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The Role of PD-1 in regulating CD8 T cell response in LCMV infection

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The Role of PD-1 in regulating CD8 T cell response in LCMV infection

By

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B.S., Sichuan University, 2008

Advisor: Rafi Ahmed, Ph.D.

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Abstract

The Role of PD-1 in regulating CD8 T cell response in LCMV infection

By Weiyan Li

Chronic infections such as human immunodeficiency virus (HIV), human hepatitis B virus (HBV) and human hepatitis C virus (HCV) afflict more than 500 million people worldwide. Functionally impaired (exahusted) antigen-specific CD8 T cell is a hallmark of many chronic infections including the HIV HBV and HCV, and also a major cause leading to the failure of the host in antigen clearance. Programmed death-1 (PD-1) is highly expressed on exhausted T cells and is an inhibitory receptor for T cell receptor signaling. Manipulation of PD-1 inhibitory signal has been shown to have great therapeutic potential in vaccine design and chronic disease treatment.

In this study I utilized a PD-1 deficient (PD-$1^{-/-}$) P14 transgenic mouse model in conjunction with chronic and acute lymphocytic choriomeningitis virus (LCMV) infection to investigate the role of PD-1 in regulating antigen-specific CD8 T cell response. During chronic LCMV infection, PD-$1^{-/-}$ P14 CD8 T cells peaked at a higher frequency. However, an exaggerated contraction followed this increase and resulted in an overall decrease of antigen-specific CD8 T cell number. In contrast to the dramatic kinetics change in chronic infection, PD-$1^{-/-}$ antigen-specific CD8 T cells in an acute LCMV infection behaved similarly compared to wild type controls. However, a gradual loss in PD-$1^{-/-}$ CD8 T cells was observed during later memory phase, revealing a defect in memory CD8 T cell differentiation and survival within the PD-$1^{-/-}$ antigen-specific CD8 T cell population.

My work has examined antigen-specific CD8 T cell response in the absence of PD-1 signaling under physiologic conditions. Novel findings in cell number kinetics demonstrated that complete loss of PD-1-mediated inhibition is detrimental to T cell survival in both acute and chronic LCMV infection, with an earlier and more severe effect seen in chronic LCMV infection. PD-1 signaling has been a key target for artificial interference to optimize immune response. My studies provide important information for numerous PD-1 based strategies in vaccine design, chronic disease treatment and tumor immunotherapy.

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Introduction

Programmed Death-1 (PD-1; also known as CD279), a cell surface transmembrane protein of the CD28 superfamily, was first discovered on a T cell hybridoma undergoing apoptosis[1]. The inhibitory role of PD-1 in regulating immune response was later suggested when PD-1 deficient (PD-$1^{-/-}$) mice developed accelerated spontaneous autoimmunity[2,3]. In recent years, PD-1 has demonstrated a key role in regulating T cell response, implicating PD-1 as an attractive target for therapeutic intervention. Numerous efforts have been made to optimize T cell response by manipulating PD-1 inhibitory signaling for applications in anti-tumor and anti-microbial treatment. Understanding PD-1 mediated inhibition of CD8 T cell response will not only greatly enhance our knowledge in the fundamental process of T cell response regulation, but also provide targets for therapeutic research. The goal of this study is to investigate the role of PD-1 signaling in regulating CD8 T cell response under physiologic conditions. Based on previous data supporting PD-1 as an inhibitory regulator, I hypothesized that lack of PD-1 signaling in CD8 T cells would lead to an increased number of antigen-specific effector and memory CD8 T cells, resulting in better control of infection. I tested this hypothesis by examining PD-$1^{-/-}$ antigen-specific CD8 T cell response in both acute and chronic lymphocytic choriomeningitis virus (LCMV) infection using a chimeric mouse model.

To provide background for this thesis, I will discuss PD-1 and its ligands in the context of immune response in the first part of my introduction. Then I will discuss memory CD8 T cells in viral infection in the second part of my introduction.

PD-1 and its ligands

Structure and expression profile

PD-1 is a type I transmembrane protein of 288 amino acids (aa) and a member of the CD28 family. PD-1 exists as monomers both in solution and on the cell surface, in contrast to other CD28 family members that are disulfide-linked homodimers[4]. It consists of 4 structural domains: an extracellular immunoglobulin (Ig) superfamily domain, a ~20 aa stalk, a transmembrane domain, and an intracellular domain of approximately 95 aa[4]. The intracellular domain of PD-1 is known to harbor 2 functional motifs,: an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibitory motif (ITIM) [5,6]. PD-1 is encoded by the *Pdcd-1* gene, in both humans and mice. The murine and human PD-1 orthologs share ~69% identity. The gene sequence contains 5 exons in both humans and mice but differs in its chromosome location: chromosome 1 in mice and chromosome 2 in humans.

PD-1 has two known ligands, programmed death-ligand 1 (PD-L1) and a closely related molecule, PD-L2; both are members of the B7 family. PD-L1, also known as B7-H1 or CD274, is a 290 aa type I transmembrane protein. The intracellular tail of PD-L1 is short, --about 30 aa, and has no confirmed functions, yet it is highly conserved across all reported species. The gene that encodes PD-L1 is *Cd279, which is* located on chromosome 19 in mouse and chromosome 9 in humans. PD-L1 can also interact with B7-1 with an affinity of ~1.7 μM. The interaction between B7-1 with CD28 occurs at 4 μM affinity, while the PD-1: PD-L1 affinity is 0.5 μM[7]. *In vitro* stimulation assay using antibody coated beads demonstrated that PD-L1 can exert an inhibitory effect on T cells

either through B7-1 or PD-1 ligation. Data from studies with PD-L1$^{-/-}$ mice suggest that PD-L1 downregulates cytokine production on T cells[8].

PD-L2, also known as B7-DC or CD273, is a type I transmembrane protein encoded by the *Pdcd1lg2* gene, which is adjacent to the *Cd274* gene. The protein is comprised of 273 aa in humans and 247 aa in mice. In humans, exons 6 and 7 contain coding sequence for a cytoplasmic tail of 30 aa while in mice the length of the cytoplasmic tail is 4 aa due to a stop codon in exon 5.

PD-1 can be expressed on T cells, B cells, natural killer T cell, activated monocytes and dendritic cells (DCs) upon activation[9-11]. PD-1 expression is induced at least partially through binding with NFATc1 upon T cell activation[12]. PD-1 expression on the cell surface can be detected as early as 24 h post stimulation and previous studies have shown PD-1 functions within hours after T cell activation[13]. PD-1 is not expressed on resting T cells and there is no evidence for PD-1 function in the absence of T cell signaling.

The two PD-1 ligands differ in their expression patterns. PD-L1 is constitutively expressed on mouse T and B cells, DCs, macrophages, mesenchymal stem cells[14], and bone marrow-derived mast cells[15]. PD-L1 expression can be further upregulated upon activation. PD-L2 has a more restricted expression profile and is inducibly expressed on DCs, macrophages, and bone-marrow derived mast cells[15]. PD-L2 is also expressed on some peritoneal B1 B cells but not on B2 B cells. PD-L1 is also widely expressed on nonhematopoietic cells, including vascular endothelial cells, epithelial cells and sites of

immune privilege. These nonlymphoid sites suggest a role for PD-L1 in regulating self-reactive lymphoid cells and providing protection from local inflammatory responses. Both PD-L1 and PD-L2 upregulation can be induced by interferon-alpha (IFNα), interferon-beta (IFNβ), and interferon-gamma (IFNγ)[16,17]. However, the effect on PD-L2 upregulation is less significant and PD-L2 can also be induced on monocytes and macrophages by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4)[18,19].

PD-1: PD-L pathway in autoimmunity and peripheral tolerance

A role of PD-1: PD-L pathway in autoimmunity was suggested by the phenotype of PD-1 deficient mice. These animals showed accelerated autoimmunity when crossed to autoimmune-prone backgrounds, and C57BL/6 mice with *Pdcd*-1$^{-/-}$ genotype spontaneously developed mild glomerulonephritis with low frequency when aged[2]. These data suggest a critical role of PD-1 in controlling self-reactive T cell response and maintaining tolerance.

Further studies with animal autoimmunity models confirmed the role of PD-1 in regulating autoimmunity and tolerance. When PD-L1 was upregulated on pancreatic islet cells from non-obese diabetic (NOD) mice, the disruption of PD-1: PD-L1 pathway resulted in accelerated insulitis and an increase in proinflammatory cytokine production[20-22]. Similar acceleration in diabetes was also seen in models of tolerance with PD-1 or PD-L1 blockade[23]. Recent studies with adoptive transfer of islet-antigen specific T cells showed PD-L1 blockade increased activation of TCR downstream signaling, and

prolonged T cell-DC interaction[24]. However, blockade of PD-L2 in either NOD or adoptive transfer models showed no changes in diabetes progression[23,25]. Bone marrow chimera experiments showed that PD-L1 expression on islet cells inhibited self-reactive T cell in tissues[22,26]. Also, PD-L1 expression on islet cells provided protection from graft rejection in islet transplantation[22,27]. Furthermore, PD-1: PD-L1 interaction was found to be necessary to induce and maintain CD4 T cell tolerance in an antigen-specific therapy model of NOD[28]. Taken together, these data demonstrate that PD-1: PD-L pathway regulates the initiation and progression of autoimmune diabetes in NOD mice.

PD-1 pathway also has been shown to regulate self-reactive T cells in a mouse encephalomyelitis (EAE) model for human multiple sclerosis (MS). Both PD-1 and its ligands are expressed on cellular infiltrates within the meninges during ongoing EAE in mice of C57BL/6 background[29]. Neutralizing antibody treatment with anti-PD-1 or anti-PD-L2 during induction of EAE accelerated disease onset and severity. Additionally, CNS inflammatory infiltrates, myelin oligodendrocyte glycoprotein (MOG)-reactive T cells and antibodies were all increased[30]. Further studies with blocking antibodies confirmed that PD-1: PD-L1 pathway was the major contributor to the severity of disease[8,31]. In a bone marrow chimera experiment with lymphocytic choriomeningitis virus (LCMV) infection, priming instead of tolerance was observed in the absence of PD-1, supporting the role of PD-1 in maintaining peripheral tolerance[32].

In humans, polymorphism of PDCD1 has been correlated with multiple autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and

multiple sclerosis (MS)[33]. Recent studies also suggested an association between *Pdcd1lg2* polymorphism and SLE. Most of these polymorphisms are located in intronic regions and may interact with transcription factors. One example is G7146A, an intronic single nucleotide polymorphism, found in some German patients with MS. G7146A is correlated with alleviated PD-1-mediated inhibition of IFNγ production[34]. This polymorphism is located at the binding site for hematopoietic transcription factor Runx1 and may alter PD-1 mRNA stability or expression level[35]. Interestingly, the effect of PDCD1 polymorphism varies among ethnic groups, similar to the effect of PD-1 deficiency seen in mice of different genetic backgrounds.

PD-1: PD-L pathway in immunopathology

PD-1 appears to be a key factor in regulating the balance between effective immune response and immunopathology. Enhanced inflammation and severe tissue damage has been reported in autoimmune models where PD-1: PD-L pathways are disrupted[20,24,36,37]. *Pdcd-1*$^{-/-}$ mice with acute adenovirus infection showed enhanced protective immunity and better control of virus infection, but also suffered greater tissue damage in the liver[38]. *CD274*$^{-/-}$ mice with chronic LCMV infection died from severe immunopathology while some mice with acute infection exhibited normal immune response and controlled infection[39]. Recent studies on PD-L1 expression in vascular endothelial cells suggested that PD-L1 down regulates local immune response by interacting with PD-1 on T cells that comes into contact with the vessel wall. *In vitro* blockade of PD-L1 on vascular endothelial cells showed increased level of cytokine production and cytolytic activity on CD8 T cells[40], further supporting this hypothesis.

PD-1: PD-L signaling pathway in T cells

The PD-1: PD-L pathway exerts its effect through inhibition of T cell receptor signaling (Fig.1). Close proximity to TCR is required for PD-1-mediated inhibition. During T cell-APC (antigen presenting cell) interaction, PD-1 is redistributed from an uniform cell surface expression to immune synapses[41]. PD-1 is phosphorylated at its functional motifs, ITIM and ITSM, upon ligation. The phosphorylated motifs serve as docking sites and recruit phosphateses into immune synapses, which subsequently lead to downregulation of TCR signaling through dephosphorylation of signaling intermediates[5]. Previous studies showed that a single mutation disrupting the phosphorylation site within ITSM led to loss of PD-1 mediated inhibition, suggesting a major role for ITSM[6,13]. Two phosphatases, SH-2 domain containing phosphatase 1 (SHP-1) and 2 (SHP-2) can both be recruited by binding to PD-1, although SHP-1 recruitment occurs at a lesser extent.

PD-1 engagement inhibits CD28-mediated activation of PI3K and downstream signaling of Akt. CTLA-4 also inhibits Akt activation but has no effect on PI3K, indicating these two molecules have synergistic effects but functions through distinct pathways. PD-1 also inhibits the TCR induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ[5].

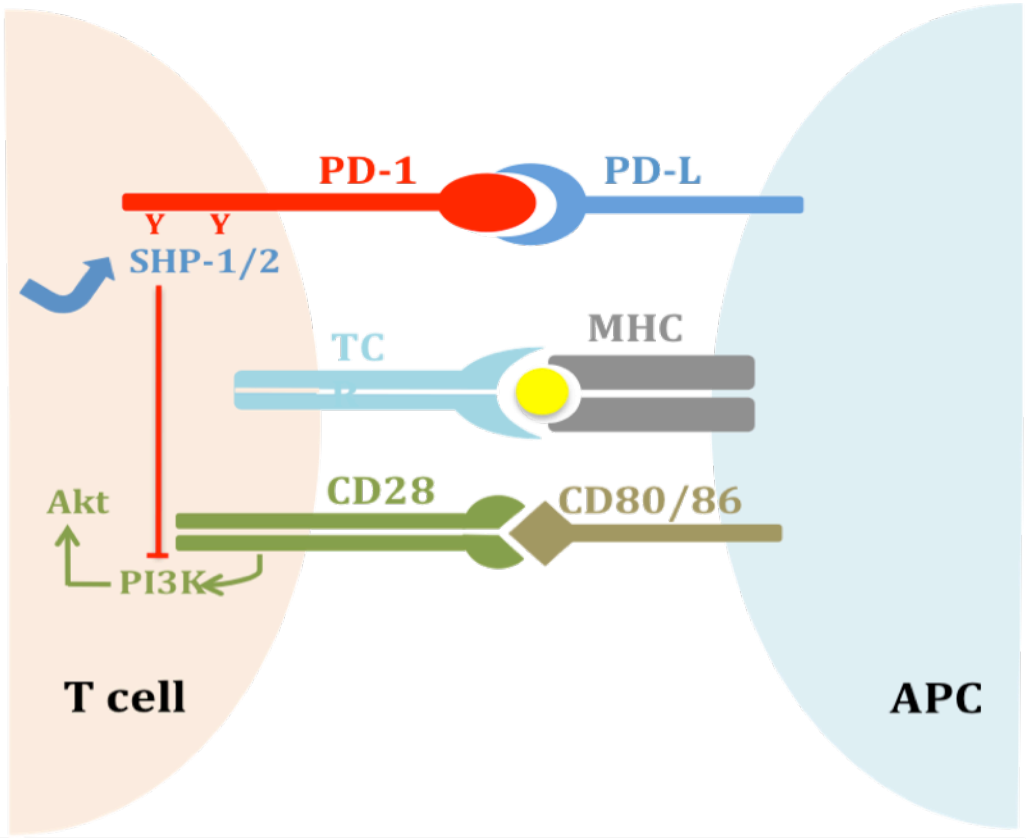

Figure 1. The PD-1: PD-L signaling. PD-1 exerts its effect through inhibition of TCR downstream signaling. Upon interactions with PD-L on antigen-presenting cells, PD-1 recruits SHP-1/2 and inhibits Akt activity through PI3K.

The importance of the PD-1: PD-L pathway in chronic infection was first revealed by studies in the LCMV model. PD-1 is rapidly upregulated in CD8 T cells in both acute and chronic LCMV infections. However, sustained high level of PD-1 expression was only seen in chronic infection with persistent high viral load and functionally impaired (exhausted) CD8 T cells. In acute LCMV infection, PD-1 is downregulated by day 8 post infection. *In vivo* administration of either anti-PD-L1 or anti-PD-1 mAb demonstrated partial recovery of exhausted CD8 T cells, increased cyokine production and cytotoxicity, and more importantly, a significant decrease in viral load[39]. Also, PD-1$^{-/-}$ mice with acute adenovirus infection showed greater immune response and better control of infection[38].

Soluble PD-1 administration together with an adenovirus-based vaccine augmented primary and secondary CD8 T cell response[42]. Collectively, these data demonstrated that PD-1 is an inhibitory regulator of T cell response.

Since the initial discovery of PD-1 in chronic LCMV model of infection, there has been great interest and effort to target PD-1 for therapeutic intervention in human infectious diseases. PD-1 expression is also high in HIV[43-46]-, HBV[47]- and HCV[48]-specific T cells. *In vitro* blockade of PD-1 restored proliferation and function of these antigen-specific T cells[43-45,48,49]. Moreover, HIV long-term nonprogressors that develop functional antigen-specific memory CD8 T cells are associated with lower PD-1 expression compared to typical progressors with decreased memory CD8 T cells and increased viral load[50]. CD4 activation in HIV infection is also impaired by PD-1 induced IL-10 production by monocytes[51]. This association between PD-1 expression level and T cell functionality suggests PD-1 to be a potential marker for progression of T cell exhaustion and disease development.

PD-1-mediated inhibition may have evolved to protect host from uncontrolled or sustained inflammation, which causes tissue damage. However, several pathogens have exploited this pathway to establish persistent infection. Comprehensive examination of PD-1: PD-L1 pathway may not only provide us with strategies to control viral infections, but to manipulate the immunosuppressive environment in general, which can have benefits for other diseases treatments such as tumor immunotherapy.

Memory CD8 T cell in viral infection

Memory CD8 T cells are antigen-specific T cells generated during primary infection that persist long after antigen clearance. Fully differentiated memory CD8 T cells are maintained in the absence of antigen at a relatively constant frequency through homeostatic turnover. In humans, long-term persistence (up to 75 years) of memory CD8 T cells post smallpox vaccination provides the hosts with almost life-time protective immunity[52]. Upon secondary challenge, memory CD8 T cells respond more rapidly and elicit an higher magnitude immune response, contributing to faster resolution of infection. Characteristics of memory CD8 T cells such as long persistence after antigen clearance, and faster and stronger recall response make memory CD8 T cell an extremely important component in protective immunity and vaccine design.

Viral infections can be categorized as acute or chronic based on time of antigen clearance. In acute infections, virus is eliminated within a short time period. In chronic infections prolonged antigen presence as well as an immunosuppressive environment are frequently observed, both of which affect differentiation of memory CD 8 T cells. Consequently, CD8 T cell fate in these two types of infections differ significantly from each other.

LCMV infection

Substantial work on CD8 T cell characterization and differentiation has been done with the lymphocytic choriomeningitis virus (LCMV) infection model. LCMV is a murine

ambisense RNA virus that can establish either acute or chronic infections by administration of Armstrong or clone-13 virus strain[53]. One advantage of the LCMV system is that these two virus strains differ in only 2 amino acids[54], which does not affect any known epitopes of LCMV, making it feasible to track CD 8 T cells of the same antigen-specificity in either acute or chronic scenarios of LCMV infection. The Armstrong strain of LCMV causes an acute infection that is resolved within a week of infection. An initial increase of viral load is observed for 3-4 days post inoculation before it starts to decline and virus is eliminated. In contrast, the clone-13 strain establishes a chronic infection and maintains a high viral load for several months.

LCMV receptor is an extracellular matrix protein receptor named α-dystroglycan[55]. A single amino acid change in the viral glycolipid[56] ligand that binds to α-dystroglycan results in a much higher binding affinity (2-3 log difference) and is observed in persistent LCMV infection[55]. This increased receptor-ligand binding affinity most likely leads to preferential infection of splenic DCs in the marginal zone and white pulp of the spleen[56-58]. Also, LCMV clone-13 but not Armstrong can infect most of the hematopoietic progenitors originating from the bone-marrow[59], leading to impaired replenishment of DCs. Splenic CD11+ DC isolated from mice with LCMV clone-13 infection showed significant decrease in expression of MHC molecules as well as co-stimulatory molecules including CD40, CD80 and CD86[59,60]. Cytokine production is also affected in LCMV infection and is involved in establishment of viral persistence. Prolonged production of IFNα/β by immature and mature DCs from the spleen and bone marrow have been observed in clone-13 but not Armstrong infected mice[61-65]. Immunosuppressive cytokine

IL-10, which inhibits antigen-presenting cells and T cells by reducing proinflammatory cytokine production, is also increased in LCMV clone13 infection[66,67]. Importantly, neutralization of IL-10 in mice with LCMV chronic infection restored functions of impaired T cells and led to viral clearance[68]. In general, multiple mechanisms are adopted by LCMV clone-13 strain to alter DC function and maintain an immunosuppressive environment, which eventually led to impaired host adaptive immunity and establishment of persistent infection.

Memory CD8 T cell differentiation during acute infection

Naïve CD8 T cells with antigen receptor specific for a given peptide-MHC (p-MHC) complex are maintained at a low frequency *in vivo*. In mice, the estimated frequency of T cells with a certain p-MHC specificity is ~$1/10^5$ T cells, corresponding to ~10^2 cells/spleen[69,70]. However, during the initial activation phase of an acute infection, naïve CD8 T cells respond to antigen stimulation and start to proliferate rapidly (Fig 2A). In mice, the initial ~10^2 cells/spleen CD8 T cells can reach as many as 10^7 cells/spleen[70]. This massive expansion occurs within a week of infection, indicating that antigen-specific CD8 T cells can divide as fast as once every 6-8 hours[70]. During this expansion phase, CD8 T cells undergo significant changes in function and globally start to express genes necessary for effector CD8 T cell function. During the expansion phase, effector CD8 T cell characteristics start to emerge, such as: upregulation in the production of cytokines IFN-γ and tumor necrosis factor (TNF), secretion of cytotoxic proteins such as granzymes and perforin (Fig 2B)[70-76], and upregulation of certain chemokine receptors and cell adhesion molecules (eg: CCR5) in coordination with decreased levels of lymph

node homing proteins (eg: L-selectin and CCR7) to enable the migration of CD8 T cells from lymphoid to nonlymphoid tissue[76,77]. Except for classic effector molecule levels, the overall expression profile of effector CD8 T cells is also dramatically different than their naïve progenitors. Microarray analysis revealed elevated mRNA levels of genes involved in TCR signaling, actin polymerization, and protein translation[78]. In summary, naïve CD8 T cell undergo rapid proliferation and differentiation upon antigen stimulation, forming a large pool of effector CD8 T cells with distinct killer functions and migration patterns.

Following the peak of expansion, most of the effector CD8 T cells (90-95%) die through apoptosis in the next couple of weeks[73,79]. The surviving subset (5-10%) is not simply a remaining population of previous effector T cells. Instead, this small pool of remaining effector CD8 T cells undergo a series of differentiation events to obtain self-renewal and memory capabilities (Fig 2C). Traditionally, effector CD8 T cells can be divided into two sub-groups according to their distinct fates: memory precursor effector cells (MPECs), which survive and undergo further programming to become long-lived memory cells, and short lived effector cells (SLECs), which die by apoptosis. High expression of IL-7R (CD127) was first identified as a marker for MPEC, and KLRG1 was later discovered as an early marker that can be detected early in infection to indentify the MPEC subset. $KLRG1^{hi}IL\text{-}7R^{lo}$ effector subset (SLECs) has a substantially reduced chance to form memory CD8 T cells compared to $KLRG1^{lo}IL\text{-}7R^{hi}$ cells (MPECs)[80,81]. A gradient of transcription factor T-bet determines the fate between SLEC and MPEC. T-bet expression is induced by IL-12 in a dose-dependent manner; high levels of T-bet lead to SLEC fate while low levels correspond to MPEC fate[80].

The current model of CD8 T cell formation indicates a linear, progressive manner of differentiation (Fig 2A, B, C). Although MPECs can be distinguished early in the effector phase, these cells need to go through further programming to gain memory properties. Memory CD8 T cells lose active cytokine production but retained the potential to produce IFNγ and TNFα along with other granule proteins rapidly upon antigen re-encounter. This fast and strong secondary response is a characteristic of memory CD8 T cells, which is beneficial to the design of effective vaccines. Furthermore, MPECs gradually upregulate IL-2 expression during progression to memory phase[80]. Both IL-7 and IL-15 are required for the antigen-independent homeostasis of memory CD8 T cell[82-87]. The role of these two cytokines differ in that IL-7 is responsible for the initial generation of memory cells while IL-15 appear to be important for the survival of these cells[83,84]. Re-expression of lymphoid homing molecule such as CCR7 and L-selectin enables memory CD8 T cell to redistribute to central lymphoid organs once infection is resolved[88].

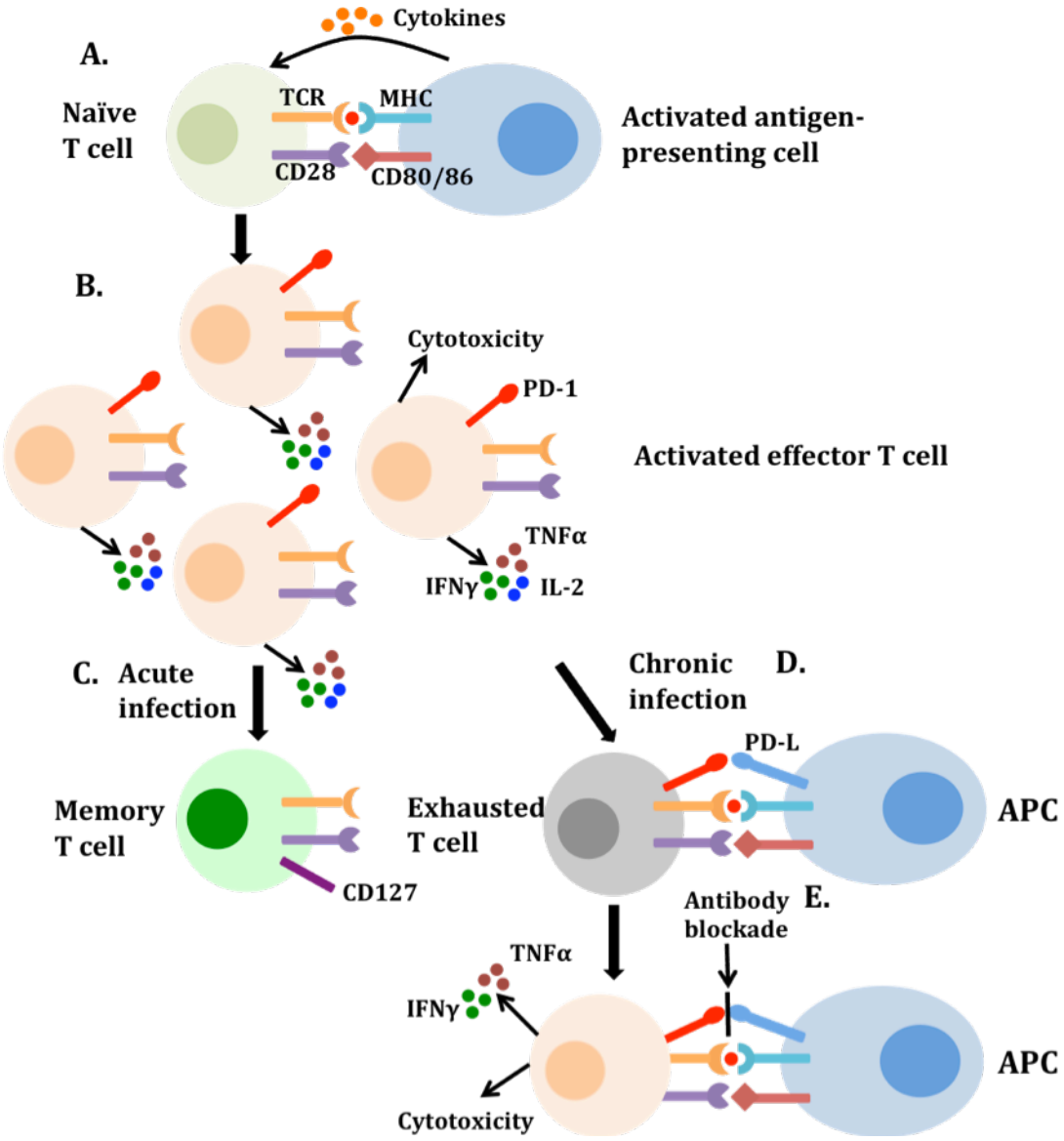

Figure 2. **CD8 T cell response in acute and chronic infection**. Naïve CD8 T cells (A) become activated upon interaction with antigen-presenting cells and start to proliferate into a large pool of effector CD8 T cells (B) producing cytokines and exerting cytotoxicity. In an acute infection where antigen is cleared (C), a small subset (5—10%) of effector CD8 T cells differentiate into memory CD8 T cells that can survive in the absence of antigen for a long period of time. However, in chronic infection (D), CD8 T cells become exhausted, fail to clear the antigen or become memory T cells, and express high levels of PD-1. Antibody blockade of PD-1—PD-L (E) restored the function of exhausted CD8 T cells.

Memory CD8 T cells can be divided into two subsets according to their different expression profile of homing molecules and consequently, different localization patterns. The $CD62L^{lo}CCR7^{lo}$ effector memory T cells (T_{EM}) are enriched in spleen, blood and nonlymphoid tissues[89,90]. In contrast, the $CD62L^{hi}CCR7^{hi}$ central memory T cell (T_{CM}) population is mainly found in lymphoid tissues. The difference in terms of function and fate of these two subsets vary among different infection scenarios and is under heavy investigation. Conversion of $CD62L^{lo}CCR7^{lo}$ to $CD62L^{hi}CCR7^{hi}$ has been observed, and is correlated to increased IL-2 production, greater protective immunity, and self-renewal capacity[88].

CD8 T cells in chronic viral infection

The fate of CD8 T cells in chronic viral infection where antigen persists can be dramatically different from what is seen in acute models (Fig 2D, E). The frequency, phenotype, function, and distribution of CD8 T cells are all under the influence of continuous antigen stimulation. During the effector phase, CD8 T cells can be divided into 3 groups according to their ability to produce cytokines following *ex vivo* antigen restimulation. The most competent subset secretes all 3 cytokines including IFNγ, TNFα, and IL-2. The intermediate group produces both IFNγ and TNFα while the least competent subset secretes only IFNγ. Progressive loss of cytokine production in CD8 T cells occurs during persistent antigen stimulation. In this process IL-2 production appear to be most vulnerable, followed sequentially by TNFα and IFNγ. Partial to complete loss of CD8 T cell functions is positively correlated with antigen load and inversely correlated with help from CD4 T cells. In an extreme case, CD8 T cells can reach a state of

nonresponsiveness to *ex vivo* peptide stimulation and become functionally exhausted[53,91-93].

Functionally impaired (exhausted) CD8 T cells sustained high levels of PD-1 expression (Fig 2D)[39,44,94]. *In vivo* administration of antibody blocking PD-1 signaling showed significant recovery of exhausted CD8 T cells in terms of proliferation, cytokine production, and cytotoxicity, which eventually resulted in a substantial reduction in viral load (Fig 2E)[39].

The immunodominance pattern of antigen-specific CD8 T cells can also be altered significantly in chronic infections. Subdominant T cells with a low strength of response can often overrule the ones found dominant in the acute model, resulting in inverted immunodominance. This phenomenon has been observed in both mouse LCMV and macaque SIV infections[53,93]. Moreover, the tissue distribution of CD8 T cells can be altered in chronic infection; expression of different homing molecules and persistence of virus resulted in the retention of large numbers of antigen-specific CD8 T cells in nonlymphoid tissues[53,95,96].

Materials & Methods

General experimental outline

Chimeric mice were generated by adoptive transfer of Thy1.1 P14 transgenic splenocytes into wild type Thy1.2 C57BL/6 recipients. Recipients were given an 8-12 hr recovery period to allow donor cells to home to lymphoid tissue. Chimeras were then given either LCMV Armstrong or clone-13 infection. Spleen, liver, lung and PBMC were collected for analysis at different time points post infection. Surface Thy1.1 or Thy1.2 expression was used as a marker to distinguish transferred cells from recipient's own cells.

Mice

Wild type Thy1.2/Thy1.2 C57BL/6 mice at age 6-8 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME). P14 mice are homozygous for a transgene that encodes a T-cell receptor specific for LCMV peptide GP_{33-41} presented by the MHC class I molecule H-2D^{b}. PD-1$^{-/-}$ mice lacking exons 2 and 3 were generated in the Sharpe Lab at Harvard[26]. PD-1$^{-/-}$ mice were crossed to P14 transgenic mice and the resulting mice colony was maintained by our lab. Thy1.1 PD-1$^{-/-}$ P14 mice were used as donors in this study.

Donor mice were screened for PD-1$^{-/-}$ genotype by PCR. Genomic DNA was obtained from mouse tail biopsies using a tissue DNA extraction kit (Qiagen).

PD-1 deletion specific primers used in genotyping is stated below:

PD-1 primer 1 5'ACAACACAGGGTAGGCATGTAGCA 3',

PD-1 primer 2 5'TCCTGCCAAACCTTGTAGTCA3'

PD-1 primer 3 5'gctagccaaccagaagtctaa'.

PCR reactions were run at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s, then 72°C for 5 min. Primers 1 and 2 yield a 325 bp product from the knockout allele, while primers 1 and 3 yield a 234bp product from the wild type allele. Thy1.1 P14 mice that were homozygous for the knockout allele were used as donors in this study. All mice were housed at the Emory Animal Facility at Rollins Building of Biomedical Research.

Infections

Chimeric mice were infected at 6-8 weeks of age. Virus stocks of high titer were preserved at -80 C°. Virus stock was thawed in a 37 C° water bath and diluted to working concentrations in pure RPMI before infection. Chronic LCMV infection was established by intravenous injection of 10^6 p.f.u. (plaque forming unit) of clone-13 virus in 500µl of RPMI. Acute infection was established by intraperitoneal injection of 2×10^5 p.f.u. of Armstrong virus in 500 µl of RPMI.

Generation of chimeric mice

Naïve female Thy1.1 PD-$1^{-/-}$ P14 mice were sacrificed and their spleen was harvested. Single cell suspension of splenotyes was prepared as described below. Cells were then counted and stained for CD8, CD44, and LCMV-GP33 tetramer to determine frequency and calculate transfer cell number. 2,000 LCMV D^bGP_{33-41}-specific CD8 T cells (referred to as P14 CD8 T cells) were transferred to naïve C57BL/6 donors through intravenous injection in 500 µl of RPMI.

Tissue collection and single cell suspension preparation

Spleen

Mice were sacrificed by cervical dislocation under anesthesia. Spleen was harvested and placed in RPMI +2% fetal bovine serum (FBS).

To isolate cells, spleen was forced through a cell strainer placed inside a 50ml conical tube (Falcon) using the barrel of a 5-cc syringe. The cell strainer was then rinsed twice with cold RPMI+2%FBS to recover remaining cells. Cells were then treated with 2ml of ACK lysis buffer for ~1 min at room temperature to lyse red blood cells. Excess amount of cold RPMI+2%FBS was then added to neutralize ACK buffer and terminate lysing reaction. Cells were then pulled down by spinning at 2,000 rpm for 5min. Cell pellets were then disrupted, washed twice and resuspended in RPMI+2% FBS until further analysis.

Liver

Liver was first perfused with 5-10 ml cold PBS through the white duct/vein that runs between the main lobes to avoid interference of PBMCs. Fading of the dark red color of the tissue indicates successful perfusion. Liver is then harvested from the mouse and kept in RPMI +2% FBS.

To isolated cells, liver tissue was forced through a cell strainer placed inside a 50ml conical tube. Tissue chunks were pressed through the strainer using the barrel of a 5-cc syringe. The cell strainer was then rinsed twice with cold RPMI+2% FBS to recover

remaining cells. Cells were then centrifuged at 2,000 rpm for 10 min and resuspend in 6 ml of 44% Percoll (in RPMI) and transferred to a 15 ml conical tube. Four ml of 67% Percoll (in PBS) was underlaid and the conical tube was spun at 2,000 rpm for 20 min at room temperature. After spinning, tissue at the top of the gradient was discarded and lymphocytes at the interface were collected and resuspended in RPMI+2% PBS. Lymphocyte pellet was obtained through additional centrifugation at 2,000 rpm for 10 min. Cells were then washed twice with RPMI + 2% FBS and then resuspended in RPMI+10% FBS until further analysis.

Lung

Lung was perfused with 3-5 ml cold PBS from the right side of the heart, and then harvest and kept in RPMI+2% FBS.

Lungs were cut into small pieces, resuspended in 40 ml HBSS (no calcium) + 1.3 mM EDTA and incubated at 37°C for 30 min on a shaker bed. Pieces of lung tissue were then collected through a cell strainer. Tissue pieces were resuspend in 25 ml of RPMI + 5% FBS + 0.02% (or 150 U/ml) Collagenase ± 10 U/ml DNase I and incubated at 37 °C for 60 min. Collagenase treated lung prep was pressed through a cell strainer placed inside a 50 ml conical tube and centrifuged at 2,000 rpm for 5 min. Cell pellets were then disrupted and resuspend in 6 ml of 44% Percoll (in RPMI) and loaded on top of a layer of 4 ml of 67% Percoll (in PBS). Lymphocyte layer was obtained by spinning at 2,000 rpm for 20 min at room temperature. Fat/insoluble material on the top of the gradient was discarded before the lymphocyte interface was collected. Lymphocytes were then pulled

down by spinning at 2,000 rpm for 10 min, washed twice and resuspended in RPMI+10% FBS until further analysis.

Blood

Approximately 250µl of whole blood was collected into a FACS tube containing 1ml of 4% sodium citrate and 2 ml of RPMI+2% FBS. Blood was then mixed by brief vortex, then loaded on top of 2 ml of Histopaque (Sigma). Tubes were then centrifuged at 2,000 rpm for 20 min at room temperature. Lymphocytes at the interface were collected and pulled down by spinning at 2,000 rpm for 5min. Cell pellets were then washed twice and resuspended in RPMI+2% FBS until further analysis.

Surface & Intracellular staining

Single cell suspension prepared as described above was distributed to a 96-well round bottom plate at a concentration of ~1 million cell/well. Cells were spun down at 2,000 rpm for 1 min and washed twice with FACS buffer (phosphate-buffered saline containing 2% fetal bovine serum and 0,01% sodium azide). Cells were then stained with antibodies at a previously optimized dilution, in a volume of 50 µl. APC-anti-Thy1-1 was used at 1:1000 dilution, while all other antibodies were used at 1:100 dilution. Cells were mixed by pipetting and then incubated on ice for 30 min in the dark. After incubation, cells were spun down at 2,000 rpm for 1 min. Cell pellets were disrupted by pipetting up and down several times and 200µl of FACS buffer was added for washing. For surface staining, washing step was repeated twice, and 75 µl of Cytofix buffer (BD) was added to each well for 10 min in the dark. Cells were then washed twice with FACS buffer and

resuspended in 200 µl of 2% PFA and kept in the dark until analysis. For intracellular staining, cells were washed twice after incubation with surface antibodies and incubated in Cytofix for 20 min. Cells were then washed twice with Perm wash buffer (BD), and stained with 50 µl working solution of intracellular antibodies in Perm wash for 30 min on ice in the dark. FITC-anti-Bcl2 was diluted at 1: 2.5 and PE-anti-granzyme B was diluted at 1: 20. All other intracellular antibodies were diluted at 1:100. After incubation, cells were washed twice with Perm wash buffer and then twice with FACS buffer. Cell pellets were then disrupted and resuspended in 2% PFA until further analysis.

***Ex vivo* stimulation assay**

Splenocytes were re-stimulated *ex vivo* in order to evaluate cytokine production capacity. Splenocytes were plated in a 96-well flat-bottomed plate (Costar, Cambridge, MA) at a concentration of 10^6 cells/well in a volume of 200 µl complete medium supplemented with 1 µl/ml Brefeldin A (Golgistop, Pharmingen), either with or without 0.1 µg/ml peptides. The plate were then cultured at 37 C° with 5% CO_2 for 5 hr. After incubation, cells were transferred to round-bottomed plates and stained for surface markers and then intracellular cytokines as described above.

Flow Cytometry

200 µl of single cell suspension preserved in 2% PFA was diluted with 200 µl FACS buffer. Samples were acquired on FACSCanto flow cytometor (BD Biosciences) at a speed of 2 µl per second for 3 min. Flow cytometry data were analyzed by FlowJo software (Tree Star)

Statistic Analysis

Prism Software (GraphPad Software) was used for statistical analyses. Normally distributed data were analyzed with the Student's test. Asterisks indicate the p-values that are statistically significant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

Results

Overview

This study utilized the PD-$1^{-/-}$ P14 transgenic mouse along with LCMV infection model. The rationale of this experimental design is to allow tracking of CD8 T cells with the same antigen specificity in both acute and chronic infection. The P14 transgenic T cell receptor specifically recognizes the LCMV GP_{33-41} epitope. Both acute and chronic infection can be achieved by administration of either the LCMV Armstrong or clone-13 strain virus. Importantly, these two strains differ in only 2 amino acids and this change does not affect any known LCMV epitopes. GP33-specific CD8 T cells derived from both transferred P14 TCR transgenic cells and the recipient's own naïve T cell reservoir were detected by tetramer staining. LCMV acute infection, elicited by Armstrong virus, reaches the peak of CD8 response around eight days post-infection and viruses are cleared shortly after. Normally, functional memory CD8 T cells are generated and maintained. However, clone-13 virus establishes a long-term persistence of high viral load that can last for several months, and CD8 T cells suffer a progressive exhaustion and fail to differentiate into the memory state. GP33-specific CD8 T cell response in both infection models is examined and will be discussed below.

PD-$1^{-/-}$ P14 CD8 T cells in chronic LCMV infection

PD-1 is a known inhibitory molecule of TCR signaling and has been shown to play a central role in regulation of tolerance and immunopathology. Complete loss of PD-1 inhibition in mice can lead to severe tissue damage/death, caused by uncontrolled inflammation. In order to examine CD8 T cells lacking in PD-1 inhibition under

physiological conditions, chimeric mice harboring either wild type (served as a control) P14 or PD-$1^{-/-}$ P14 CD8 T cells were generated (Fig 3A). Thy1.1 Donor mice were sacrificed and P14 cell frequency/number was counted and calculated. 2,000 donor cells were transferred into naïve female Thy1.2 B6 recipients at 6-8 weeks of age. The number of cells transferred was determined given that approximately ~10% of the 2,000 transferred cells (100~200) would migrate to the spleen, resulting in a frequency close to endogenous levels of T cells with the same antigen-specificity. Endogenous GP33-specific CD8 T cell subsets (always wild type in terms of PD-1 genotype since they are from the wild type recipients) was detected by tetramer staining and served as an elegant internal control for individual variables among different recipient mice. Chimeras were rested overnight to allow time for donor lymphocyte homing to the recipient's lymphoid tissue. Standard Armstrong or clone-13 infection was given to mice the next morning following adoptive transfer. Mice were later euthanized to collect spleen/liver/lung samples, or maintained alive for longitudinal bleeding at different time points. Various assays were carried out for examination of CD8 T cell phenotypes and functions. Flow cytometry was conducted to acquire data. Analysis was performed with gating strategy identical or similar to Figure 3C. Each group of mice contained at least 3 animals and 3+ independent experiments were carried out. Results obtained from the chronic infection model are summarized below.

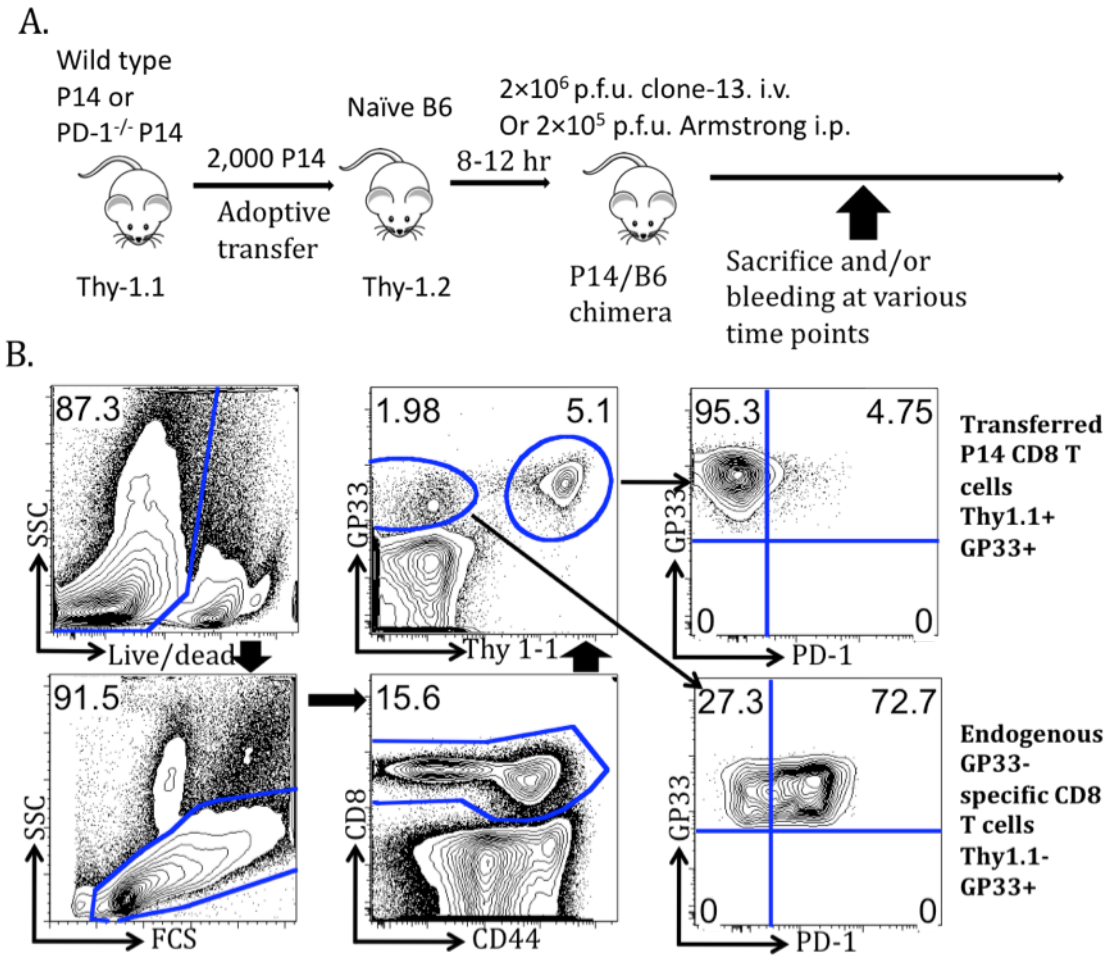

Figure 3. A. General experimental design. Thy1.1 wild type or PD-1$^{-/-}$ P14 transgenic mice were sacrificed and splenocytes were transferred to naive B6 recipients through intravenous injection. Chimeras were then given a 8-12 hr rest before infection to allow homing of donor cells to lymphoid tissue. 2×10^6 p.f.u. clone-13 virus were given intravenously to establish a chronic infection. Acute LCMV infection was elicited by intraperitoneal injection of 2×10^5 p.f.u. Armstrong virus. Different tissues were harvested at various time points post infection for analysis. **B. Gating strategy.** Gating was done in the following or similar order: Live cells → lymphocytes → total CD8 T cells → endogenous vs transferred P14 → functional and phenotypic analysis. Numbers on graph indicates the portion of cells within the gate(s)

PD-1$^{-/-}$ P14 CD8 T cells showed an exaggerated expansion in LCMV clone-13 infection

Chimeric mice, generated as described, were given standard LCMV clone-13 infection. Spleen, liver, lung and blood were collected at 8 days post-infection for examination of CD8 T cells.

The most surprising finding was the enhanced expansion of PD-1$^{-/-}$ CD8 T cells. Tetramer staining allied with the Thy1.1 surface marker revealed a significant increase in cell frequency at day 8 post infection in the spleen (Fig 4A, C), while endogenous GP33-specific CD8 T cell frequency between wild type and PD-1$^{-/-}$ groups maintained similar (Fig 4A, C,). The total number of splenocyte (data not shown), total CD8 T cell number and frequency were maintained as largely similar (Fig 4B), resulting in a final 5~10 fold increase in absolute PD-1$^{-/-}$ P14 cell number in spleen, as compared to wild type, transferred P14 cells (Fig 4C). A similar trend was observed for cells isolated from liver and PBMC.

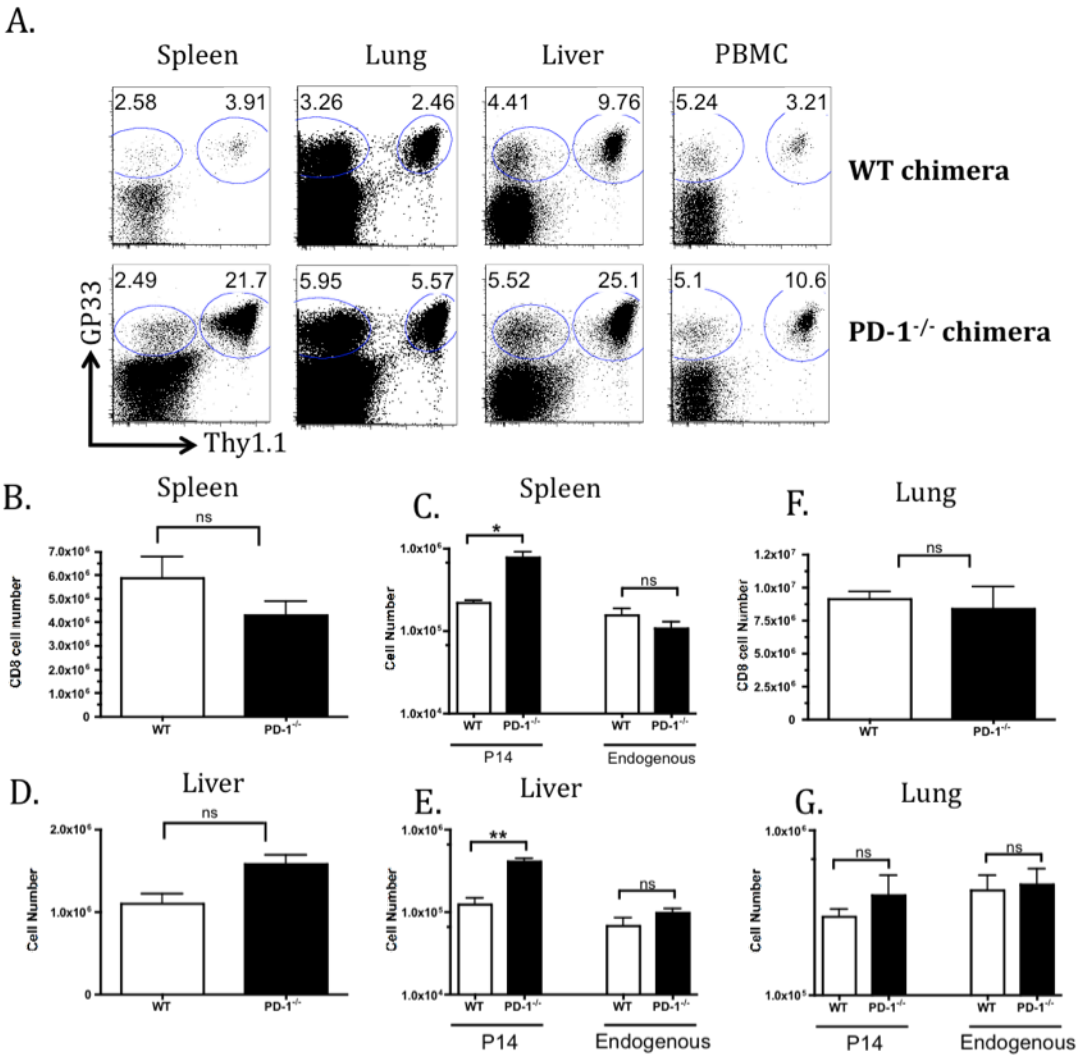

Figure 4. PD-1$^{-/-}$ P14 CD8 T cells showed an increase in frequency and absolute cell number at day 8 post clone-13 infection. A. P14 CD8 T cell frequency across different tissues. Upper panel shows cells isolated from control chimeras made with wild type P14 donor cells. Lower panel shows cells isolated from chimeras made with PD-1$^{-/-}$ P14 donor cells. P14 CD8 T cells are distinguished as the GP33+Thy1.1+ population. GP33+Thy1.1- population represents endogenous LCMV-GP33 specific CD8 T cells derived from recipient's naïve T cell reservoir. Gated on total CD8 T cells. **B, D, F.** Total CD8 T cell number from either WT P14 chimeras or PD-1$^{-/-}$ P14 chimeras. **C, E, G.** P14 and endogenous GP33-specfic CD8 T cell number isolated from either WT or PD-1$^{-/-}$ chimeras. **H.** Comparison of P14 and endogenous GP33-specfic CD8 T cell number/10^6 total cell number for PBMC. *: p<0.05; **:p<0.01; ***: p<0.001

Phenotype characterization was performed by staining a series of classic CD8 T cell surface markers including CD127, KLRG1, and CD62L. CD127 and CD62L (L-selectin) are expressed at high levels on naïve CD8 T cells and are rapidly down-regulated upon antigen stimulation. KLRG1 is up-regulated after activation and down-regulated again on memory precursors. Lower level of KLRG1 in effector CD8 T cells along with high CD127 expression is an important indicator of memory precursor fate while $KLRG1^{hi}CD127^{lo}$ population represents short-lived effector cells that die through apoptosis after viral clearance. CD62L is a lymph node homing molecule and is down-regulated on activated CD8 T cells to allow migration to non-lymphoid tissue during infection. CD62L is also an important marker for central memory CD8 T cells (T_{CM}). In contrast to the striking differences we found in terms of cell frequency and number, no significant changes in phenotypic markers were discovered (Fig 5A), indicating that none of these surface proteins were under direct surveillance of PD-1 signaling.

Abrogation of PD-1 signaling has been shown to improve CD8 T cell functions. Cytotoxicity of CD8 T cells in the absence of PD-1 signaling was evaluated by staining of cytolytic protein granzyme B. To measure the granzyme B producing activity, whole splenocytes were cultured at 37 C° +5% CO_2 with or without GP33 peptide re-stimulation. Brefeldin A was added to the culture media to block secretion of protein to the environment. Granzyme B expression levels were slightly increased in $PD\text{-}1^{-/-}$ P14 CD8 T cells, suggesting a greater cytotoxicity (Fig 5B).

Expression of anti-apoptotic protein Bcl-2 was also examined, to estimate the survival potential of CD8 T cells. However, PD-$1^{-/-}$ P14 cells demonstrated similar level of Bcl-2 expression compared to both wild type P14 and endogenous GP33-specific CD8 T cells (Fig 5B).

Ex vivo stimulation with GP33 peptide was performed as described above to examine cytokine producing ability of P14 cells. PD-$1^{-/-}$ P14 CD8 T cells isolated from spleen showed a slight increase in the frequency of IFNγ producing cells (Fig 5C). IL-2 expression was low on day 8 post infection and no changes in terms of number of IL-2 producing cell or secretion level on an individual basis was observed (Fig 5C). However, a decrease in TNFα production was detected. Frequency of IFNγ+TNFα+ double producing cell is lower in PD-1 deficient P14 population (Fig 5C). Splenocytes cultured without peptide stimulation showed no production of cytokines.

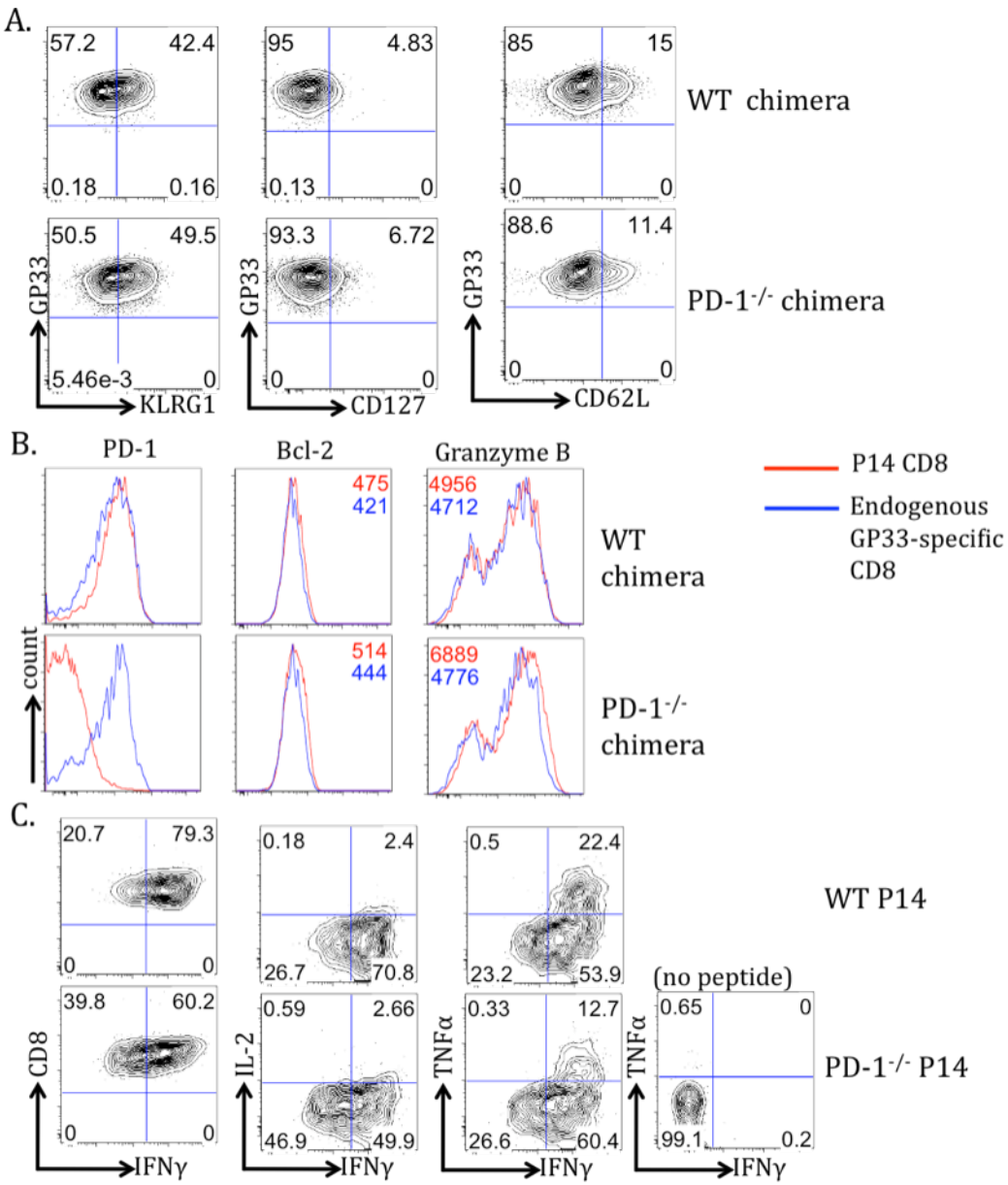

Figure 5. Phenotypic and functional analysis of spleen P14 CD8 T cells at day 8 post clone-13 infection. **A.** Comparison of cell surface marker expression on P14 CD8 T cells isolated from either wild type or PD-1$^{-/-}$ P14 chimeras. **B.** Left: Histogram confirms PD-1 surface expression deficiency in PD-1$^{-/-}$ P14 cells. Right: Histograms of cytolytic protein survival protein expression. Graphs plotted on either P14 CD8 T cells (Red line) or endogenous GP-33 specific CD8 (Blue line) **C.** Intracellular cytokine staining of splenocytes isolated from day 8 infected animal after 5 hr stimulation with GP_{33-41} peptide. Gated on P14 cells.

Gp33-specific CD8 T cells recovered from liver, lung and blood showed similar patterns in expression profile of the phenotypic markers compared to splenocytes (Fig 6A, B, C). No significant changes in expression of KLRG-1, CD127 or CD62L were detected at day

8 post clone-13 infection. Deficiency in PD-1 expression was demonstrated in all tissue (Fig 6 left columns.).

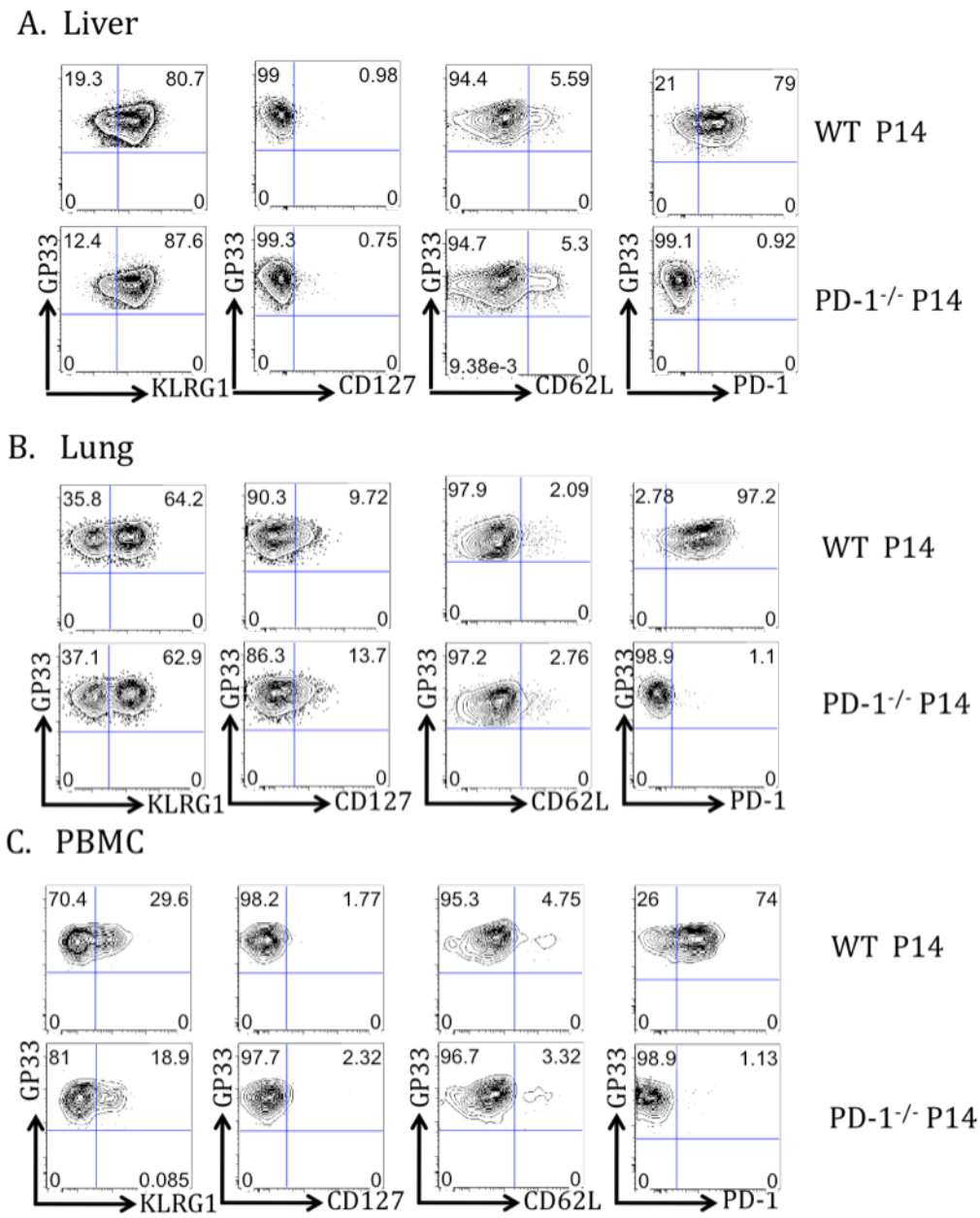

Figure 6. Phenotypic analysis of P14 CD8 T cells from liver, lung and PBMC.
A. liver; B. lung; C. PBMC. Graphs show expression level of KLRG1, CD127, CD62L and PD-1 in P14 CD8 T cells isolated from either wild type or PD-1$^{-/-}$ chimeras. Graphs are representative of groups of 3+ animals. Upper panels show wild type P14 cells; lower panels show PD-1$^{-/-}$ P14 cells. Gated on GP33+Thy1.1+ P14 cells.

PD-1$^{-/-}$ P14 CD8 T cells showed enhanced contraction in LCMV clone-13 infection

Similar experiments as described above were performed on day 15 post clone-13 infection for examination of CD8 T cells during contraction phase.

Strikingly, PD-1$^{-/-}$ P14 cells were found at a much lower frequency compared to wild type P14 and endogenous GP33-specific CD8 T cells (Fig 7A). This phenomenon was consistent across all tissues samples, regardless of the different magnitude of increase observed previously at day 8. No significant change was found in terms of total CD8 T cell number (Fig 7B, D, F) or total lymphocyte number (data not shown). These results added to a final decrease in absolute number of PD-1$^{-/-}$ P14 CD8 T cells. Despite this drastic change in cell frequency/number, another interesting finding was that in PD-1$^{-/-}$ P14 chimeras, endogenous GP33-specific CD8 T cells (PD-1 wild type) derived from each recipient's own naïve T cell reservoir also showed a significant decrease of cell frequency and number (Fig 7C, E G, H). Since both subsets of CD8 T cells recognized the GP33 epitope, whether the decrease was due to this specific epitope we were tracking, or a global effect in the environment harboring PD-1$^{-/-}$ P14 donor cells became an interesting and valued question. Subsequent replica experiments included GP276 tetramer staining and revealed a similar decrease of GP276-specific CD8 T cells at day 15 (data not shown), supporting the view that the decrease in endogenous cells was caused by the environment in PD-1$^{-/-}$ P14 chimeric mice.

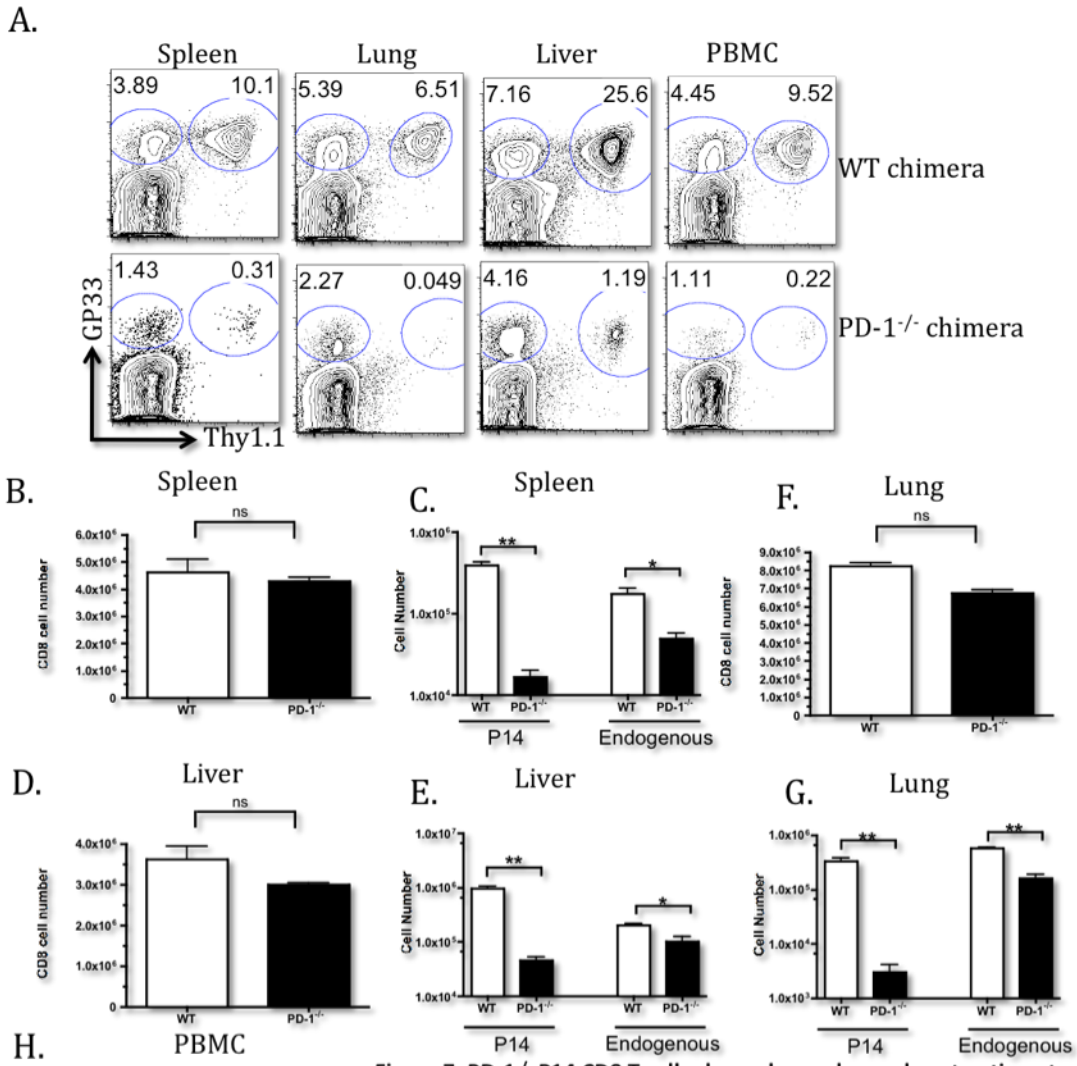

Figure 7. PD-1$^{-/-}$ P14 CD8 T cells showed an enhanced contraction at day 15 post clone-13 infection.

A. P14 CD8 T cell frequency across different tissues. Upper panel shows cells isolated from control chimeras made with wild type P14 donor cells. Lower panel shows cells isolated from chimeras made with PD-1$^{-/-}$ P14 donor cells. Transferred P14 CD8 T cell subset is distinguished as the GP33+Thy1.1+ population. GP33+Thy1.1- population represents endogenous LCMV-GP33 specific CD8 T cells derived from recipient naïve T cell reservoir. **B, D, F.** Total CD8 T cell number obtained from either WT P14 chimeras or PD-1$^{-/-}$ P14 chimeras. **C, E, G.**P14 and endogenous GP33-specfic CD8 T cell number. **H.** P14 and endogenous GP33-specfic CD8 T cell number per 10^6 total cell number for PBMC. *: $p<0.05$; **:$p<0.01$; ***: $p<0.001$

PD-1$^{-/-}$ P14 CD8 T cell number remained low in chronic phase of LCMV clone-13 infection

As shown in Figure 6A, frequency of PD-1$^{-/-}$ P14 CD8 T cell maintained lower compared wild type P14 cells after day 15 post infection. By 60 days post infection, the frequency of PD-1$^{-/-}$ P14 CD8 T cell was less than 0.1% of total CD8 in both spleen and blood. Total lymphocyte number (data not shown) and CD8 T cell number (Fig 8C) showed no significant changes between wild type and PD-1$^{-/-}$ P14 chimeras. Absolute cell numbers of PD-1$^{-/-}$ P14 CD8 T cell in spleen and blood were consistent with the decrease in frequency (Fig 8D, E. Left bars). At this time point, no significant changes in endogenous GP33-specific CD8 T cells were seen in either spleen or blood (Fig 8D, E. Right bars.).

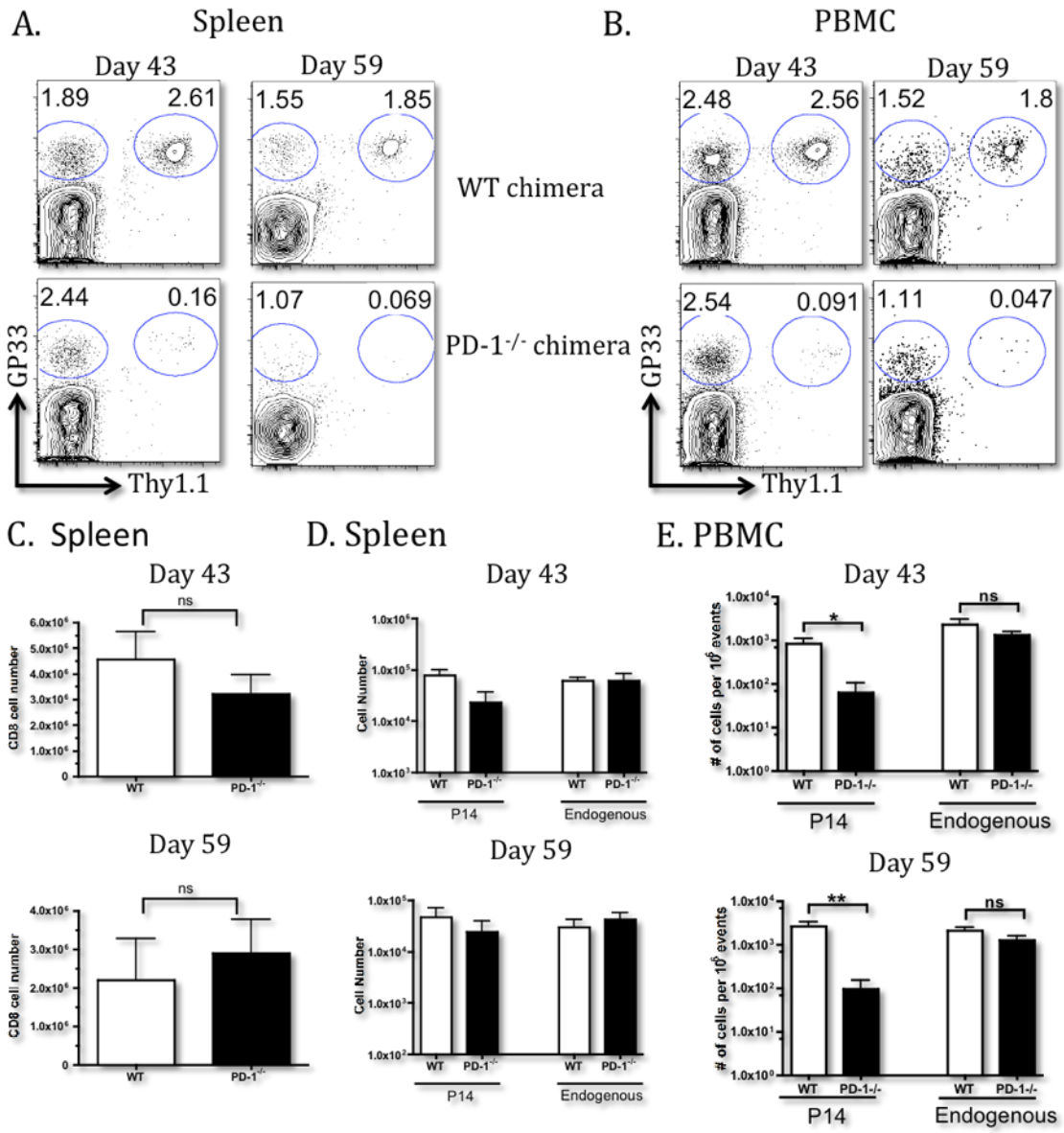

Figure 8. Continuous loss of PD-1$^{-/-}$ antigen-specific CD8 T cells during late clone-13 infection in spleen and blood. A & B. P14 CD8 T cell frequency in spleen and blood. Upper panel shows CD8 T cells isolated from control chimeras made with wild type P14 donor cells. Lower panel shows CD8 T cells isolated from chimeras made with PD-1$^{-/-}$ P14 donor cells. Transferred P14 CD8 T cell subset is distinguished as the GP33+Thy1.1+ population. GP33+Thy1.1- population represents endogenous LCMV-GP33 specific CD8 T cells derived from recipient naïve T cell reservoir **C.** Spleen total CD8 T cell numbers in wild type and PD-1$^{-/-}$ P14 chimeras. D. Comparison of both spleen P14 (left) and endogenous GP33-specific CD8 T cell (right) numbers between wild type and PD-1$^{-/-}$ chimeras. **E.** Comparison of P14 and endogenous GP33-specfic CD8 T cell number/10^6 total cell for PBMC. Left bars show data collected from wild type P14 chimeras. Right bars show data collected from PD-1$^{-/-}$ P14 chimeras. *: p<0.05; **:p<0.01; ***: p<0.001

PD-1$^{-/-}$ P14 CD8 T cell kinetics from matched longitudinal analysis

Blood was drawn from separate groups of chimeras to obtain longitudinal data for matched analysis. Two groups of bleeding mice were set up: wild type P14/B6 chimeras and PD-1$^{-/-}$ P14/B6 chimeras generated as described before with 2×10^6 p.f.u. clone-13 intravenous infection. Up to 250 μl of whole blood was drawn from each mouse at each time point with minimal time interval of 72 hr.

A summary of the longitudinal data is plotted by mean of absolute number with error bars to indicate SEM (Fig 9A). Individual kinetics of each mouse were also shown (Fig 9B). Blue line indicates that cells were isolated from wild type P14 chimeras while red line represents cells from PD-1$^{-/-}$ P14 chimeras. Longitudinal data in PBMC confirmed that an initial increase in cell number seen at day 8 was followed by a sudden, severe contraction. Data obtained from later time points also revealed a continuous decline in PD-1$^{-/-}$ P14 CD8 T cell number, restricting this subset at an increasingly low frequency compared to wild type P14 cells.

Summary of data from chronic LCMV infection

Transferred PD-1$^{-/-}$ P14 CD8 T cells reach a significantly higher frequency at day 8 post clone-13 infection, as compared to wild type P14 cells. However, this increased population of antigen-specific CD8 T cells was not maintained but underwent more drastic contraction, ending at a lower frequency compared to wild type P14 cells at day 15 post infection. This lower frequency/number of CD8 T cells was maintained in chronic phase of clone-13 infection. A continuous decline of cell number in this PD-1$^{-/-}$

population was seen in PBMC, and caused an increasingly low cell frequency. The effector PD-1$^{-/-}$ P14 cells showed slight increase in terms of cytotoxicity and interferon-gamma production at day 8 post infection. Frequency of tumor necrosis factor-alpha producing cells was also found decreased within the PD-1 deficient population. However, no classic surface marker of CD8 (KLRG1, CD127, CD62L) examined showed significant/consistent changes. A decrease of endogenous LCMV-specific CD8 T cell frequency seen in PD-1$^{-/-}$ P14 chimeras at day 15 suggested a role of environment in the severe decrease of activated CD8 T cells.

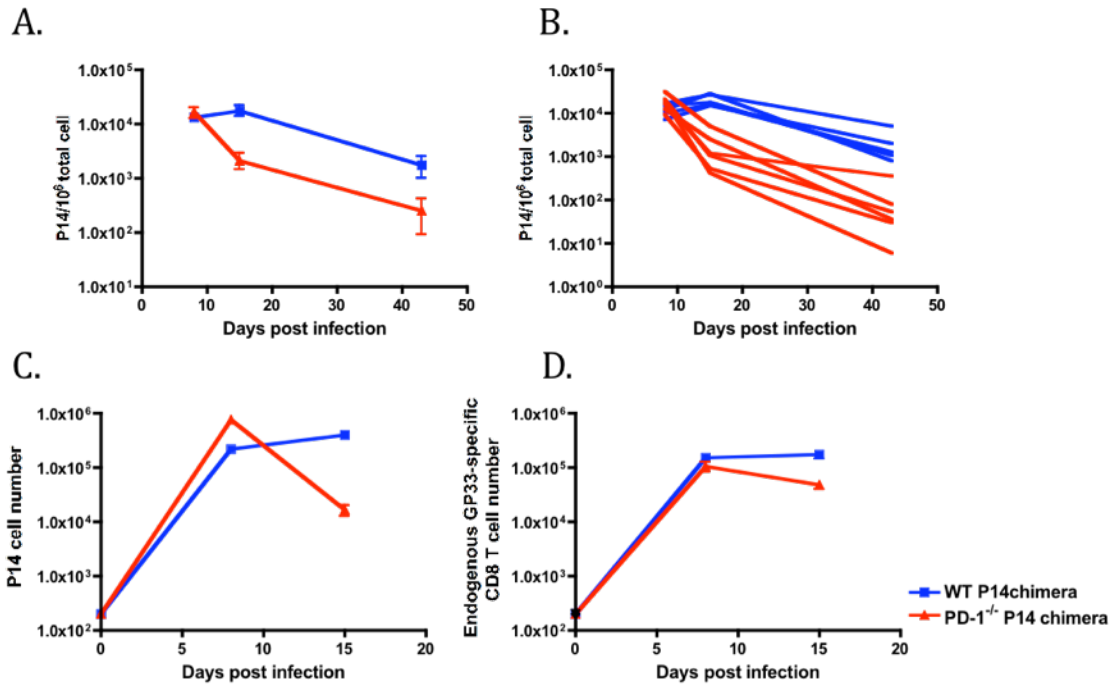

Figure 9. Kinetics of P14 CD8 T cells during LCMV clone-13 infection.
A. Longitudinal analysis of P14 CD8 T cell number. Left graph shows summarized data (mean+SEM). **B** Individual kinetics of each animal from same data shown in **A**. **C.** Kinetics of spleen PD-1$^{-/-}$ P14 CD8 T cells post clone-13 infection. **D.** Kinetics of spleen endogenous GP_{33-41} –specific CD8 T cells post clone-13 infection. Blue line represents cells isolated from control chimeras (wild type P14/B6), either P14 or endogenous as marked by the Y axis of each graph. Red line represents cells isolated from PD-1$^{-/-}$ P14 chimeras in a similar fashion. Data generated from groups of 3 or more animals.

PD-1$^{-/-}$ P14 CD8 T cells in acute LCMV infection

PD-1 is upregulated on CD8 T cells upon TCR stimulation. In acute LCMV infection, PD-1 was down regulated by day 8 post infection. In order to evaluate PD-1$^{-/-}$ antigen-specific CD8 T cell response during acute LCMV infection, chimeric mice with either wild type of PD-1$^{-/-}$ P14 donor cells were given 2×10^5 p.f.u. LCMV Armstrong virus intraperitoneally. Tissue sample was collected at day 5, 8, 15 and later time points for analysis.

PD-1$^{-/-}$ P14 CD8 T cells showed similar behavior during early LCMV Armstrong infection compared to wild type P14 cells

In contrast to the dramatic changes seen in the clone-13 response, PD-1$^{-/-}$ P14 CD8 T cells behaved almost identical to wild type P14 cells during the first two weeks of infection. Transferred PD-1$^{-/-}$ P14 CD8 T cells showed very similar frequency at all three time points demonstrated (Fig 10A). Total lymphocyte number (data not shown) and total CD8 T cell number (Fig 10B) were also similar between control and PD-1$^{-/-}$ chimeras. Further analysis of absolute cell number confirmed that there was no significant changes between donor-derived wild type and PD-1$^{-/-}$ P14 CD8 T cells (Fig 10C). Kinetics of transferred population in spleen was almost identical between wild type and PD-1$^{-/-}$ P14 chimeric animals as far as by day 15 (Fig 10E). Endogenous GP33-specific CD8 T cells of recipient origins also behaved similarly between groups regardless of the PD-1$^{-/-}$ P14 presence in host system (Fig 10D, F). Characterization of cell surface markers and

functional analysis also exhibited highly identical features of PD-1$^{-/-}$ P14 cells compared to wild type cells (data not shown).

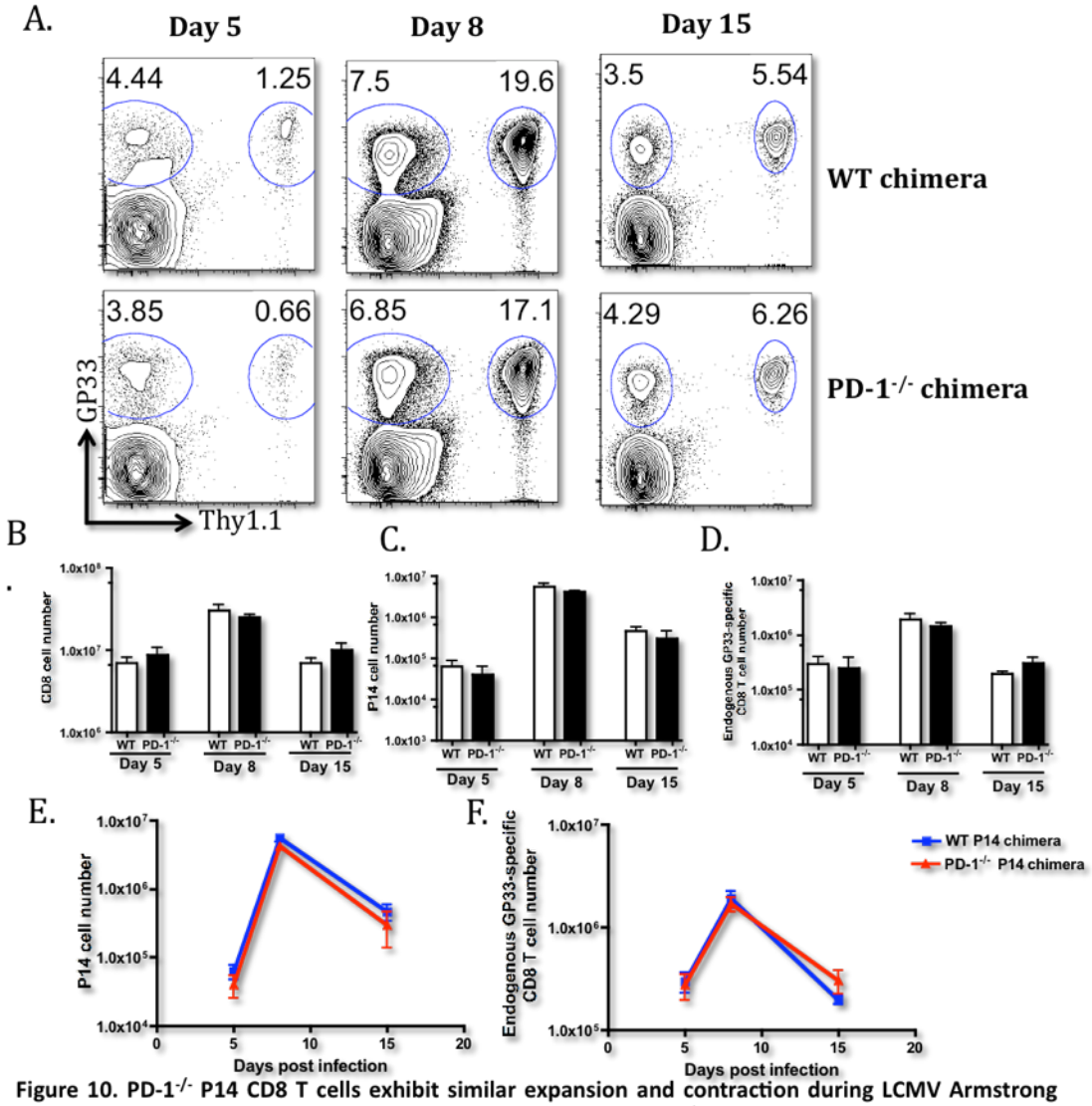

Figure 10. PD-1$^{-/-}$ P14 CD8 T cells exhibit similar expansion and contraction during LCMV Armstrong infection. **A**. Flow cytometry data showing either wild type or PD-1$^{-/-}$ P14 CD8 T cell frequency at various time points. Upper panel shows CD8 T cells isolated from control chimeras made with wild type P14 donor cells. Lower panel shows CD8 T cells isolated from chimeras made with PD-1$^{-/-}$ P14 donor cells. **B.** Numbers of total CD8 T cells from wild type or PD-1$^{-/-}$ chimeras at various time points. **C.** Number of P14 CD8 T cells from wild type or PD-1$^{-/-}$ chimeras at various time points. **D**. Numbers of endogenous GP33-specific CD8 T cells from wild type or PD-1$^{-/-}$ chimeras at various time points. **E.** Kinetics of transferred P14 CD8 T cell number at day 5, 8 and 15 post infection. **F.** Kinetics of endogenous GP33-specific CD8 T cell number at day 5, 8 and 15 post infection. E & F: blue line with squares represents cells isolated from wild type P14 chimeras and red line with triangles represents cells from PD-1$^{-/-}$ P14 chimeras.

Frequency of PD-1$^{-/-}$ P14 CD8 T cells declines gradually during memory phase of LCMV Armstrong infection

Although the PD-1$^{-/-}$ P14 and wild type P14 cells were almost indistinguishable during first 2 weeks of Armstrong infection, observation made at later time points revealed a decline in cell number occurred gradually over a period of time (Fig 11C). The loss of antigen-specific CD8 T cell is very similar with that of clone-13 infections past day 15. The behavioral pattern of wild type and PD-1$^{-/-}$ P14 CD8 T cells started to diverge around 3rd week of infection (Fig 11A) and the gap between these two subset continued to increase over time. Thus, although similar amount of effector CD8 T cells were initially formed, PD-1$^{-/-}$ P14 cells were found at a significantly lower frequency 40 days after infection (Fig 11C).

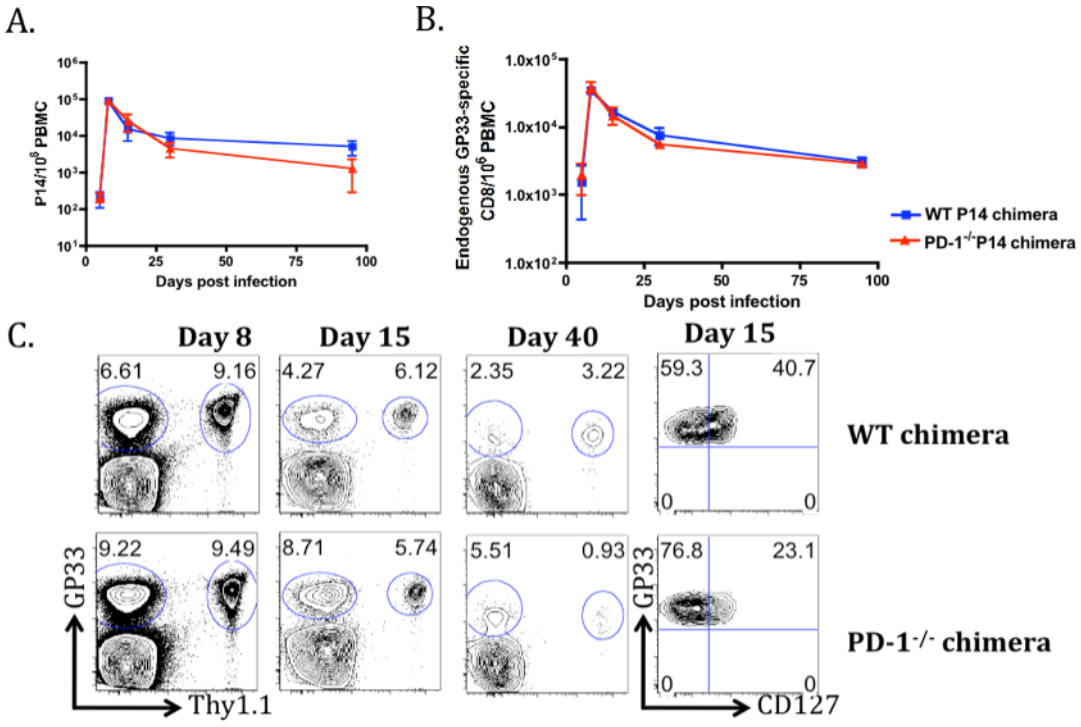

Figure 11. PD-1$^{-/-}$ P14 CD8 T cell frequency in PBMC gradually declines over time during memory phase of LCMV Armstrong infection. A. Kinetics of transferred P14 CD8 T cell frequency. Blue line with squares represents cells isolated from wild type P14 chimeras and red line with triangles represents cells from PD-1$^{-/-}$ P14 chimeras. **B.** Kinetics of endogenous GP33-specific CD8 T cell frequency. **C.** Comparison of transferred wild type and PD-1$^{-/-}$ P14 CD8 T cell frequency at various time points. Upper panel shows CD8 T cells isolated from control chimeras made with wild type P14 donor cells. Lower panel shows CD8 T cells isolated from chimeras made with PD-1$^{-/-}$ P14 donor cells. . Right column shows CD127 expression in PD-1$^{-/-}$ P14 CD8 T cells.

Discussion

The PD-1 pathway is well known for regulating CD8 T cell response through inhibition of TCR downstream signaling. T cells may have evolved to down regulate its responsiveness during viral persistence in order to avoid severe tissue damage caused by prolonged immune response. Unfortunately, some pathogens have taken advantage of this protective mechanism to establish long-term infection. As an inhibitory receptor of TCR signaling, PD-1 appears to play a key role in balancing effective immune response and immunopathology. PD-1 deficient animals demonstrated better control of infection but also suffered more severe hepatocellular injury in adenovirus infections[38]. Antibody blockade of PD-1: PD-L1 pathway in mice with chronic LCMV infection restored functions and proliferation of exhausted CD8 T cells and consequently, resulted in a decreased viral load[39]. PD-1 has also been reported in SIV[94,97], HIV[44,98] and HBV[99] studies as being associated with dysfunctional T cells that are characteristic of a immunosuppressive environment where viral infection persists. More dramatically, PD-L1$^{-/-}$ mice with LCMV clone-13 infection died due to severe immunopathlogy, indicating a key role of PD-1 inhibition in preventing immune-mediated self-injury[39]. However, the same mice with acute LCMV Armstrong infection behaved just like wild type animals in terms of CD8 T cell functions and viral clearance[39]. It is well demonstrated that PD-1 signaling pathway leads to different outcomes depending on infection scenario, and regulation of T cell response may be achieved by manipulating PD-1 pathway with a clinical purpose.

In this thesis, I examined PD-1 deficient CD8 T cell kinetics in both acute and chronic LCMV infections. PD-1 has been shown to function differently in chronic compared to acute infections. Manipulation of PD-1 pathway has been done in many infection models, yet little information is available for parallel comparisons between acute and chronic infections. I took advantage of the LCMV Armstrong and chronic infection model and examined PD-$1^{-/-}$ antigen-specific CD8 T cells under physiologic conditions. My findings are summarized and discussed below.

Disruption of PD-1 inhibition has been shown to enhance T cell proliferation in previous studies. PD-$1^{-/-}$ P14 effector CD8 T cells in chronic LCMV infection also showed exaggerated expansion and reached a much higher frequency compared to wild type cells of the same antigen specificity at day 8 post infection (Fig 4). However, this larger population was not maintained. These effector CD8 T cells went through drastic contraction in the following week and crashed to a lower frequency compared to wild type (Fig 7). Not only transferred PD-$1^{-/-}$ P14 cells, but also endogenous GP33-specific CD8 T cells that shared the *in vivo* environment showed an enhanced contraction compared to cells from wild type chimeras (Fig 7). How did this enhance contraction occur, what molecules and pathways are involved, and what clinical implication could it offer? These are intriguing and important questions to answer. This decrease of endogenous GP276-specific CD8 T cells suggest a change in the environment of PD-$1^{-/-}$ P14 chimeric CD8 T cells, which is likely to also contribute partially to the enhanced contraction of PD-$1^{-/-}$ P14 CD8 T cells. Moreover, increased production of the cytolytic protein granzyme B indicates an increased activation and enhanced T cell response.

Staining of inhibitory molecules other than PD-1, such as 2B4 and Tim-3 showed increased expression levels, suggesting an over-activated T cell status. Co-transfer of wild type and PD-$1^{-/-}$ P14 CD8 T cells into the same host followed by clone-13 infection also showed more severe contraction in the PD-$1^{-/-}$ subset, supporting a cell-intrinsic mechanism for the severe death of antigen-specific CD 8 T cells (Ahmed lab, unpublished data). The number of PD-$1^{-/-}$ P14 cells showed no signs of recovering to the wild type level during late clone-13 infection (Fig 8). This finding was true in all tissues examined (spleen, liver, lung, PBMC), with variations in level of significance.

Antibody blockade experiments done by Barber, D. *et al* showed PD-1 signaling was reduced during the chronic phase of clone-13 infection (day 30+). Antibody blockade led to a recovery of exhausted T cells while PD-$1^{-/-}$ P14 effector CD8 T cell crashed and showed no signs of coming back. The difference in outcomes may be explained by the difference in experimental settings. Exhausted T cells that were later rescued had experienced PD-1 signaling during acute phase of clone-13 infection while PD-1 mediated inhibition never occurred in PD-$1^{-/-}$ P14 cells. PD-1 was rapidly upregulated in both acute and chronic infections upon TCR stimulation, suggesting a positive role of PD-1 in regulating immune response from an evolutionary aspect. Also, gradual loss of PD-$1^{-/-}$ P14 cells during late Armstrong infection suggested a defect in long-term survival of cells lacking PD-1 expression during the 1^{st} week of infection. Thus, it is likely that the absence of PD-1 signaling may be harmful for initial T cell priming and causes defects in memory differentiation and long-term survival.

PD-1$^{-/-}$ cells experienced complete loss of PD-1 while antibody blockade achieved a less absolute but more global effect. Previous studies demonstrated that PD-1 inhibits T cell activation through recruitment of phosphatases SHP-1 and SHP-2 to its ITSM motif and dephosphorylation of TCR downstream signaling molecules in the signalosome. *In vitro* studies showed increased level of T cell activation by blocking PD-1. In my model, data of increased cytolytic proteins and surface inhibitory receptor expression in clone-13 infection pointed to over-activation. Complete loss of PD-1 inhibition may lead to over-activation that subsequently causes activation induced cell death (AICD). Given that T cells in clone-13 infection experienced much greater antigen-stimulation, over-activation induced cell death is expected to be severe, and indeed was observed in my studies.

PD-1$^{-/-}$ P14 cells in Armstrong infection behaved almost identical to wild type P14 cells (Fig 10). Antigen-specific CD8 T cells are initially fully functional and controls virus efficiently even in the presence of PD-1 inhibition. The absence of any enhanced cell death, as compared to clone-13 infection, may be due to a transient and much lower magnitude of antigen stimulation. This low level of stimulation may not be sufficient to induce over-activation of T cells, which can lead to apoptosis. However, when later time points of infection were examined, slow but steady loss of PD-1$^{-/-}$ P14 CD8 T cells was observed, demonstrating a defect in memory CD8 T cell differentiation and survival (Fig 11). IL-7R is required for homeostatic turnover of memory CD8 T cells and was found to be expressed at lower levels on day 15 post Armstrong infection, further supporting a differentiation and survival defect in PD-1$^{-/-}$ P14 cells (Ahmed lab, unpublished data). Further investigation evaluating the quality of these PD-1$^{-/-}$ memory CD8 T cells is

needed. Direct secondary challenge may be difficult due to the diverse cell frequencies, but by pooling memory PD-$1^{-/-}$ P14 CD8 T cells before adoptive transfer and then readministrating a re-challenge may reveal more information on these cells.

A few slight changes in cell surface markers were detected, indicating the interaction between PD-1 signaling and the markers tested were slight. PD-$1^{-/-}$ P14 CD8 T cells showed little change in cytokine production in *ex vivo* peptide stimulation assay, and serum ELISA data showed no significant changes in cytokine levels (Ahmed lab, unpublished data). However, even at the peak of its expansion, the PD-$1^{-/-}$ P14 CD8 T cell subset was still a small portion in a multi-epitope response against LCMV virus. Increased secretion of cytokines could be diluted once they enter circulation. Indeed, chimeras made with Rag deficient mice showed increased level of TGF-beta around day 7 post infection (Ahmed lab, unpublished data), supporting the view that cellular function as well as survival potential was altered.

Numerous studies have showed altered infection outcomes by manipulating PD-1: PD-L pathway, and PD-1 has been placed under intensive investigation given its great potential for clinical purposes. However, one must proceed with extra caution in this field since PD-1 appears to be a very powerful regulator in many aspects of immunity. More importantly, the complex and balanced immune network made PD-1 signaling protective to immune response in certain cases, as shown in my study.

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