroviral over expression of constitutively active EOMES (EO^{VP16}) or Dominant negative Eomes (EO^{DN}) compared to empty vector (MIG-empty) in Transgenic LCMV specific SMARTA+ CD4 T cells (congenic CD45.1$^{+)}$ transferred into C57BL/6 mice 24hr before infection and analyzed 9 days later. **C)** Number of SMARTA T cells in the blood 9 d.p.i. **D)** Expression of PSGL1 and Ly6C in Eomes modified T cells from (C) **E)** Co-transfer of MIG-Eomes and Thy1.1+ empty vector transduced SMARTA+ T cells analyzed 9 days post-infection by microarray **F)** Venn diagram of overlap between genes upregulated in absence of TGFbRII and Eomes over-expression (left) and heatmap of 16 overlapping genes. **G)** Granyzme A and B expression in cells from (E). **H)** Normalized enrichment score (NES) from GSEA analysis of TGFßRII regulated genes against, SMARTA-EOMES, CD4^{+} CD8^{+} DP cells from thPOK-/-, and CD4 T cells from Ezh2-/- mice. **I)** Viremia of mice from (A) determined by plaque assay. Representative of 2-3 independent experiments of n=4-5 mice per group.

2.7 Cell-intrinsic TGFβRII signaling in adults does not limit LCMV-specific CD8 T cell accumulation after established chronic infection

We found sustained TGFβ signaling in T cells during chronic infection. Many T cell fate decisions including Th1 vs Tfh can be made as early as 3 days p.i. in acute LCMV (196, 197). To determine cell-intrinsic effects of TGFßRII signaling on individual cells types during a therapeutic window of established chronic infection we also performed TGFßRII deletion two weeks after LCMV CL13 infection using mixed bone marrow chimeras of 50% WT BM and 50% ERcre^{+}TGFßRII bone marrow as above. TGFßRII deletion was initiated starting 12 days p.i. and efficiently deleted in blood leukocytes by day 20 p.i. (Fig 2-7a). Spleens were further analyzed at 30 days p.i. Late deletion of TGFßRII does not allow accumulation of virus specific D^{b}:GP_{33-41} CD8 T cells in the Spleen or peripheral tissues (Fig 2-7b). Antiviral CD8 T cells without TGFßRII are also similarly exhausted as WT CD8 T cells in producing IFNγ with only minimal differences in TNFα and IL-2 upon cognate peptide stimulation (Fig 2-7c). Virus specific CD8 T cells do not have increased KLRG, Granzyme B or EOMES expression sans TGFßRII signaling (Fig 2-7d). Overall these data again indicate minimal regulation of antiviral CD8 T cells by TGFß late during established chronic infection, in contrast to developmental deletion of TGFßRII in CD8 T cells which accumulated anti-viral T cells late in chronic infection.

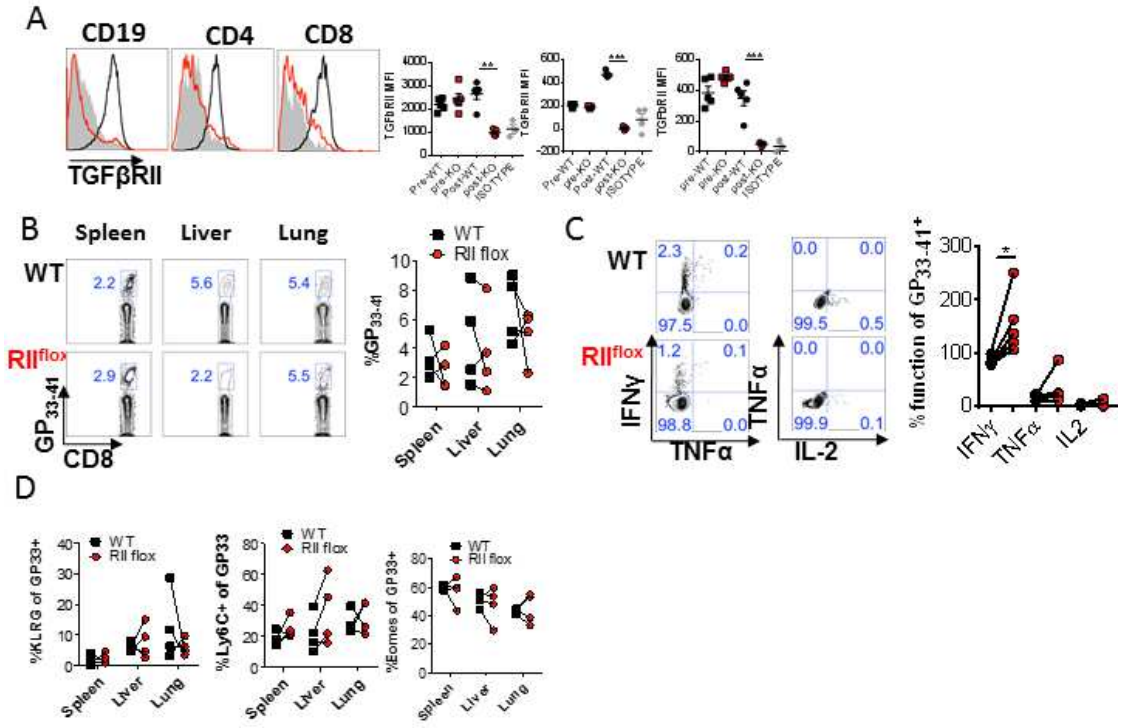

Figure 2-7. Cell-intrinsic TGFβRII signaling in adults does not limit LCMV-specific CD8 T cell accumulation after established chronic infection. 8 weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+}TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow, mice were first infected with 2x10^{6} PFU of LCMV Cl13 then tamoxifen treated between Days 12-17p.i. and spleens analyzed at day 30 p.i. for the presence of LCMV specific T cells by flow cytometry. **A)** Representative overlays of TGFβRII expression of CD19^{+} B cells, CD4^{+} and CD8^{+} T cells post-Tamoxifen in RII^{flox} and WT animals, MFI of cell types pre and post tamoxifen is graphed (right). **B**) Representative plots and percentage D^{b}:GP$_{31-41}$$^{+}$ of CD8 T cells in indicated tissues. **C)** Co-production of intracellular IFNγ, TNFα, IL-2 after 5 hr stimulation with GP$_{31-41}$ specific peptide **D**) Percent of D^{b}:GP$_{31-41}$$^{+}$ T cells expressing KLRG1, Ly6C and Eomes. Representative of 2-3 independent experiments of n=5 mice/group.

2-8. Cell-intrinsic TGFβRII signaling in adults limits LCMV-specific CD4 T cell accumulation after established chronic infection

Next we further compared CD4 T cells in absence of TGFß signaling late after LCMV CL13 infection. CD4 T cell proliferation and effector differentiation have largely subsided late in infection. , we cin proportions of TGFßRII deficient CD4 T cell compared to the WT T cell compartment. While the proportion of proliferating cells was decreased compared to 8 days p.i., activated PD1^{+} TGFβRII deficient CD4 T

cells have significantly increased proliferation compared to WT cells (Fig 2-8b), as described above for early TGFßRII deletion. This increased proliferation was likely insufficient to increase the numbers of virus specific I-A^b:GP_{61-80}^+ T cells which were comparable in WT versus ERcreTGFßRII compartments (Fig 2-8c). Similarly to early TGFßR deletion, we observed no differences in IFNγ expression and again minimal increases in TNFα and IL-2 secretion in TGFßRII deficient CD4 T cells upon cognate peptide stimulation (Fig 2-8d). Consistently, we again observed no difference in TBET expression between WT and TGFβRII deficient CD4 T cell late in infection (Fig 2-8e). We further analyzed Tfh differentiation by BCL6 expression by I-A^b:GP_{61-80}^+ LCMV specific CD4 T cells and found similar BCL6 expression with TGFßRII deletion (Fig 2-8f), as well as SLAM vs CXCR5 proportions of Th1 and Tfh cells between WT and TGFßRII deficient T cell compartments (Fig 2-8g). In contrast to the early TGFßRII deletion, there was no significant difference in Treg proportions in CD4 $PD1^+$ T cells without TGFß RII late in infection (Fig 2-8h). There is however increased TGFBRII KO T cell differentiation towards $PSGL1^+Ly6C^+$ cells (Fig 2-8i) with increased Eomes, (albeit similarly low Granzyme B) in both $PD1^+$ and I-A^b:GP_{61-80}^+ T cells (Fig 2-8j). These data indicate that similar to TGFßRII deletion before infection, receptor deletion after infection does not influence canonical T helper differentiation but does repress terminal differentiation and Eomes expression.

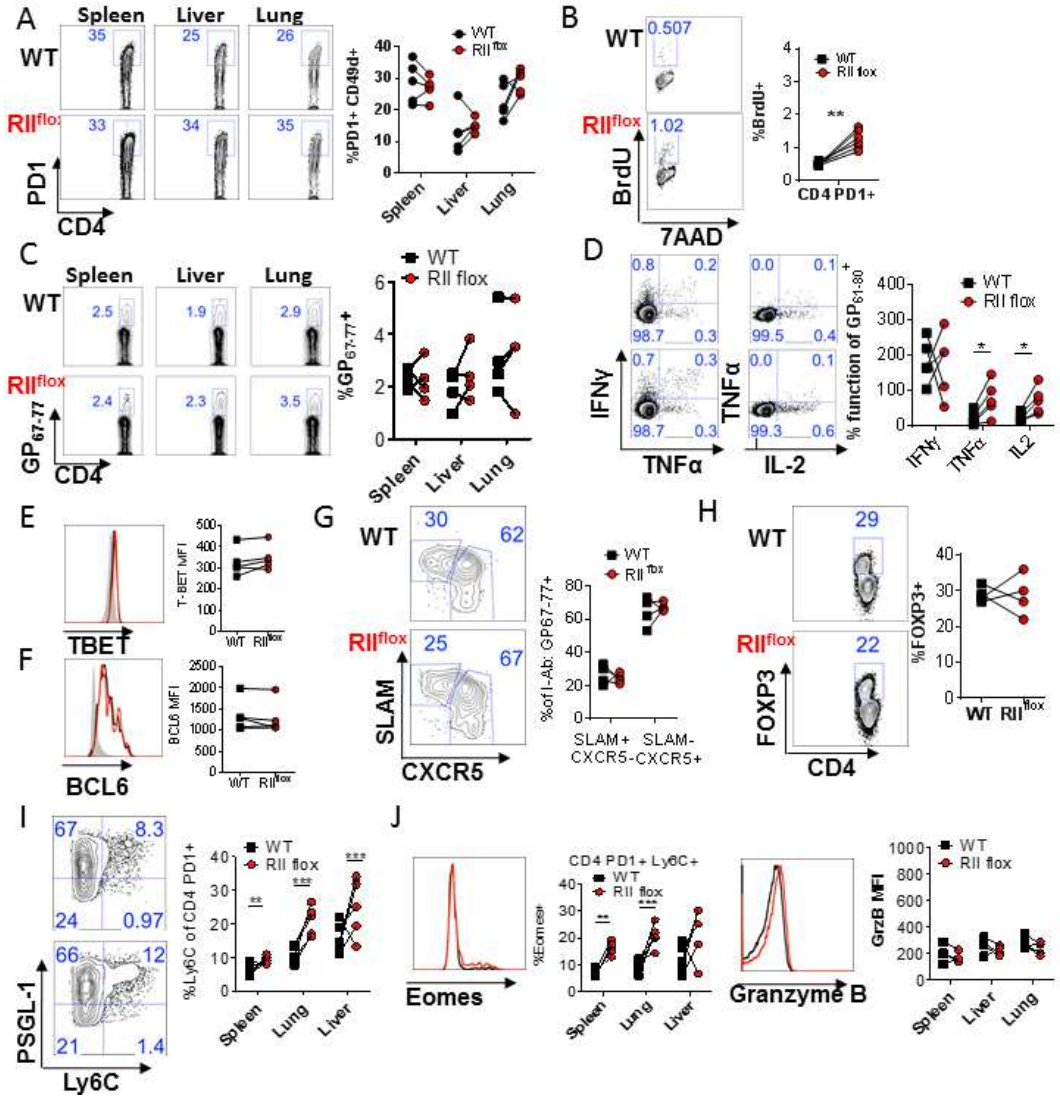

Figure 2-8. Cell-intrinsic TGFβRII signaling in adults limits LCMV-specific CD4 T cell differentiation after established chronic infection. 8 weeks post-bone marrow reconstitution with 1:1 mix of WT(CD45.1) and ERcre^{+}TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow, mice were first infected with 2x10^{6} PFU of LCMV Cl13 then tamoxifen treated between Days 12-17p.i. and spleens analyzed at day 30 p.i. for the presence of LCMV specific T cells by flow cytometry. **A)** Percentage of activated PD1^{+} CD4 T cells from each compartment in indicated tissues. **B**) BrdU incorporation after 16hours in CD4 PD1^{+} T cells. **C)** Accumulation of CD4 I-A^{b}:GP$_{61-80}$ T cells by tetramer staining in indicated tissues from either WT compartment (upper) or ERcre+TGFßRII$^{flox/flox}$ (lower). **D)** Co-production of intracellular IFNγ, TNFα, IL-2 after 5 hr splenocyte stimulation with GP$_{67-77}$ specific peptide. **E&F)** Overlays and MFI plotted for TBET and BCL6 expression in CD4 I-A^{b}:GP$_{61-80}$ T cells, Naïve CD4 T cells(Grey), WT after infection (Black) and ERcre^{+} TGFβRII^{flox} (Red). **G)** SLAM vs CXCR5 staining on CD4 I-A^{b}:GP$_{61-80}$ T cells. **H)** FOXP3 expression on CD4 PD1^{+} cells **I)** PSGL1 and Ly6C gated on CD4 PD1^{+} **J)** Eomes and Granzyme B expression gated on CD4 PD1^{+} cells. Representative of 2-3 independent experiments of n=5 mice/group.

CD4 restricted TGFβRII signaling does not influence the course of chronic infection.

Multiple immune functions including CD4 T cell IL-21 production and Tfh escalation, together with CD8 T cell and antibody responses are necessary to control chronic LCMV infection (18, 198, 199). To further validate the effect of TGFßRII signaling in CD4 T cells in a non-chimeric setting and investigate its impact on CD8 T cells, antibody responses, and viral control we used CD4:ERcre TGFβRII^{flox} mice, which allow restricted TGFßRII deletion in CD4 expressing cells upon tamoxifen treatment(95). These mice have absence of detectable surface TGFβRII in CD4 T cells, but not CD8 T cell or B cells after tamoxifen compared to WT or heterozygous mice (Fig 2-9a) and maintained low expression of CD44 activation prior to infection (Fig 2-9b).

We then infected mice with LCMV Cl13 and investigated CD4 T cell responses in blood throughout the course of infection. Again we observed enhanced numbers of activated PD1+ CD49d+ T cells in absence of CD4-TGFßRII signaling (Fig 2-9c). These cells also expressed increased PSGL1^{+} and Ly6C^{+} in the blood after Cl13 infection than WT littermate controls, enriching for terminally differentiated Th1 T cells. Although there was a reduced percentage, the total number of progenitor/memory and Tfh subsets, enriched by differential PSGL1 and Ly6C expression were not different (Fig 2-9d). Furthermore, we found GranzymeB and Eomes expression was increased in TGFßRII deficient CD4 T cells and maintained throughout infection (Fig 2-9e). We also observed virus specific CD8 T cell number over the course of infection and did not observe significant differences between CD4:ERcre TGFβRII mice and littermate controls (Fig 2-9f). However we did see a

reduction in anti-LCMV specific antibody responses at 30 days post-infection. While CD4-TGFßRII deficient mice had similar total anti-LCMV Immunoglobin (Ig), levels of IgG1 were substantially decreased from levels in WT mice. Whereas levels of IgM and IgG2a against LCMV were similar between groups (Fig 2-9g). Notably, viremia persisted to similar degree in CD4-TGFßRII deficient versus WT mice (Fig 2-9h) indicating the defect in antibody responses was not significant enough to delay control of infection.

Overall these data suggest TGFß signaling is continuously necessary to repress CD4 T cell proliferation and differentiation towards terminal differentiated Th1 cells both early and late during chronic viral infection. Relief of Th1 terminal differentiation via TGFβR ablation alone is, however, insufficient to change the outcome of LCMV Cl13 infection and may ultimately compromise Tfh function and antibody responses.

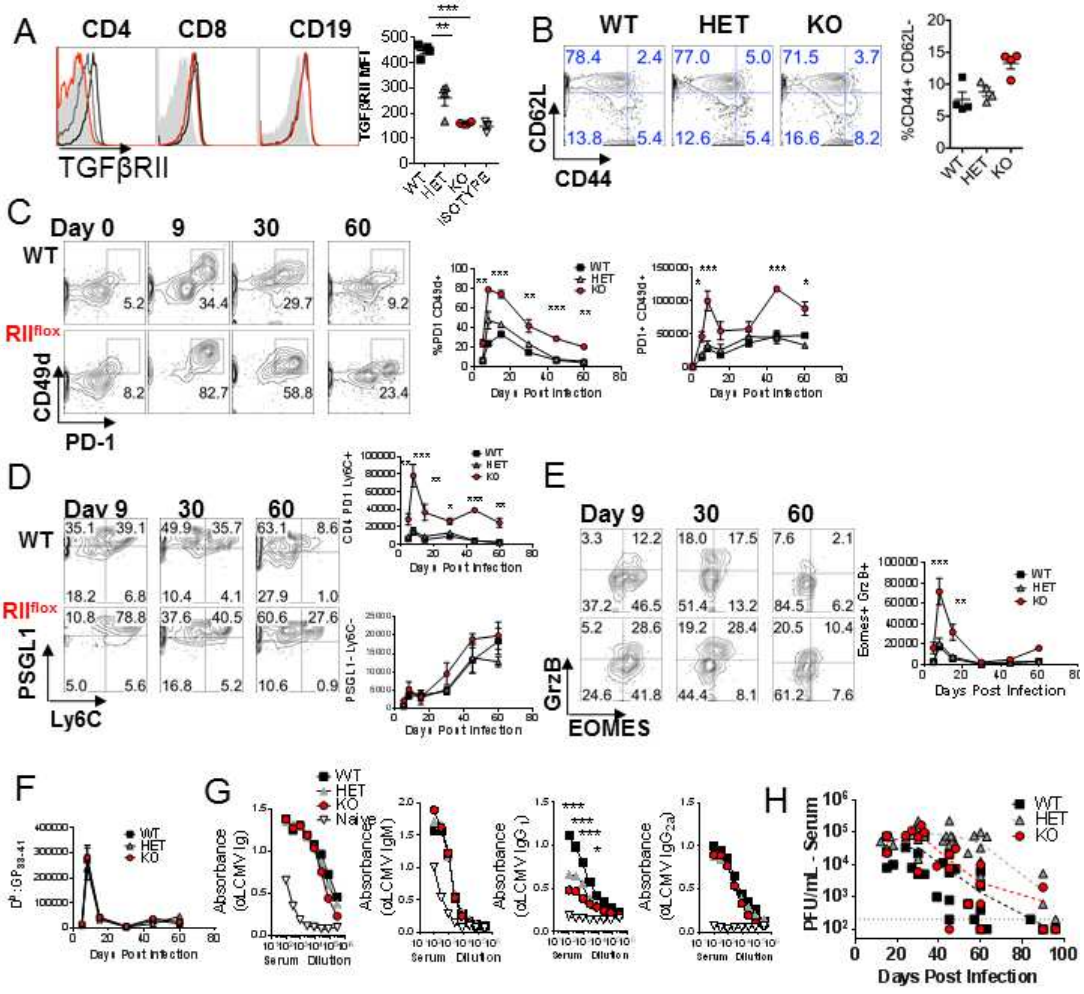

Figure 2-9. CD4 restricted TGFβRII signaling does not influence the course of chronic infection. **A)** CD4-ERcre^{+} TGFβRII$^{flox/flox}$ (RII^{flox}) mice, CD4-ERcre^{+} TGFβRII$^{flox/+}$ (HET) heterozygous and Cre- littermate controls were infected with 2x10^{6} PFU of LCMV Cl13 . Blood was monitored for the presence of LCMV specific T cells by flow cytometry. **A**) TGFβRII expression on B cells CD4 T cell and CD8 T cells prior to infection, MFI for TGFβRII on CD4 T cells is graphed. **B**) Percentage of activated CD44^{+} CD62L^{-} CD4 T cells prior to infection**. C)** Percentage and number of virus specific PD1^{+} CD49d^{+} cells over time post infection. **D)** Percentage and number PSGL1+ Ly6C+ activated CD4 T cells **D)** Percentage and number of Eomes+ GranzymeB+ over time**. F)** Percentage and number of virus specific D^{b}:GP$_{33-41}$$^{+}$ cells over time post infection **G)** Anti-LCMV Ig levels in serum at Day 30p.i. **H)** Serum viremia by plaque assay. Representative of 3 independent experiments of n=4-5 mice/group.

2.10 TGFβ suppression of Eomes-driven CD4 T cell responses is common to chronic viral infections in mice

We next examined if TGFß suppression of Eomes, terminal differentiation and cytotoxicity-associated molecules may be extended to chronic infections other than LCMV CL13. For that, we infected ERcreRII:WT mixed chimeras with Murine cytomegalovirus, MCMV, a latent herpesvirus that is commonly used as model for human CMV. Indeed, compared to WT counterparts, there were more activated TGFßRII deficient CD4 T cells in the blood 14 days after infection (Fig 2-10a). These activated CD4 T cells expressed more KLRG and Eomes, but not Granzyme B (Fig 2-10b). These results suggest TGFβ mediated suppression of CD4 T cell expansion and Eomes associated terminal differentiation are common to both LCMV and MCMV chronic infections.

CD4 CTL activity has also been shown by HIV-specific CD4 T cells ex-vivo (200). Indeed, Granzyme A expression in CD4 T cells correlates with HLA dependent CTL activity in-vitro and predates better outcomes during acute HIV infection (201). These data indicate that TGFβ may also restrict CD4 T CTL activity in human T cells during chronic infection.

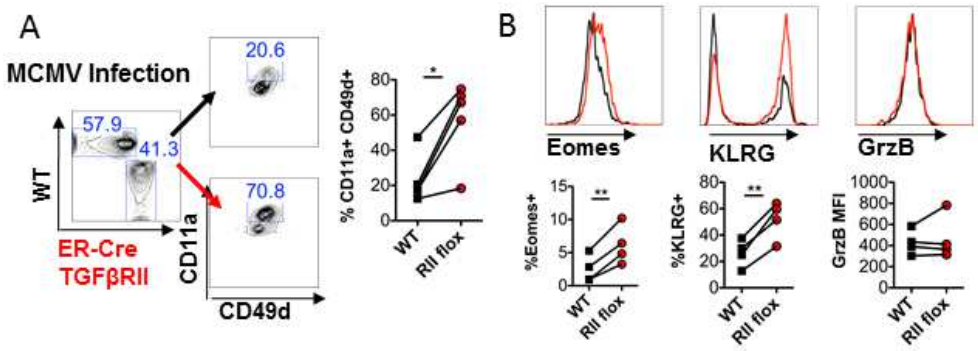

Figure 2-10. TGFß limits CD4 CTL phenotype in MCMV infection. A) Mixed chimeras with TGFßRII deletion prior to $2x10^4$ MCMV infection i.p., CD4 T cells analyzed by compartment for indicated markers 14 days p.i. in the blood.

Discussion

Exogenous TGFβ mediated suppression of cytotoxic NK and T cell responses to viral infection has been appreciated for some time(202, 203). Indeed enhanced TGFβ signaling in T cell is associated with many chronic infections including LCMV in mice and HIV in humans (74, 82, 170). However genetic models of TGFβ1 and TGFβRII deficiency succumb to CD4 T cell mediated inflammatory disease early in life, precluding analysis of peripheral T cell responses. Here we show inducible, cell type specific deletions of TGFβR signaling vs adaptor SMAD4 have opposing roles regulating CD4 T cell numbers and an Eomes-driven terminal differentiation program characterized by Eomes, KLRG, Ly6C and Granzymes and other cytotoxic molecules. We show that TGFß mediated suppression of CD4 T cell number and Eomes expression is common in chronic LCMV and MCMV infections.

Previous genetics models with inducible deletion TGFßRII signaling have indicated a blood based transplantable disease develops in about 2-3 months (204) that is largely CD4 T cell dependent in C57BL/6 mice (95, 205). Increased self/microbiome reactivity of CD4 T cells underlie colitis and multi-organ T cell infiltration, as antibiotics or non-self TCR Transgenes can alleviate disease progression (126, 205, 206). In addition, ablation of TGFßRII during bone marrow reconstitution or transfer of TGFßRII deficient CD4 T cells into a severe but not mild lymphopenic environment induced pathology in about 6-9 weeks(95, 96, 204). Otherwise post-thymic development, deletion of TGFßRII in T cells does not induce autoimmunity(95, 96).

In multiple infectious and tumor models, dominant negative TGFßRII transgenic mice (CD4-dnTGFßRII) have increased responsiveness and function (80,

106, 121, 154, 207). Indeed we have shown complete control of LCMV-CL13 infection by CD4-dnTGFßRII mice(74). However, this model drives aberrant activation of T cells(145). Indeed progressive auto-immunity, sterility, and reduced lifespan are present in many TGFß deficient genetic models; highlighting the cost/benefit trade of increased immune activation to defend against pathogens, yet protect self (91-93, 96, 145, 151, 156, 176, 205, 208, 209). Interestingly, a TGFβRAP1 allele is also associated with disease progression in HIV infection as well as IFN-I responsiveness in HCV infection(210, 211), whereas TGFß1 alleles are also associated with susceptibility to Crohn's disease in humans (212).

Recently, use of dLCKcre TGFBRII^{flox} to minimize autoimmunity, delayed exhaustion in tumor responding OT-1 cells but did not substantially change outcome in contrast to CD4-dnTGFβRII results(106, 213). Use of this genetic model also did not lead to increased CD8 T cell response in B16-OVA tumors or LCMV specific P14 Transgenic CD8 T cells transferred into C57BL/6 mice before LCMV CL13 infection, in contrast to CD4-dnTGFßRII mice above (96, 214). This group further showed that mutant OT-1 epitopes of lower affinity were more sensitive to TGFβ inhibition in-vitro than strong agonist peptide epitopes, like LCMV epitopes(96). Similarly, over-expression of TGFβ1 in the pancreas can suppress T cell mediated diabetes in NOD mice but not in Pancreas driven-LCMV transgenic mice (RIP-LCMV) when triggered with LCMV (208, 215). Indeed, initial attempts using early anti-TGFβ or late therapeutic of TGFβR chemical inhibition during chronic LCMV have only modestly amplified T cell responses reaching insufficient levels to improve viral control, leaving the net effect of TGFβ during infection unclear(107, 108).

Here we found absence of TGFßRII on endogenous LCMV specific CD8 T cells alone does not increase control of chronic viral infection. Mice deficient in TGFßRII signaling in CD8 T cells from birth (CD8-cre) also have increased PD1 activated T cells as well as KLRG, Granzyme B and Eomes expression prior to infection, indicating TGFß does regulate this pathway during development of CD8 T cells. We further show that adult deletion of TGFβRII using ERcre did not influence endogenous CD8 T cells responses to multiple viral epitopes. Together these results suggest TGFß signaling minimally alters CD8 T cell response driven by high avidity antigen signaling of LCMV epitopes, consistent with studies indicating antigen is sufficient to drive exhaustion of CD8 T cells. CD8+ Tregs also depend on TGFβ signaling (216). Indeed CD8cre TGFBRII mice resemble transgenic mice lacking non-classical MHC-Ib Qa-1 recognition by CD8 Tregs (but not NK cells) and have increased Granzyme B and KLRG expression in CD8 T cells but only slightly enhanced viral control of chronic LCMV at 45 d.p.i. (217). Whether there is increased autoimmunity in absence of CD8-TGFßRII signaling model, as in the CD8 Treg deficient Transgenic animal model above is unknown.

CD4 T cell responses are dampened in chronic infection yet are critical to ultimately clear the persisting virus providing both CD8 T cell and B cell help (18, 218). TGFβ signaling is known to regulate many T helper subtypes in-vitro. Here, acute deletion of TGFß signaling in-vivo during chronic viral infection directly repressed both expansion and terminal differentiation of a CTL gene program in CD4 T cells normally found in CD8 T cells and NK cells. TGFß signaling minimally effected Tregs or Tfh development as well as protocypic Th1 genes TBET and IFNγ.

CD4 T regulatory cells (Treg) that express FOXP3 rely on TGFß for development in-vitro and in-vivo (175-177). TGFβ mediated induction of $Foxp3^+$ Tregs maintain immune system quiescence and prevent reactivity that would otherwise promote immuno-pathology (reviewed in (219)). Recent work has shown that TGFβ signaling is not necessary to maintain FOXP3 suppressive activity ex-vivo and TGFβRII deletion in adult mice does not lead to spontaneous autoimmunity, similar to well-tolerated adult chemical inhibition of TGFβ signaling (95, 101, 102). However the proportion of activated Tregs is also increased in chronic LCMV over acute infection and Tregs are important to prevent pathology in absence of anti-viral CD4 T cell help(178). Here we see modest reduction in Tregs expressing activation markers in absence of TGFβ signaling, whereas Total Treg numbers are not affected. However virus specific I-A^b:GP_{61-80} T cells do not develop into Tregs and the specificity of activated Tregs during LCMV CL13 infection remains unknown.

Chronic LCMV infection leads to the pronounced accumulation of T_{FH} cells driven by antigen and increased late IL-6 signaling to promote germinal center B cells and virus-specific antibodies(70, 72). At steady state TGFß signaling prevents accumulation of Tfh and auto-reactive B cells through CD4 survival and CD8 dependent mechanisms in-vivo (216). During acute LCMV infection, TGFβRII suppressed Tfh proportions via inhibiting early Cd25 and IL-2 signaling at Day 3 p.i. to drive effector differentiation in LCMV-ARM infection, however this phenotype was gone by Day 8 p.i.(220). IL-2 is known to promote Th1 expansion and Granzyme B expression while decreasing Tfh representation during acute infections in-vivo. We also see increased proliferation and Granzymes in Th1 cells but significantly decreased CD25 expression by microarray.

In our model, expanded CD4 effector numbers slightly decreases Tfh and Treg proportions, but not overall numbers, early during infection. Consistently, late in infection when proliferation is reduced, we saw no intrinsic change in Treg or Tfh cell proportions in absence of TGFßR. BCL6 levels were also the same of TGFßR sufficient and deficient virus specific I-A^b:GP_{61-80}^+ CD4 T cells. Virus specific CD8 T cell responses are also intact with CD4 T cells sans TGFß signaling, despite CD4 terminal differentiation, however anti-LCMV specific IgG1, but not IgG2a, responses were significantly diminished in absence of CD4-specifc TGFßRII signaling. This suggests Tfh function is somewhat compromised by CD4 terminal differentiation.

Interestingly CD4 T cells have lower antigen affinity than CD8 T cells perhaps making them more sensitive to TGFß signaling. In fact, TGFβ regulates the 3 common signals needed for T cell activation suggesting a more general mode of action in T cell differentiation (80, 95, 126, 206, 221-225). TGFß signaling and SMAD proteins have relatively low binding affinity (86, 226) and associate with different master regulators in different cells types (89) to influence gene transcription by recruitment of different epigenetic modifiers to gene promoters (227). One epigenetic modifier, EZH2, of the Polycomb repressive complex 2 (PRC2) puts down the silencing H3K27me3 histone mark and is important in determining CD4 T cell fate (165). Indeed, we found a EZH2 gene network by microarray analysis enriched in TGFßRII deficient CD4 T cells, highlight a potential lack of targeting EZH2 to these TGFß regulated loci. In support of this hypothesis, a recent report showed Eomes and Granzyme B are upregulated in absence of Ezh2 in CD4 T cells in-vitro (169). EZH2/methyltransferase inhibitor DNzep also increase Eomes in CD4 T cells in-vivo during graft vs host disease (228).

In addition, using CD4cre SMAD2 or SMAD4 conditional KO CD4 T cell in-vitro, Wang et al (229) showed TGFß/SMAD4 signaling (in the presence of IL-4) bound to and displaced EZH2 and H3K27me3 to activate IL-9 production. Indeed, SMAD proteins also recruit JMJ3D3 demethylase (of the PRC2-H3K27me3) to Eomes during development (230) and JMJD3 is required for TGFβ dependent IL-17 induction in CD4 T cells(231). TGFß mediated repression of EOMES is also required for IL-17 production in-vitro(189). Overall these suggest TGFß/SMAD signaling modulates Eomes through positive and negative H3K27me3 dynamics.

Master regulator Th-POK is important for enforcing CD4 identity and repressing Eomes, Runx3, and cytotoxic molecules normally induced by Runx3 in CD8 T cells like GrzB and Perforin (233-235). CD4-Th1 cells, however, do express Granzyme B and high levels of Runx3 (236). By microarray comparison we found TGFβRII gene network resembles THPOK deficient CD4 T cells. SMAD2/3 proteins are known to complex with ThPOK, RUNX3 and EOMES, therefore TGFβ signaling may be in part responsible for ThPOK mediated maintenance of CD4 T cell fate during inflammatory responses driving T cell cytotoxicity.

TGFßRI inhibition upregulates Eomes and CD8 CTL activity against B16 melanomas through SMAD4 degradation (188). We found SMAD4 was unexpectedly required for accumulation of activated CD4 and CD8 T cells in response to infection as well as increased Eomes and Granzyme B expression. Similarly, published work using inducible GrzB-Cre in CD8 T cells found deletion of SMAD4 reduces accumulation of activated T cells substantially due to failure to upregulate c-myc and cellular proliferation(191). Interestingly, CD4-cre SMAD4 deficient mice have enhanced T cell response to B16 tumor cells (and increased colon pathology and

cancer), perhaps highlighting differences in developmental deletion vs acute deletion of an important scaffold protein (162, 191). Indeed SMAD4 serves as a co-factor for both Notch and Wnt signaling which upregulate c-myc as well as Eomes in T cells in-vitro as well as compromise effector differentiation in-vivo when absent(229, 232). This crosstalk might preclude analysis of the role of TGFβRII related SMAD4 during T cell differentiation.

CD4 T cells expressing CD8 associated CTL markers have been described in human chronic infections, yet their contribution to viral control has yet to be shown conclusively (13). Recently Brown et al have shown CD4-Perforin dependent control in lungs of influenza infected mice(237, 238). Antigen, IL-12, and IL-2 induce Eomes and Granzyme expression in CD8 T cells in-vitro and CD4 T cells in-vivo (239, 240), however IL-12 and IFN-I inflammation rather than IL-2 is necessary for Eomes expression during CD4 CTL differentiation in Influenza infection(241) where Eomes is required for CD4 CTL activity after OX40L and CD134 agonist treatment in-vivo (242). Here we show reduced CD4 Granzyme expression and CD4 CTL function in chronic infection compared to acute LCMV infection that correlates with high and low TGFß signaling in T cells.

Interestingly, Granzyme A production and in-vitro cytotoxicity by CD4 T cells correlates with improved outcome after acute HIV infection (201). Low TGFß production in HCV specific CD4 T cells during acute co-infection of HIV patients also is predictive of HCV clearance. Here we show a clear decrease of CD4 CTL activity during chronic infection. However, relief of TGFß signaling was not sufficient to restore CD4 CTL activity during chronic infection. The relevance of CD4 granzyme expression and TGFβ signaling to chronic LCMV infection remains unknown, but

might be subordinate to existing negative regulators, like PD1, to dampen T cell function. Importantly reduced CD4-TGFβ signaling also does not cause loss of control of LCMV CL13 infection. Overall we found TGFβ signaling on either CD4 or CD8 T cells does not alter the viral load during infection.

High Eomes expression is associated with CD8 T cell exhaustion where antigen-dependent expression is necessary for CD8 T cells to control viral infection (41) and is also expressed late but not early in exhausted CD4 T cells in chronic LCMV. Eomes has also been shown to drive terminal differentiation of CD8 exhausted T cells from $Tbet^{HI}$ effectors (193, 194). In our hands, Eomes is sufficient to drive terminal differentiation in a CD4-Th1 response. Recent microarray analysis of virus specific $CD4^+$ T cells from LCMV Cl13 infected mice shows a transcriptional signature distinct from the canonical $CD4^+$ T cell fates(69). Interestingly, Crawford *et al* (69) found that high expression of EOMES was restricted to distinct $BLIMP1^{hi}$ (Th1) populations of $CD4^+$ T cells late during infection that were most exhausted, lacking TNF and IL-2 production (181). We do see enrichment of TGFβRII deficient CD4 T cell gene network in both exhausted CD4 and CD8 T cells late in chronic infection that express high Eomes, suggesting TGFβ signaling could prevent some exhaustion, likely by down-regulating antigen singling. The role of Eomes in promoting CD4 T cell exhaustion later during infection remains unknown.

Therapeutic treatments blocking TGFß signaling are currently being tested in a variety of human cancers. Anti-TGFß treatment did not change the outcome of chronic LCMV viral infection. Early blockade led to small increases in CD4 and CD8 T cell responses in part similar to our proximal receptor deletion(108), whereas late blockade saw no increase in anti-viral CD8 T cell responses or viremia but CD4 T

cells were not examined (107). Similarly we did not see positive anti-viral activity during deletion of TGFßRII in either CD8 or CD4 T cells. We have previously shown that direct TGFßRII signaling on NK cells or Dendritic cells does not influence the outcome of chronic LCMV(171). It remains open whether therapeutic blockade of TGFß signaling would be effective anti-viral treatment without inducing organ pathology.

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Chapter 3: TGFβ mediated pathology during chronic viral infection

Chronic infections cause marked increased in pathology due to both ongoing viral replication as well as inflammation and host immune response. Chronic infection induced Immune exhaustion is important for preventing death during chronic LCMV infection. Here we found TGFß/SMAD4 signaling is essential to prevent mortality after LCMV and MCMV persistent viral challenge. TGFβ signaling was continuously necessary during infection as this pathology appeared upon TGFβ-R deletion both early before viral challenge and late during an established chronic infection. Importantly this pathology was hematopoietic based but independent of TGFβ signaling on the adaptive immune response.

Introduction

Chronic viral infections induce pronounced bone marrow aplasia, liver, lung kidney and gut dysfunction. Indeed dampening of immune response is important to protect from pathology during the extended immune activation caused by chronic infection (243-245). TGFß is a pleiotropic cytokine with critical roles in the development of the hematopoietic system (84, 85). TGFß1 null mice or T cell specific targeting of TGFß-RII deficiency results in lethal multifocal inflammatory diseases by 3-4 weeks of age (90-92). Less complete abrogation of signal using a T cells specific dominant negative receptor transgene or conditional deletion of TGFß1 ligand allowed early onset autoimmunity symptoms and colitis apparent at 4-6 months of age (91, 93, 94, 126, 205, 206). Innate cells such as NK cells and macrophages are also affected by TGFß signaling in-vivo but without spontaneous disease(121, 246) however Dendritic cell specific TGFßRII deletion increased IFNγ production and long-term autoimmunity(156).

Ligation of TGFß signals ALK5 phosphorylation of SMAD2/3 and subsequent binding to nuclear shuttling adapter SMAD4(87). TIF1γ also competes for SMAD2/3 binding and has opposing roles to SMAD4 in hematopoiesis (88). SMAD DNA binding activity is weak and overlays strongly with 'master' regulators of any given cell type (89), hence the gene targets and role in regulation seems unique to many cell types. While conditional T cell specific $SMAD2^{flox}$/SMAD3 double KO mice recapitulate inflammatory phenotype (190, 247), CD4cre-SMAD4 mice do not develop spontaneous autoimmunity. Hence the role of TGFß/SMAD signaling in the adult immune response to pathogens remains incompletely understood..

Importantly, long-term treatment of mice or humans with TGFß antagonists does not lead to the severe autoimmune phenotype of TGFß deficiency during development. Hence, the developmental abnormalities in T cells has precluded analysis of the direct effects of TGFß signaling in-vivo on individual cell types, especially during immune response to pathogens.

Here we utilize a temporal system to modulate TGFß/SMAD signaling in adult mice and examine the contribution of TGFß signaling to the immune response to chronic LCMV infection. We show that inducible genetic ablation of TGF-β receptor (RII) or SMAD4 before infection resulted in rapid mortality upon chronic (but not acute) LCMV infection. Genetic TGFßRII ablation late after an established chronic infection also induced morbidity. This mortality was T cell independent.

Materials and Methods

Mice and viral stocks. C57BL/6 (CD45.2), B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1) and ERt2-cre mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). CD8-cre;TGFβRII^{flox} (Exon2) were generously provided by Dr. M. Li (Sloan Kettering). SMAD4^{flox} mice on a C57BL/6 background were provide by Dr. Frank Jirik (University of Calgary)(162). Hemizygous ER-cre mice were bred to TGFβRII$^{flox/flox}$ mice (126) or SMAD4$^{flox/flox}$ both on C57BL/6 backgrounds. CD4-ERcre knock-in mice crossed to TGFβRII$^{flox/flox}$ (Exon3/4) were generously provided by Dr. T. Buch (95). CD11c-dnTGFßRII mice were kindly provided by Dr. Flavell (Yale)(121). 1:1 mixed chimeras were generated by transferring 2x10^{6} bone marrow cells of each indicated genotype into irradiated CD45.1 mice and allowed 8 weeks to reconstitute. For gene deletion, 1mg/day Tamoxifen (Sigma) emulsified in Sunflower seed Oil

(Sigma) was injected *intraperitoneal* (i.p.) for 5 days and mice were used for experiments 7 days after last treatment. For gene deletion after infection mice were injected for 5 days starting 12 d.p.i.. 6-12 week old mice were infected *intravenously* (i.v.) with $2x10^6$ plaque forming units (PFU) of LCMV Cl13 or $2x10^4$ PFU of MCMV (Smith strain) i.p. All viruses were grown, identified, and quantified as previously described (24, 127). Mice were maintained in a closed breeding facility in compliance with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego.

Serum analysis. Whole blood was centrifuged for 15 min at 2000g and serum collected at indicated time point post-infection. Mouse 23 parameter serum chemistry panel was run by UCSD core veterinary services. Serum cytokine production was analyzed by 20-plex mouse cytokine panel using Luminex beads (life technologies).

Statistics. Survival curves were compared using log-rank(Mantel-Cox) test. All analyses were carried out using GraphPad Prism 6.0. $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.001$. Animal groups were matched unblended, by litter, age and sex, no statistical methods were used to predetermine sample size.

Results

3.1 Early TGFßRII signaling prevents pathology during chronic, not acute viral infection

To examine the contribution of TGFß signaling toward immune regulation during chronic viral infection we used a temporally controlled ERcre strain crossed to TGFßRII conditional allele ($TGFßRII^{flox/flox}$) to proximately delete the TGFßRII using injection of Tamoxifen (TAM) in adult mice as performed in chapter 2 above (123).

Mice were subsequently infected with $2x10^6$ PFU high dose LCMV CL13 to establish chronic infection. Both Tamoxifen treated WT and TGFßRII heterozygous mice survive the duration of infection past 120 days. In contrast, approximately 10-15 days post infection age and sex-matched littermate TGFßRII deficient mice succumbed to infection displaying excessive hunching, scruffiness, and shivering (Fig 3-1a).

To test whether general acute inflammation post-infection was sufficient to cause death we took similar groups of tamoxifen treated ERcre+ and ERcre- littermates and infected them with acute strain LCMV-ARM. Acutely infected ERcre+ TGFßRII deficient mice survived the course of acute infection similar to WT littermate controls (Fig 3-1b), indicating a transient strong anti-viral response was not sufficient to cause rapid death, compared to continuous inflammation in chronic viral infection. Of note, TGFßRII deletion in adult mice followed by acute infection does eventually lead to a lethal inflammatory disease at about 3 months of age, consistent with previously published results using Mx1-Cre mice inected with polyI:C to delete the TGFßRII, and seemingly unrelated to initial acute infection. However both polyI:C for Mx1 and LCMV infection used here induce strong Type-I Interferon responses that could be a trigger for late onset inflammatory disease.

To address the nature of pathology induced by chronic viral infection we measured serum levels of multiple clinical chemistry parameters of organ function. While chronic infection induced general pathology we found TGFßRII deficient mice had increased ALT, AST, and Bilrubin indicating slightly more Liver and Kidney pathology (Fig 3-1e). We next measured a panel of inflammatory cytokine levels in serum of both acute and chronically infected WT and TGFßRII deficient mice. TGFßRII deficient mice infected with chronic LCMV had increased levels of many

cytokines associated with innate IL-1 responses at 9 days post-infection (Fig 3-1f). Cytokines were hierarchically clustered by expression pattern as individual mice after indicated infection and genotype. Importantly, this inflammatory increase was not present during acute infection of TGFßRII deficient mice compared to their WT littermate controls (Fig 3-1f). Overall these data indicate TGFß suppressed a generalized inflammatory environment with increased organ damage during chronic, but not acute LCMV infection.

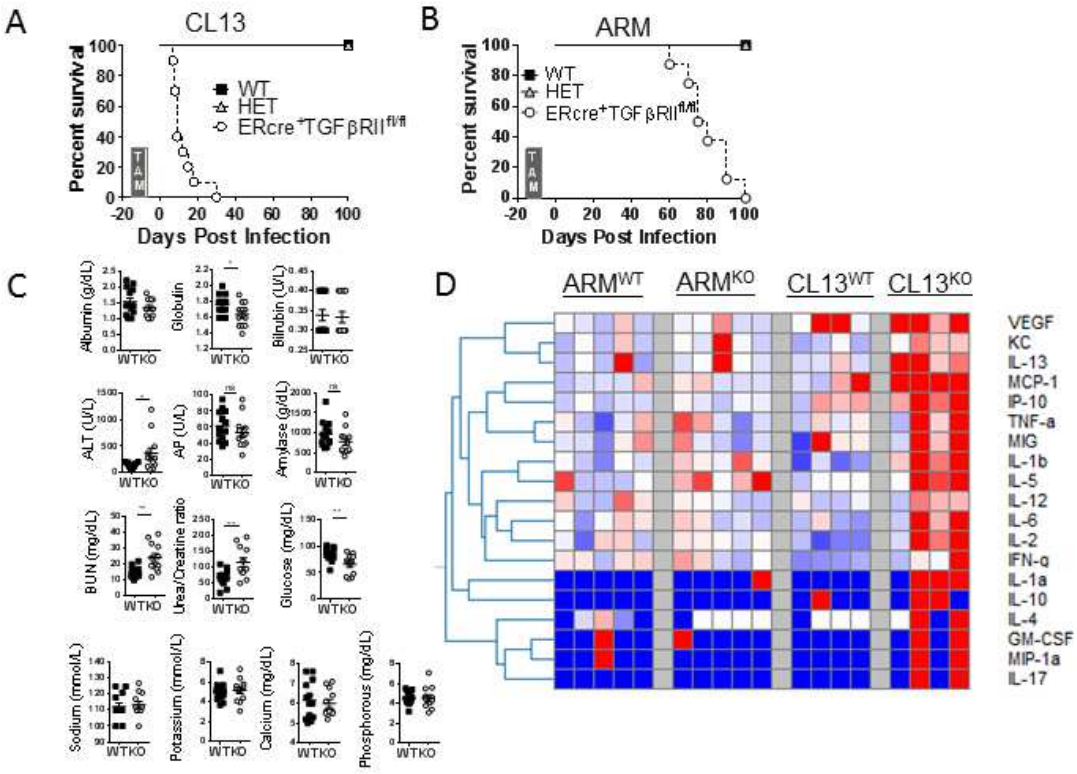

Figure 3-1. Early TGFßRII signaling prevents pathology during chronic, not acute viral infection. ERcre^{+} TGFβRII$^{flox/flox}$ mice (KO) and littermate controls ERcre^{+} TGFβRII$^{flox/+}$(HET) or ERcre^{-}TGFβRII$^{flox/flox}$ (WT) mice were treated for 5 days with 1mg/day Tamoxifen I.P., then 5 days later infected with 2x10^{6} PFU of LCMV ARM or LCMV Cl13 I.V. Blood was analyzed for **A)** Survival curve after chronic infection LCMV-CL13. **B)** survival after acute infection LCMV-ARM. **C)** Serum chemistry profile of WT and KO mice during LCMV-CL13 infection. **D)** Expression in pg/mL of indicated cytokine (rows-right) is shown as a relative heatmap normalized to each row mean and standard deviation where columns (top) are individual mice in LCMV ARM or CL13 infection. Hierarchical clustering of Serum Inflammatory profile is shown. Pooled results of 2-3 independent experiments of n=4-7 mice/group.

3.2 TGFßRII signaling does not suppress viral replication

Intermediate doses of LCMV Cl13 can fail to induce T cell exhaustion and cause enhanced mortality and tissue pathology partially dependent on NK cells and CD4 T cells (113, 245) for CD8 mediated Liver (ALT) and Lung pathology (244). During acute LCMV-ARM infection, WT and TGFßRII deficient mice both efficiently controlled virus in 1 week (Fig 3-2a). Chronic LCMV persists in the blood at high titer for up to 60 days. Importantly, deletion of TGFßRII did not influence viremia before death in chronic infection(Fig 3-2b). TGFßRII deficiency also did not influence viral titers in peripheral tissues 9 days after infection (Fig3-2c). These data indicate undetectable net increase in anti-viral activity in absence of TGFß signaling.

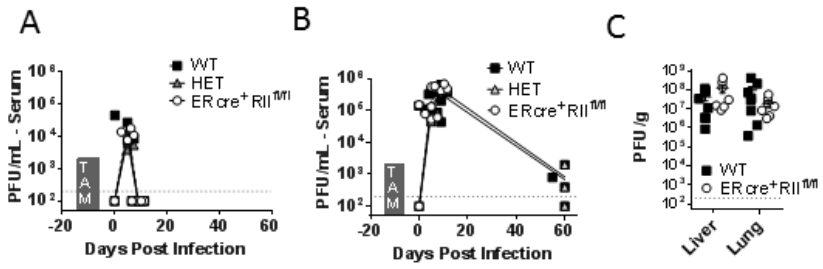

Figure 3-2. TGFßRII signaling does not suppress viral replication. ERcre^{+} TGFβRII$^{flox/flox}$ mice (KO) and littermate controls ERcre^{+} TGFβRII$^{flox/+}$(HET) or ERcre^{-}TGFβRII$^{flox/flox}$ (WT) mice were treated for 5 days with 1mg/day Tamoxifen I.P., then 5 days later infected with 2x10^{6} PFU of LCMV ARM or LCMV Cl13 I.V. Blood was analyzed for **A)** Viremia by plaque assay at days 5 and 9 post-infection (p.i.) with LCMV-ARM **B)** Viremia after LCMV-CL13 infection. **C)** Viral load in tissues 9 days p.i. with LCMV-CL13. Representative of 2-3 independent experiments of n=4-7 mice/group.

3.3 TGFßRII signaling late in established chronic infection to prevent pathology

The immune response to chronic LCMV is very dynamic becoming both exhausted after a canonical anti-viral IFN-I response and continuously responding with late waves of inflammatory molecules such as IL-6 at later time points (32, 248). To test if pathology caused by induced TGFßRII deficiency during chronic infection is

unique to early inflammatory events we ablated TGFßRII signaling after the establishment of chronic infection and T cell exhaustion during a time of potential therapeutic intervention. ERcre+TGFßRII$^{fl/fl}$ mice and littermate controls infected with 2x10^6 PFU of LCMV CL13 were injected starting 12 days p.i. with Tamoxifen for 5 days and checked for receptor expression at 20 days p.i.. We have previously shown TGFßRII deficient mice lost over 90% of receptor expression with this treatment (Chapter 2). Again, WT and TGFßRII heterozygous mice survived the course of infection, whereas TGFßRII deficient mice presented with similar wasting disease of hunched, scruffiness and shivering and succumbed to death with only slightly delayed kinetics compared to TGFßRII deletion before infection, approximately 25 days after receptor deletion (40 days post-infection, (Fig 3-3a). Similar to early deletion of TGFßRII, pathology was not accompanied by uncontrolled viral replication as WT and TGFßRII deficient mice had similar viremia after late deletion (Fig 3-3b). This indicates that TGFß signaling is essential for host survival during the continuous inflammatory course of chronic infection.

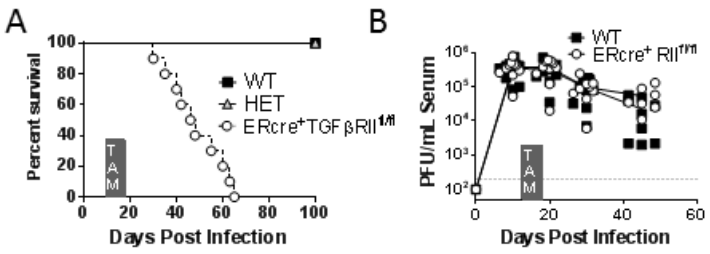

Figure 3-3. TGFßRII signaling late in established chronic infection to prevent pathology. ERcre$^+$ TGFβRII$^{flox/flox}$ mice (KO) and littermate controls ERcre$^+$ TGFβRII$^{flox/+}$(HET) or ERcre^{--} TGFβRII$^{flox/flox}$ (WT) mice were first infected with 2x10^6 PFU LCMV-CL13 and tamoxifen treated Days 12-17 to delete TGFβRII. **A)** Survival curve of mice after infection. **B)** Viremia after late deletion of TGFβRII starting Day 12 post- chronic infection with LCMV CL13. Pooled results of 2 independent experiments of n=4-5 mice/group.

3.4 Cell intrinsic, hematopoietic TGFßRII and SMAD4 signaling prevent pathology during chronic infection

To further isolate direct effects of TGFß on the immune response to LCMV we generated mixed bone marrow chimeras of 50 percent WT (CD45.1) bone marrow and 50 percent ERcre+TGFßRII$^{fl/fl}$ (RII^{flox}) bone marrow into lethally irradiated CD45.1 host mice. Eight weeks post reconstitution, mice were tamoxifen injected to delete TGFßRII and subsequently infected with high dose LCMV CL13. Interestingly, chimeric mice also succumbed to infection with similar kinetics as intact TGFßRII deficient mice (above), whereas tamoxifen treated chimeras without infection survived past 120 days (Fig 3-4a). This indicates a pathology that is a hematopoietic based, dominant phenotype induced by LCMV chronic infection.

To address whether this pathology was unique to LCMV chronic infection, we infected similar mixed chimeric animals with MCMV. While MCMV persists at a much lower level, it still drives accumulation of adaptive immune effectors over time. Indeed MCMV infection led to wasting and death of WT:ERcre RII flox mixed chimeras within 60 days of infection (Fig 3-4b). This is significantly delayed from rapid death following LCMV CL13 infection, however might be explained by the lower overall inflammation during MCMV infection.

Adapter molecules SMAD4 and TIFγ mediate different pSMAD2/3 effects of TGFß on hematopoiesis. We next wanted to ask whether canonical signaling adapter SMAD4 was necessary for TGFßRII related pathology during infection. Similar to published inducible model using mx1-Cre, Tamoxifen treated ERcre^{+} SMAD4^{flox} mice rapidly wasted away within 2-3 weeks (not shown), likely due to non-hematopoietic defects in endothelial cells and colon(249), where hematopoietic reconstitution is

normal without SMAD4, unlike pathological absence of TGFßRII(250). To address SMAD4 in the hematopoietic immune response we created mixed bone marrow chimeras of 1:1 ERcre+SMAD4$^{fl/fl}$ (SM4^{flox}) bone marrow with WT bone marrow. Chimeric mice were treated as above to delete SMAD4 gene before LCMV CL13 infection. ERcre^{+}SMAD4$^{fl/fl}$ mixed chimeras also succumbed to death with two weeks of infection (Fig3-4c). Again, uninfected chimeras survived past 100 days post SMAD4 deletion. Overall these data indicate TGFßRII and SMAD4 are required in the hematopoietic compartment to protect against lethal pathology during chronic infections.

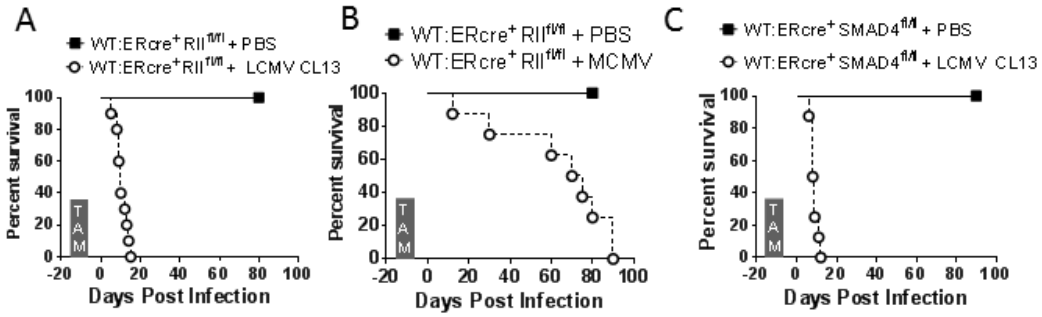

Figure 3-4. Cell intrinsic, hematopoietic TGFßRII and SMAD4 signaling prevent pathology during chronic infection. 8 weeks post-bone marrow reconstitution with 1:1mix of WT(CD45.1) and ERcre^{+} TGFβRII$^{flox/flox}$ (RII^{flox}-CD45.2) bone marrow or ERcre^{+} SMAD4$^{flox/flox}$, mice were tamoxifen treated before infection as above and infected with 2x10^{6} PFU of LCMV Cl13, 2x10^{4} PFU MCMV, or PBS as indicated. **A)** Percent survival WT:RII^{flox} chimeras post LCMV CL13 infection. **B)** Percent survival post MCMV infection. **C)** Percent survival of WT:SMAD4^{flox} chimeras after CL13 infection. Pooled results of 2 independent experiments of n=3-5 mice/group.

3.5 CD8 T cells, CD4 T cells, NK cells and Dendritic cells alone are not responsible for TGFßRII induced pathology

Virus induced death 10-15 days p.i. is consistent with an exaggerated adaptive immune response, namely cytotoxic CD8 T cell mediated killing of target cells. However we previously found the magnitude of CD8 T cell responses against both the acute and chronic strain of LCMV were similar between intact WT mice and

TGFßRII deficient mice (Chapter 2). To further eliminate lack of TGFßRII in CD8 T cells as a cause of death we infected CD8cre TGFßRII deficient mice that lack TGFßRII solely in CD8 T cells from birth. TGFßRII expression in blood lymphocytes is only absent on CD8 T cells, not CD4 T cells or B cells. CD8-TGFßRII deficient mice survive infection similar to WT littermate controls (Fig 3-5a).

Constitutive ablation of TGFß from birth result in a CD4 based lethal inflammatory disease resulting in multi-organ T cell infiltrates and pathology (90, 92, 205). To assess the role of CD4 T cells in infection induced pathology again utilized a temporal system, now restricted to Cd4 promoter driver ERcre protein. CD4-ERcre+TGFßRII deficient mice were tamoxifen treated prior to infection with LCMV Cl13. CD4-TGFßR deficient mice survive chronic infection similar to WT littermates indicating CD4 T cells are not required for this pathology (Fig 3-5b). NK cells are important rheostats of chronic infection and can prevent CD4 accumulation and death in chronic LCMV. Using transgenic mice expressing a dominant negative TGFßRII in CD11c+ Dendritic cells and NK cells we found reduced TGFß signaling in innate cells also does not lead to pathology (Fig 3-5c). Overall these data indicate rule out many known causes of pathology during chronic infection and leave open the possibility of defects in monocytes and erythrocyte lineages.

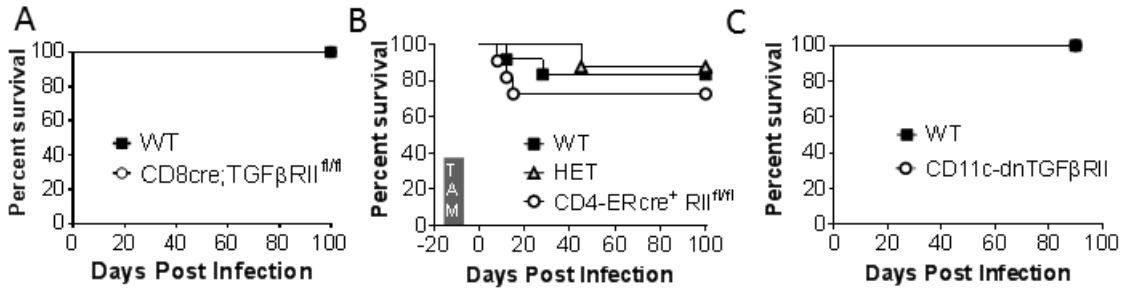

Figure 3-5. CD8 T cells, CD4 T cells, NK cells and Dendritic cells alone are not responsible for TGFßRII induced pathology. Mice of indicated genotype and cre- littermate controls (WT) were infected with $2x10^6$ PFU of LCMV Cl13 I.V. **A**) Survival of $CD8cre^+$ $TGF\beta RII^{flox/flox}$ **B)** Survival curve $CD4\text{-}ERcre^+$ $TGF\beta RII^{flox/flox}$ after Tamoxifen treatment and infection as before **C)** Survival of CD11c-dnTGFßRII transgenic mice to littermate controls. Pooled results of 2-3 independent experiments of n=3-6 mice/group.

Discussion

Previous genetics models with inducible deletion TGFßRII signaling have indicated a blood based transplantable disease develops in about 2-3 months (204), which is largely CD4 T cell dependent (95, 205). In addition, ablation of TGFßRII during bone marrow reconstitution or transfer of TGFßRII deficient CD4 T cells into a severe but not mild lymphopenic environment induced pathology in about 6-9 weeks.

We see TGFßRII deficient mice acutely infected with LCMV-ARM succumb to death at 3 months of age, seemingly unrelated to initial infection and similar to inducible Mx1-cre TGFßRII mice. Using Cd11c-Cre, Raminglan et al recently showed TGFßRII is necessary for DC homeostasis in-vivo and ultimately leads to autoimmunity in 4-6 months(156). Whereas others have shown monocyte deletion using LysM-Cre TGFßRII flox mice to be normal and healthy but have increased tumor metastasis(251). Both models have increased IFNγ from myeloid cells, albeit at different tissue sites, lung and spleen/tumor respectively.

During chronic infection we show rapid mortality upon chronic infection in absence of TGFß signaling on hematopoietic cells. We further show TGFßRII

deficiency in Cd4 or Cd8 T cells alone was not sufficient to induce pathology of TGFßRII deficiency. We also show that TGFß signaling on dendritic cells or NK cells alone, using a dominant negative receptor transgene expressed by CD11c+ cells, does not cause excess pathology during chronic infection (171). In addition to anti-viral CD4 and CD8 T cell responses, inflammation in chronic infection puts a large strain on the hematopoietic system, causing bone marrow aplasia, thymic atrophy, as well as large inflammatory monocyte generation and all possibly contribute to increased pathology seen here(29, 243, 252, 253). Indeed we see a strong inflammatory profile in the blood of activated innate responses in absence of TGFßRII, similar to cytokine storms in septic mice. Indeed a large inflammatory monocyte population is generated during chronic but not acute LCMV infection (29). This population resembles suppressive MDSCs and limits Cd8 T cell responses. TGFß may be important for suppressing ROS activity in these cells. The hematopoietic cellular target of TGFß signaling during chronic infection remains unknown, but is distinctly different than self-reactivity during development or long-term autoimmunity.

Therapeutic treatments blocking TGFß signaling are currently being tested in a variety of human cancers. Interestingly, in a melanoma model, anti-tumor effects were dependent on Neutrophil enhancement of CD8 activity. Anti-TGFß treatment did not change the outcome of chronic LCMV viral infection (107, 108). Importantly, neither treatment study reported pathology or death, nor reported the extent to which TGFßR signaling was abrogated. In contrast we find both early and late deletion of TGFßRII leads to lethality during LCMV CL13 and MCMV chronic infections.

However, similar to previous reports we see do not see positive anti-viral activity during early or late deletion of TGFßRII.

SMAD4 deficiency is also embryonic lethal, but mx1-cre conditional mice show a non-hematopoietic intestinal hemorrhage in the colon and death 5 weeks post-gene deletion without systemic lymphocyte infiltration of TGFßRII deficiency, where SMAD4 deficient T cell also cause similar colon pathology (162, 254). Indeed we also found deletion of SMAD4 via ER-cre in adult mice led to rapid lethality within two weeks without infection (not shown). In isolating the blood compartment through mixed bone marrow chimeras we found non-infected SMAD4:WT chimeras are healthy, but waste away upon chronic infection. Previously, inducible SMAD4 deletion in hematopoietic cells does not lead to overt disease, similar to what we find in non-infected mixed chimeras (250). Similarly SMAD7 overexpression to dampen TGFß signaling does not lead to spontaneous blood based inflammatory disease (255).

In contrast deletion of T cell SMAD4 can actually rescue TGFßR deficiency induced spontaneous autoimmunity (191). Indeed we have previously found (Chapter 2) SMAD4 deficiency limits both activated CD4 and CD8 T cell accumulation and effector differentiation, a opposite phenotype from TGFßRII in T cells(188). In contrast we find that during chronic infection, absence of SMAD4 leads to excessive pathology similar to TGFßRII, and not dependent on T cells lacking TGFß signaling.

Our data clearly highlight differences between developmental and inducible genetic models as a window into potential therapeutics. By understanding the cell specific roles of TGFß/SMAD in-vivo in the context of therapeutic treatment of

persistent infections we may be able to maximize therapeutic potential while limiting unintended pathology.

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Conclusion

Chronic viral infections remain a global health burden. Both preventative and therapeutic vaccination strategies have not proved successful for HCV, HIV chronic infections largely due to the evolved nature of the pathogen to continuously elude T cell and neutralizing antibody responses (256, 257). Study of the immune response in animal models of chronic infection has led to many new avenues of immune based therapies to amplify immune responses and reduce viral loads. These same molecules might also potentiate vaccine strategies to treat chronically infected individuals.

Here we study the role of immunosuppressive TGFß signaling on the immune response to chronic infection in mice. TGFß was originally identified as a tumor-promoting molecule secreted by tumors, however it also has a role signaling on the immune system to suppress T cell responses. As such anti-TGFß molecules are in clinical trials to treat melanoma and a variety of targeting strategies have been developed(103). We and others have reported increased TGFß signaling in the immune system during human and mouse chronic infections.

Prophylactic attempts to block TGFß signaling in mice at the start of chronic LCMV infection showed increased adaptive CD4 and CD8 T cell responses in the first week, but this was not sufficient to reduce viral burden(108). In addition therapeutic chemical inhibition of TGFßRII signaling later during established LCMV infection did not increase T cell responses substantially(107). As TGFß has a complex activation process that occurs during antigen contact at cell synapses, the ability to block TGFß signaling on individual cell types is unknown(87, 103).

Here we use genetic systems to modulate TGFßRII and SMAD4 availability in a cell type specific and inducible manner similar to therapeutic attempts. We found that TGFß signaling minimally influenced Dendritic cell or NK cell responses early during infection. We further found TGFß signaling did not substantially alter antiviral CD8 T cell number and function during chronic infection. TGFß signaling did however influence CD4 T cell differentiation. Preventing TGFß signaling on any of these cell types alone did not provided protection from viral persistence. Importantly, we found TGFß signaling did prevent hematopoietic-based pathology during chronic infection, which should be considered in therapy design.

Many immune stimulatory and inhibitory molecules have been tried therapeutically in chronic LCMV infection without success. Indeed many stimulatory molecules cause pathology early in infection similar to death we observe in absence of TGFßRII signaling. Yet only PD1 blockade has been sufficient to alleviate CD8 T cell exhaustion and lead to durable reduction in viral load. However combination therapies of many flavors synergize with PD1. Therefore it is likely that while TGFß does have immunosuppressive effects during chronic infection, other dominant signals such as antigen and PD1 continue to suppress T cell function.

REFERENCES

1. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296:1323-1326.

2. Li, Q., P. J. Skinner, S. J. Ha, L. Duan, T. L. Mattila, A. Hage, C. White, D. L. Barber, L. O'Mara, P. J. Southern, C. S. Reilly, J. V. Carlis, C. J. Miller, R. Ahmed, and A. T. Haase. 2009. Visualizing antigen-specific and infected cells in situ predicts outcomes in early viral infection. *Science* 323:1726-1729.

3. Tishon, A., P. Borrow, C. Evans, and M. B. Oldstone. 1993. Virus-induced immunosuppression. 1. Age at infection relates to a selective or generalized defect. *Virology* 195:397-405.

4. Stelekati, E., and E. J. Wherry. Chronic bystander infections and immunity to unrelated antigens. *Cell Host Microbe* 12:458-469.

5. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. Innate or adaptive immunity? The example of natural killer cells. *Science* 331:44-49.

6. Aoshi, T., S. Koyama, K. Kobiyama, S. Akira, and K. J. Ishii. Innate and adaptive immune responses to viral infection and vaccination. *Curr Opin Virol* 1:226-232.

7. Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke, and S. Vucikuja. 2006. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J Immunol* 176:4525-4529.

8. Ben-Sasson, S. Z., J. Hu-Li, J. Quiel, S. Cauchetaux, M. Ratner, I. Shapira, C. A. Dinarello, and W. E. Paul. 2009. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A* 106:7119-7124.

9. Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D. F. Tough. 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99:3263-3271.

10. Crawford, A., and E. J. Wherry. 2009. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr Opin Immunol* 21:179-186.

11. Garcia-Sastre, A., and C. A. Biron. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 312:879-882.

12. Schenten, D., and R. Medzhitov. The control of adaptive immune responses by the innate immune system. *Adv Immunol* 109:87-124.

13. Swain, S. L., K. K. McKinstry, and T. M. Strutt. Expanding roles for CD4(+) T cells in immunity to viruses. *Nat Rev Immunol* 12:136-148.

14. Planz, O., S. Ehl, E. Furrer, E. Horvath, M. A. Brundler, H. Hengartner, and R. M. Zinkernagel. 1997. A critical role for neutralizing-antibody-producing B cells, CD4(+) T

cells, and interferons in persistent and acute infections of mice with lymphocytic choriomeningitis virus: implications for adoptive immunotherapy of virus carriers. *Proc Natl Acad Sci U S A* 94:6874-6879.

15. Thomsen, A. R., J. Johansen, O. Marker, and J. P. Christensen. 1996. Exhaustion of CTL memory and recrudescence of viremia in lymphocytic choriomeningitis virus-infected MHC class II-deficient mice and B cell-deficient mice. *J Immunol* 157:3074-3080.

16. Hangartner, L., R. M. Zellweger, M. Giobbi, J. Weber, B. Eschli, K. D. McCoy, N. Harris, M. Recher, R. M. Zinkernagel, and H. Hengartner. 2006. Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread. *J Exp Med* 203:2033-2042.

17. Bergthaler, A., L. Flatz, A. Verschoor, A. N. Hegazy, M. Holdener, K. Fink, B. Eschli, D. Merkler, R. Sommerstein, E. Horvath, M. Fernandez, A. Fitsche, B. M. Senn, J. S. Verbeek, B. Odermatt, C. A. Siegrist, and D. D. Pinschewer. 2009. Impaired antibody response causes persistence of prototypic T cell-contained virus. *PLoS Biol* 7:e1000080.

18. Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68:8056-8063.

19. de Jong, Y. P., M. Dorner, M. C. Mommersteeg, J. W. Xiao, A. B. Balazs, J. B. Robbins, B. Y. Winer, S. Gerges, K. Vega, R. N. Labitt, B. M. Donovan, E. Giang, A. Krishnan, L. Chiriboga, M. R. Charlton, D. R. Burton, D. Baltimore, M. Law, C. M. Rice, and A. Ploss. Broadly neutralizing antibodies abrogate established hepatitis C virus infection. *Sci Transl Med* 6:254ra129.

20. Balazs, A. B., J. Chen, C. M. Hong, D. S. Rao, L. Yang, and D. Baltimore. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* 481:81-84.

21. Barton, L. L., and M. B. Mets. 2001. Congenital lymphocytic choriomeningitis virus infection: decade of rediscovery. *Clin Infect Dis* 33:370-374.

22. Charrel, R. N., and X. de Lamballerie. Zoonotic aspects of arenavirus infections. *Vet Microbiol* 140:213-220.

23. Bonthius, D. J. Lymphocytic choriomeningitis virus: an underrecognized cause of neurologic disease in the fetus, child, and adult. *Semin Pediatr Neurol* 19:89-95.

24. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160:521-540.

25. Salvato, M., P. Borrow, E. Shimomaye, and M. B. Oldstone. 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. *J Virol* 65:1863-1869.

26. Matloubian, M., S. R. Kolhekar, T. Somasundaram, and R. Ahmed. 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single

amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 67:7340-7349.

27. Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K. P. Campbell, J. C. de La Torre, and M. B. Oldstone. 2000. Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells. *J Exp Med* 192:1249-1260.

28. Mueller, S. N., M. Matloubian, D. M. Clemens, A. H. Sharpe, G. J. Freeman, S. Gangappa, C. P. Larsen, and R. Ahmed. 2007. Viral targeting of fibroblastic reticular cells contributes to immunosuppression and persistence during chronic infection. *Proc Natl Acad Sci U S A* 104:15430-15435.

29. Norris, B. A., L. S. Uebelhoer, H. I. Nakaya, A. A. Price, A. Grakoui, and B. Pulendran. Chronic but not acute virus infection induces sustained expansion of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. *Immunity* 38:309-321.

30. Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone. 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J Clin Invest* 113:737-745.

31. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27:670-684.

32. Zuniga, E. I., L. Y. Liou, L. Mack, M. Mendoza, and M. B. Oldstone. 2008. Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. *Cell Host Microbe* 4:374-386.

33. Goodrum, F., K. Caviness, and P. Zagallo. Human cytomegalovirus persistence. *Cell Microbiol* 14:644-655.

34. Andrews, D. M., C. E. Andoniou, A. A. Scalzo, S. L. van Dommelen, M. E. Wallace, M. J. Smyth, and M. A. Degli-Esposti. 2005. Cross-talk between dendritic cells and natural killer cells in viral infection. *Mol Immunol* 42:547-555.

35. Andrews, D. M., M. J. Estcourt, C. E. Andoniou, M. E. Wikstrom, A. Khong, V. Voigt, P. Fleming, H. Tabarias, G. R. Hill, R. G. van der Most, A. A. Scalzo, M. J. Smyth, and M. A. Degli-Esposti. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *J Exp Med* 207:1333-1343.

36. Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J Exp Med* 194:29-44.

37. Dokun, A. O., S. Kim, H. R. Smith, H. S. Kang, D. T. Chu, and W. M. Yokoyama. 2001. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol* 2:951-956.

38. Walton, S. M., S. Mandaric, N. Torti, A. Zimmermann, H. Hengel, and A. Oxenius. Absence of cross-presenting cells in the salivary gland and viral immune evasion confine cytomegalovirus immune control to effector CD4 T cells. *PLoS Pathog* 7:e1002214.

39. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857-860.

40. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 189:991-998.

41. Paley, M. A., D. C. Kroy, P. M. Odorizzi, J. B. Johnnidis, D. V. Dolfi, B. E. Barnett, E. K. Bikoff, E. J. Robertson, G. M. Lauer, S. L. Reiner, and E. J. Wherry. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338:1220-1225.

42. Thimme, R., S. Wieland, C. Steiger, J. Ghrayeb, K. A. Reimann, R. H. Purcell, and F. V. Chisari. 2003. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 77:68-76.

43. Wherry, E. J. T cell exhaustion. *Nat Immunol* 12:492-499.

44. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.

45. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10:29-37.

46. Radziewicz, H., C. C. Ibegbu, M. L. Fernandez, K. A. Workowski, K. Obideen, M. Wehbi, H. L. Hanson, J. P. Steinberg, D. Masopust, E. J. Wherry, J. D. Altman, B. T. Rouse, G. J. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 81:2545-2553.

47. Boni, C., P. Fisicaro, C. Valdatta, B. Amadei, P. Di Vincenzo, T. Giuberti, D. Laccabue, A. Zerbini, A. Cavalli, G. Missale, A. Bertoletti, and C. Ferrari. 2007. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 81:4215-4225.

48. Bengsch, B., B. Seigel, M. Ruhl, J. Timm, M. Kuntz, H. E. Blum, H. Pircher, and R. Thimme. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* 6:e1000947.

49. Jo, J., B. Bengsch, B. Seigel, S. J. Rau, J. Schmidt, E. Bisse, P. Aichele, U. Aichele, L. Joeckel, C. Royer, K. Sa Ferreira, C. Borner, T. F. Baumert, H. E. Blum, V. Lohmann, R. Fischer, and R. Thimme. Low perforin expression of early differentiated HCV-specific CD8+ T cells limits their hepatotoxic potential. *J Hepatol* 57:9-16.

50. Zhang, D., P. Shankar, Z. Xu, B. Harnisch, G. Chen, C. Lange, S. J. Lee, H. Valdez, M. M. Lederman, and J. Lieberman. 2003. Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood* 101:226-235.

51. Blackburn, S. D., H. Shin, G. J. Freeman, and E. J. Wherry. 2008. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* 105:15016-15021.

52. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.

53. Doering, T. A., A. Crawford, J. M. Angelosanto, M. A. Paley, C. G. Ziegler, and E. J. Wherry. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity* 37:1130-1144.

54. Larsson, M., E. M. Shankar, K. F. Che, A. Saeidi, R. Ellegard, M. Barathan, V. Velu, and A. Kamarulzaman. Molecular signatures of T-cell inhibition in HIV-1 infection. *Retrovirology* 10:31.

55. Schietinger, A., and P. D. Greenberg. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends Immunol* 35:51-60.

56. Wirth, T. C., H. H. Xue, D. Rai, J. T. Sabel, T. Bair, J. T. Harty, and V. P. Badovinac. Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. *Immunity* 33:128-140.

57. Quigley, M., F. Pereyra, B. Nilsson, F. Porichis, C. Fonseca, Q. Eichbaum, B. Julg, J. L. Jesneck, K. Brosnahan, S. Imam, K. Russell, I. Toth, A. Piechocka-Trocha, D. Dolfi, J. Angelosanto, A. Crawford, H. Shin, D. S. Kwon, J. Zupkosky, L. Francisco, G. J. Freeman, E. J. Wherry, D. E. Kaufmann, B. D. Walker, B. Ebert, and W. N. Haining. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat Med* 16:1147-1151.

58. Shin, H., S. D. Blackburn, A. M. Intlekofer, C. Kao, J. M. Angelosanto, S. L. Reiner, and E. J. Wherry. 2009. A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31:309-320.

59. Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27:281-295.

60. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol* 79:10514-10527.

61. Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 41:529-542.

62. Oxenius, A., R. M. Zinkernagel, and H. Hengartner. 1998. Comparison of activation versus induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. *Immunity* 9:449-457.

63. Aubert, R. D., A. O. Kamphorst, S. Sarkar, V. Vezys, S. J. Ha, D. L. Barber, L. Ye, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2011. Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proc Natl Acad Sci U S A* 108:21182-21187.

64. Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari. 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 194:1395-1406.

65. Diepolder, H. M., R. Zachoval, R. M. Hoffmann, E. A. Wierenga, T. Santantonio, M. C. Jung, D. Eichenlaub, and G. R. Pape. 1995. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 346:1006-1007.

66. Schulze Zur Wiesch, J., D. Ciuffreda, L. Lewis-Ximenez, V. Kasprowicz, B. E. Nolan, H. Streeck, J. Aneja, L. L. Reyor, T. M. Allen, A. W. Lohse, B. McGovern, R. T. Chung, W. W. Kwok, A. Y. Kim, and G. M. Lauer. 2012. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med* 209:61-75.

67. Fuller, M. J., and A. J. Zajac. 2003. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 170:477-486.

68. Battegay, M., D. Moskophidis, A. Rahemtulla, H. Hengartner, T. W. Mak, and R. M. Zinkernagel. 1994. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J Virol* 68:4700-4704.

69. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. 2014. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40:289-302.

70. Fahey, L. M., E. B. Wilson, H. Elsaesser, C. D. Fistonich, D. B. McGavern, and D. G. Brooks. 2011. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 208:987-999.

71. Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. *Nature* 422:307-312.

72. Harker, J. A., G. M. Lewis, L. Mack, and E. I. Zuniga. 2011. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* 334:825-829.

73. Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern, and M. B. Oldstone. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12:1301-1309.

74. Tinoco, R., V. Alcalde, Y. Yang, K. Sauer, and E. I. Zuniga. 2009. Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity* 31:145-157.

75. Clerici, M., T. A. Wynn, J. A. Berzofsky, S. P. Blatt, C. W. Hendrix, A. Sher, R. L. Coffman, and G. M. Shearer. 1994. Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J Clin Invest* 93:768-775.

76. Kekow, J., W. Wachsman, J. A. McCutchan, W. L. Gross, M. Zachariah, D. A. Carson, and M. Lotz. 1991. Transforming growth factor-beta and suppression of humoral immune responses in HIV infection. *J Clin Invest* 87:1010-1016.

77. Hirsch, C. S., J. J. Ellner, R. Blinkhorn, and Z. Toossi. 1997. In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against Mycobacterium tuberculosis by natural inhibitors of transforming growth factor beta. *Proc Natl Acad Sci U S A* 94:3926-3931.

78. Nelson, D. R., R. P. Gonzalez-Peralta, K. Qian, Y. Xu, C. G. Marousis, G. L. Davis, and J. Y. Lau. 1997. Transforming growth factor-beta 1 in chronic hepatitis C. *J Viral Hepat* 4:29-35.

79. Omer, F. M., and E. M. Riley. 1998. Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. *J Exp Med* 188:39-48.

80. Sanjabi, S., M. M. Mosaheb, and R. A. Flavell. 2009. Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. *Immunity* 31:131-144.

81. Lotz, M., and P. Seth. 1993. TGF beta and HIV infection. *Ann N Y Acad Sci* 685:501-511.

82. Wiercinska-Drapalo, A., R. Flisiak, J. Jaroszewicz, and D. Prokopowicz. 2004. Increased plasma transforming growth factor-beta1 is associated with disease progression in HIV-1-infected patients. *Viral Immunol* 17:109-113.

83. Carlson, C. M., E. A. Turpin, L. A. Moser, K. B. O'Brien, T. D. Cline, J. C. Jones, T. M. Tumpey, J. M. Katz, L. A. Kelley, J. Gauldie, and S. Schultz-Cherry. Transforming growth factor-beta: activation by neuraminidase and role in highly pathogenic H5N1 influenza pathogenesis. *PLoS Pathog* 6:e1001136.

84. Larsson, J., M. J. Goumans, L. J. Sjostrand, M. A. van Rooijen, D. Ward, P. Leveen, X. Xu, P. ten Dijke, C. L. Mummery, and S. Karlsson. 2001. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J* 20:1663-1673.

85. Oshima, M., H. Oshima, and M. M. Taketo. 1996. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* 179:297-302.

86. Shi, Y., and J. Massague. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113:685-700.

87. Massague, J. TGFbeta signalling in context. *Nat Rev Mol Cell Biol* 13:616-630.

88. He, W., D. C. Dorn, H. Erdjument-Bromage, P. Tempst, M. A. Moore, and J. Massague. 2006. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. *Cell* 125:929-941.

89. Mullen, A. C., D. A. Orlando, J. J. Newman, J. Loven, R. M. Kumar, S. Bilodeau, J. Reddy, M. G. Guenther, R. P. DeKoter, and R. A. Young. Master transcription factors determine cell-type-specific responses to TGF-beta signaling. *Cell* 147:565-576.

90. Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward, and S. Karlsson. 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90:770-774.

91. Marie, J. C., D. Liggitt, and A. Y. Rudensky. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25:441-454.

92. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, and et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359:693-699.

93. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12:171-181.

94. Lucas, P. J., S. J. Kim, S. J. Melby, and R. E. Gress. 2000. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 191:1187-1196.

95. Sledzinska, A., S. Hemmers, F. Mair, O. Gorka, J. Ruland, L. Fairbairn, A. Nissler, W. Muller, A. Waisman, B. Becher, and T. Buch. TGF-beta Signalling Is Required for CD4 T Cell Homeostasis But Dispensable for Regulatory T Cell Function. *PLoS Biol* 11:e1001674.

96. Zhang, N., and M. J. Bevan. TGF-beta signaling to T cells inhibits autoimmunity during lymphopenia-driven proliferation. *Nat Immunol* 13:667-673.

97. Siddiqui, K. R., S. Laffont, and F. Powrie. E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity* 32:557-567.

98. Belladonna, M. L., C. Volpi, R. Bianchi, C. Vacca, C. Orabona, M. T. Pallotta, L. Boon, S. Gizzi, M. C. Fioretti, U. Grohmann, and P. Puccetti. 2008. Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. *J Immunol* 181:5194-5198.

99. Borkowski, T. A., J. J. Letterio, C. L. Mackall, A. Saitoh, A. G. Farr, X. J. Wang, D. R. Roop, R. E. Gress, and M. C. Udey. 1997. Langerhans cells in the TGF beta 1 null mouse. *Adv Exp Med Biol* 417:307-310.

100. Contractor, N., J. Louten, L. Kim, C. A. Biron, and B. L. Kelsall. 2007. Cutting edge: Peyer's patch plasmacytoid dendritic cells (pDCs) produce low levels of type I interferons: possible role for IL-10, TGFbeta, and prostaglandin E2 in conditioning a unique mucosal pDC phenotype. *J Immunol* 179:2690-2694.

101. Ruzek, M. C., M. Hawes, B. Pratt, J. McPherson, S. Ledbetter, S. M. Richards, and R. D. Garman. 2003. Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. *Immunopharmacol Immunotoxicol* 25:235-257.

102. Yang, Y. A., O. Dukhanina, B. Tang, M. Mamura, J. J. Letterio, J. MacGregor, S. C. Patel, S. Khozin, Z. Y. Liu, J. Green, M. R. Anver, G. Merlino, and L. M. Wakefield. 2002. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 109:1607-1615.

103. Connolly, E. C., J. Freimuth, and R. J. Akhurst. Complexities of TGF-beta targeted cancer therapy. *Int J Biol Sci* 8:964-978.

104. Fridlender, Z. G., J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G. S. Worthen, and S. M. Albelda. 2009. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16:183-194.

105. Nam, J. S., M. Terabe, M. J. Kang, H. Chae, N. Voong, Y. A. Yang, A. Laurence, A. Michalowska, M. Mamura, S. Lonning, J. A. Berzofsky, and L. M. Wakefield. 2008. Transforming growth factor beta subverts the immune system into directly promoting tumor growth through interleukin-17. *Cancer Res* 68:3915-3923.

106. Donkor, M. K., A. Sarkar, P. A. Savage, R. A. Franklin, L. K. Johnson, A. A. Jungbluth, J. P. Allison, and M. O. Li. T cell surveillance of oncogene-induced prostate cancer is impeded by T cell-derived TGF-beta1 cytokine. *Immunity* 35:123-134.

107. Boettler, T., Y. Cheng, K. Ehrhardt, and M. von Herrath. TGF-beta blockade does not improve control of an established persistent viral infection. *Viral Immunol* 25:232-238.

108. Garidou, L., S. Heydari, S. Gossa, and D. B. McGavern. Therapeutic blockade of transforming growth factor beta fails to promote clearance of a persistent viral infection. *J Virol* 86:7060-7071.

109. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2:1144-1150.

110. Dalod, M., R. Chelbi, B. Malissen, and T. Lawrence. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J* 33:1104-1116.

111. Dalod, M., T. P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Briere, G. Trinchieri, and C. A. Biron. 2002. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *J Exp Med* 195:517-528.

112. Kallies, A., S. Carotta, N. D. Huntington, N. J. Bernard, D. M. Tarlinton, M. J. Smyth, and S. L. Nutt. A role for Blimp1 in the transcriptional network controlling natural killer cell maturation. *Blood* 117:1869-1879.

113. Waggoner, S. N., M. Cornberg, L. K. Selin, and R. M. Welsh. Natural killer cells act as rheostats modulating antiviral T cells. *Nature* 481:394-398.

114. Lang, P. A., K. S. Lang, H. C. Xu, M. Grusdat, I. A. Parish, M. Recher, A. R. Elford, S. Dhanji, N. Shaabani, C. W. Tran, D. Dissanayake, R. Rahbar, M. Ghazarian, A. Brustle, J. Fine, P. Chen, C. T. Weaver, C. Klose, A. Diefenbach, D. Haussinger, J. R. Carlyle, S. M. Kaech, T. W. Mak, and P. S. Ohashi. Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. *Proc Natl Acad Sci U S A* 109:1210-1215.

115. Yamaguchi, Y., H. Tsumura, M. Miwa, and K. Inaba. 1997. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* 15:144-153.

116. Fitzgerald-Bocarsly, P., and E. S. Jacobs. Plasmacytoid dendritic cells in HIV infection: striking a delicate balance. *J Leukoc Biol* 87:609-620.

117. Sun, C., B. Fu, Y. Gao, X. Liao, R. Sun, Z. Tian, and H. Wei. TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. *PLoS Pathog* 8:e1002594.

118. Alter, G., N. Teigen, B. T. Davis, M. M. Addo, T. J. Suscovich, M. T. Waring, H. Streeck, M. N. Johnston, K. D. Staller, M. T. Zaman, X. G. Yu, M. Lichterfeld, N. Basgoz, E. S. Rosenberg, and M. Altfeld. 2005. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. *Blood* 106:3366-3369.

119. Herzer, K., C. S. Falk, J. Encke, S. T. Eichhorst, A. Ulsenheimer, B. Seliger, and P. H. Krammer. 2003. Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p53 and TAP1 impairs natural killer cell cytotoxicity. *J Virol* 77:8299-8309.

120. Su, H. C., R. Ishikawa, and C. A. Biron. 1993. Transforming growth factor-beta expression and natural killer cell responses during virus infection of normal, nude, and SCID mice. *J Immunol* 151:4874-4890.

121. Laouar, Y., F. S. Sutterwala, L. Gorelik, and R. A. Flavell. 2005. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 6:600-607.

122. Marcoe, J. P., J. R. Lim, K. L. Schaubert, N. Fodil-Cornu, M. Matka, A. L. McCubbrey, A. R. Farr, S. M. Vidal, and Y. Laouar. TGF-beta is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy. *Nat Immunol* 13:843-850.

123. Feil, R., J. Wagner, D. Metzger, and P. Chambon. 1997. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* 237:752-757.

124. Ruzankina, Y., C. Pinzon-Guzman, A. Asare, T. Ong, L. Pontano, G. Cotsarelis, V. P. Zediak, M. Velez, A. Bhandoola, and E. J. Brown. 2007. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* 1:113-126.

125. Li, M. O., S. Sanjabi, and R. A. Flavell. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25:455-471.

126. Ouyang, W., S. A. Oh, Q. Ma, M. R. Bivona, J. Zhu, and M. O. Li. TGF-beta cytokine signaling promotes CD8+ T cell development and low-affinity CD4+ T cell homeostasis by regulation of interleukin-7 receptor alpha expression. *Immunity* 39:335-346.

127. Chalmer, J. E., J. S. Mackenzie, and N. F. Stanley. 1977. Resistance to murine cytomegalovirus linked to the major histocompatibility complex of the mouse. *J Gen Virol* 37:107-114.

128. Zuniga, E. I., D. B. McGavern, J. L. Pruneda-Paz, C. Teng, and M. B. Oldstone. 2004. Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. *Nat Immunol* 5:1227-1234.

129. Jiang, Z., P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos, M. Freudenberg, and B. Beutler. 2005. CD14 is required for MyD88-independent LPS signaling. *Nat Immunol* 6:565-570.

130. Lin, A. H., J. Luo, L. H. Mondshein, P. ten Dijke, D. Vivien, C. H. Contag, and T. Wyss-Coray. 2005. Global analysis of Smad2/3-dependent TGF-beta signaling in living mice reveals prominent tissue-specific responses to injury. *J Immunol* 175:547-554.

131. Swiecki, M., Y. Wang, W. Vermi, S. Gilfillan, R. D. Schreiber, and M. Colonna. Type I interferon negatively controls plasmacytoid dendritic cell numbers in vivo. *J Exp Med* 208:2367-2374.

132. Swiecki, M., S. Gilfillan, W. Vermi, Y. Wang, and M. Colonna. Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* 33:955-966.

133. Nguyen, K. B., T. P. Salazar-Mather, M. Y. Dalod, J. B. Van Deusen, X. Q. Wei, F. Y. Liew, M. A. Caligiuri, J. E. Durbin, and C. A. Biron. 2002. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* 169:4279-4287.

134. Nguyen, K. B., W. T. Watford, R. Salomon, S. R. Hofmann, G. C. Pien, A. Morinobu, M. Gadina, J. J. O'Shea, and C. A. Biron. 2002. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science* 297:2063-2066.

135. Chaix, J., M. S. Tessmer, K. Hoebe, N. Fuseri, B. Ryffel, M. Dalod, L. Alexopoulou, B. Beutler, L. Brossay, E. Vivier, and T. Walzer. 2008. Cutting edge: Priming of NK cells by IL-18. *J Immunol* 181:1627-1631.

136. Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292:934-937.

137. Orr, M. T., W. J. Murphy, and L. L. Lanier. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* 11:321-327.

138. Gerosa, F., M. Scardoni, M. Tommasi, C. Benati, L. Snelli, G. Gandini, M. Libonati, G. Tridente, and G. Carra. 1991. Interferon alpha induces expression of the CD69 activation antigen in human resting NK cells, while interferon gamma and tumor necrosis factor alpha are ineffective. *Int J Cancer* 48:473-475.

139. Bukowski, J. F., and R. M. Welsh. 1985. Inability of interferon to protect virus-infected cells against lysis by natural killer (NK) cells correlates with NK cell-mediated antiviral effects in vivo. *J Immunol* 135:3537-3541.

140. Jung, A., H. Kato, Y. Kumagai, H. Kumar, T. Kawai, O. Takeuchi, and S. Akira. 2008. Lymphocytoid choriomeningitis virus activates plasmacytoid dendritic cells and induces a cytotoxic T-cell response via MyD88. *J Virol* 82:196-206.

141. Macal, M., G. M. Lewis, S. Kunz, R. Flavell, J. A. Harker, and E. I. Zuniga. Plasmacytoid dendritic cells are productively infected and activated through TLR-7 early after arenavirus infection. *Cell Host Microbe* 11:617-630.

142. Cervantes-Barragan, L., K. L. Lewis, S. Firner, V. Thiel, S. Hugues, W. Reith, B. Ludewig, and B. Reizis. Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proc Natl Acad Sci U S A* 109:3012-3017.

143. Blasius, A. L., P. Krebs, B. M. Sullivan, M. B. Oldstone, and D. L. Popkin. Slc15a4, a gene required for pDC sensing of TLR ligands, is required to control persistent viral infection. *PLoS Pathog* 8:e1002915.

144. Montoya, M., M. J. Edwards, D. M. Reid, and P. Borrow. 2005. Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN. *J Immunol* 174:1851-1861.

145. Ishigame, H., M. M. Mosaheb, S. Sanjabi, and R. A. Flavell. Truncated form of TGF-betaRII, but not its absence, induces memory CD8+ T cell expansion and lymphoproliferative disorder in mice. *J Immunol* 190:6340-6350.

146. Chytil, A., M. A. Magnuson, C. V. Wright, and H. L. Moses. 2002. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. *Genesis* 32:73-75.

147. Tay, C. H., and R. M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J Virol* 71:267-275.

148. Salazar-Mather, T. P., J. S. Orange, and C. A. Biron. 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways. *J Exp Med* 187:1-14.

149. Kanzler, S., M. Baumann, P. Schirmacher, V. Dries, E. Bayer, G. Gerken, H. P. Dienes, and A. W. Lohse. 2001. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-beta. *J Viral Hepat* 8:430-437.

150. Kornfeld, C., M. J. Ploquin, I. Pandrea, A. Faye, R. Onanga, C. Apetrei, V. Poaty-Mavoungou, P. Rouquet, J. Estaquier, L. Mortara, J. F. Desoutter, C. Butor, R. Le Grand, P. Roques, F. Simon, F. Barre-Sinoussi, O. M. Diop, and M. C. Muller-Trutwin. 2005. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest* 115:1082-1091.

151. Boomershine, C. S., A. Chamberlain, P. Kendall, A. R. Afshar-Sharif, H. Huang, M. K. Washington, W. E. Lawson, J. W. Thomas, T. S. Blackwell, and N. A. Bhowmick. 2009. Autoimmune pancreatitis results from loss of TGFbeta signalling in S100A4-positive dendritic cells. *Gut* 58:1267-1274.

152. Fainaru, O., E. Woolf, J. Lotem, M. Yarmus, O. Brenner, D. Goldenberg, V. Negreanu, Y. Bernstein, D. Levanon, S. Jung, and Y. Groner. 2004. Runx3 regulates mouse TGF-beta-mediated dendritic cell function and its absence results in airway inflammation. *EMBO J* 23:969-979.

153. Laouar, Y., T. Town, D. Jeng, E. Tran, Y. Wan, V. K. Kuchroo, and R. A. Flavell. 2008. TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 105:10865-10870.

154. Lievens, D., K. L. Habets, A. K. Robertson, Y. Laouar, H. Winkels, T. Rademakers, L. Beckers, E. Wijnands, L. Boon, M. Mosaheb, H. Ait-Oufella, Z. Mallat, R. A. Flavell, M. Rudling, C. J. Binder, N. Gerdes, E. A. Biessen, C. Weber, M. J. Daemen, J. Kuiper,

and E. Lutgens. Abrogated transforming growth factor beta receptor II (TGFbetaRII) signalling in dendritic cells promotes immune reactivity of T cells resulting in enhanced atherosclerosis. *Eur Heart J*.

155. Allen, S. J., K. R. Mott, S. L. Wechsler, R. A. Flavell, T. Town, and H. Ghiasi. Adaptive and innate transforming growth factor beta signaling impact herpes simplex virus 1 latency and reactivation. *J Virol* 85:11448-11456.

156. Ramalingam, R., C. B. Larmonier, R. D. Thurston, M. T. Midura-Kiela, S. G. Zheng, F. K. Ghishan, and P. R. Kiela. Dendritic cell-specific disruption of TGF-beta receptor II leads to altered regulatory T cell phenotype and spontaneous multiorgan autoimmunity. *J Immunol* 189:3878-3893.

157. Robbins, S. H., G. Bessou, A. Cornillon, N. Zucchini, B. Rupp, Z. Ruzsics, T. Sacher, E. Tomasello, E. Vivier, U. H. Koszinowski, and M. Dalod. 2007. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog* 3:e123.

158. Sheth, P. M., A. Danesh, A. Sheung, A. Rebbapragada, K. Shahabi, C. Kovacs, R. Halpenny, D. Tilley, T. Mazzulli, K. MacDonald, D. Kelvin, and R. Kaul. 2006. Disproportionately high semen shedding of HIV is associated with compartmentalized cytomegalovirus reactivation. *J Infect Dis* 193:45-48.

159. Fisher-Hoch, S., J. B. McCormick, D. Sasso, and R. B. Craven. 1988. Hematologic dysfunction in Lassa fever. *J Med Virol* 26:127-135.

160. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 7:1118-1122.

161. Zhang, Q., X. Yang, M. Pins, B. Javonovic, T. Kuzel, S. J. Kim, L. V. Parijs, N. M. Greenberg, V. Liu, Y. Guo, and C. Lee. 2005. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* 65:1761-1769.

162. Kim, B. G., C. Li, W. Qiao, M. Mamura, B. Kasprzak, M. Anver, L. Wolfraim, S. Hong, E. Mushinski, M. Potter, S. J. Kim, X. Y. Fu, C. Deng, and J. J. Letterio. 2006. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. *Nature* 441:1015-1019.

163. Sohn, S. J., G. M. Lewis, and A. Winoto. 2008. Non-redundant function of the MEK5-ERK5 pathway in thymocyte apoptosis. *EMBO J* 27:1896-1906.

164. Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102:15545-15550.

165. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T. Y. Roh, W. T. Watford, D. E. Schones, W. Peng, H. W. Sun, W. E. Paul, J. J. O'Shea, and K. Zhao. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30:155-167.

166. Yusuf, I., R. Kageyama, L. Monticelli, R. J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is

dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* 185:190-202.

167. Safford, M., S. Collins, M. A. Lutz, A. Allen, C. T. Huang, J. Kowalski, A. Blackford, M. R. Horton, C. Drake, R. H. Schwartz, and J. D. Powell. 2005. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat Immunol* 6:472-480.

168. Mucida, D., M. M. Husain, S. Muroi, F. van Wijk, R. Shinnakasu, Y. Naoe, B. S. Reis, Y. Huang, F. Lambolez, M. Docherty, A. Attinger, J. W. Shui, G. Kim, C. J. Lena, S. Sakaguchi, C. Miyamoto, P. Wang, K. Atarashi, Y. Park, T. Nakayama, K. Honda, W. Ellmeier, M. Kronenberg, I. Taniuchi, and H. Cheroutre. Transcriptional reprogramming of mature CD4(+) helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. *Nat Immunol* 14:281-289.

169. Tumes, D. J., A. Onodera, A. Suzuki, K. Shinoda, Y. Endo, C. Iwamura, H. Hosokawa, H. Koseki, K. Tokoyoda, Y. Suzuki, S. Motohashi, and T. Nakayama. The polycomb protein Ezh2 regulates differentiation and plasticity of CD4(+) T helper type 1 and type 2 cells. *Immunity* 39:819-832.

170. Elrefaei, M., C. M. Burke, C. A. Baker, N. G. Jones, S. Bousheri, D. R. Bangsberg, and H. Cao. HIV-specific TGF-beta-positive CD4+ T cells do not express regulatory surface markers and are regulated by CTLA-4. *AIDS Res Hum Retroviruses* 26:329-337.

171. Lewis, G. M., M. Macal, C. R. Hesser, and E. I. Zuniga. Constitutive but not inducible attenuation of transforming growth factor beta signaling increases natural killer cell responses without directly affecting dendritic cells early after persistent viral infection. *J Virol* 89:3343-3355.

172. Gutcher, I., M. K. Donkor, Q. Ma, A. Y. Rudensky, R. A. Flavell, and M. O. Li. Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation. *Immunity* 34:396-408.

173. Park, I. K., J. J. Letterio, and J. D. Gorham. 2007. TGF-beta 1 inhibition of IFN-gamma-induced signaling and Th1 gene expression in CD4+ T cells is Smad3 independent but MAP kinase dependent. *Mol Immunol* 44:3283-3290.

174. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006-1010.

175. Ouyang, W., O. Beckett, Q. Ma, and M. O. Li. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity* 32:642-653.

176. Du, W., F. S. Wong, M. O. Li, J. Peng, H. Qi, R. A. Flavell, R. Sherwin, and L. Wen. 2006. TGF-beta signaling is required for the function of insulin-reactive T regulatory cells. *J Clin Invest* 116:1360-1370.

177. Peng, Y., Y. Laouar, M. O. Li, E. A. Green, and R. A. Flavell. 2004. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A* 101:4572-4577.

178. Penaloza-MacMaster, P., A. O. Kamphorst, A. Wieland, K. Araki, S. S. Iyer, E. E. West, L. O'Mara, S. Yang, B. T. Konieczny, A. H. Sharpe, G. J. Freeman, A. Y.

Rudensky, and R. Ahmed. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med* 211:1905-1918.

179. Marshall, H. D., A. Chandele, Y. W. Jung, H. Meng, A. C. Poholek, I. A. Parish, R. Rutishauser, W. Cui, S. H. Kleinstein, J. Craft, and S. M. Kaech. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity* 35:633-646.

180. Hale, J. S., B. Youngblood, D. R. Latner, A. U. Mohammed, L. Ye, R. S. Akondy, T. Wu, S. S. Iyer, and R. Ahmed. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity* 38:805-817.

181. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40:289-302.

182. Fahey, L. M., E. B. Wilson, H. Elsaesser, C. D. Fistonich, D. B. McGavern, and D. G. Brooks. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* 208:987-999.

183. Jellison, E. R., S. K. Kim, and R. M. Welsh. 2005. Cutting edge: MHC class II-restricted killing in vivo during viral infection. *J Immunol* 174:614-618.

184. Hildemann, S. K., J. Eberlein, B. Davenport, T. T. Nguyen, F. Victorino, and D. Homann. High efficiency of antiviral CD4(+) killer T cells. *PLoS One* 8:e60420.

185. Brien, J. D., J. L. Uhrlaub, and J. Nikolich-Zugich. 2008. West Nile virus-specific CD4 T cells exhibit direct antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. *J Immunol* 181:8568-8575.

186. Brown, D. M., A. M. Dilzer, D. L. Meents, and S. L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *J Immunol* 177:2888-2898.

187. Thomas, D. A., and J. Massague. 2005. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8:369-380.

188. Yoon, J. H., S. M. Jung, S. H. Park, M. Kato, T. Yamashita, I. K. Lee, K. Sudo, S. Nakae, J. S. Han, O. H. Kim, B. C. Oh, T. Sumida, M. Kuroda, J. H. Ju, K. C. Jung, D. K. Kim, and M. Mamura. Activin receptor-like kinase5 inhibition suppresses mouse melanoma by ubiquitin degradation of Smad4, thereby derepressing eomesodermin in cytotoxic T lymphocytes. *EMBO Mol Med* 5:1720-1739.

189. Ichiyama, K., T. Sekiya, N. Inoue, T. Tamiya, I. Kashiwagi, A. Kimura, R. Morita, G. Muto, T. Shichita, R. Takahashi, and A. Yoshimura. Transcription factor Smad-independent T helper 17 cell induction by transforming-growth factor-beta is mediated by suppression of eomesodermin. *Immunity* 34:741-754.

190. Takimoto, T., Y. Wakabayashi, T. Sekiya, N. Inoue, R. Morita, K. Ichiyama, R. Takahashi, M. Asakawa, G. Muto, T. Mori, E. Hasegawa, S. Saika, T. Hara, M. Nomura, and A. Yoshimura. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J Immunol* 185:842-855.

191. Gu, A. D., S. Zhang, Y. Wang, H. Xiong, T. A. Curtis, and Y. Y. Wan. A critical role for transcription factor Smad4 in T cell function that is independent of transforming growth factor beta receptor signaling. *Immunity* 42:68-79.

192. Eshima, K., S. Chiba, H. Suzuki, K. Kokubo, H. Kobayashi, M. Iizuka, K. Iwabuchi, and N. Shinohara. Ectopic expression of a T-box transcription factor, eomesodermin, renders CD4(+) Th cells cytotoxic by activating both perforin- and FasL-pathways. *Immunol Lett* 144:7-15.

193. Gordon, S. M., J. Chaix, L. J. Rupp, J. Wu, S. Madera, J. C. Sun, T. Lindsten, and S. L. Reiner. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* 36:55-67.

194. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, H. Shen, N. Cereb, S. Y. Yang, T. Lindsten, J. Rossant, C. A. Hunter, and S. L. Reiner. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302:1041-1043.

195. Oxenius, A., M. F. Bachmann, R. M. Zinkernagel, and H. Hengartner. 1998. Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur J Immunol* 28:390-400.

196. Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* 315:1687-1691.

197. Choi, Y. S., J. A. Yang, I. Yusuf, R. J. Johnston, J. Greenbaum, B. Peters, and S. Crotty. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. *J Immunol* 190:4014-4026.

198. Chevalier, M. F., B. Julg, A. Pyo, M. Flanders, S. Ranasinghe, D. Z. Soghoian, D. S. Kwon, J. Rychert, J. Lian, M. I. Muller, S. Cutler, E. McAndrew, H. Jessen, F. Pereyra, E. S. Rosenberg, M. Altfeld, B. D. Walker, and H. Streeck. HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol* 85:733-741.

199. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. *Science* 324:1569-1572.

200. Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones, and A. D. Kelleher. 2002. Characterization of CD4(+) CTLs ex vivo. *J Immunol* 168:5954-5958.

201. Soghoian, D. Z., H. Jessen, M. Flanders, K. Sierra-Davidson, S. Cutler, T. Pertel, S. Ranasinghe, M. Lindqvist, I. Davis, K. Lane, J. Rychert, E. S. Rosenberg, A. Piechocka-Trocha, A. L. Brass, J. M. Brenchley, B. D. Walker, and H. Streeck. HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome. *Sci Transl Med* 4:123ra125.

202. Fontana, A., K. Frei, S. Bodmer, E. Hofer, M. H. Schreier, M. A. Palladino, Jr., and R. M. Zinkernagel. 1989. Transforming growth factor-beta inhibits the generation of cytotoxic T cells in virus-infected mice. *J Immunol* 143:3230-3234.

203. Su, H. C., K. A. Leite-Morris, L. Braun, and C. A. Biron. 1991. A role for transforming growth factor-beta 1 in regulating natural killer cell and T lymphocyte proliferative responses during acute infection with lymphocytic choriomeningitis virus. *J Immunol* 147:2717-2727.

204. Leveen, P., J. Larsson, M. Ehinger, C. M. Cilio, M. Sundler, L. J. Sjostrand, R. Holmdahl, and S. Karlsson. 2002. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood* 100:560-568.

205. Letterio, J. J., A. G. Geiser, A. B. Kulkarni, H. Dang, L. Kong, T. Nakabayashi, C. L. Mackall, R. E. Gress, and A. B. Roberts. 1996. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 98:2109-2119.

206. Chen, C. H., C. Seguin-Devaux, N. A. Burke, T. B. Oriss, S. C. Watkins, N. Clipstone, and A. Ray. 2003. Transforming growth factor beta blocks Tec kinase phosphorylation, Ca2+ influx, and NFATc translocation causing inhibition of T cell differentiation. *J Exp Med* 197:1689-1699.

207. Zhang, Q., X. J. Yang, S. D. Kundu, M. Pins, B. Javonovic, R. Meyer, S. J. Kim, N. M. Greenberg, T. Kuzel, R. Meagher, Y. Guo, and C. Lee. 2006. Blockade of transforming growth factor-{beta} signaling in tumor-reactive CD8(+) T cells activates the antitumor immune response cycle. *Mol Cancer Ther* 5:1733-1743.

208. Moritani, M., K. Yoshimoto, S. F. Wong, C. Tanaka, T. Yamaoka, T. Sano, Y. Komagata, J. Miyazaki, H. Kikutani, and M. Itakura. 1998. Abrogation of autoimmune diabetes in nonobese diabetic mice and protection against effector lymphocytes by transgenic paracrine TGF-beta1. *J Clin Invest* 102:499-506.

209. Ishigame, H., L. A. Zenewicz, S. Sanjabi, P. Licona-Limon, M. Nakayama, W. J. Leonard, and R. A. Flavell. Excessive Th1 responses due to the absence of TGF-beta signaling cause autoimmune diabetes and dysregulated Treg cell homeostasis. *Proc Natl Acad Sci U S A* 110:6961-6966.

210. Hwang, Y., E. Y. Chen, Z. J. Gu, W. L. Chuang, M. L. Yu, M. Y. Lai, Y. C. Chao, C. M. Lee, J. H. Wang, C. Y. Dai, M. Shian-Jy Bey, Y. T. Liao, P. J. Chen, and D. S. Chen. 2006. Genetic predisposition of responsiveness to therapy for chronic hepatitis C. *Pharmacogenomics* 7:697-709.

211. Le Clerc, S., S. Limou, C. Coulonges, W. Carpentier, C. Dina, L. Taing, O. Delaneau, T. Labib, R. Sladek, C. Deveau, H. Guillemain, R. Ratsimandresey, M. Montes, J. L. Spadoni, A. Therwath, F. Schachter, F. Matsuda, I. Gut, J. D. Lelievre, Y. Levy, P. Froguel, J. F. Delfraissy, S. Hercberg, and J. F. Zagury. 2009. Genomewide association study of a rapid progression cohort identifies new susceptibility alleles for AIDS (ANRS Genomewide Association Study 03). *J Infect Dis* 200:1194-1201.

212. Almeida, N. P., G. O. Santana, T. C. Almeida, M. T. Bendicho, D. C. Lemaire, M. Cardeal, and A. C. Lyra. 2013. Polymorphisms of the cytokine genes TGFB1 and IL10 in a mixed-race population with Crohn's disease. *BMC Res Notes* 6:387.

213. Chou, C. K., A. Schietinger, H. D. Liggitt, X. Tan, S. Funk, G. J. Freeman, T. L. Ratliff, N. M. Greenberg, and P. D. Greenberg. Cell-intrinsic abrogation of TGF-beta signaling delays but does not prevent dysfunction of self/tumor-specific CD8 T cells in a murine model of autochthonous prostate cancer. *J Immunol* 189:3936-3946.

214. Zhang, N., and M. J. Bevan. Transforming Growth Factor-beta Signaling Controls the Formation and Maintenance of Gut-Resident Memory T Cells by Regulating Migration and Retention. *Immunity*.

215. Lee, M. S., S. Sawyer, M. Arnush, T. Krahl, M. von Herrath, M. A. Oldstone, and N. Sarvetnick. 1996. Transforming growth factor-beta fails to inhibit allograft rejection or virus-induced autoimmune diabetes in transgenic mice. *Transplantation* 61:1112-1115.

216. McCarron, M. J., and J. C. Marie. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest* 124:4375-4386.

217. Holderried, T. A., P. A. Lang, H. J. Kim, and H. Cantor. 2013. Genetic disruption of CD8+ Treg activity enhances the immune response to viral infection. *Proc Natl Acad Sci U S A* 110:21089-21094.

218. Mothe, B. R., B. S. Stewart, C. Oseroff, H. H. Bui, S. Stogiera, Z. Garcia, C. Dow, M. P. Rodriguez-Carreno, M. Kotturi, V. Pasquetto, J. Botten, S. Crotty, E. Janssen, M. J. Buchmeier, and A. Sette. 2007. Chronic lymphocytic choriomeningitis virus infection actively down-regulates CD4+ T cell responses directed against a broad range of epitopes. *J Immunol* 179:1058-1067.

219. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30:531-564.

220. Marshall, H. D., J. P. Ray, B. J. Laidlaw, N. Zhang, D. Gawande, M. M. Staron, J. Craft, and S. M. Kaech. The transforming growth factor beta signaling pathway is critical for the formation of CD4 T follicular helper cells and isotype-switched antibody responses in the lung mucosa. *Elife* 4:e04851.

221. Koehler, H., D. Kofler, A. Hombach, and H. Abken. 2007. CD28 costimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. *Cancer Res* 67:2265-2273.

222. Delisle, J. S., M. Giroux, G. Boucher, J. R. Landry, M. P. Hardy, S. Lemieux, R. G. Jones, B. T. Wilhelm, and C. Perreault. The TGF-beta-Smad3 pathway inhibits CD28-dependent cell growth and proliferation of CD4 T cells. *Genes Immun* 14:115-126.

223. Lucas, P. J., S. J. Kim, C. L. Mackall, W. G. Telford, Y. W. Chu, F. T. Hakim, and R. E. Gress. 2006. Dysregulation of IL-15-mediated T-cell homeostasis in TGF-beta dominant-negative receptor transgenic mice. *Blood* 108:2789-2795.

224. McKarns, S. C., and R. H. Schwartz. 2005. Distinct effects of TGF-beta 1 on CD4+ and CD8+ T cell survival, division, and IL-2 production: a role for T cell intrinsic Smad3. *J Immunol* 174:2071-2083.

225. McKarns, S. C., R. H. Schwartz, and N. E. Kaminski. 2004. Smad3 is essential for TGF-beta 1 to suppress IL-2 production and TCR-induced proliferation, but not IL-2-induced proliferation. *J Immunol* 172:4275-4284.

226. Shi, Y., Y. F. Wang, L. Jayaraman, H. Yang, J. Massague, and N. P. Pavletich. 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 94:585-594.

227. Gaarenstroom, T., and C. S. Hill. TGF-beta signaling to chromatin: how Smads regulate transcription during self-renewal and differentiation. *Semin Cell Dev Biol* 32:107-118.

228. He, S., J. Wang, K. Kato, F. Xie, S. Varambally, S. Mineishi, R. Kuick, K. Mochizuki, Y. Liu, E. Nieves, R. S. Mani, A. M. Chinnaiyan, V. E. Marquez, and Y. Zhang. Inhibition of histone methylation arrests ongoing graft-versus-host disease in mice by selectively inducing apoptosis of alloreactive effector T cells. *Blood* 119:1274-1282.

229. Guo, X., and X. F. Wang. 2009. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* 19:71-88.

230. Kartikasari, A. E., J. X. Zhou, M. S. Kanji, D. N. Chan, A. Sinha, A. Grapin-Botton, M. A. Magnuson, W. E. Lowry, and A. Bhushan. The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation. *EMBO J* 32:1393-1408.

231. Li, Q., J. Zou, M. Wang, X. Ding, I. Chepelev, X. Zhou, W. Zhao, G. Wei, J. Cui, K. Zhao, H. Y. Wang, and R. F. Wang. Critical role of histone demethylase Jmjd3 in the regulation of CD4+ T-cell differentiation. *Nat Commun* 5:5780.

232. Cho, O. H., H. M. Shin, L. Miele, T. E. Golde, A. Fauq, L. M. Minter, and B. A. Osborne. 2009. Notch regulates cytolytic effector function in CD8+ T cells. *J Immunol* 182:3380-3389.

233. Cruz-Guilloty, F., M. E. Pipkin, I. M. Djuretic, D. Levanon, J. Lotem, M. G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J Exp Med* 206:51-59.

234. Egawa, T., and D. R. Littman. 2008. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat Immunol* 9:1131-1139.

235. Wang, L., K. F. Wildt, E. Castro, Y. Xiong, L. Feigenbaum, L. Tessarollo, and R. Bosselut. 2008. The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral CD4+ T cells. *Immunity* 29:876-887.

236. Djuretic, I. M., F. Cruz-Guilloty, and A. Rao. 2009. Regulation of gene expression in peripheral T cells by Runx transcription factors. *Adv Immunol* 104:1-23.

237. Brown, D. M., S. Lee, L. Garcia-Hernandez Mde, and S. L. Swain. Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection. *J Virol* 86:6792-6803.

238. Curran, M. A., T. L. Geiger, W. Montalvo, M. Kim, S. L. Reiner, A. Al-Shamkhani, J. C. Sun, and J. P. Allison. Systemic 4-1BB activation induces a novel T cell phenotype driven by high expression of Eomesodermin. *J Exp Med* 210:743-755.

239. Brown, D. M., C. Kamperschroer, A. M. Dilzer, D. M. Roberts, and S. L. Swain. 2009. IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4+ T cells. *Cell Immunol* 257:69-79.

240. Pipkin, M. E., J. A. Sacks, F. Cruz-Guilloty, M. G. Lichtenheld, M. J. Bevan, and A. Rao. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32:79-90.

241. Workman, A. M., A. K. Jacobs, A. J. Vogel, S. Condon, and D. M. Brown. Inflammation enhances IL-2 driven differentiation of cytolytic CD4 T cells. *PLoS One* 9:e89010.

242. Qui, H. Z., A. T. Hagymasi, S. Bandyopadhyay, M. C. St Rose, R. Ramanarasimhaiah, A. Menoret, R. S. Mittler, S. M. Gordon, S. L. Reiner, A. T. Vella, and A. J. Adler. CD134 plus CD137 dual costimulation induces Eomesodermin in CD4 T cells to program cytotoxic Th1 differentiation. *J Immunol* 187:3555-3564.

243. Binder, D., M. F. van den Broek, D. Kagi, H. Bluethmann, J. Fehr, H. Hengartner, and R. M. Zinkernagel. 1998. Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. *J Exp Med* 187:1903-1920.

244. Cornberg, M., L. L. Kenney, A. T. Chen, S. N. Waggoner, S. K. Kim, H. P. Dienes, R. M. Welsh, and L. K. Selin. Clonal exhaustion as a mechanism to protect against severe immunopathology and death from an overwhelming CD8 T cell response. *Front Immunol* 4:475.

245. Stamm, A., L. Valentine, R. Potts, and M. Premenko-Lanier. An intermediate dose of LCMV clone 13 causes prolonged morbidity that is maintained by CD4+ T cells. *Virology* 425:122-132.

246. Doisne, J. M., L. Bartholin, K. P. Yan, C. N. Garcia, N. Duarte, J. B. Le Luduec, D. Vincent, F. Cyprian, B. Horvat, S. Martel, R. Rimokh, R. Losson, K. Benlagha, and J. C. Marie. 2009. iNKT cell development is orchestrated by different branches of TGF-beta signaling. *J Exp Med* 206:1365-1378.

247. Gu, A. D., Y. Wang, L. Lin, S. S. Zhang, and Y. Y. Wan. Requirements of transcription factor Smad-dependent and -independent TGF-beta signaling to control discrete T-cell functions. *Proc Natl Acad Sci U S A* 109:905-910.

248. Harker, J. A., G. M. Lewis, L. Mack, and E. I. Zuniga. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* 334:825-829.

249. Pan, D., T. Schomber, C. P. Kalberer, L. M. Terracciano, K. Hafen, W. Krenger, H. Hao-Shen, C. Deng, and R. C. Skoda. 2007. Normal erythropoiesis but severe polyposis and bleeding anemia in Smad4-deficient mice. *Blood* 110:3049-3055.

250. Karlsson, G., U. Blank, J. L. Moody, M. Ehinger, S. Singbrant, C. X. Deng, and S. Karlsson. 2007. Smad4 is critical for self-renewal of hematopoietic stem cells. *J Exp Med* 204:467-474.

251. Novitskiy, S. V., M. W. Pickup, A. Chytil, D. Polosukhina, P. Owens, and H. L. Moses. Deletion of TGF-beta signaling in myeloid cells enhances their anti-tumorigenic properties. *J Leukoc Biol* 92:641-651.

252. Binder, D., J. Fehr, H. Hengartner, and R. M. Zinkernagel. 1997. Virus-induced transient bone marrow aplasia: major role of interferon-alpha/beta during acute infection with the noncytopathic lymphocytic choriomeningitis virus. *J Exp Med* 185:517-530.

253. Miller, N. E., J. R. Bonczyk, Y. Nakayama, and M. Suresh. 2005. Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. *J Virol* 79:9419-9429.

254. Hahn, J. N., V. G. Falck, and F. R. Jirik. Smad4 deficiency in T cells leads to the Th17-associated development of premalignant gastroduodenal lesions in mice. *J Clin Invest* 121:4030-4042.

255. Blank, U., G. Karlsson, J. L. Moody, T. Utsugisawa, M. Magnusson, S. Singbrant, J. Larsson, and S. Karlsson. 2006. Smad7 promotes self-renewal of hematopoietic stem cells. *Blood* 108:4246-4254.

256. Ip, P. P., H. W. Nijman, J. Wilschut, and T. Daemen. Therapeutic vaccination against chronic hepatitis C virus infection. *Antiviral Res* 96:36-50.

257. Richman, D. D., D. M. Margolis, M. Delaney, W. C. Greene, D. Hazuda, and R. J. Pomerantz. 2009. The challenge of finding a cure for HIV infection. *Science* 323:1304-1307.