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Epigenetic and Signaling Pathways Regulating the Maintenance of CD8 T Cell Identity and Function

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by

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ABSTRACT

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In response to infection, antigen specific CD4 and CD8 T cells rapidly divide to provide help to the immune system and promote cytotoxicity of infected cells, respectively. Through this rapid division, CD4 and CD8 T cells maintain silencing of the opposing lineage's genes, which is essential to acutely eliminating pathogens. However, not all pathogens are acutely eliminated even when silencing is maintained, and the pathogen persists in the presence of activated CD8 T cells. CD8 T cells chronically exposed to antigen are phenotypically different than CD8 T cells acutely exposed to antigen, but CD8 T cell still exert control over chronic infections and cancers. Two unanswered questions regarding the maintenance CD8 T cell responses are: 1. How do CD8 T cells maintain the silencing of alternative lineage genes through division in the periphery, and 2. How do these cells maintain viral control through chronic stimulation. To shed light on these questions, two specific aims were developed for this thesis.

The first specific aim was to determine whether the epigenetic factor G9a is required to maintain silencing of helper lineage genes in proliferating CD8 T cells. To this end, genetic

deletion of G9a in CD8 T cells resulted in de-repression of *Cd4* and other helper T-related genes during lymphopenia- or tumor antigen-induced proliferation. In response to *Listeria monocytogenes* infection, G9a deficient CD8 T cells maintained silencing of *Cd4*. These data highlight that proliferating CD8 T cells employ multiple gene silencing mechanisms including G9a–mediated epigenetic modifications to maintain silencing of T helper-associated genes.

The second specific aim of this study was to determine how increasing PI3K signaling affects the maintenance of a functional CD8 T cell pool during chronic viral stimulation. During chronic *Lymphocytic choriomeningtis* virus (LCMV) infection, overexpression of a constitutively active form of PI3K in CD8 T cells caused lethal immunopathology reminiscent of chronic infection of PDL1 knockout mice. Inducible overexpression of PI3K after CD8 T cell priming depleted the memory- and stem-like CD8 T cell pool, which is required to sustain the CD8 T cell response. These data highlight an epistatic relationship between PI3K and PD1 in chronic CD8 T cells, and inhibitory signals may protect the chronic CD8 T cell progenitors from depletion throughout the course of infection. Future work will determine whether the responsiveness of CD8 T cells to PI3K signaling or PD1 blockade requires the transcription factor AP4.

Chapter 1:

Introduction

1.1 T lymphocytes

T and B lymphocytes constitute the adaptive immune system, which utilizes an extensive repertoire of antigen specific receptors for targeting infectious agents. The antigen specific receptor expressed by T lymphocytes is the T cell receptor (TCR), which requires a concomitant co-receptor, CD4 or CD8, for recognition of foreign antigens (1, 2). While CD4 expressing T cells function to provide help to other immune cells such as macrophages, B cells, and CD8 T cells, CD8 expressing T cells mainly function as cytotoxic cells (3).

1.2 T cell development

Helper T and cytotoxic T cells are derived from a common precursor cell in the thymus. Early thymocyte progenitors lack expression of both co-receptors and are designated as double negative (DN) cells (4). During this stage of development, the gene for CD4, *Cd4*, is actively silenced by the transcription factor RUNX1, which binds to a silencer *cis*-element within the first intron of the gene (5). As the developing cell successfully rearranges the β chain locus of the T cell receptor (TCRβ), expression of RUNX1 is decreased and silencing of *Cd4* is relieved (5, 6). The cell expresses both co-receptors as the locus for the complementary TCR chain, the α chain, undergoes rearrangement (7).

After successful TCRα rearrangement, the co-receptor double positive (DP) cell is subjected to positive selection, whereby the TCR is selected for reactivity to major histocompatibility complex (MHC) class I and II with self-peptides expressed by thymic medullary cells (8). Whereas MHC class II-selected cells differentiate into helper T cells, MHC class I-selected cells differentiate into cytotoxic T cells. Developing helper T cells upregulate *Zbtb7b* expression and maintain CD4 expression, while developing cytotoxic T cells upregulate *Runx3*

expression and silence *Cd4* (9–12). RUNX3 is an orthologue of RUNX1 and binds to the same *Cd4* silencer cis-element as RUNX1, and silencing of *Cd4* during the transition to the single positive CD8 T cell requires RUNX3 and the silencer (5, 12, 13). The *Cd4* gene remains silenced in peripheral CD8 T cells, however, RUNX3 and the silencer *cis*-element are not required to maintain silencing in peripheral CD8 T cells (14). The factors required to maintain silencing of *Cd4* and other helper related genes such as *Zbtb7b* and *Cd40lg* in peripheral CD8 T cells are not completely known, and the maintenance of alternative lineage gene silencing may require other transcription factors, epigenetic factors, or chromatin remodeling enzymes. The DNA methyltransferases DNMT3a, DNMT3b, and DNMT1 are necessary but not sufficient for *Cd4* silencing in peripheral CD8 T cells (15). How these ubiquitously expressed factors are specifically recruited to alternative lineage loci remains to be determined. These DNA modifying factors and other epigenetic factors may be recruited by the lineage-specific factor RUNX3 during the establishment of heritable silencing of helper lineage genes in CD8 T cells.

1.3 Acute vs chronic viral infections

Upon viral infection, naive CD4 and CD8 T cells are activated through TCR and co-stimulatory signals from professional antigen presenting cells (APCs) (16). These signals induce metabolic and transcriptional reprogramming required for differentiation and rapid proliferation, which enriches rare antigen specific T cells on the order of tens to hundreds of cells to millions of cells (17). The process of metabolic and transcriptional reprogramming requires the transcription factor c-Myc, which globally amplifies gene transcription (18–20). This factor is not sustained till the completion of amplification process (18, 21), and another transcription factor AP4 is required to sustain the transcriptional and metabolic program initiated by c-Myc (22).

The clonally expanding T cells compete with the replicating virus, which results in one of three outcomes: 1. Death of the host, 2. Acute elimination of the pathogen, or 3. Establishment of persistent or chronic infection (23). When the host survives, the immune system may acutely eliminate the pathogen as observed in the mouse models for acute infections *Lymphocytic choriomeningitis* virus (LCMV) Armstrong (Arm), or low dose Docile strain (24, 25). After elimination, the responding antigen specific T cell population contracts leaving behind a fraction of previously activated cells (26, 27). The cells that persist through contraction provide rapid protection to subsequent infection of the same pathogen, which is termed a memory response (28–30).

Not all pathogens are acutely eliminated from surviving hosts, and some pathogens may persist for months, years, or the lifespan of the host as observed in the mouse models for chronic infection LCMV clone 13 (c13), T1b, or high dose Docile (25, 31, 32). The utility of the LCMV mouse model of infection is the ability to measure T cell responses with the same TCR specificities to different viral outcomes (26). LCMV-specific CD8 T cells are phenotypically different between acute and chronic infections. Although the expression of TCR, CD3, and CD8 are similar between acute and chronic CD8 T cells, chronic CD8 T cells expand and persist at lower absolute numbers than acute CD8 T cells (33, 34). Compared to acute CD8 T cells, chronic CD8 T cells exhibit a decrease *in vitro* killing capacity, a decrease in production of effector cytokines such as IFNγ and TNFα, and an increased in expression of inhibitory receptors such as PD1, TIM3, LAG3, 2B4, and CD160 (32–37). These differences in the CD8 T cell phenotype between acute and chronic infections are attributed to an immune tolerance mechanism to overwhelming chronic infections known as T cell exhaustion (33, 34). Whether viral persistence causes the phenotypic changes to the CD8 T cell or the phenotypic changes to the CD8 T cell allow the virus to persist is not known.

The latter is not favored because chronic CD8 T cells after adoptive transfer into T cell deficient mice are sufficient to clear acute LCMV-Armstrong infection (38).

1.4 PD1 signaling in CD8 T cells

The mechanism that causes the CD8 T cell phenotypic changes in chronic infection compared to acute infection remains elusive. Candidate genes required in the development of these phenotypic changes were determined by transcriptional profiling of acute and chronic CD8 T cells from LCMV infections. One of the most differentially expressed inhibitory receptors is *Pdcd1*, which encodes programmed cell death protein 1 (PD1) (35). PD1 is structurally categorized in the same family of receptors as the costimulatory receptor CD28 (39). Instead of containing cytoplasmic tail motifs associated with activating signaling pathways, PD1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine switch motif (ITSM), which dampen or inhibit signaling pathways (40, 41). Beyond the structural differences between PD1 and CD28, these receptors exhibit different expression kinetics and ligands. CD28 is constitutively expressed by naive and activated T cells, but PD1 is only expressed by activated T cells, limiting inhibition to activated T cell states (42–44). Rather than binding to the CD28 ligands, CD80 and CD86, PD1 binds to PDL1, which is expressed by hematopoietic and non-hematopoietic cells, and PDL2, which is restricted to the hematopoietic compartment (45–47).

To study the role of PD1 in the development and maintenance of T cell exhaustion, PD1 signals were inhibited by antibody blockade or by genetic deletion of *Cd274*, the gene encoding PDL1, during LCMV clone 13. Mice deficient for *Cd274* succumb to lethal immunopathology after infection with LCMV-c13 (35), which caused difficulty for testing the requirement of PD1 signals in the development of exhaustion. However, blocking PD1 signals *in vivo* after T cell priming was not sufficient to cause lethal immunopathology, and antagonistic antibodies to PD1-

PDL1 interaction increased the numbers of antigen-specific CD8 T cells that produced effector cytokines (35). These changes correlated with an increased rate of viral elimination from the blood, spleen, and liver. These results highlighted the benefit of blocking PD1 inhibitory signals in CD8 T cells, which led to exploring the effects of blocking other inhibitory receptors separately and in combination during chronic infections and cancers (48–51). The requirement of PD1 in the development of T cell exhaustion was answered by utilizing adoptive transfer of small numbers of PD1 deficient LCMV-specific CD8 T cells into wildtype mice and subsequently infecting with chronic LCMV (52). PD1 deficient CD8 T cells expanded to numbers much greater than wildtype CD8 T cells. However, PD1-deficient CD8 T cells produced less IFNγ and TNFα and expressed more inhibitory receptors (52). These data implicated that PD1 is not required for the development of exhaustion, and unexpectedly, PD1 signals in CD8 T cells reduced the severity of exhaustion.

PD1 suppresses proliferation and exhaustion of CD8 T cells during chronic viral infection, but how PD1 signals to mediate these effects remains to be completely elucidated. Since PD1 contains an ITIM and an ITSM, PD1 association with the phosphatases SHP-1, SHP-2 or SHIP could mediate inhibition of TCR and co-stimulatory signaling. By utilizing FcγR and PD1 tail chimeric receptors, the co-ligation of BCR and the chimeric receptor resulted in preferential association with SHP-2 over SHP-1 or SHIP (53). However, using extracellular CD28 receptor and PD1 tail chimeric receptors in human CD4 T cells, PD1 associated with SHP-2 and SHP-1 (54). In both chimeric receptor expressing cells, mutations of the ITSM not the ITIM caused non-responsiveness to chimeric receptor ligation implicating the inhibitory activity through the ITSM not the ITIM.

More recently, *in vitro* reconstitution assays determined PD1 preferentially associated with SHP-2 compared to SHIP-1 or SHP-1 when measured by Forster resonance energy transfer

(FRET) (55). In contrast to *in vitro* studies, SHP-2 is not required for the development of exhaustion and is dispensable for PD1 blockade responsiveness (56). SHP-2 deficient CD8 T cells outcompeted wildtype CD8 T cells, which suggests that part of the proliferative effect of PD1 blockade is mediated by decreased SHP-2 activity (56). These data suggest that other phosphatases may compensate for the loss of SHP-2 in CD8 T cells during chronic infection.

1.5 Sustaining CD8 T cell responses to chronic viral infections

The responsiveness to PD1 blockade is not equivalent in all chronic CD8 T cells because responsiveness correlates with the expression level of PD1. In response to PD1 blockade, chronic CD8 T cells expressing intermediate levels of PD1 ($PD1^{int}$) expand significantly more than cells expressing high levels of PD1 ($PD1^{Hi}$) (57). These data highlight that a subset of chronic CD8 T cells are responsive to PD1 blockade.

The PD1 responsive and PD1 nonresponsive T cells subsets represent transcriptionally distinct CD8 T cell populations. $PD1^{int}$ CD8 T cells express higher levels of the transcription factor T-BET than $PD1^{Hi}$ CD8 T cells whereas $PD1^{Hi}$ CD8 T cells express higher levels of the transcription factor EOMESODERMIN (Eomes) than $PD1^{int}$ CD8 T cells (58). Through a series of transfer experiments, reporters, and knockouts, a progenitor-progeny relationship was established in that CD8 T cell progenitors expressing high levels of T-BET give rise to terminally differentiated cells expressing high levels of EOMES (58).

Recently, an alternative progenitor-progeny relationship of PD1 responsive and nonresponsive cells was defined on the expression of the transcription factors TCF-1 and BLIMP-1 (**Figure 1.1**). Since the transcription factor TCF-1 is required by CD8 T cells to persist after acute infection and proliferate in response to secondary infection (59), TCF-1 was implicated in

sustain chronic CD8 T cell responses. Indeed, TCF-1 is required to maintain the CD8 T cell numbers throughout the course of chronic viral infection (60–64). The cells expressing TCF-1 give rise to BLIMP-1 expressing cells, and TCF-1+ cells expand more greatly than BLIMP-1+ cells in response to PD1 blockade (60, 61, 64). These results highlight that transcriptionally distinct subsets of chronic CD8 T cells exist in the chronically infected mouse, and these subsets have different proliferative capacities in response to PD1 blockade.

1.6 IL21R signaling in CD8 T cells

The signals that are required for generating and maintaining the progenitor CD8 T cell subset during chronic infections are not completely known. Signals such as IL21R signals are required to sustain the chronic CD8 T cell response, and mice deficient for IL21R or IL21 are unable to clear chronic LCMV from the blood or spleen as seen in wildtype mice (65–67). Multiple immune cell types might require IL21R signals to aid in the clearance of chronic infection, but CD8 T cells deficient for IL21R are not able to persist at similar numbers as wildtype CD8 T cells as seen with TCF-1 knockout CD8 T cells. These data suggest that IL21R knockout CD8 T cells may provide signals for generating or replenishing the TCF-1 population. Alternatively, IL21R signals may be required by the BLIMP-1 population for survival or proliferation. Therefore, the mechanism by which IL21R signals sustain the CD8 T cell population during chronic infection should be determined.

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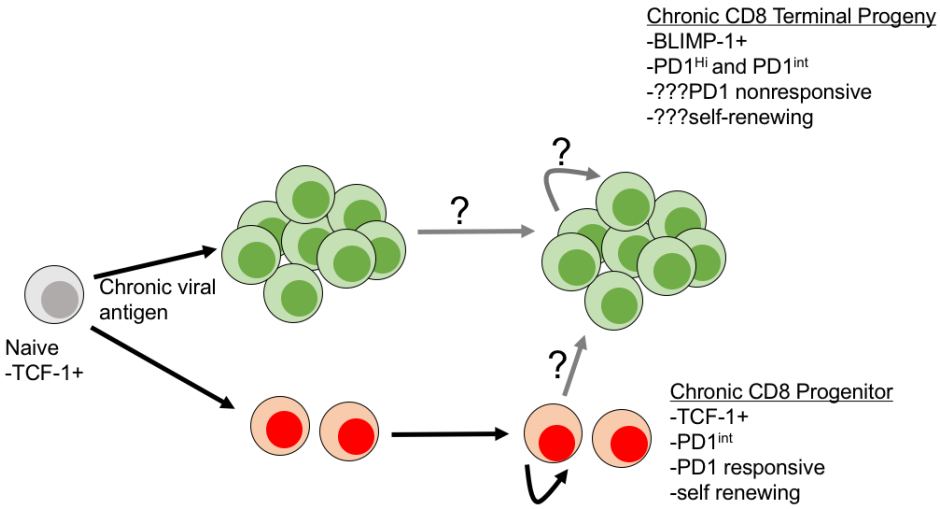

Figure 1.1 A schematic of CD8 T cell differentiation in response to chronic infection.

In response to chronic viral infections, antigen specific CD8 T cells clonally expand, and two distinct activated CD8 T cell populations exist in the host. One population expresses the transcription factor TCF-1 and proliferates in response to PD1 blockade. The other population expresses the transcription factor BLIMP-1 and responds poorly to PD1 blockade.

Chapter 2:

The histone methyltransferase G9a is required for silencing of helper T lineage-associated genes in proliferating CD8 T cells

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2.1 Abstract

Helper versus cytotoxic T lineage decision in the thymus has been studied as a model for silencing of alternative lineage genes. While the transcription factor RUNX3 is required for the initiation of *Cd4* silencing in developing CD8 T cells, it is unknown how silencing of *Cd4* and other helper T lineage genes is maintained. We show that the histone methyltransferase G9a is necessary for silencing of helper T lineage genes in proliferating mouse CD8 T cells. Despite normal initial *Cd4* downregulation, G9a-deficient CD8 T cells de-repress *Cd4* and other helper lineage genes during repeated division in lymphopenia or in response to tumor Ag. However, G9a was dispensable for continued silencing of those genes in CD8 T cells that respond to infection by *L. monocytogenes*. These results demonstrate that G9a facilitates maintenance of cellular identity of CD8 T cells during cell division, which is further reinforced by inflammatory signals.

2.2 Introduction

During a binary fate decision, genes related to the opposing lineage are heritably silenced (1, 2). This silencing is achieved through the constitutive activity of transcription factors involved in the lineage determining process or by recruitment of epigenetic machinery in a locus-specific manner, presumably by those lineage-specific transcription factors. The differentiation of the common thymocyte precursor to the helper or the cytotoxic T cell lineage in the thymus has been studied to understand the requirements for transcription factors and epigenetic gene regulation for stable lineage decisions (1-9). $CD4^+$ $CD8^+$ double positive (DP) thymocytes are subjected to positive selection of rearranged TCR$\alpha\beta$ by self-peptides presented on MHC class II or I (MHC-II or -I), and differentiate into cells in the helper or cytotoxic T lineages, respectively. MHC-I-selected thymocytes express the transcription factor RUNX3 that establishes the silencing of helper T lineage genes, including *Cd4* and *Zbtb7b* (10-14).

However, it is poorly understood how helper T lineage-associated genes are heritably silenced in mature cytotoxic T cells. During thymocyte development, *Cd4* is transiently repressed by RUNX1, an orthologue of RUNX3, in $CD4^-$ $CD8^-$ double negative (DN) thymocytes via direct binding to the silencer *cis*-element in the locus (11). This repression is subsequently reversed upon selection of a successfully rearranged *Tcrb* locus (15). *Cd4* is expressed uniformly in DP thymocytes that give rise to helper and cytotoxic T lineage cells after positive selection. While *Cd4* continues to be expressed in helper lineage T cells, $CD8^+$ cytotoxic T cells terminate *Cd4* transcription by upregulating RUNX3, which binds the identical *cis*-element as RUNX1 (11). Deletion of the silencer element or disrupting RUNX binding sites in the silencer results in continued *Cd4* expression in CD8 T cells (16, 17). However, deletion of the silencer or *Runx3* in differentiated CD8 T cells does not reactivate *Cd4*, indicating that the initial repression but not

maintenance of *Cd4* silencing requires RUNX3 (16, 18). RUNX3 may therefore recruit epigenetic modifications to the *Cd4* locus and loci encoding helper-lineage genes, which are maintained independently of RUNX3. Since the *Cd4* locus is only reversibly repressed in DN thymocytes but irreversibly silenced in CD8 T cells, we hypothesized that the irreversible silencing is mediated by epigenetic modifiers that specifically interact with RUNX3 but not RUNX1.

In this study, we identified the histone methyltransferase (HMT) G9a as an epigenetic modifier that preferentially interacts with RUNX3 to RUNX1 and is necessary for continued silencing of helper lineage genes in dividing CD8 T cells under non-inflammatory conditions. G9a-deficiency resulted in de-repression of several genes, which are otherwise expressed only in CD4 T cells, while it was compensated for by the inflammatory cytokine IL-12. These results suggest that G9a and inflammatory cues cooperatively maintain the identity of CD8 T cells during their division.

2.3 Results and Discussion

G9a forms a complex with RUNX3

To identify candidate epigenetic modifiers that are recruited by RUNX3, we analyzed RUNX1- and RUNX3-intractomes in 1200M thymoma cells, which have active *Cd4* silencing machinery (15). Among 71 DNA-binding proteins and epigenetic modifiers identified in RUNX1- or RUNX3-interactomes, all three components of the G9a HMT complex, G9a, GLP and WIZ as well a known G9a-interacting transcription factor CUX1 (31) were found predominantly in the RUNX3-interactome (**Fig. 2.1A, 2.1B**). Preferential interaction between G9a and RUNX3 compared to RUNX1 was confirmed by co-immunoprecipitation against FLAG-G9a and immunoblotting with anti-pan-RUNX Ab that detects both RUNX1 and RUNX3 (**Fig. 2.1C**). These data show that G9a and RUNX3 form a complex, which may deliver the HMT activity to *Cd4* and other helper lineage-related genes repressed by RUNX3 in developing CD8 T cells.

G9a is required for silencing of helper lineage-associated genes in proliferating CD8 T cells *in vivo under non-inflammatory conditions*

To define the role of G9a in CD8 T cells, we conditionally inactivated *Ehmt2*, encoding G9a, at the DP stage of thymocyte development using *Cd4*-cre. *Ehmt2* mRNA was barely detectable in $CD8^+$ mature thymocytes from $Ehmt2^{F/F}$ *Cd4*-cre mice (data not shown) (referred to as $Ehmt2^{-/-}$ CD8 T cells hereafter). As previously reported using p*Lck*-cre (32), numbers and frequencies of total thymocytes and $CD4^+$ and $CD8^+$ splenic T cells were comparable between $Ehmt2^{-/-}$ and control $Ehmt2^{+/+}$ mice (data not shown). Different from the reported phenotype in $Runx3^{-/-}$ thymocytes (11, 14), CD4 was normally downregulated in mature CD8 thymocytes, and was not expressed in splenic naive or memory CD8 T cells under steady state conditions (data not shown).

To determine whether G9a is required for maintaining *Cd4* silencing during cell division, we adoptively transferred *Ehmt2*$^{-/-}$ or control *Ehmt2*$^{+/+}$ naive CD4^{-} CD8^{+} T cells expressing the OT-I TCR transgene into *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice, in which donor-derived CD8 T cells divide under non-inflammatory conditions. By four weeks after transfer, both *Ehmt2*$^{-/-}$ CD8 T cells expanded at similar rates if not faster than control *Ehmt2*$^{+/+}$ cells as determined by CFSE, and repopulated in the recipients' peripheral blood (**Fig. 2.2A**, data not shown). While *Ehmt2*$^{+/+}$ CD8 T cells remained CD4-negative, approximately 30% of *Ehmt2*$^{-/-}$ CD8 T cells that had diluted CFSE beyond the limit of detection upregulated CD4 (**Fig. 2.2A, 2.2B**). Since a similar result was observed with transferred Vα2^{+} CD8 T cells from *Ehmt2*$^{F/F}$ *CD8*-cre OT-I mice, in which *Ehmt2* was deleted after positive selection (**Fig. 2.2B**), it is unlikely that *Cd4* de-repression is secondary to deregulated thymocyte selection in the absence of G9a.

To determine whether *Ehmt2*$^{-/-}$ CD8 T cells de-repressed additional helper lineage genes, global gene expression in CD4^{+} CD8^{+} and CD4^{-} CD8^{+} *Ehmt2*$^{-/-}$ T cells as well as control *Ehmt2*$^{+/+}$ CD8^{+} T cells 4 weeks after transfer was profiled by RNA-seq. Approximately 1,100 genes were differentially expressed by greater than 2-fold with the majority (637 genes) being upregulated in *Ehmt2*$^{-/-}$ CD8 T cells compared to *Ehmt2*$^{+/+}$ CD8 T cells (**Fig. 2.2C, 2.2D**). Among the genes that were differentially expressed between CD4 and CD8 memory T cells in the Immgen datasets (>1.8-fold difference), 92 genes that are more highly expressed in CD4 T cells, including *Cd4*, *Foxp3*, *Cd40lg*, *Rorc*, *Rora*, *Zbtb7b* and *Il21*, were de-repressed in the *Ehmt2*$^{-/-}$ CD4^{+} CD8^{+} T cells (**Fig. 2.2E**). We also observed downregulation of 18 genes that are more highly expressed in CD8 T cells in the absence of G9a (**Fig. 2.2E**), suggesting that G9a also contributes to turning-on genes in CD8 T cells directly or indirectly. A similar change in gene expression, albeit to lesser extent, was also observed in *Ehmt2*$^{-/-}$ CD4^{-} CD8^{+} T cells in which we have confirmed *Ehmt2*

deletion was also nearly complete (**Fig. 2.2E, data not shown**). In *Ehmt2*$^{-/-}$ CD8 T cells, the level of total H3K9me2 was substantially reduced whereas that of H3K9me3 was unchanged (**Fig. 2.3A**). In addition, we did not observe a reduction in H3K9me3 deposition near transcriptional start sites of the genes that were upregulated in CD4^{+} CD8^{+} *Ehmt2*$^{-/-}$ T cells compared to *Ehmt2*$^{+/+}$ CD8 T cells (**Fig. 2.3B**). *Cd4* upregulation was also observed in *Ehmt2*$^{-/-}$ OT-I T cells in response to E.G7-OVA tumor cells transplanted to WT mice (**Fig. 2.2F, 2.2G**). These results suggest that de-repression of helper-lineage genes in proliferating *Ehmt2*$^{-/-}$ CD8 T cells occurs also in lymphocyte-repleted mice although it may not continue once they slow down or stop division, such as memory CD8 T cells under steady-state conditions. Collectively, these data indicate that the G9a is required for continued silencing of a subset of helper lineage-associated genes in dividing CD8 T cells, which appears independent of H3K9me3. Since the G9a/GLP complex recruits PRC2 to its repressive target loci in embryonic stem cells (33), these helper lineage-associated genes may be kept silenced through the G9a-mediated recruitment of PRC2 activity.

G9a is dispensable for silencing of helper lineage genes in the presence of strong TCR or IL-12R signals

To determine whether G9a is required for continued *Cd4* silencing in dividing CD8 T cells in response to infection, *Ehmt2*$^{-/-}$ or *Ehmt2*$^{+/+}$ OT-I T cells (Thy1.2/CD45.2) mixed with internal control congenic OT-I T cells (Thy1.1/CD45.2) were transferred into CD45.1 WT mice, which were subsequently infected with Lm-OVA. In contrast to lymphopenia- and tumor-driven proliferation, *Ehmt2*$^{-/-}$ OT-I T cells remained CD4-negative (**Fig. 2.4A**). In addition, we observed comparable expansion and the ability to produce IFN-γ of *Ehmt2*$^{-/-}$ and *Ehmt2*$^{+/+}$ OT-I T cells relative to control OT-I T cells (**Fig. 2.4B,** data not shown). These results indicate that G9a is

dispensable for CD8 T cells under inflammatory conditions to maintain *Cd4* silencing and express IFN-γ, which is also RUNX3-dependent (34).

Distinct dependency of silencing of helper lineage genes on G9a of CD8 T cells between inflammatory and non-inflammatory conditions suggests that cell extrinsic signals through TCR, co-stimulatory molecules or cytokine receptors engage compensatory pathways that reinforce gene silencing. To define such cell-extrinsic determinants, we first cultured naive polyclonal CD8 T cells with varying concentrations of anti-CD3 and anti-CD28 Abs and determined whether distinct intensities of signaling through TCR or CD28 alter *Cd4* de-repression in *Ehmt2*$^{-/-}$ CD8 T cells. As seen in the lymphopenic condition, a substantial fraction of *Ehmt2*$^{-/-}$ CD8 T cells de-repressed CD4 when they were cultured with low anti-CD3 and high anti-CD28 Ab concentrations ("CD28^{Hi}" condition), whereas CD4 was barely expressed in *Ehmt2*$^{+/+}$ CD8 T cells (**Fig. 2.4C-E**). *Ehmt2*$^{-/-}$ CD8 T cells also upregulated additional helper lineage-related genes, such as *Il21* and *Rorc* (**Fig. 2.4F**). In contrast, when *Ehmt2*$^{-/-}$ CD8 T cells were stimulated with high anti-CD3 and low anti-CD28 Ab concentrations ("CD28^{Lo}" condition), de-repression of the helper lineage genes was markedly reduced (**Fig. 2.4D-F**).

Furthermore, *Cd4* de-repression in *Ehmt2*$^{-/-}$ CD8 T cells cultured in the CD28^{Hi} condition was significantly inhibited by the cytokine IL-12 (**Fig. 2.5A, 2.5B**). We detected elevated H3K9me2 in *Ehmt2*$^{-/-}$ CD8 T cells cultured in the presence of IL-12 compared to those without IL-12 (**Fig. 2.5C, 2.5D**). The IL-12 treatment upregulated the H3K9me3 demethylase *Kdm4c* as well as GLP/*Ehmt1* by 3-fold (**Fig 2.5E**), thus possibly maintaining H3K9me2-dependent gene regulation by increasing demethylation of H3K9me3 by KDM4C or by elevating residual HMT activity of GLP. These results suggest that the inflammation-dependent compensation may

reinforce stable lineage-specific gene expression signature in CD8 T cells that proliferate in response to infection.

Our study has demonstrated that G9a is required for maintaining silencing of multiple helper lineage-associated genes, such as *Cd4*, in dividing CD8 T cells in response to lymphopenia or tumor Ag. In developing CD8 T cells *Cd4* is shut-off by RUNX3 in a G9a-independent manner. However, the continued silenced state, which is independent of RUNX3, is not maintained in the absence of G9a, suggesting that transient RUNX3-dependent recruitment of G9a establishes the heritably silenced states of the locus in cooperation with other factors, such as additional methyltransferases. Alternatively, G9a is constitutively recruited to the *Cd4* locus initially by RUNX3 and subsequently by a RUNX3-independent mechanism. All the G9a-dependent repression targets are not RUNX3 targets (18, 34), and *Ehmt2*$^{-/-}$ CD8 T cells are also able to proliferate and express IFN-γ, which is dependent on RUNX3. Therefore, there are multiple distinct RUNX3- or G9a-containing complexes that regulate gene activation or repression in CD8 T cells, while *Cd4* silencing and repression of some of TFH-signature genes(18), including *Icos*, *Cxcr5*, and *Il21,* appear to be dependent on a complex containing both.

The absence of *Cd4* de-repression in CD8 T cells responding to Lm-OVA infection may be explained by high levels of Ag and IL-12 both of which compensate for G9a-deficiency in CD8 T cells *in vitro*. Although the exact mechanism is unknown, our data suggest that IL-12R signaling alters the balance between histone methylation and demethylation to increase the levels of H3K9me2 independent of G9a, possibly facilitating heritable gene silencing in dividing CD8 T cells through the compensatory pathways. CD8 T cells may thus engage multiple epigenetic pathways in a context-dependent manner to shape their gene expression signature.

2.4 Materials and methods

Mice

C57BL/6N (B6N) and B6-CD45.1 mice were purchased from Charles River. OT-I mice (19) were purchased from Taconic. *Cd4*-cre (10) and *CD8*-cre (*E8I*-cre) (20) mice were obtained from D. Littman (New York University). *Ehmt2*-flox mice were previously described (21). All mice were generated in or have been backcrossed more than 8 times to B6. Unless otherwise specified, littermate cre^{+} $Ehmt2^{+/+}$ or cre^{-} $Ehmt2^{F/F}$ were used as control. All mice were maintained in the specific pathogen-free facility at Washington University School of Medicine. All experiments were conducted following a protocol approved by the Washington University Animal Studies Committee.

Co-immunoprecipitation

1200M and AKR1 cell lines were transduced with MSCV-based retrovirus as described (22). For interactome analyses, 1200M cells in which endogenous *Runx1* expression had been knocked down (22), were transduced with FLAG-HA-tagged RUNX1 or RUNX3. RUNX1- and RUNX3-interacting proteins in nuclear extract were immunoprecipitated with anti-FLAG beads (M2, Sigma), eluted with 3xFLAG peptide (GenScript), and analyzed by mass spectrometry at the Taplin Mass Spectrometry Facility at Harvard University. For analytical immunoprecipitation, nuclear proteins were extracted from AKR1 cells that were transduced with RUNX1, RUNX3, RUNX1 and FLAG-tagged G9a retrovirus (FLAG-G9a), or RUNX3 and FLAG-G9a. Immune complexes containing FLAG-tagged protein were precipitated with anti-FLAG, followed by immunoblotting using anti-FLAG and anti-pan-RUNX Abs (12).

Flow cytometry

The following mAbs were purchased from Biolegend: APC conjugated anti-CD62L (MEL-14); APC-Cy7 conjugated CD45.2 (104); FITC conjugated anti-CD62L (MEL-14), -Vα2 (B20.1); Pacific Blue conjugated anti-CD44 (IM7); PE conjugated anti-Vβ5 (MR9-4), -IFN-γ (XMG1.2); PE-Cy7 conjugated anti-CD8a (53.6.7); PerCP-Cy5.5 conjugated anti-CD4 (GK1.5), -CD90.1 (OX-7). Cells were analyzed with an LSR II or an LSR Fortessa or sorted with a FACS Aria II (BD). Dead cells were excluded by staining with DAPI (Sigma) or Aqua Live/Dead (Life Technologies). Data were analyzed on FlowJo software (TreeStar).

T cell transfer, tumor innoculation, and *L. monocytogenes* (Lm) infection

Naive CD8 cells from OT-I TCR transgenic mice were sorted by flow cytometry as $V\alpha2^+$ $V\beta5^+$ $CD62L^+$ $CD44^{lo/-}$ $CD4^-$ $CD8^+$ cells. 2 x 10^5 cells were transferred i.v. to *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice. PBMCs and splenocytes were isolated from the recipient mice 4 weeks after transfer and analyzed for surface marker and gene expression. For experiments with transplanted tumors, 1 x 10^6 E.G7-OVA (ATCC #CRL-2113) cells were injected s.c. in the flank of B6-CD45.1 mice. 5 days later, 1 x 10^6 OT-I cells were transferred i.v. to the tumor bearing mice followed by analysis of T cells collected from the draining lymph node 7 days later. For Lm infection, 5 x 10^3 OT-I cells were transferred i.v. into B6-CD45.1 mice, which were infected i.v. with 2 x 10^4 CFU of Lm expressing OVA (Lm-OVA) on the next day as described (23).

Quantitative RT-PCR.

Total RNA was purified using Trizol (Life Technologies) and reverse-transcribed using qScript (QuantaBio). Gene expression was quantitated using a Luminaris SYBR green reagent (Thermo Fisher) and a Roche LightCycler 480. Primer sequences are listed in **Table 2.1**.

RNA-sequencing (RNA-seq)

RNA-seq was done essentially as described previously (24) using total RNA extracted from 5 x 10^4 *Ehmt2*$^{-/-}$ and *Ehmt2*$^{+/+}$ Vα2$^+$ CD8$^+$ T cells purified from *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ recipient mice 4 weeks after transfer. Sequenced tags were mapped to the mouse genome mm9 using Tophat (25) with default setting, followed by transcript assembly and estimation of expression levels using Cufflinks (26-29) on Galaxy (https://usegalaxy.org/).

Chromatin immunoprecipitation (ChIP)

Mono nucleosomes were prepared from cultured CD8 T cells by micrococcal nuclease digestion as described (30). H3K9me3-modified nucleosomes were immunoprecipitated using anti-H3K9me3 (Abcam 8898) conjugated with Protein G magnetic beads (Life Technologies). For genome-wide analysis, purified DNA from precipitated nucleosomes was sequenced with a HiSeq 2500 sequencer (Illumina) with a 50-bp single end read option as described (23).

Statistical Analysis

All statistics were performed using Graphpad Prism (version 7.0) using non-parametric two-tailed student T-tests for comparing 2 groups. Multiple groups were analyzed using two-tailed ANOVA. All other statistics were performed as described in the manuscript. Statistical analyses are shown with the mean ± SD. *p*-values smaller than 0.05 were considered significant.

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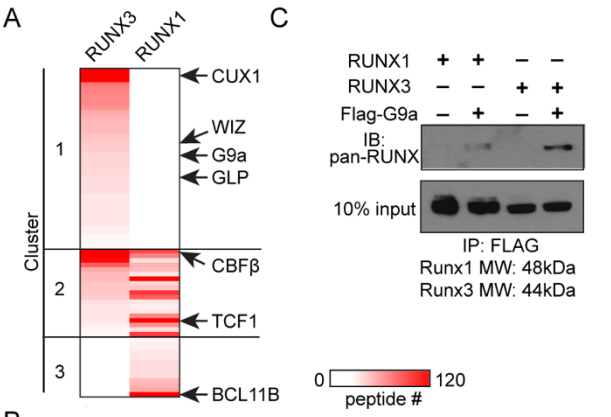

B

	Transcription factors	DNA Methyl-transferases	Histone Modifying Enzymes
Cluster 1	ADNP, CUX1, FOXO1, MYB,SMAD4	DNMT1 DNMT3A	G9A, GLP WIZ
Cluster 2	TCF1, CBFβ, FOXP4, GATA3, SATB1	–	KDM2A HDAC1
Cluster 3	BCL11B, ETV3, FOXP1, KLF4, ZBTB3	–	HDAC3, KDM1, KDM3A

FIGURE 2.1. G9a preferentially forms a complex with RUNX3 to RUNX1.

(**A**) A heat map showing the 71 DNA-interacting proteins that were co-immunoprecipitated with only RUNX3 (cluster 1), RUNX1 and RUNX3 (cluster 2), or only RUNX1 (cluster 3) from transduced 1200M cells. (**B**) A list of the transcription factors, DNA methyltransferases, and histone modifying enzymes in (A). (**C**) Immunoblotting (IB) for RUNX proteins co-immunoprecipitated with FLAG-G9a from lysates of AKR1 cells transduced with RUNX1, RUNX3 or FLAG-G9a. Blots are representative of 2 experiments.

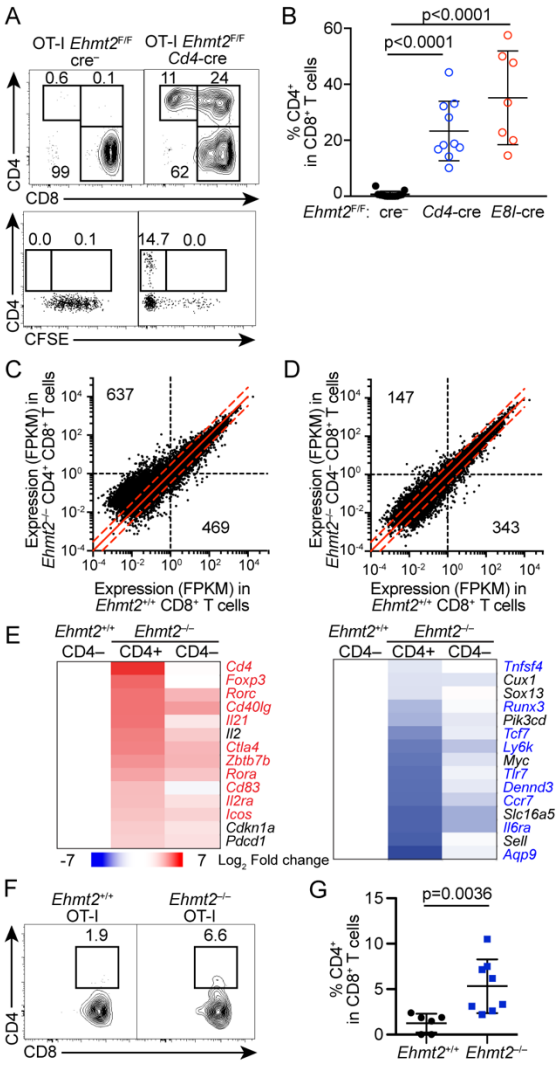

FIGURE 2.2. G9a is required to maintain silencing of helper lineage genes in CD8 T cells during lymphopenia- or tumor Ag-driven proliferation.

(**A**, **B**) CD4 expression and CFSE dilution of CD8 T cells in PBMC of *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice that received *Ehmt2*$^{-/-}$ or *Ehmt2*$^{+/+}$ OT-I T cells 4 weeks prior to the analysis. Data are pooled from 3 experiments in which one donor of each genotype was transferred into 2-3 recipients. (**C**, **D**) RNA-seq analysis of CD4^{+} CD8^{+} *Ehmt2*$^{-/-}$, CD4^{-} CD8^{+} *Ehmt2*$^{-/-}$ and CD4^{-} CD8^{+} *Ehmt2*$^{+/+}$ OT-I T cells harvested from *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$ mice 4 weeks after transfer. Quantification of genes with ≥1 FKPM in *Ehmt2*$^{-/-}$ or *Ehmt2*$^{+/+}$ samples and >2-fold difference in expression is indicated for each genotype. Dashed red lines: 2-fold change between genotypes. (**E**) Heat maps showing genes differentially expressed between CD4^{+} CD8^{+} or CD4^{-} CD8^{+} *Ehmt2*$^{-/-}$ and control *Ehmt2*$^{+/+}$ CD8 T cells. Values represent the $\log_2$ fold change of the mean of 2-4 mice compared to *Ehmt2*$^{+/+}$ CD8 T cells. (**F**, **G**) Expression of CD4 of OT-I T cells in the lymph node draining transplanted E.G7-OVA tumors. n=6-8 in 2 experiments.

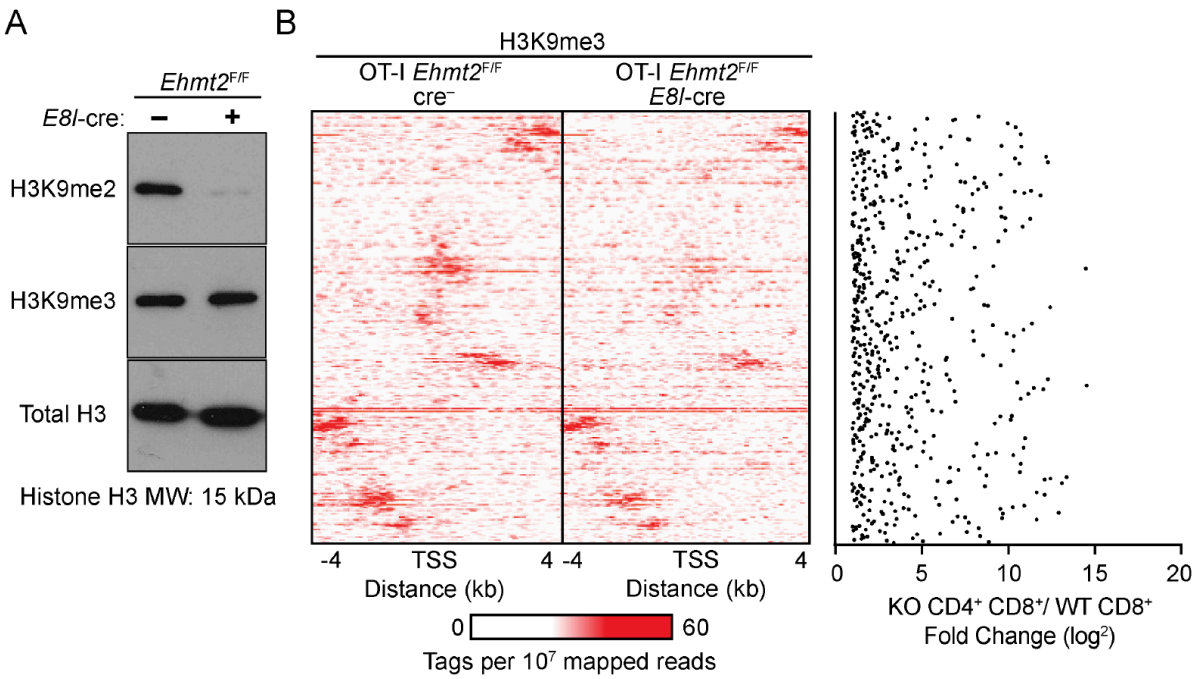

FIGURE 2.3. De-repression of the helper lineage-associated genes in Ehmt2–/– cells is independent of H3K9 trimethylation.

(A) Western blots showing amounts of H3K9me2, H3K9me3 or total H3 in CD8 T cells from *Ehmt2*$^{F/F}$ *Cd8-E8I*-cre mice and control *Ehmt2*$^{F/F}$ cre– mice. Data are representative of two experiments. (B) A heat map displaying the distribution of H3K9me3 ChIP-seq tags at the 637 genes that were identified from the RNA-sequencing data with at least a 2-fold higher expression in the CD4+CD8+ *Ehmt2*$^{F/F}$ *Cd4*-cre mice compared to CD8+ cells from control *Ehmt2*$^{F/F}$ cre– in Fig. 2.2C. Fold-changes of expression of those genes are plotted on the right panel.

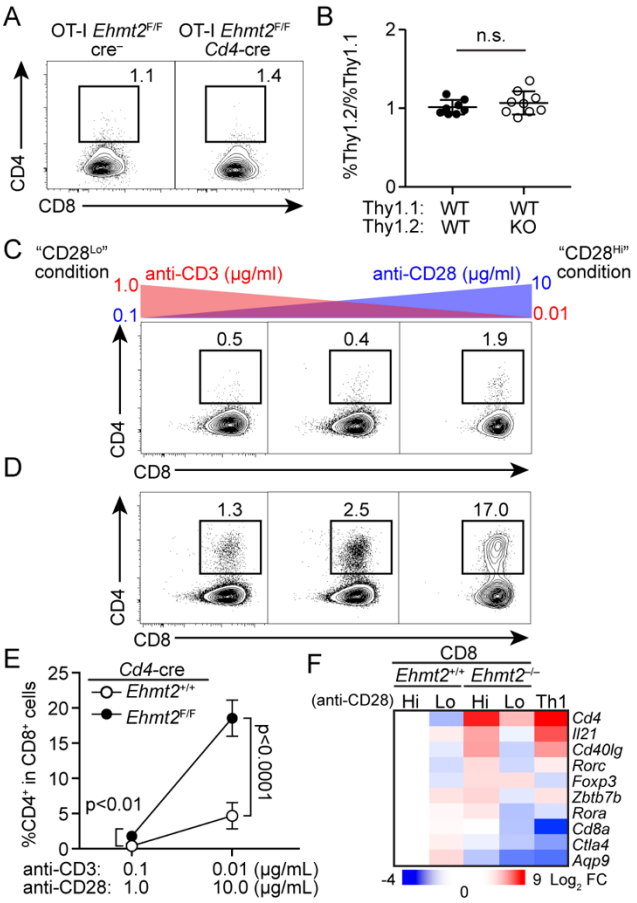

FIGURE 2.4. Increased TCR signaling compensates for G9a-deficiency in silencing of helper lineage genes in CD8 T cells.

(**A**, **B**) CD4 and CD8 expression of *Ehmt2*$^{-/-}$ and *Ehmt2*$^{+/+}$ OT-I T cells (Thy1.2/CD45.2) that were co-transferred as a 1:1 mixture with Thy1.1/CD45.2 OT-I T cells into CD45.1 mice 4 days after Lm-OVA infection. (**B**) The ratios of Thy1.2^{+} to Thy1.1^{+} donor cells 4 days after infection. Data points represent individual recipient in three experiments, in which cells from one donor per genotype were transferred into 3 recipients. (**C**, **D**) Expression of CD4 and CD8 on *Ehmt2*$^{+/+}$ (C) or *Ehmt2*$^{-/-}$ (D) T cells cultured in the presence of indicated concentrations of anti-CD3 and anti-CD28 Abs. Data are representative of 3 experiments (n=2 per genotype per experiment). (**E**) Percentage of CD4^{+} cells in cultured *Ehmt2*$^{-/-}$ or *Ehmt2*$^{+/+}$ CD8 T cells shown by mean ± SD. (**F**) qPCR analysis of gene expression in *Ehmt2*$^{-/-}$ and *Ehmt2*$^{+/+}$ CD8 T cells cultured in either CD28^{Hi} or CD28^{Lo} condition. *Ehmt2*$^{+/+}$ Th1 CD4 T cells were used as control (Th1).

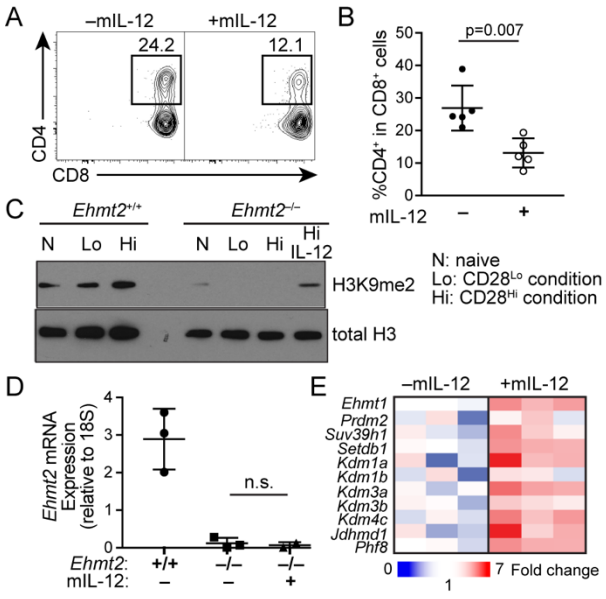

FIGURE 2.5. Signals through IL-12 receptor compensate for G9a-deficiency in repression of helper lineage genes in CD8 T cells.

(**A**, **B**) Percentages of $CD4^+$ cells in *Ehmt2*$^{-/-}$ or control *Ehmt2*$^{+/+}$ CD8 T cells cultured in the $CD28^{Lo}$ condition with or without mIL-12 (10 ng/ml). Plots are representative of 2 independent experiments (2-3 mice per experiment). (**C**) Immunoblotting for total H3 and H3K9me2 of lysates from *Ehmt2*$^{-/-}$ or *Ehmt2*$^{+/+}$ CD8 T cells cultured in the $CD28^{Hi}$ or $CD28^{Lo}$ condition. Data are representative of 2 experiments. (**D**) Relative expression of *Ehmt2* in CD8 T cells from *Ehmt2*$^{-/-}$ and *Ehmt2*$^{+/+}$ mice cultured with the $CD28^{Hi}$ condition with or without mIL-12. (**E**) qPCR analysis of expression of H3K9 HMTs and demethylases in *Ehmt2*$^{-/-}$ CD8 T cells cultured in the $CD28^{Hi}$ condition with or without mIL-12. n=3.

Gene	Forward Primer	Reverse Primer
Cd4	5'-GGGCTGTGGCAGTGTCTACT-3'	5'-GACACAGCAGAGGATGCAGA-3'
Rorc	5'-TCTACGCTATGAGGAAGGAAGGC-3'	5'-GACTATGGAGGAGAAACAGGTCCC-3'
Il21	5'-GCTCCACAAGATGTAAAGGGGC-3'	5'-CCACGAGGTCAATGATGAATGTC-3'
Cd40lg	5'-GTGAGGAGATGAGAAGGCAA-3'	5'-CACTGTAGAACGGATGCTGC-3'
Foxp3	5'-AGAAGCTGGGAGCTATGCAG-3'	5'-TACTGGTGGCTACGATGCAG-3'
Ctla4	5'-GGATCCTTGTCGCAGTTAGC-3'	5'-TCACATTCTGGCTCTGTTGG-3'
Aqp9	5'-TGGGGATTTGAGGTCTTCAC-3'	5'-GTTCGAGTGATGCATTTGGA-3'
Zbtb7b	5'-TGCTTCCGCATGTGGATC-3'	5'-GTGAGAAGCCCTTTGCCTGT-3'
Ehmt2	5'-TCATCCCTGTCCGGGTTTTC-3'	5'-TCACCGTAGTCAAAGCCCA-3'
Ehmt1	5'-AAGCAAGAGACCAAGCAGGA-3'	5'-TGTGGAACCTTCATCAGCAG-3'
Cd8a	5'-CACAGGAGCCGAAAGCGT-3'	5'-GGGCTTGCCTTCCTGTCTG-3'
Rora	5'-CGCATTGATGGATTTATGGAG-3'	5'-TCGCATACTTCCCGTCAAAG-3'
Kdm1a	5'-TGGGATGGATGTCACACTTC-3'	5'-CTCGTCCACCTACTCGATCC-3'
Kdm1b	5'-GAGTATGCTTGTGGCAGCAG-3'	5'-GGGTATGGTCACCAGCAAAC-3'
Kdm3a	5'-TGTCGACTATTGAGCCACACA-3'	5'-TCCTTTGACAGCTCGTTTCC-3'
Kdm3b	5'-TGGAAGGCTCACTTGTTTGG-3'	5'-GGTATTCAACTGGCACCACAG-3'
Kdm4c	5'-ATGGATTGACTACGGCAAGG-3'	5'-CCATGTCATTCCGACAAGTG-3'
Kdm7a	5'-CAGCTCTACACGGCTCTTCC-3'	5'-ACAGGTTTGGAGCCATCATC-3'
Phf8	5'-GTGTGACATGTGCCAGGACT-3'	5'-TCCTCCTCAACACCAACACA-3'
Setdb1	5'-CACAAAGGCACCCTTATTGC-3'	5'-CGGGAGGGTGGTAATCATAG-3'
Suv39h1	5'-AGGGGAGGAAGAAGTGGAAC-3'	5'-CAGGTCCTGCAGTTGATTCC-3'
Prdm2	5'-CTCCACCTCTGCAAACATGA-3'	5'-ACTGCATCCTGGCTTACCAC-3'
18s rRNA	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'

Table 1. Sequences of primers used for quantitative PCR.

Chapter 3:

PI3K signals mediate proliferation and terminal differentiation of the memory- and stem-like CD8 T cells during chronic viral infection

3.1 Abstract

Immunotherapy targeting PD1 requires co-stimulatory signals to reinvigorate diminished CD8 T cell responses in chronic infections and cancers. PD1 inhibits the co-stimulatory signals from activating phosphoinositide 3-kinase (PI3K), which signal activation of cell survival and proliferative pathways. Whether enhanced PI3K activity in chronically stimulated CD8 T cells is sufficient to restore function or prevent functional exhaustion is not known. In this study, we utilized a conditionally and inducibly constitutively active PI3K mouse model to enhance activity of PI3K specifically in CD8 T cells during chronic LCMV infection. Although PI3K activity caused lethal immunopathology, PI3K signals were sufficient to drive proliferation and differentiation of the chronic CD8 T cell progenitor, which these processes required the transcription factor AP4. These data demonstrate that the chronic CD8 T cell response is sustained by AP4-dependent replenishment of the terminal CD8 T cell population, and dampened PI3K signals protect the progenitors from depletion.

3.2 Introduction

In response to viral or intracellular bacterial infections, CD8 T cells proliferate and differentiate into effector T cells to promote acutely sterilizing responses (1). However, not all pathogens are acutely eliminated, leading to persistent infections. During persistent stimulation, CD8 T cells exhibit the loss of effector cytokine production, the loss of killing capacity, the loss of proliferative capacity, and the gain of inhibitory receptors compared to acute CD8 T cells (2, 3). The constellation of these traits is termed exhaustion or chronic phenotype, which is a stable differentiated state (4). The signals that drive or impede the differentiation to the chronic phenotype or exhausted state remain to be fully elucidated. Signals from the inhibitory receptors such as PD1, Tim3, Lag3, CD160, and 2B4 are implicated in the development or modulation of the chronic phenotype because blocking these signals has shown improvement in control of chronic infections and other chronic antigen exposures such as in cancers (5–7).

The inhibitory receptor PD1 is the most extensively studied inhibitory receptor in the development and modulation of T cell function during chronic stimulation because blocking PD1 signals in chronic CD8 T cells increases T cell numbers and increases the rate of chronic viral clearance (5). The pathways inhibited by PD1 signaling are hypothesized to be TCR and co-stimulatory, and indeed, the efficacy of anti-PD1 blockade requires co-stimulatory signals from CD28. Co-stimulatory signals through CD28 are inhibited by the PD1 activation of phosphatases. SHP-2, which dephosphorylates the CD28 tail is preferentially recruited to PD1 than other phosphatases (8–10). However, SHP-2 is dispensable for PD1 signaling and the development of exhaustion in CD8 T cells *in vivo* (11). Other phosphatases such as SHP-1, SHIP-1, and PTEN may provide compensatory inhibitory signals.

One consequence of CD28 dephosphorylation is the diminished activity of phosphoinositide 3-kinase (PI3K)(10). As compared to acute effector CD8 T cells, the phosphorylation of PI3K targets such as AKT, S6, FOXO1, and m-TOR is diminished in chronic CD8 T cells (12, 13). Phosphorylation of AKT and m-TOR in acute CD8 T cells promotes terminal differentiation while nuclear FOXO1 promotes memory differentiation and maintenance through transcriptional upregulation of memory related genes such as *Il7ra*, *Klf2*, *Sell*, *Tcf7*, and *Bcl2* (14). A memory-like and stem-like population of CD8 T cells that expresses many of these memory associated genes maintains the CD8 T cell response to chronic viral infection (15–19). These TCF-1 expressing CD8 T cells require constitutive FOXO1 expression for maintenance (20). Although one target of PI3K has been studied in chronic CD8 T cells, the role of PI3K in chronic CD8 T cells remains unknown. Therefore, determining what effects that restoring PI3K activity to chronic CD8 T cells will shed light on the development of exhaustion and provide a better understanding of the therapeutic effects of PD1 blockade.

In this study, we have utilized a conditional and inducible constitutively active PI3K mouse model to investigate the role of restoring PI3K signaling in CD8 T cells during the chronic infection, lymphocytic choriomeningitis virus clone 13 (LCMV-c13). Mice over-expressing a constitutively active form of PI3K in CD8 T cells succumbed to immunopathology within two weeks of infection, which phenocopies PDL1/PD1 knockout and early blockade. Death correlated with an increase in the numbers of low affinity TCR bearing CD8 T cells. While PI3K signaling failed to prevent the development of the exhaustion phenotype, cell-intrinsic PI3K signaling drove the differentiation and proliferation of chronic CD8 T cells to more terminal states at the expense of depleting the memory-like ($TCF1^{+}Tim3^{low}$). Furthermore, the replenishment of terminally differentiated cells through PI3K signals requires the transcription factor AP4. These data support

that the chronic CD8 T cell response integrate co-stimulatory and cytokine signals with inhibitory signals to balance renewal of chronic CD8 T cell progenitors with replenishment of terminal effectors.

3.3 Results

Constitutive activity of PI3K in CD8 T cells is sufficient to cause lethal immunopathology in chronically infected mice.

Previous studies have shown that phosphorylation of PI3K targets, S6, AKT, FoxO1, and mTOR, is reduced in LCMV-specific CD8 T cells in chronic infection compared to acute infection (12, 13). We tested whether restoring PI3K activity in chronically exposed CD8 T cells prevents exhaustion and permits faster viral clearance. Therefore, we crossed the *R26STOP^{FL}P110** mouse, which harbors a constitutively active form of PI3K (CA-PI3K) under the control of the ROSA26 promoter and a lox flanked STOP cassette, with *Cd8-E8I*-cre mice to generate CD8 specific expression of CA-PI3K. After infection with LCMV-c13, *R26STOP^{FL}P110*; CD8-E8I*-cre mice exhibited a mean survival of 8 days whereas wildtype mice survived and became chronically infected as previously reported (**Figure 3.1A**) (5). The weights of *R26STOP^{FL}P110*; CD8-E8I*-cre mice and *CD8-E8I*-cre mice were not significantly different at any time point before death. (**Figure 3.1B**). On day 6 of infection, the lungs of *R26STOP^{FL}P110*; CD8-E8I*-cre mice had more cellular infiltration and accumulation of hyaline causing reduced air space compared to wildtype mice (**Figure 3.1C**). To determine whether immunopathology correlated with viral replication, we measured the viral titers and found no differences between mice expressing CA-PI3K and wildtype mice (**Figure 3.1D**). These data show that PI3K activity in CD8 T cells causes lethal immunopathology of lungs and livers, which phenocopies the pathology seen in *Pdcd1* or *Cd247* knockout mice infected with LCMV-c13 (5, 21).

Constitutive activity of PI3K in CD8 T cells enables low affinity TCR bearing CD8 T cells to accumulate during chronic LCMV infection

To determine whether CA-PI3K signals increased the numbers of CD8 T cells during initial clonal expansion, we measured the percentage and absolute number of LCMV-specific CD8 T cells at day 6 after infection. The percentage and absolute number of GP33, GP276, and NP396 was equivalent between CA-PI3K expressing mice or wildtype mice (**Figure 3.2A, B**) as seen with PDL1 knockout mice. Upon *ex vivo* peptide re-stimulation, the percentage and absolute number of IFNγ producing cells in response to GP33, GP276, and NP396 peptides was increased in *R26STOP^{FL}P110*; CD8-E8I*-cre mice compared to *CD8-E8I*-cre mice (**Figure 3.2C, D**). These data suggest that overexpression of CA-PI3K is sufficient to drive expansion of low affinity TCR bearing LCMV-specific CD8 T cells. To determine whether this expansion is dependent on antigen load, we infected *R26STOP^{FL}P110*; CD8-E8I*-cre with LCMV-Armstrong, which is causes an acute infection with lower antigen burden compared to clone 13. The expansion of LCMV-peptide tetramer positive cells and cytokine producing cells was similar between wildtype and CA-PI3K expressing mice (**Figure 3.2E, F**). These data show that initial clonal expansion of high affinity TCR bearing LCMV-specific CD8 T cells is unaffected by increasing PI3K activity. Low affinity TCR bearing CD8 T cells are recruited to high antigen load responses with increased PI3K activity.

PI3K activity is sufficient to drive differentiation of chronic CD8 T cell progenitors to terminal progeny.

Since overexpression of CA-PI3K in CD8 T cells phenocopied PDL1 knockout, we hypothesized that PI3K activity is sufficient to drive differentiation of the chronic CD8 T cell progenitor as observed with PD1 blockade. The percent of CA-PI3K expressing progenitors was significantly lower than the percentage of wildtype progenitors (**Figure 3.3A, B**). Consistent with

differentiation to terminal progeny, the MFI and percentage of granzyme B were increased in the LCMV-specific CD8 T cells expressing CA-PI3K (**Figure 3.3C, D**). With similar absolute LCMV-specific CD8 T cell numbers, these data highlight that PI3K activity drove differentiation of chronic CD8 T cell progenitors without renewal to sustain the progenitor population. Although PI3K signals drove differentiation to terminal states, the fraction of cells going through cell cycle was similar between CA-PI3K expressing CD8 T cells and wildtype CD8 T cells (**Figure 3.3E, F**), which was similarly observed in chronic CD8 T cells receiving or not receiving PD1 signals (21). These data show that dampening PI3K signals may protect the chronic CD8 T cell progenitor population from terminal differentiation, and PD1 signals may therefore help sustain the chronic CD8 T cell response through diminishing PI3K activity.

PI3K signaling intrinsically causes proliferation and differentiation of chronically stimulated CD8 T cells beyond the initial activation phase.

We next tested whether PI3K-mediated differentiation of CD8 T cells is cell intrinsic or extrinsic. We crossed the P14 transgenic mice to *R26STOP^{FL}P110*; R26*-Cre-ER^{T2} to generate LCMV-specific inducible CA-PI3K CD8 T cells. We transferred an equivalent amount of P14 *R26STOP^{FL}P110*; R26*-Cre-ER^{T2} (CD45.2, Thy1.1.2) and P14 *R26*-Cre-ER^{T2} (CD45.2, Thy1.1) to naive CD45.1 mice and subsequently infected with LCMV-c13. To circumvent lethal immunopathology, tamoxifen was given on day 7 after infection. After one week from starting tamoxifen, CA-PI3K expressing CD8 T cells outcompeted wildtype CD8 T cells (**Figure 3.4A, B**). The competitive advantage could be caused by enhanced proliferation or survival of the CA-PI3K CD8 T cells, and cell extrinsic mechanisms may repress the total CD8 T cell numbers in

R26STOP^{FL}*P110*; CD8-E8I*-cre mice. Similar percentages of CA-PI3K- or non-expressing CD8 T cells stained for the proliferation marker protein, Ki67, suggesting a similar percentage of cells are cycling (**Data not shown**). Additionally, PI3K drove differentiation of the chronic CD8 T cell in a cell intrinsic manner (**Figure 3.4C, D**). These findings correlate with an increase in MFI and percentage of granzyme B expression in CA-PI3K expressing CD8 T cells compared to wildtype cells in the same environment (**Figure 3.4E, F**). Furthermore, CA-PI3K signaling did not reverse the exhaustion characteristic of reduced effector cytokine production, and CA-PI3K expressed less IFNγ than wildtype CD8 T cells (**Figure 3.4 G, H**). These data show that PI3K signaling intrinsically promotes the proliferation and differentiation of activated CD8 T cells in response to LCMV-c13. These data further confirm epistasis between PD1 and PI3K because PD1 deficient P14 T cells outcompete and produce less effector cytokines compared to wildtype P14 T cells responding to LCMV-c13 .

The transcription factor AP4 is required for PI3K-mediated differentiation of chronic CD8 T cells progenitors.

The transcription factors required for PI3K-mediated differentiation of chronic CD8 T cell progenitors to terminal progeny are not known. Since the transcription factor AP4 is required for sustaining effector differentiation during acute viral infections (22), we hypothesized that PI3K mediated differentiation of CD8 T cell progenitors requires AP4. CD8 T cells express AP4 after activation, and AP4 expression is reduced by inhibition of PI3K or m-TOR (**Figure 3.5A**). To determine whether AP4 is required for establishment and differentiation of progenitor cells during chronic LCMV infection, we chronically infected CD8-specific AP4 knockout mice, *Tfap4*$^{F/F}$; *Cd8-E8I*-cre mice. AP4 was dispensable for the generation of chronic CD8 T cell progenitors

(**Figures 3.5B, C**); however, BrdU incorporation by the chronic CD8 T cell progenitors was significantly reduced in AP4 deficient CD8 T cells compared to wildtype (**Figures 3.5D, E**). The differentiation block during the chronic phase of LCMV-c13 correlated with impaired viral clearance (**Figure 3.5F)**. These data show that AP4 is required by the chronic CD8 T cell progenitor to sustain the response to chronic viral infection. These data highlight that activating PI3K causes AP4-dependent differentiation of the chronic CD8 T cell progenitor.

3.4 Discussion

During chronic viral infections, CD8 T cells exhibit the loss of effector cytokine production, the loss of proliferative capacity, and the gain of inhibitory receptor expression (2, 3, 23, 24). These changes constitute an immunological tolerance process known as T cell exhaustion. The development of exhaustion is hypothesized to involve signals from inhibitory receptors because antibody blockade of inhibitory receptors during chronic phases of infection increases the rate of viral clearance and the proliferation of CD8 T cells (5). Of the inhibitory receptors, PD1 is more highly expressed in chronic CD8 T cells compared to acute CD8 T cells. Although PD1 and a PD1-associated phosphatase SHP-2 are not required for the development of exhaustion (11, 21), the mechanism by which PD1 blockade increases chronic CD8 T cell responses is not completely understood. We hypothesized that PD1 inhibits PI3K signaling in chronic CD8 T cells, and we tested the effects of restoring PI3K activity in chronic CD8 T cells.

Our work shows that PI3K signals are sufficient to drive the proliferating and differentiation of the chronic CD8 T cell progenitor during LCMV-c13 infection. In addition, these data establish an epistatic relationship between PD1 and PI3K in chronically activated CD8 T cells. Inhibitory receptor signals may act as a rheostat in chronic CD8 T cells to resist positive signals from co-stimulatory or cytokine receptors promoting terminal differentiation. Our data suggests that PD1 inhibits PI3K-mediated differentiation and proliferation of chronic CD8 T cell progenitors. This progenitor population expresses the transcription factor TCF-1 and sustains the chronic CD8 T cell response (15–19). Without inhibitory signals, the progenitor population is at risk of depletion as observed in CA-PI3K expressing mice. These observations might explain why some cancer patients become insensitive to PD1 blockade therapies (25).

The transcription factors required for differentiation and proliferation of the chronic CD8 T cell progenitor during the chronic phase of infection are not completely known, but our work shows that the transcription factor AP4 is required for this process. AP4 is required to maintain clonal expansion of CD8 T cell effectors responding to acute viral infections. Additionally, acute CD8 T cells deficient for AP4 significantly upregulate *Tcf7* expression, and AP4 binds within the *Tcf7* locus. Therefore, co-stimulatory or cytokine signals may increase the expression of AP4 causing this factor to promote differentiation through direct transcription repression of *Tcf7*. Whether AP4 is sufficient to repress *Tcf7* and cause differentiation of chronic CD8 T cell progenitors remains to be tested.

The signals that sustain or induce AP4 expression in chronic CD8 T cells are not known. Whether co-stimulatory and cytokine signaling induce AP4 expression in chronically stimulated CD8 T cells was not assessed in our study. Co-stimulatory signals from CD28 are required for responses to PD1 blockade, and these positive signals may increase AP4 expression in chronically activated CD8 T cells. Cytokines such as IL-21 are also required to sustain the chronic CD8 T cell response (26–28), but whether these cytokine and co-stimulatory signals are sufficient to increase proliferation or differentiation of chronic CD8 T cell progenitors needs to be tested. Furthermore, IL-21R signals are sufficient to sustain AP4 expression in B lymphocytes (29), and IL-21R signals may increase AP4 expression in chronic CD8 T cells, thus promoting terminal differentiation. AP4-dependent proliferation and terminal differentiation are required for clearance of the chronic viral infection. Therefore, the turnover of memory-like and terminal CD8 T cells is essential to the clearance of chronic viral infection.

3.5 Materials and methods

Mice

Cd8-E8I-cre, *Tfap4*$^{F/F}$, and P14 mice have been previously described (Maekawa et al., 2008; Pircher et al., 1989; Chou et al., 2014). *R26STOP*^{FL}*P110** and *R26*-Cre-ER^{T2} were purchased from the Jackson Laboratories (Srinivasan et al., 2009; Ventura et al., 2007), and C57Bl/6N and B6-CD45.1 mice were purchased from Charles River Laboratories. All animals studied were between the ages of 6-8 weeks with experiments conducted in accordance with an approved protocol from the Washington University in St. Louis Animal Studies Committee.

Infection

Mice were infected with $2x10^6$ PFU/mouse of LCMV-c13 retro-orbitally or $2x10^5$ PFU/mouse of LCMV-Arm intraperitoneally. Mice that received tamoxifen (Sigma) were gavaged daily for 7 days with 4mg of tamoxifen in corn oil (Sigma).

Ex vivo peptide stimulation

Splenocytes were cultured at $1.2x10^6$ cells per well with or without GP33, GP276 or NP396 peptides were added at a concentration of 10μM for 5hrs. At 3hrs of stimulation, Brefeldin A (Sigma) was added.

Hematoxylin & eosin staining

Lungs and livers were immediately fixed in 10% formaldehyde. After 2 days of fixation, organs were dehydrated in 70% ethanol and were embedded in paraffin before sectioning at 8μm. Then slides were stained with hematoxylin and eosin.

BrdU labeling

Mice received two intraperitoneal injections of 4mg/mouse BrdU (Sigma, St. Louis) at 24hr and 12hr before analysis. BrDU FACS staining was carried out using an BD BrdU FACS Flow Kit.

FACS

The following antibodies were purchased from Biolegend or BD Bioscience: Peridinin chlorophyll protein-cyanin 5.5 conjugated anti-CD4 (GK1.5; Biolegend), anti-CD8α (53-6.7, Biolegend), peridinin chlorophyll protein-cyanin-eFluor 710 anti-eomesodermin (Dan11mag, eBioscience), fluorescein isothiocyanate-conjugated anti-Ki67 (), allophycocyanin-conjugated anti-Tim3 (RMT3-23, Biolegend), anti-PD1 (29F.1A12, Biolegend), anti-TNFa, anti-Tbet (4B10, Biolegend) phycoerythrin-indotricarbocyanine-conjugated anti-CD8α (53-6.7, Biolegend), anti-PD1 (29F.1A12, Biolegend), Brilliant Violet 421 conjugated anti-Tim3 (RMT3-23, Biolegend). TCF1 (C63D9) antibody was purchased from cell signaling, and Alexa Fluor 488 conjugated goat anti-rabbit IgG (ThermoFisher). Dead cells were excluded by DAPI (4,6-diamidino-2-phenylindole; Sigma) or Aqua Live/Dead (Life Technologies). Analysis was performed on a BD Fortessa or BD Fortessa X20, and data were analyzed on FlowJo Software (TreeStar).

Statistics

All statistics were performed using Graphpad Prism version 7. Nonparametric student T tests were performed on 2 group analysis. All other statistics are described in the text above.

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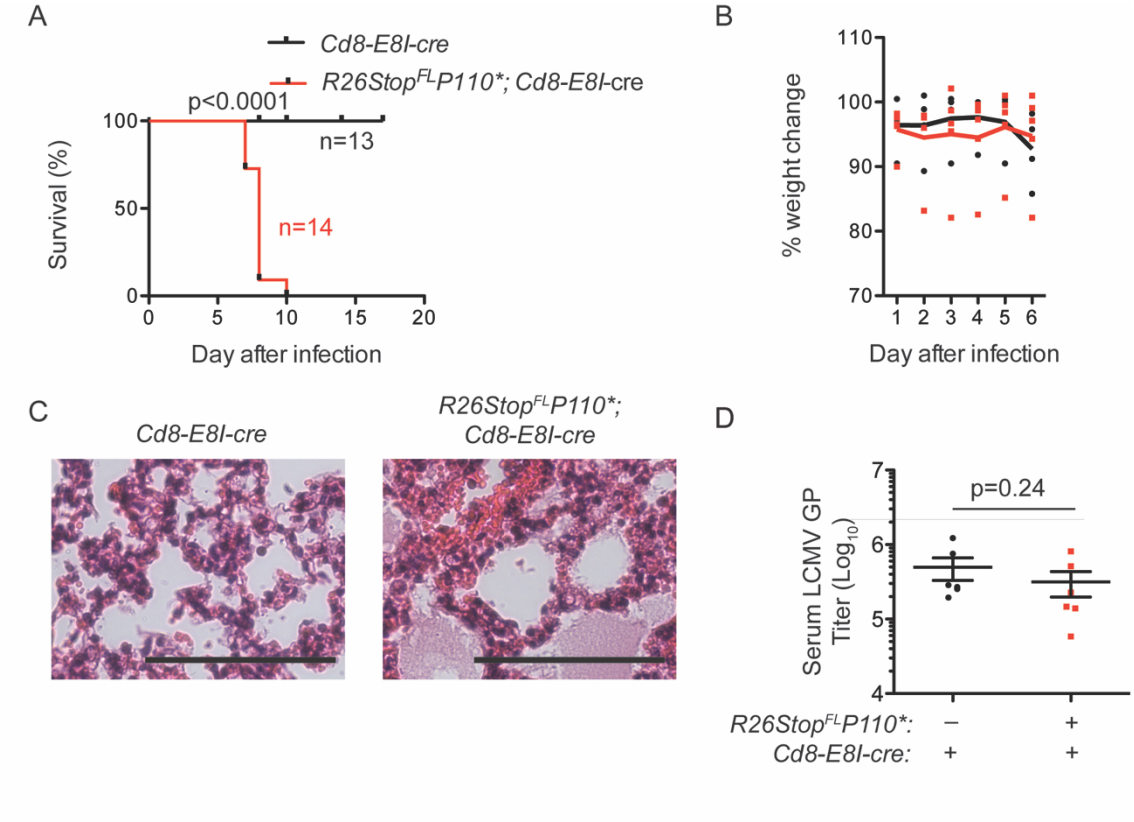

Figure 3.1. CA-PI3K activity in CD8 T cells is sufficient to cause lethal immunopathology during chronic LCMV infection.

A. Survival of *R26STOP*^{FL}*P110*; Cd8-E8I*-cre and *Cd8-E8I*-cre mice after infection with LCMV-c13 is graphed. Log-rank (Mantel-Cox) test was used to calculate p-value. Data is pooled from three experiments, in which 3-4 mice of each genotype were infected. **B**. The percent change in body weight between indicated day after infection and before infection is illustrated till the day before death. **C**. Representative images of hematoxylin and eosin (H&E) stained lungs and livers sections from mice on day 6 of LCMV-c13 infection are displayed. Scale bars represent 100µm and images are representative of 4 mice of each genotype. **D**. The serum LCMV titer was determined at day 6 after infection by QPCR of viral GP compared to an RNA spike-in control, ERCC108.

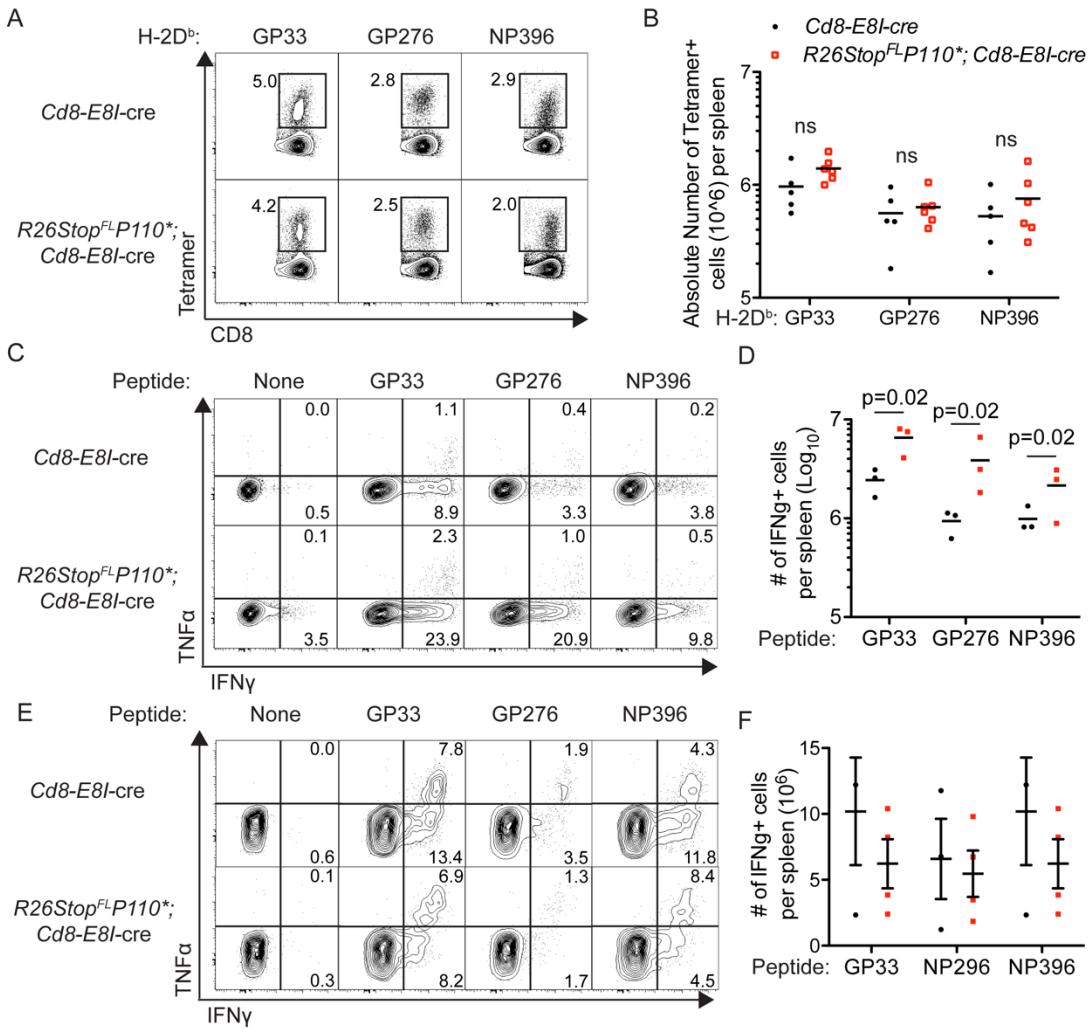

Figure 3.2. PI3K activity in CD8 T cells enables low affinity LCMV-specific CD8 T cells to accumulate early in LCMV-c13 infection.

A. **B**. Representative FACS plots and absolute numbers of LCMV-specific tetramer binding within CD8+ gated splenocytes from *R26STOP^{FL}P110*; Cd8-E8I*-cre and *Cd8-E8I*-cre mice on day 6 of infection with LCMV-c13 are illustrated. Numbers on FACS plots indicate the percentage of cells within the drawn gate. **C**. Representative FACS plots of IFNγ and TNFα expression within CD8+ splenocytes from day 6 after infection with LCMV-c13 upon *ex vivo* stimulation with indicated LCMV-specific peptides. Numbers indicate the percentages within the drawn gates. Plots are representative of 5-6 mice of each genotype from two independent experiments. **D**. The absolute number of IFNγ expressing CD8+ splenocytes were calculated for individual mice. **E, F**. Representative FACS plots of IFNγ and TNFα expression and quantification of IFNγ producing CD8 T cells 8 days after infection with LCMV-Armstrong upon *ex vivo* stimulation with indicated LCMV-specific peptides.

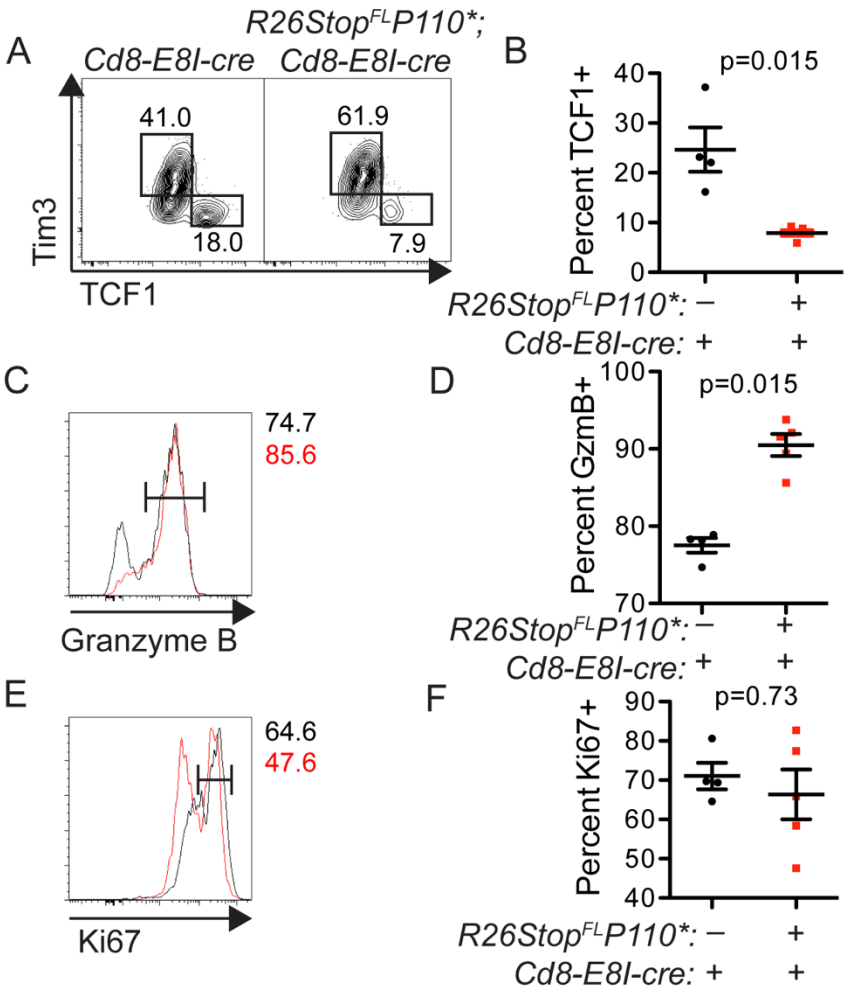

Figure 3.3. PI3K signaling promotes conversion of TCF1^{+}Tim3^{low} to TCF1^{-}Tim3^{High} CD8 T cells.

A. Representative FACS plots of Tim3 and TCF1 expression on GP33 tetramer+ CD8+ from *R26STOP^{FL}P110*; Cd8-E8I*-cre and *Cd8-E8I*-cre splenocytes on day 6 of infection with LCMV-c13. Plots are representative of 5-6 mice of each genotype. **B**. The percentage of TCF1+ GP33+ tetramer CD8+ T cells from the spleens of day 6 infected mice. **C-F**. Histograms and percentages of granzyme B and Ki67 expression in *R26STOP^{FL}P110*; Cd8-E8I*-cre and *Cd8-E8I*-cre splenocytes on day 6 of infection with LCMV-c13.

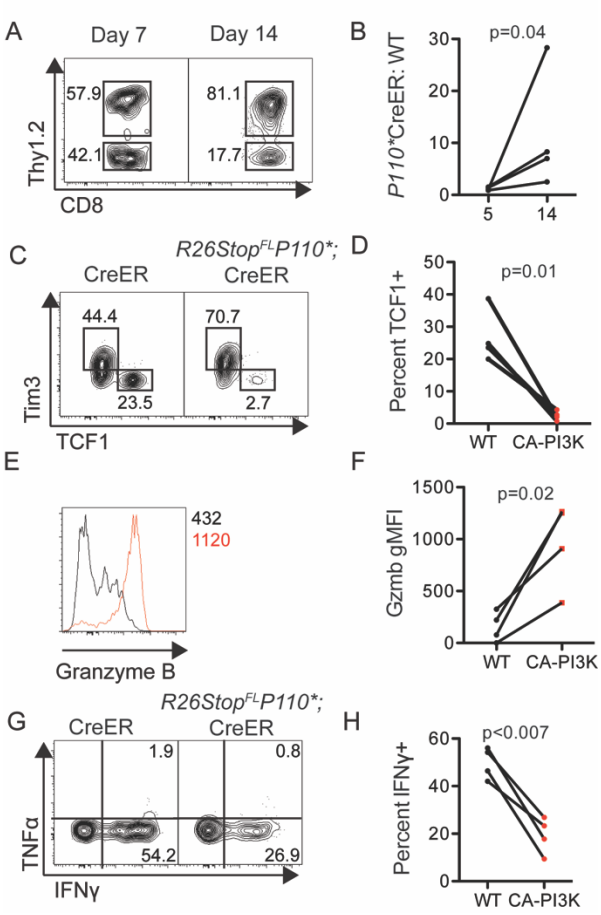

Figure 3.4. Intrinsic PI3K activity is sufficient to drive CD8 proliferation and differentiation of chronic CD8 T cells progenitors.

Thy1.1 P14 CreER and Thy1.1.2 P14 *R26STOP^{FL}P110*;* CreER cells (CD45.2+) were transferred at ratio of 1:1 into CD45.1 host mice, which were subsequently infected with LCMV-c13. Mice received 4μg of tamoxifen daily from day 7 till day 14 of infection. **A**. Representative FACS plots illustrate the percentage of Thy1.2 expressing and non-expressing CD45.2+ splenocytes from day 14 of LCMV-c13 infection. Plots are representative of 5 recipient mice per experiment. **B**. The ratio of Thy1.1.2 P14 *R26STOP^{FL}P110*;* CreER to Thy1.1 P14 CreER cells at days 5 and 14 of infection is plotted. **C, D**. Representative FACS plots and quantification of Tim3 and TCF1 expression on CD8+CD45.2+ Thy1.1+ or Thy1.1.2+ cells from the same recipient mouse at day 14 of LCMV-c13 infection. **E, F**. A representative histogram and quantification of the expression of granzyme B within Thy1.1 P14 CreER (black) and Thy1.1.2 P14 *R26STOP^{FL}P110*;* CreER (red) cells from the same recipient mouse are displayed. **G, H**. Representative FACS plots and quantification of TNFα and IFNγ expression from CD8+CD45.2+ Thy1.1+ or Thy1.1.2+ cells from the same recipient mouse at day 14 of LCMV-c13 infection.

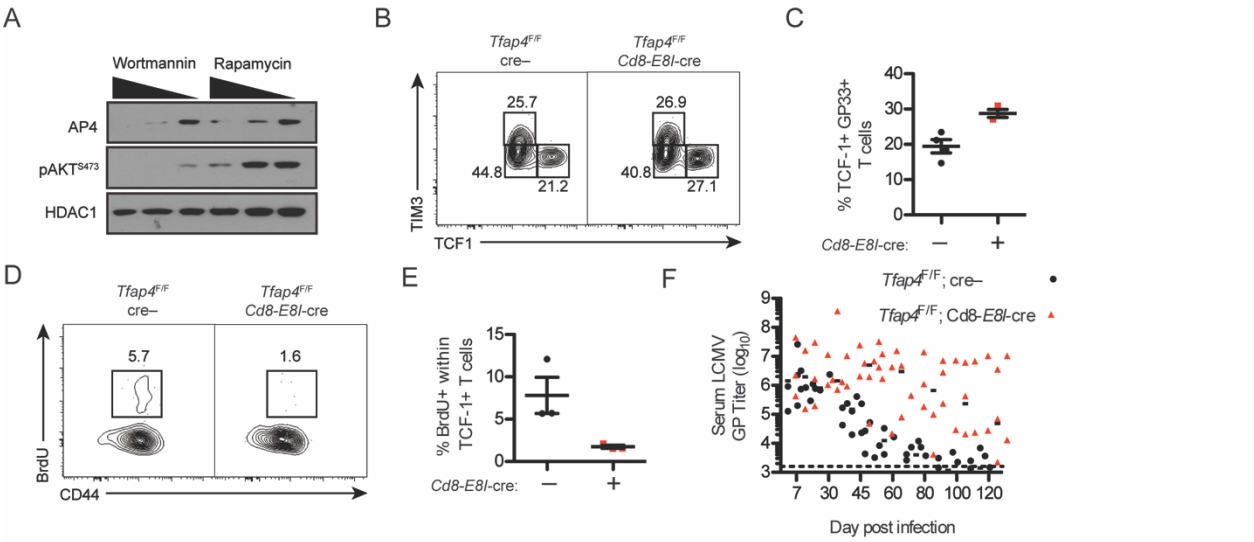

Figure 3.5 Chronic memory- and stem-like CD8 T cell require AP4 for proliferation and sustaining the chronic viral response.

A. Western blots of AP4, pAKTS473, and HDAC1 from antibody activated CD8 T cells, which received increasing concentrations of wortmannin or rapamycin. **B, C.** Representative FACS plots and percentages of GP33+ CD8 T cells expressing TIM3 or TCF-1 from *Tfap4*$^{F/F}$; cre– or *Tfap4*$^{F/F}$; *Cd8-E8I*-cre mice on day 30 of LCMV-c13 infection. D, E. Representative FACS plots and percentages of BrdU+ TCF-1+ GP33+ CD8 T cells at day 30 of LCMV-c13 infection. **F**. The serum viral titers measured from days 7 till 120 after infection by QPCR of viral GP compared to an RNA spike-in control, ERCC108.

Chapter 4:

Discussion and Future Directions

4.1 Heritable silencing of helper lineage associated genes in CD8 T cells

The lineage decision between helper T and cytotoxic T cells has been used as a model system to study the requirements of transcription factors and epigenetic factors for establishing and maintaining gene expression or repression. The lineage specific transcription factor RUNX3 is required for establishment of *Cd4* silencing in developing CD8 T cells (1–3), but RUNX3 and the silencer cis-element, where this transcription factor binds, are not required for the maintenance of *Cd4* silencing in proliferating CD8 T cells (4). Therefore, we hypothesized that RUNX3 recruits other factors to maintain the silencing of *Cd4*. Regarding the *Cd4* locus, previous studies showed that DNA methylation also plays important roles in the maintenance of epigenetic silencing, in part through inactivating an enhancer (5–7). Our work showed that RUNX3 associates with multiple epigenetic factors such as DNMT1 and DNMT3a, which are required for *Cd4* silencing in proliferating CD8 T cells (7). The interaction between RUNX3 and DNMT1 and DNMT3a was not confirmed by co-immunoprecipitation assays, but these RUNX3 containing complexes could explain how lineage nonspecific DNA methyltransferases are recruited to the *Cd4* locus.

We also demonstrated that RUNX3 formed a complex with the histone lysine methyltransferase G9a. G9a is required for *Cd4* silencing in CD8 T cells proliferating in response to lymphopenia or tumor antigens. These data highlight that RUNX3 forms multiple complexes such as with G9a or DMNT1 to mediate gene silencing through demethylation of H3K9 or DNA methylation, respectively. However, G9a is dispensable for *Cd4* silencing in CD8 T cells proliferating in response to inflammatory bacterial infection. Signals through the receptor for the inflammatory cytokine IL-12 were sufficient to compensate for *Cd4* silencing in the absence of G9a. The compensatory mechanism is not known, but IL-12R signals increased the total dimethylation of histone 3 lysine 9 (H3K9me2). IL-12R signals also increased the expression of

H3K9 methylases and demethylases, which may cause the total increase in H3K9me2. Whether the compensatory silencing *Cd4* are mediated through H3K9me2 dependent or independent pathways is not known. Double knockouts of the compensatory histone dimethylases, trimethyl demethylases, or DNA methyltransferase and G9a would directly test whether these are the required compensatory factors. In addition, whether the H3K9me2 changes with or without IL-12 in G9a deficient T cells occur at silenced genes is not known. Reliable chromatin immunoprecipitation of G9a, RUNX3, and H3K9me2 would assess whether G9a and RUNX3 bind to similar regions of genes that coincide with H3K9me2 marks. These data would provide more evidence that RUNX3 recruits G9a to the *Cd4* locus to mediate H3K9me2 dependent silencing.

Recent studies also implied that CD8 T cell immune responses require another repressive histone methyltransferase EZH2, which is a component of the PRC2 complex and mediates H3K27 methylation, for effective antigen/inflammation-stimulated responses (8, 9). How EZH2 is specifically recruited to genes associated with memory in terminally differentiated CD8 T cells is not known. However, the transcription factor FOXO1 restrains H3K27 methylation at memory associated genes (8), and FOXO1 may inhibit the binding of the transcription factor that recruits PRC2 complex to memory T cell associated genes. Determining the interactomes of transcription factors required for memory or terminal CD8 T cell differentiation would shed light on how memory CD8 T maintain identity through the lifetime of the host. Thus, $CD8^+$ T cells engage multiple epigenetic pathways in a context-dependent manner to shape their gene expression signature, which is further supported by our current study.

4.2 Sustaining the CD8 T cell response during chronic infection

During chronic viral infections, CD8 T cells exhibit loss of effector cytokine production, loss of proliferative potential, and gain in inhibitory receptor expression as compared to acute

effector CD8 T cells (10–12). The phenotypic differences between acute and chronic CD8 T cells have been attributed to an immune tolerance mechanism known as T cell exhaustion (13, 14). CD8 T cells examined at late time points of chronic infection produce less effector cytokines and have more inhibitory receptor expression than CD8 T cells examined at early time points of chronic infection (15). Also, early chronic CD8 T cells retain the ability to convert into memory CD8 T cells after adoptive transfer into acutely infected hosts whereas late chronic CD8 T cells do not retain the ability to convert into memory CD8 T cells (16). These observations imply that the CD8 T cell population progressively changes throughout the course of chronic viral infection. However, whether the progressive change happens at the population level or at a per cell level is not known. The wildtype CD8 T cell population dynamics are still uncertain. Whether CD8 T cells that exist at day 8 after LCMV-c13 infection are the same cells at day 30 after infection is not known. Therefore, pulsed lineage tracing experiments would shed light on these unknowns.

Lineage tracing experiments involving a tamoxifen inducible Cre driven by the *Tcf7* or *Prdm1* regulatory elements and a LOX-STOP-LOX-fluorescent protein would allow for pulse labeling of the progenitor or progeny CD8 T cells during chronic infection. The terminally differentiated cells labeled by *Prdm1*-CreER at day 8 would be followed over the course of infection by peripheral blood analysis of the fluorescent protein. If the percentage of cells remains constant, then there would be no death or replenishment of the terminal population over the course of infection. If the percentage of labeled cells increases overtime, these data would imply that the terminal differentiated cells proliferates and renews the terminal population over the course of viral infection. Alternatively, since a progenitor population exist, these cells could give rise to terminal cells, which would replenish the terminal CD8 T cells over time. The *Tcf7* driven CreER would be used to confirm that TCF-1 expressing cells do differentiation into BLIMP-1 expressing

cells throughout the course of chronic infection. If BLIMP-1 cells are labeled by fluorescent proteins by *Tcf7* driven CreER, then these data would directly show that TCF-1 cells differentiate into BLIMP-1 cells during chronic infection. Otherwise, these TCF-1 cells may be quiescent while BLIMP-1 cells may proliferate as indicated by BrdU experiments (17).

Despite the progressive loss of effector function, CD8 T cells exert control over persistent or chronic viral infections (18, 19). How CD8 T cells sustain viral control over the course of chronic infection is not completely understood, but genetic knockouts, antibody blockade, and adoptive transfer studies are starting to shed light on how this response is sustained (17, 20–23). A prevailing model describing the ability of CD8 T cells to maintain responsiveness to chronic viral infections is the establishment of activated stem- and memory-like CD8 T cell population that replenishes the terminally differentiated CD8 T cell population (17, 20, 24). This model argues in favor of cellular turnover throughout the course of chronic viral infection, but this has yet to be rigorously tested as previously discussed.

The lineage tracing depends on the progenitor-progeny relationship of TCF-1 and BLIMP-1; however, two separate progenitor-progeny relationships exists for chronic CD8 T cells. Both relationships are defined on expression of transcription factors. CD8 T cells expressing high levels of T-BET are classified as progenitor cells to terminal CD8 T cells expressing high levels of EOMES. The T-BET progenitor and EOMES progeny relationship has not been directly tested with adoptive transfer of cells expressing T-BET via a T-BET reporter. Moreover, the requirement of T-BET in CD8 T cells is not clear from *Tbx21*$^{F/F}$; Cd4-cre mice as these mice have deficient CD4 T cell responses, and CD4 T cell help is required to limit the severity of CD8 T cell exhaustion (11). The cell intrinsic role of T-BET of should be tested with CD8 specific knockout of T-BET and competitive P14 *Tbx21*$^{F/F}$ CreER^{T2}: P14 *Tbx21*$^{+/+}$ CreER^{T2}. In the latter experiments, T-BET

can be inducibly deleted after the initial priming phase. During the priming phase in acute infections, T-BET is required for short lived effector cell generation (25, 26). The ratio of wildtype to T-BET knockout P14 T cells can be measured over the course of infection. These data will clarify the cell intrinsic role of T-BET in chronic CD8 T cells, and whether T-BET plays any role in maintaining a progenitor cell population in the later phases of chronic infection.

As previously described, the alternative progenitor-progeny relationship is defined on the expression of the transcription factors TCF1 and BLIMP-1. Like the studies with T-BET and EOMES, the CD8 intrinsic requirement of TCF-1 during chronic infection is not clear. There is a discrepancy as to whether TCF-1 is required by P14 T cells for initial expansion at day 8 (17, 20). Additionally, a caveat of the TCF-1 studies is that the role of TCF-1 in chronic CD4 T cells is not clear. Therefore, utilization of *Tcf7*$^{F/F}$; *Cd4*-cre or *Tcf7* germline knockout mice could impair CD4 T cell help (20, 27). The intrinsic requirement of TCF-1 in chronic CD8 T cells should be tested with *Tcf7*$^{F/F}$; *Cd8-E8I*-cre and *Tcf7*$^{F/F}$; CreER^{T2} P14 transfers. These data will also shed light on the requirement of TCF-1 during the maintenance of CD8 T cell responses chronic viral infection.

Our work shows that the TCF1+ population is depleted with a cell intrinsic increase in PI3K activity, which raises the potential problem that continuous PD1 blockade could deplete the CD8 T cell progenitors sustaining the immune response. Similarly, PD1 knockout P14 T cells contracted significantly more than wildtype cells in the same host mouse (28), which suggests that the *Pdcd1*$^{-/-}$ CD8 T cell response is not maintained to the same extent as wildtype. Could the stem-like population be depleted in patients on long term anti-PD1 or anti-PDL1 treatment? This observation could explain why some patients can become insensitive to PD1 blockade (29). Whether PD1 blockade depletes the TCF-1 population should be directly test by administering PD1 blockade and measuring the numbers of TCF-1 expressing cells.

The question of how the TCF-1 population is protected from PI3K signals remains unanswered. The answer is not simply expression of PD1 because TCF-1+ and TCF-1– cells have overlapping PD1 expression, and the TCF-1– cells are not responsive to PD1 blockade. Like PD1 expression, CD28 and TCR expression is not significantly different between the subsets of CD8 T cells that are responsive or nonresponsive to PD1 blockade (17). Besides PD1, CD28, and TCR signals, possible modulators of PI3K activity are cytokines signals such as IL21R signals and type 1 interferon signals, these signals may tip the balance of positive and negative signals in favor of active PI3K activity. These signals may increase PI3K activity causing the differentiation to TCF1- CD8 T cells that then may replenish the dying terminal cells. The process for turn-over is not understood either, and whether competition plays a role in the replenishment of TCF1- cells is not known.

The signaling pathway of PD1 has been extensively studied *in vitro*, but only a few studies have tried to determine the signaling molecules required in vivo. Our study shows that many of the chronic CD8 phenotype changes with PD1 blockade occur with overexpression of PI3K establishing an epistatic relationship. The requirement of the phosphatases SHP-1 and SHIP-1 and the immunoreceptor tyrosine-based switch motif (ITSM) for mediating PD1 inhibition should be determined. The functional roles of the PD1 ITSM and ITIM in chronic CD8 T cells has yet to be determined. Developing a mouse model that has genetic mutations in the ITSM or the ITIM is essential to understanding potentially two signaling pathways by which PD1 works in chronic CD8 T cells.

4.3 References

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