University of Pennsylvania
ScholarlyCommons

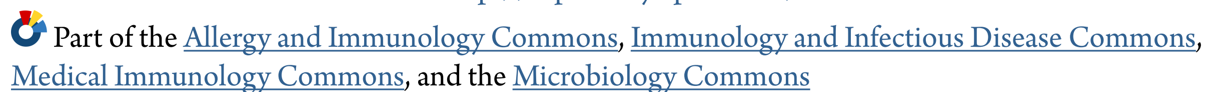
Publicly Accessible Penn Dissertations

1-1-2015

Regulation of Anti-Viral Cd8+ T Cell Responses by the Pd-1 Pathway

Pamela Odorizzi
University of Pennsylvania, odorizzi@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations


Part of the Allergy and Immunology Commons, Immunology and Infectious Disease Commons, Medical Immunology Commons, and the Microbiology Commons

Recommended Citation
Odorizzi, Pamela, "Regulation of Anti-Viral Cd8+ T Cell Responses by the Pd-1 Pathway" (2015). *Publicly Accessible Penn Dissertations*. 1109.
http://repository.upenn.edu/edissertations/1109

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1109
For more information, please contact libraryrepository@pobox.upenn.edu.

Regulation of Anti-Viral Cd8+ T Cell Responses by the Pd-1 Pathway

Abstract
PD-1 and its ligands, PD-L1 and PD-L2, constitute a critical immunoregulatory pathway for modulating CD8+ T cell responses. This pathway has become an important therapeutic target in cancer and chronic viral infections, where blockade of PD-1/PD-L1 reverses CD8+ T cell exhaustion and improves outcomes. However, it remains unclear how PD-1 pathway signals shape the development and maintenance of memory CD8+ T cell responses following acutely-resolved infection or exhausted CD8+ T cell responses following chronic infection. Absence of PD-1 results in early over-activation, excessive proliferation and diminished survival of virus-specific CD8+ T cells during both acutely-resolved and chronic viral infections. Following influenza infection, PD-1 pathway deficiency led to sub-optimal CD8+ T cell memory development. Defects were observed in the magnitude of the influenza-specific CD8+ T cell responses, as well as in cytokine production and recall capacity. Similarly, during chronic LCMV infection, PD-1 deficient CD8+ T cells were more dysfunctional than their WT counterparts. Permanent absence of PD-1 dramatically dysregulated lineage dynamics and long-term stability of exhausted CD8+ T cell populations through a mechanism involving the transcription factors T-bet and Eomesodermin. As a result, PD-1 deficiency led to the accumulation of terminally-differentiated, but more cytotoxic, exhausted CD8+ T cells. These findings reveal a novel dual role for PD-1 signals during acutely-resolved and chronic viral infections. PD-1 pathway signals clearly restrict CD8+ T cell activation, expansion and function early, consistent with previous work. However, in doing this, PD-1 also preserves the quantity and quality of long-term memory or exhausted CD8+ T cell responses. Thus, PD-1 acts as a critical "rheostat" on CD8+ T cells, balancing the need for activation to achieve pathogen clearance with the establishment and maintenance of long-term memory or exhausted T cell responses.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
E. John Wherry

Keywords
CD8 T cells, Immunology, Infectious disease, PD-1

Subject Categories
Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology | Microbiology

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1109

REGULATION OF ANTI-VIRAL CD8+ T CELL RESPONSES BY

THE PD-1 PATHWAY

Pamela M. Odorizzi

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

Supervisor of Dissertation	Graduate Group Chairperson
_______________________	_______________________
E. John Wherry, Ph.D.	Daniel Kessler, Ph.D.
Director of the Institute for Immunology	Associate Professor of Cell and
Associate Professor of Microbiology	Developmental Biology

Dissertation Committee

Christopher Hunter, Ph.D. : Professor and Chair of Pathobiology

Paula Oliver, Ph.D. : Associate Professor of Pathology and Laboratory Medicine

Sara Cherry, Ph.D. : Associate Professor of Microbiology

James Riley, Ph.D. : Associate Professor of Microbiology

REGULATION OF ANTI-VIRAL CD8+ T CELL RESPONSES BY THE PD-1 PATHWAY

COPYRIGHT

2015

Pamela Marie Odorizzi

ACKNOWLEDGEMENTS

My journey through graduate school has been full of adventure and growth, both scientifically and personally, that was fostered by mentorship, friendship, and support from some amazing people. I have to start by thanking my incredible mentor, E. John Wherry, who challenged me to become a better scientist every day. I am forever grateful for your passion for science, your enthusiasm for data, your encouragement and support, your guidance and your tough love. I also have to thank all of the members of the Wherry lab, past and present, who made science fun on a daily basis: Michael Paley, Burton Barnett, Kristen Pauken, Erietta Stelekati, Makoto Kurachi, Doug Dolfi, Ramin Herati, Vesselin Tomov, Alison Crawford, Kathleen Mansfield, Mohammed Ali, Jonathan Johnnidis, Bertram Bengsch, Alexander Huang, and Ryan Staupe. I am so grateful to have worked with such a brilliant and wonderfully weird group of scientists. Thank you to my thesis committee: Christopher Hunter, James Riley, Sara Cherry, and Paula Oliver for valuable advice and constant support. Also, thank you to Bob Doms who always had advice on-hand about my scientific future. I wish to thank Paul Bates and the HIV Pathogenesis Training Grant for two years of financial support. I am also grateful for all of the help provided by the Microbiology Department and Microbiology, Virology and Parasitology graduate group, especially Anna Kline. I would like to thank my wonderful friends, who helped me through the tough times and celebrated with me during the good times, especially Adrienne Swanstrom, Sudil Mahendra, Brianna Morgan, Saloni Malik, Jill Alspach, Stephanie Weir and Heather Turnbach. Finally, I would like to thank my amazing family. Thank you to my brother, Mark Odorizzi, and my best friend and sister, Laura Odorizzi, for always being there to lean on for support, as a kick in the butt when I

needed it or just a laugh. And, of course, thank you to my amazing parents, Mark and Mary Odorizzi, for all their love, support and guidance through the ups and downs of graduate school. Thank you for always encouraging me to reach for the stars and for being my biggest cheerleaders.

ABSTRACT

REGULATION OF ANTI-VIRAL CD8+ T CELL RESPONSES BY THE PD-1 PATHWAY

Pamela M Odorizzi

E. John Wherry

PD-1 and its ligands, PD-L1 and PD-L2, constitute a critical immunoregulatory pathway for modulating CD8+ T cell responses. This pathway has become an important therapeutic target in cancer and chronic viral infections, where blockade of PD-1/PD-L1 reverses CD8+ T cell exhaustion and improves outcomes. However, it remains unclear how PD-1 pathway signals shape the development and maintenance of memory CD8+ T cell responses following acutely-resolved infection or exhausted CD8+ T cell responses following chronic infection. Absence of PD-1 results in early over-activation, excessive proliferation and diminished survival of virus-specific CD8+ T cells during both acutely-resolved and chronic viral infections. Following influenza infection, PD-1 pathway deficiency led to sub-optimal CD8+ T cell memory development. Defects were observed in the magnitude of the influenza-specific CD8+ T cell responses, as well as in cytokine production and recall capacity. Similarly, during chronic LCMV infection, PD-1 deficient CD8+ T cells were more dysfunctional than their WT counterparts. Permanent absence of PD-1 dramatically dysregulated lineage dynamics and long-term stability of exhausted CD8+ T cell populations through a mechanism involving the transcription factors T-bet and Eomesodermin. As a result, PD-1 deficiency led to the accumulation of terminally-differentiated, but more cytotoxic, exhausted CD8+ T cells. These findings reveal a novel

dual role for PD-1 signals during acutely-resolved and chronic viral infections. PD-1 pathway signals clearly restrict CD8+ T cell activation, expansion and function early, consistent with previous work. However, in doing this, PD-1 also preserves the quantity and quality of long-term memory or exhausted CD8+ T cell responses. Thus, PD-1 acts as a critical “rheostat” on CD8+ T cells, balancing the need for activation to achieve pathogen clearance with the establishment and maintenance of long-term memory or exhausted T cell responses.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS III

ABSTRACT V

LIST OF FIGURES X

CHAPTER 1: INTRODUCTION 1

1.1: Global Heath Challenges: Infectious Disease 1

1.2: The Adaptive Immune System 2

1.3: CD8+ T cells 3

1.3.1: Initiation of CD8+ T cell Responses 4

1.3.2: Generation of CD8+ T cell Memory after Acutely-Resolved Infections 7

1.3.3: CD8+ T cell Exhaustion and the Response to Chronic Infection and Cancer 10

1.4: Inhibitory Receptors on Lymphocytes 12

1.5: The Programmed Death-1 Pathway 13

1.5.1: PD-1 Signaling in T cells 14

1.5.2: Regulation of Effector and Memory CD8+ T cells by the PD-1 Pathway 16

1.5.3: Regulation of Exhausted CD8+ T cells by the PD-1 pathway 18

1.5.4: Clinical Use of PD-1 Pathway Blockade 21

1.6: The Lymphocytic Choriomeningitis Virus Model 21

1.6.1: LCMV Strains: Armstrong and clone 13 23

1.7: Influenza Virus Infection 24

1.7.1: Influenza Virus Strains: PR8 and X31 25

CHAPTER 2: PD-1 REGULATES THE DEVELOPMENT AND FUNCTION OF MEMORY CD8+ T CELLS FOLLOWING RESPIRATORY VIRAL INFECTION 26

2.1: Introduction 26

2.2: Methods 29

2.2.1: Mouse Strains 29
2.2.2: Viral Infection 29
2.2.3: Isolation of Cells from Tissues 30
2.2.4: Antibodies, Tetramers, and Flow Cytometry 31
2.2.5: BrdU Treatment and Detection 31
2.2.6: Statistical Analysis 31

2.3: Results 32

2.3.1: Virus-specific $CD8^+$ T cell memory development is impaired in mice lacking PD-1 signals 32
2.3.2: The PD-1 pathway regulates the memory $CD8^+$ T cell compartment by controlling initial $CD8^+$ T cell expansion and contraction 37
2.3.3: Increased turnover of $CD8^+$ T cells in the absence of PD-1 signaling. 39
2.3.4: The regulation of $CD8^+$ T cell memory differentiation by PD-1 is cell-intrinsic. 43

2.4: Discussion 50

CHAPTER 3: GENETIC ABSENCE OF PD-1 PROMOTES ACCUMULATION OF TERMINALLY-DIFFERENTIATED EXHAUSTED CD8+ T CELLS DURING CHRONIC VIRAL INFECTION 55

3.1: Introduction 55

3.2: Methods 58

3.2.1: Mice and Infections 58
3.2.2: Adoptive Transfer and Lymphocyte Isolation 58
3.2.3: Flow Cytometry and Cell Sorting 59
3.2.4: BrdU Treatment and Detection 59
3.2.5: In vitro Stimulation 60
3.2.6: Statistical Analysis 60

3.3: Results 60

3.3.1: Co-adoptive transfer strategy allows for examination of PD-1 deficient virus-specific CD8+ T cells during chronic LCMV infection 60
3.3.2: CD8+ T cell exhaustion develops in the absence of PD-1 63
3.3.3: Despite early signs of dysfunction, PD-1 deficient CD8+ T cells proliferate robustly during the acute phase of infection 67
3.3.4: Decreased survival of CD8+ T cells after the effector phase in the absence of PD-1 71
3.3.5: Decreased proliferation and long-term durability of PD-1 KO exhausted $CD8^+$ T cells 73
3.3.6: PD-1 controls exhausted CD8+ T cell proliferative dynamics by regulating $Tbet^{hi}$ and $Eomes^{hi}$ subsets 76

3.3.7: Discussion **81**

CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS **85**

4.1 Overview of Results **85**

4.2 Context dependent roles of PD-1 on CD8+ T cells **86**

4.2.1 PD-1 and TCR signal strength 87

4.2.2: PD-1 and duration of antigen stimulation 88

4.2.3: PD-1 and anatomical location 89

4.3: Temporal Regulation by the PD-1 pathway **91**

4.4: Similarities between exhausted PD-1 deficient CD8+ T cells and exhausted PD-1^{hi} CD8+ T cells in chronic infection **93**

4.5: How do you most effectively target the PD-1 pathway? **95**

4.6: Biomarkers for successful PD-1-based therapies **97**

4.7: Safety of PD-1-based therapies in patients **98**

4.8: Summary **99**

BIBLIOGRAPHY **100**

LIST OF FIGURES

Figure 1.1: Model of CD8+ T cell memory versus exhaustion during acute and chronic viral infection

Figure 2.1: Magnitude of CD8+ T cell memory response is impaired in PD-1 pathway-deficient mice.

Figure 2.2: Quality of CD8+ T cell memory is impaired in PD-1 pathway-deficient mice.

Figure 2.3: Defective recall capacity of memory CD8+ T cells in PD-1 pathway deficient mice.

Figure 2.4: Altered effector CD8+ T cell expansion and contraction in the absence of PD-1 pathway signals.

Figure 2.5: Altered expression of genes representing cell division pathways in WT versus PD-L1/L2 DKO virus-specific CD8+ T cells.

Figure 2.6: CD8+ T cells undergo robust proliferation but have reduced survival in the absence of PD-1 signaling in influenza-infected mice

Figure 2.7: WT mice clear primary influenza infection more quickly than PD-1 pathway deficient mice.

Figure 2.8: PD-1 controls the magnitude of the CD8+ T cell response in a cell-intrinsic manner.

Figure 2.9: PD-1 regulates CD8+ T cell proliferation and cell death in a cell-intrinsic manner.

Figure 2.10: Cell-intrinsic defect in CD8+ T cell memory in the absence of PD-1.

Figure 2.11: Model of the role for PD-1 signals in effector and memory CD8+ T cell responses to influenza virus infection

Figure 3.1: Adoptive transfer of WT and PD-1 KO P14 cells as a model to study T cell exhaustion in the absence of PD-1.

Figure 3.2: Severe loss of exhausted CD8+ T cell cytokine production in the absence of PD-1.

Figure 3.3: Increased inhibitory receptor expression on exhausted CD8+ T cells in the absence of PD-1.

Figure 3.4: Early signs of CD8+ T cell exhaustion in PD-1 KO P14 cells.

Figure 3.5: Increased expansion and proliferation of PD-1 KO P14 cells during effector phase

Figure 3.6: Reduced survival of PD-1 KO P14 cells during T cell contraction.

Figure 3.7: Diminished long-term proliferation and stability of CD8+ T cells in the absence of PD-1.

Figure 3.8: Altered dynamics of exhausted CD8+ T cell subsets in the absence of PD-1.

Figure 3.9: PD-1 KO P14 cells accumulate as Eomes^{hi} progeny with the same cardinal properties.

Figure 3.10: Model of the role for PD-1 in the development of CD8+ T cell exhaustion during chronic viral infection.

CHAPTER 1: Introduction

1.1: Global Heath Challenges: Infectious Disease

Infectious diseases place a significant burden on the health of individuals worldwide, as well as on healthcare infrastructure and global economy. Much of this burden is borne by developing countries, where poor sanitation and unsafe drinking water propagate the spread of disease. However, even in developed countries, many infectious diseases remain a considerable threat, particularly to the young and elderly. Over the past 60 years, the public health and research communities have made significant advances in the control and elimination of infectious diseases (Panhuis et al., 2013). Arguably the most important medical intervention implemented has been vaccinations. It is estimated that vaccines have prevented over 100 million cases of disease in the United States and more than 2.5 million deaths worldwide (WHO, 2012). Perhaps one of the most notable successes of vaccinations is the elimination of smallpox in 1980 (Fenner et al.1988). However, infectious diseases are continually emerging and re-emerging. In fact, since 1980, there has been an increase in the number of new infectious agents, the number of outbreaks worldwide and also a resurgence of known diseases (Smolinski, Hamburg, & Lederberg, 2003). Many of these diseases cause significant mortality worldwide and challenge the current model of vaccine development. For example, seasonal influenza, an acutely-resolved infection, causes 3,000-49,000 deaths annually in the United States (CDC, 2010). Other infections, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), often cause chronic disease and death in patients. As of 2012, 35.3 million people were living with HIV, leading to 1.6 million HIV-related deaths per year (UNAIDS, 2013). In the

United States, there are over 3.2 million people chronically infected with HCV, resulting in approximately 16,000 deaths each year (CDC, 2011). While anti-viral drugs and other strategies can lower viral load and extend life span in many patients with chronic HIV, there are currently no medical interventions that can reliably cure this infection (Piacenti, 2006). The recent introduction of direct-acting anti-viral drugs against HCV may provide a potential cure for some chronic HCV patients; however, the high cost of treatment makes this approach unattainable for many (Kiser and Flexner, 2013). Thus, a major goal remains the development of prophylactic vaccination strategies that can protect against these public health threats. Given the ineffectiveness of implementing our current vaccine platforms, an important next step is defining the adaptive immune components that contribute to effective immune responses against these diseases.

1.2: The Adaptive Immune System

The adaptive immune system possesses the unique properties of diversity, specificity and memory. These properties allow the adaptive immune response to combat many different pathogens in a specific and targeted way, maximizing pathogen clearance and minimizing damage to the host. In addition, this strategy provides adaptive immune cells with the knowledge to "remember" a pathogen encounter and results in better protection against a pathogen upon subsequent exposure (Ahmed and Gray, 1996; Williams and Bevan, 2007). There are two major cell types that make up the adaptive immune system: B cells and T cells. Both cell types undergo genomic rearrangement to generate millions of unique surface receptors, known as B cell receptors (BCR; also known as antibodies) and T cell receptors (TCR). Each B cell and T cell expresses one unique version of a BCR or TCR, respectively, which generates a

repertoire of adaptive immune cells with immense diversity (Wong and Pamer, 2003; Zhang and Bevan, 2011).

The primary role of B cells is production of soluble antibodies that specifically bind intact pathogen proteins. While B cells are critical for neutralizing infectivity of pathogens, T cells are required for clearance of a pathogen after the establishment of infection. Broadly speaking, T cells can be sub-divided into CD4+ or CD8+ T cells. CD4+ T cells, or T helper cells, determine the type of immune response that will be mounted against an invading pathogen through production of distinct cytokines, as well as via specific interactions with other immune cells. CD8+ T cells, or cytotoxic T cells (CTLs), kill pathogen-infected cells through the recognition of short viral peptide fragments presented on the surface of infected cells in the context of major histocompatibility complex (MHC) (Zinkernagel and Doherty, 1974; Rock and Goldberg, 1999). Collectively, B cells, CD4+ T cells and CD8+ T cells mount a coordinated immune response against an invading pathogen.

1.3: CD8+ T cells

The critical role of CD8+ T cells during infection has been demonstrated in many mouse models where loss of CD8+ T cell effector functions leads to impairment in pathogen control. For example, mice deficient in the cytotoxic molecule perforin or the effector cytokine IFN-γ (discussed below) have defective CD8+ T cell responses that correlate with increased pathogen burden (Walsh et al., 1994; Matloubian et al., 1999; Dorman et al., 1999; Binder and Griffin, 2001; Bartholdy et al., 2000). Similarly, depletion of CD8+ T cells during undetectable, chronic SIV infection of rhesus macaques leads to rebound of the virus to high circulating levels (Jin et al., 1999; Schmitz et al., 1999).

Restoration of viral control in this setting is associated with the re-population of the CD8+ T cell compartment. HIV and the progression to acquired immunodeficiency syndrome (AIDS) is also associated with loss of CD8+ T cell populations and effector functions (Shankar et al., 2000; Appay et al., 2000; Kostense et al., 2002; Gruener et al., 2001b; Maini et al., 1999). As a result, late-stage HIV patients are more susceptible to new infections, as well as re-activatoin of latent infections, including herpes viruses (Siegal et al., 1981; Kaplan et al., 2000). Thus, CD8+ T cell anti-viral activities are associated with control of disease in both mice and humans.

1.3.1: Initiation of CD8+ T cell Responses

Naïve CD8+ T cells become activated upon encounter with an antigen-presenting cell (APC) that is presenting pathogen-derived peptide in the context of MHC on its surface (signal 1) (Smith-Garvin et al., 2009). Co-stimulation (signal 2) fine-tunes TCR signaling through positive signals from molecules, such as CD28, ICOS, 4-1BB, CD27 and many others (Crawford and Wherry, 2009; Chen and Flies, 2013). These co-stimulatory signals augment and sustain T cell activation that is initiated by the TCR through a variety of mechanisms. For example, co-stimulation is known to augment cytokine and chemokine production, promote expression of anti-apoptosis proteins (such as Bcl-xL) and upregulate growth factor receptors (Chen and Flies, 2013; June et al., 1987; Boise et al., 1995; Acuto and Michel, 2003). In addition, co-stimulation plays a critical role in regulating the metabolism of CD8+ T cells. For example, CD28 induces signaling through the P13K-Akt-mTor pathway, which, among other things, promotes glucose transport and protein synthesis in newly activated CD8+ T cells (McAdam et al., 1998; Stein et al., 1994; Boomer and Green, 2010; Frauwirth et al., 2002). The positive signals propagated by co-stimulatory receptors are also counter-balanced by negative

signals from inhibitory receptors (discussed in detail below). Finally, optimal CD8+ T cell activation also requires signals from inflammatory cytokines (signal 3), such as IL-12, type 1 interferons, and TLR ligands (Curtsinger and Mescher, 2010; Curtsinger et al., 2003, 2005; Valenzuela et al., 2002). The coordinated integration of these three signals is critical for generation of functional CD8+ T cells and, importantly, the quantity and quality of each signal can dramatically impact the type of response that is mounted.

Following activation, naïve CD8+ T cells expand and differentiate into a pool of effector cells. Proliferation of CD8+ T cells is extremely robust after pathogen encounter, with CD8+ T cells undergoing up to a 50,000-fold expansion (Badovinac et al., 2007; Zhang and Bevan, 2011). This proliferation is accompanied by migration to sites of infection and acquisition of properties to fight an invading pathogen. These properties include cytotoxic ability and production of pro-inflammatory cytokines. The killing of infected cells by CD8+ T cells can be executed by two major mechanisms: perforin/granzyme and Fas/FasL interactions (Kägi et al., 1995, 1994). Release of perforin at a target cell leads to the formation of a pore in the target cell membrane. This pore then allows for entry of granzymes into the cell that induce cell death of a pathogen-infected cell (Heusel et al., 1994; Kägi et al., 1996). Alternatively, the Fas death receptor pathway can be activated through Fas/FasL interactions, resulting in programmed death of an infected cell (Kägi et al., 1995, 1994). Co-production of multiple pro-inflammatory cytokines is another critical function of CD8+ T cells. Cytokines such as IL-2, TNF-α, Mip-1α, and IFN-γ augment immune responses while inducing an anti-viral state (Harty and Bevan, 1999; Cocchi et al., 1995; Guidotti and Chisari, 2001). Following expansion, 90-95% of effector cells will die by apoptosis during the contraction phase (Badovinac et al., 2002). Depending on the type of infection, subsequent

differentiation of CD8+ T cells into memory cells or exhausted cells will follow (**Figure 1.1**).

Differentiation of CD8+ T cells from naïve to effector cells is accompanied by dramatic changes in gene expression profiles, which are regulated by key transcription factors. A central regulator of CD8+ T cell effector differentiation appears to be BATF, a basic leucine zipper transcprion family member (Kurachi et al., 2014). Kurachi et al. demonstrated that, in the absence of BATF, CD8+ T cells undergo a dramatic metabolic crash that prevents subsequent differentiation and expansion into effector cells. BATF, along with IRF4, regulates effector differentiation by promoting the expression of the transcription factors Tbet, Blimp-1 and Runx3 (Kurachi et al., 2014). Notch has also recently been implicated as a key "hub" for integration of signals to promote CD8+ T cell effector differentiation. These studies indicate that Notch forms a critical feedback loop with CD25 (IL-2Ra), Tbet and mTOR to drive differentiation of effector cells (Backer et al., 2014). Early expression of Tbet induced by TCR signaling, as well as BATF and Notch, has been shown to play a key role in the early induction of IFN-γ expression (Cruz-Guilloty et al., 2009; Backer et al., 2014). Subsequent expression of Runx3 and Blimp-1 re-enforces IFN-γ expression while also inducing the expression of cytotoxic molecules, such as granzyme B. Runx3 also induces the expression of the transcription factor Eomesodermin (Eomes), which controls expression of perforin and seems critical for late IFN-γ expression (Cruz-Guilloty et al., 2009; Pearce et al., 2003). Importantly, naïve CD8+ T cells that lack Tbet, Blimp-1, Notch 1/2, or Runx3 show defects in effector responses, including proliferation, cytotoxicity, and cytokine production (Chang et al., 2014; Angelosanto and Wherry, 2010; Cruz-Guilloty et al., 2009; Pearce et al., 2003; Backer et al., 2014). Thus, effector CD8+ T cell differentiation requires the temporal

induction of key transcription factors that regulate gene expression of effector molecules. In addition, these transcription factors play a central role in the fate of effector cells in acutely-resolved and chronic infections.

1.3.2: Generation of CD8+ T cell Memory after Acutely-Resolved Infections

Expansion and differentiation of CD8+ T cells results in the generation of a phenotypically and functionally heterogenous pool of effector cells during acutely-resolved infections. The majority of the effector cell population is comprised of short-lived effector cells (SLECS), while a smaller population of memory precursor cells also exist. Studies of acute lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* (*L. monocytogenes*) infections in mice found that SLECs could be readily identified by high expression of KLRG-1 and low expression of CD127 (IL-7R) (Joshi et al., 2007; Obar et al., 2011). In addition to this defining phenotype, SLECs also have higher expression of the transcription factors Tbet and Blimp-1, which corresponds with increased effector molecules, greater migration into non-lymphoid tissues and the more terminally-differentiated state of these cells (Rutishauser et al., 2009; Kallies et al., 2009; Pearce et al., 2003; Intlekofer et al., 2005). During the contraction phase, the majority of SLECs will die by apoptosis, whereas memory precursor cells will persist and differentiate into long-lived memory cells. In contrast to SLECs, memory precursor cells are KLRG-1^{lo} IL-$7R^{hi}$ and memory differentiation of these cells is associated with higher levels of the transcription factors Eomes, Tcf-1, Foxo1, and Id3 (Joshi et al., 2007; Kaech et al., 2003a; Zhou et al., 2010; Hess Michelini et al., 2013; Kim et al., 2013; Yang et al., 2011; Intlekofer et al., 2005).

Memory CD8+ T cells acquire several cardinal properties that endow them with the ability to more quickly and effectively respond to a secondary encounter with a pathogen

(**Figure 1.1**). These properties include rapid reactivation of effector functions, robust proliferation and enhanced homing to secondary lymphoid organs (Wherry and Ahmed, 2004; Kaech et al., 2002; Bevan, 2011). In addition, memory cells persist via antigen-independent, cytokine-driven homeostatic proliferation in response to IL-7 and IL-15 (Kaech et al., 2003b). Given the heterogeneity of effector CD8+ T cells, it is not surprising that many sub-populations of memory cells also exist (Huster et al., 2004; Kaech et al., 2003b; Hamann et al., 1997). The most notable of these are central memory T cells (Tcm), effector-memory T cells (Tem), tissue-resident memory cells (Trm) and stem memory T cells (Tsm) (Sallusto et al., 1999; Hamann et al., 1997; Carbone et al., 2013; Turtle et al., 2009; Zhang et al., 2005). Generally, Tcm are $CD62L^{hi}$ $CCR7^{hi}$, which leads to increased retention of these cells in lymphoid tissue. In contrast, Tem are $CD62L^{lo}$ $CCR7^{lo}$, which endows them with the ability to circulate through non-lymphoid tissues more readily. Tem are also thought to be more “effector” like, possessing greater cytotoxicity (Wherry et al., 2003b; Wherry and Ahmed, 2004).

The signals that govern and shape memory CD8+ T cell development remain poorly understood. One factor that seems to play an important role in the generation of memory cells is the strength and duration of antigenic stimulus. This includes the strength of TCR:pepide-MHC interactions, as well as the collective input from co-stimulatory, inhibitory and inflammatory signals (Iezzi et al., 1998; Tubo et al., 2013). For example, the cytokine milieu and inflammatory environment have been shown to influence the acquisition of CD8+ T cell memory properties (Plumlee et al., 2013). IL-12R-deficiency or Tbet-deficiency in mice leads to more rapid development of memory characteristics during *L. monocytogenes* infection (Takemoto et al., 2006). The mTOR pathway, which is upregulated by TCR and co-stimulatory signals, also controls effector-memory differentiation. LCMV-infected mice who received low does treatment with

rapamycin, an inhibitor of mTOR signaling, had enhanced generation of MPECs during the expansion phase as well as accelerated development of memory properties (Araki et al., 2009). Nevertheless, exactly how signals 1, 2 and 3 contribute to the differentiation of memory CD8+ T cells remains unclear.

Figure 1.1

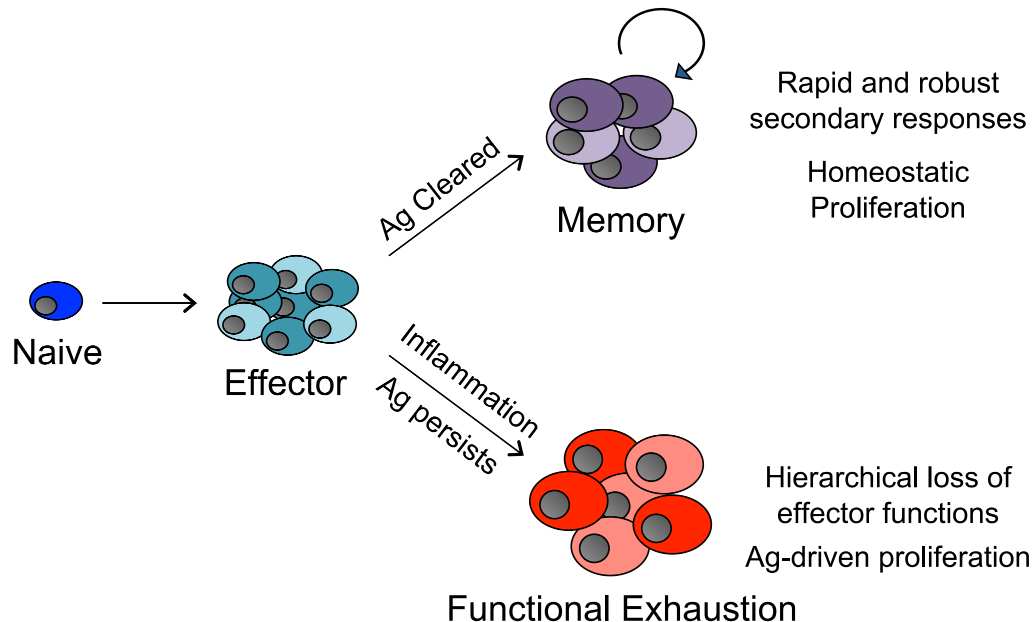


Figure 1.1: Model of CD8+ T cell memory versus exhaustion during acute and chronic viral infection. Naïve CD8+ T cells become activated, leading to proliferation and differentiation into a pool of effector CD8+ T cells. During acutely-resolved infection, effector CD8+ T cells facilitate pathogen clearance after which most short-lived effector cells will die. Memory precursor cells will transition into a pool of long-lived memory CD8+ T cells that acquire cardinal memory properties, such as homeostatic proliferation and recall capacity. In contrast, failure to achieve pathogen clearance results in sustained antigen loads and inflammation that result in exhausted CD8+ T cell responses. Exhausted CD8+ T cells are characterized by loss of effector functions and antigen-driven proliferation.

1.3.3: CD8+ T cell Exhaustion and the Response to Chronic Infection and Cancer

During chronic infections, pathogen-specific CD8+ T cells are initially activated and gain effector functions; however these cells become progressively dysfunctional, or exhausted, over time (Zajac and Ahmed, 1998; Gallimore and Zinkernagel, 1998; Wherry, 2011). Exhaustion of CD8+ T cells during chronic infection is hierarchical, with early defects in proliferation, IL-2 production, and cytotoxicity, followed by the loss of TNF-α, IFN-γ, and β-chemokine production at late stages (Wherry et al., 2003a). Ultimately, exhausted CD8+ T cells can be physically deleted (**Figure 1.1**). The severity of CD8+ T cell exhaustion correlates with the strength and duration of antigen stimulation. For example, high levels of persistent epitope presentation during chronic LCMV infection in mice leads to severe exhaustion and physical deletion of corresponding epitope-specific CD8+ T cells. In contrast, in the same mice, epitopes that are presented at lower levels of stimulation induce less severe exhaustion of their respective epitope-specific CD8+ T cells (Wherry et al., 2003a). Viral load during chronic infection also correlates with the severity of CD8+ T cell exhaustion (Fuller and Zajac, 2003; Wherry et al., 2003a; Virgin et al., 2009).

CD8+ T cell exhaustion was first described during chronic LCMV infection in mice; however, similar dysfunction has now been described in many human chronic infections, including HIV, HCV, HBV and others (Zajac and Ahmed, 1998; Gallimore and Zinkernagel, 1998; Moskophidis et al., 1993; Boni et al., 2007; Urbani et al., 2006). In addition, exhausted CD8+ T cells have also been identified in many cancer models, as well as in human cancer patients (Blank et al., 2004; Iwai et al., 2002; Muenst et al., 2013, 2010). Along with loss of poly-functionality, exhausted CD8+ T cells have several

other defining features. These include the up-regulation of negative regulatory pathways, altered expression of key transcription factors and antigen-dependent maintenance. First, exhausted CD8+ T cells aberrantly express high levels of multiple inhibitory receptors (discussed below) (Crawford and Wherry, 2009; Wherry et al., 2007; Blackburn et al., 2009). Immunoregulatory cytokines, such as IL-10 and TGF-β, have also been shown to contribute to CD8+ T cell dysfunction during chronic infection (Brooks et al., 2006; Tinoco et al., 2009; Wherry, 2011). Second, exhausted CD8+ T cells have a unique gene expression profile that is regulated by altered expression of effector- and memory-associated transcription factors (Wherry et al., 2007; Chang et al., 2014). For example, Blimp-1 is associated with terminal-differentiation of effector cells during acutely-resolved infection; however, elevated and sustained expression of Blimp-1 is associated with dysfunction of exhausted CD8+ T cells (Rutishauser et al., 2009; Kallies et al., 2009; Shin et al., 2009). Dysregulation of the central transcription factor BATF also seems to occur during T cell exhaustion (discussed below) (Quigley et al., 2010). In addition, Tbet and Eomes have been demonstrated to play critical roles in distinct subsets of exhausted CD8+ T cells during chronic infection (Kao et al., 2011; Paley et al., 2012). How these transcription factors cooperate to regulate gene expression and function of exhausted CD8+ T cells remains poorly understood. Finally, CD8+ T cell exhaustion is accompanied by the loss of classical memory properties, including poor antigen-independent maintenance and lack of responsiveness to IL-7 and IL-15 (Wherry et al., 2004). As a result, exhausted CD8+ T cells become “addicted” to antigen for survival and proliferation throughout the course of a chronic infection (Shin et al., 2007). Recent work has further characterized the mechanism responsible for long-term proliferation of exhausted CD8+ T cells. Subsets of exhausted CD8+ T cells exist in

a progenitor-progeny relationship and cooperate to balance continued proliferation with maintenance of CD8+ T cells for many years. High levels of Tbet expression and low levels of Eomes expression maintain the progenitor subset of exhausted CD8+ T cells. These $Tbet^{hi}$ cells have the ability to self-renew and also, through a process of extensive proliferation, give rise to progeny that are maintained by high expression of Eomes. In contrast to $Tbet^{hi}$ progenitor cells, $Eomes^{hi}$ cells cannot self renew and are more terminally-differentiated. These subsets have distinct phenotypic and functional properties (discussed below) and both are critical for durability of exhausted CD8+ T cell responses (Paley et al., 2012). The signals that regulate the establishment and maintenance of this progenitor-progeny relationship during chronic infection are unclear.

1.4: Inhibitory Receptors on Lymphocytes

Co-stimulatory and inhibitory receptors are critical regulators of adaptive immune cell function. In general, signal 2 from co-stimulation fine tunes TCR signaling through positive signals from molecules, such as CD28, ICOS, 4-1BB, CD27 and many others (Chen and Flies, 2013). These co-stimulatory signals can augment survival, T cell function, enhance metabolic activity of developing effector T cells and generally promote sustained responses. However, negative signals from inhibitory receptors, such as CTLA-4, PD-1 and others, balance and tune these positive signals from TCR and/or co-stimulation. A central role for inhibitory receptors is the regulation of autoimmunity and tolerance (Francisco et al., 2010). It is not surprising, therefore, that deficiencies in many inhibitory receptor pathways have been associated with autoimmunity in mice, including CTLA-4, PD-1 and others (Waterhouse et al., 1995; Tivol and and Sharpe, 2004; Nishimura and Honjo, 1998; Nishimura et al., 1999, 2001). Polymorphisms in inhibitory receptor genes are also associated with susceptibility to several human autoimmune

diseases, including diabetes, multiple sclerosis, and rheumatoid arthritis (James et al., 2005; Zhang et al., 2012; Velázquez-Cruz et al., 2007). This negative regulatory system has been co-opted, and perhaps diversified, to temper the immune response to infections, tumors, allografts, and perhaps even allergens (Keir et al., 2008). In many settings, efficient negative regulation by inhibitory receptor pathways may be critical to restrain and terminate immune responses, particularly those that could result in immunopathology. However, inhibitory receptors have also been shown to hinder the effective immune responses needed to clear some pathogens and tumors.

1.5: The Programmed Death-1 Pathway

The Programmed Death-1 (PD-1) pathway consists of the inhibitory receptor PD-1 and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) (Ishida et al., 1992; Freeman et al., 2000a; Latchman et al., 2001). PD-1, a member of the immunoglobulin (Ig) superfamily, is a 288 amino acid, type 1 transmembrane protein encoded by the *PDCD1* gene. Structurally, PD-1 is composed of an N-terminal IgV-like domain, a short stalk region, a transmembrane domain and a cytoplasmic tail. The cytoplasmic tail of PD-1 contains two tyrosine-based signaling motifs: an immunoreceptor tyrosine-based inhibition motif (ITIM) followed by an immunoreceptor tyrosine-based switch motif (ITSM) (Chemnitz et al., 2004; Okazaki et al., 2001; Zhang et al., 2004; Riley, 2009). PD-1 is expressed during thymic development on immature CD4- CD8- thymocytes and is inducibly expressed in the periphery on T cells, B cells, some NK cells and myeloid cells upon activation (Agata et al., 1996; Nishimura et al., 1996). Induction of PD-1 expression occurs by multiple mechanisms in different cell types. Signaling via the T cell receptor (TCR) or B cell receptor (BCR) induces upregulation of PD-1 expression within a few hours on T cells and B cells, respectively (Agata et al., 1996; Nishimura et al., 1996).

PD-1 expression is also induced by common γ-chain cytokines, including IL-2, IL-7, IL-15, and IL-12, or by IFN-α in some settings (Kinter et al., 2008; Terawaki et al., 2011).

The expression profiles of the PD-1 ligands, PD-L1 and PD-L2, differ dramatically. PD-L1 is expressed ubiquitously by many cell types, including myeloid cells (DCs and macrophages), B cells, T cells, stromal cells and a range of non-hematopoietic cells. PD-L2 expression is restricted primarily to professional antigen-presenting cells, such as DCs, macrophages and some B cells (Eppihimer et al., 2002; Yamazaki and Yagita, 2002; Keir et al., 2008). Of note, both PD-L1 and PD-L2 have binding partners in addition to PD-1. PD-L1 can also interact with B7-1 and PD-L2 with RGMb (Butte et al., 2007; Xiao et al., 2014). Inflammatory signals seem to regulate the expression of PD-L1 and PD-L2 in most cell types. Type I and II interferons induce expression of PD-L1 via two interferon regulatory factor-1 (IRF-1) bindings sites upstream of the transcriptional start site (Lee et al., 2006; Liang et al., 2003). Cytokine signaling also regulates expression of PD-L1 and PD-L2 (Loke and Allison, 2003).

1.5.1: PD-1 Signaling in T cells

PD-1 primarily mediates its inhibitory effects via the local and transient intracellular attenuation of positive signals from activating receptors, including TCR/CD3 and co-stimulatory receptors. Upon ligation, both the ITIM and ITSM within the cytoplasmic domain of PD-1 are phosphorylated, leading to recruitment of Src homology 2 (SH2) domain-containing tyrosine phosphatases (Parry and Riley, 2005; Okazaki et al., 2001; Riley, 2009). In T cells, mutagenesis and microscopy studies have implicated the interaction between phosphorylated ITSM and the phosphatase SHP2 as critical for PD-1 inhibitory function (Parry and Riley, 2005; Yokosuka et al., 2012). SHP2 can then

mediate the dephosphoryation of signaling molecules downstream of TCR/CD3 and CD28, including CD3z, Zap70, and PKCθ (Parry and Riley, 2005; Yokosuka et al., 2012; Riley, 2009). PD-1 also inhibits both the PI3K-Akt and Ras-MEK-ERK pathways, which dramatically impacts glucose metabolism and cell cycle regulation (Parry and Riley, 2005; Patsoukis et al., 2012). Recent work in primary CD4+ T cells has demonstrated that PD-1 inhibits cell cycle progression through the G_1 phase by preventing the activation of cyclin-dependent kinases (Cdks). PD-1 signaling can directly affect the expression, phosphorylation and degradation of key cell-cycle regulators (Patsoukis et al., 2012). Importantly, CD28 and/or IL-2 can override some, but not all, of PD-1 mediated inhibitory effects (Parry and Riley, 2005; Bennett et al., 2003; Freeman et al., 2000a). Thus, PD-1 causes a broad, quantitative reduction in TCR/CD3 activation-induced signal transduction and downstream gene expression. In addition, by interfering with co-stimulatory signaling pathways, PD-1 also hampers T cell metabolism, which has profound consequences on proliferation and survival.

Recently, other mechanisms of PD-1 mediated inhibition in T cells have also been elucidated. Using integrated genomic approaches, PD-1 signaling was shown to upregulate the expression of BATF in exhausted T cells from humans chronically infected with HIV and mice chronically infected with LCMV. Over-expression of BATF in primary human T cells reduced proliferation and IL-2 secretion upon stimulation. Conversely, effector functions could be rescued in exhausted T cells by shRNA-mediated silencing of BATF, further supporting an inhibitory function of BATF in T cells (Quigley et al., 2010). Thus, a mechanism of PD-1 inhibition may be to promote the aberrant upregulation of BATF expression in exhausted CD8+ T cells, which, in turn, has negative functional consequences. These findings suggest that inhibitory receptors not

only blunt positive signaling events from TCR and co-stimulatory receptors, but may also be capable of inducing transcriptional pathways that actively regulate immune cell function. Since BATF might not only act as a dominant negative regulator of normal AP-1 activity, but also have unique transcriptional activity, this mechanism of inhibitory receptor activity also suggests the potential to influence cellular differentiation.

1.5.2: Regulation of Effector and Memory CD8+ T cells by the PD-1 Pathway

PD-1 and its ligands are rapidly upregulated upon activation and throughout the effector phase of immune responses to acutely-resolved infections. Following antigen clearance, however, expression of PD-1 decreases to low levels similar to that on naïve CD8+ T cells (Barber et al., 2006; Brown et al., 2010). The potent ability of PD-1 to modulate TCR and co-stimulatory signals suggests that the PD-1 pathway could have both short-term and/or long-term consequences on CD8+ T cell responses to acutely-resolved infections. For example, PD-1 could regulate the activation and expansion of CD8+ T cells during the effector phase, as well as the differentiation of CD8+ T cells into long-lived memory cells. In addition, PD-1 pathway signals on CD8+ T cells, as well as other immune cells, could substantially alter the pathogenesis and outcome of infection.

Many groups have investigated the role of the PD-1 pathway in various settings of acutely-resolved infection using mice genetically deficient in PD-1 or PD-L1, as well as through the use of monoclonal blocking antibodies. In some cases, loss of PD-1 signals increases the effectiveness of anti-pathogen immune responses. For example, knocking out or blocking the PD-1 pathway in mice increases effector CD8+ T cell responses and survival following infection with *Histoplasma capsulatum*, rabies virus, human metapneumovirus (HMPV), influenza virus or respiratory syncytial virus (Lázár-Molnár et

al., 2008; Lafon and Wiendl, 2008; Erickson et al., 2012; Telcian et al., 2011). However, PD-1 pathway deficiency has also been shown to be detrimental in some settings of acutely-resolved infection. Although PD-1–deficient mice can more efficiently clear adenovirus from the liver than wild-type mice, they also develop more severe hepatocellular injury, likely due to an overaggressive adaptive immune response (Iwai et al., 2003). Other studies showed that the absence or blockade of PD-L1 reduced effector CD8+ T cell responses to *L. monocytogenes* in mice. In these examples, PD-L1 on innate immune cells, such as DCs and macrophages, plays an important role in CD8+ T cell activation and survival (Rowe and Sing Way, 2008; Talay et al., 2009; Yamazaki and Okumura, 2005; Yao et al., 2009). Thus, the PD-1 pathway seems to restrict effector CD8+ T cell responses during acutely-resolved infection. In some settings, this prolongs pathogen clearance, however, can promote the survival of highly activated effector CD8+ T cells and prevent detrimental immunopathology.

The PD-1 pathway has also been shown to regulate the differentiation and development of memory CD8+ T cells following acutely-resolved infections. Similar to regulation of the effector response by PD-1, there is conflicting data about how PD-1 pathway signals skew memory differentiation. Transient blockade of PD-1 signals can improve recall responses of memory $CD8^+$ T cells in some settings (Erickson et al., 2012, 2014). There is also evidence that genetic absence of PD-1 can skew memory CD8+ T cell differentiation towards either effector memory or central memory phenotypes in different contexts. Allie et al demonstrated that PD-1 pathway deficiency leads to enhancement of the quantity and quality of memory CD8+ T cell responses. In addition, these memory CD8+ T cells were skewed towards a central memory phenotype, with elevated expression of CD62L and CCR7 (Allie et al., 2011). In contrast,

other studies have demonstrated that PD-1 deficiency results in accumulation of effector memory CD8+ T cells that are CD62L^{lo} CCR7^{lo} (Charlton et al., 2013).

Thus, the precise role of PD-1 in regulating CD8+ T cell responses to acutely-resolved infections remains unclear. It is likely that the strength and duration of antigen stimulation, as well as the amount of inflammation, induced by different pathogens dramatically impacts how PD-1 regulates CD8+ T cell effector and memory formation (Obar and Lefrançois, 2010; Joshi et al., 2007; Kaech and Ahmed, 2001). In addition to regulating CD8+ T cell responses, the PD-1 pathway also appears critical for prevention of immunopathology during many acutely resolved infections, particularly for pathogens that infect sensitive tissues, such as the lung, liver, and brain (Iwai et al., 2003; Barber et al., 2006; Frebel et al., 2012; Lafon and Wiendl, 2008). **Chapter 2** in this thesis will investigate the role of PD-1 in effector and memory development to acutely-resolved viral infection.

1.5.3: Regulation of Exhausted CD8+ T cells by the PD-1 pathway

In contrast to acutely-resolved infections where PD-1 expression is transient, expression of PD-1 is highly elevated and sustained on exhausted CD8+ T cells for many months or years during chronic infection and cancer (Barber et al., 2006; Freeman et al., 2006; Blank et al., 2005; Wherry, 2011). Expression of PD-L1 and PD-L2 also remains elevated in these settings of sustained inflammation, particularly in the tumor microenvironment or in organs that harbor high pathogen loads (Blank et al., 2005; Blackburn et al., 2010b). This high level of PD-1/PD-L expression has profound consequences on anti-viral and anti-tumoral CD8+ T cell responses, which, in turn, influences the outcome of chronic infections and cancers. For example, high PD-1 expression on T cells has been shown to correlate with disease progression in several

human chronic infections, including HIV and HCV (Day et al., 2006; Freeman et al., 2006; Urbani et al., 2006). In addition, expression of PD-L1 on tumors corresponds with poor prognosis in many cancer settings (Muenst et al., 2013, 2010; Kozako et al., 2009; Topalian et al., 2012). The profound negative consequences of high PD-1/PD-L expression on exhausted CD8+ T cells has been demonstrated in many settings of chronic infection and cancer. Barber et al demonstrated that *in vivo* antibody-mediated blockade of the PD-1 pathway during chronic LCMV infection in mice significantly improves the magnitude and functionality of virus-specific CD8+ T cell responses. Administration of anti-PD-L1 blocking antibody resulted in virus-specific CD8+ T cells with improved cytokine production and cytotoxicity, which corresponded with a significant decline in viral loads (Barber et al., 2006). Soon thereafter, several groups showed upregulation of PD-1 expression on exhausted CD8+ T cells during human chronic viral infections, such as HIV, HCV, and HBV, and demonstrated improved function of T cells following *in vitro* PD-1/PD-L1 blockade or *in vivo* blockade of the PD-1 pathway in SIV-infected primates (Day et al., 2006; Freeman et al., 2006; Urbani et al., 2006; Maini et al., 1999; Velu et al., 2009). Increased expression of PD-1 and its ligands also impairs the effector responses against other persisting pathogens, such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Schistosoma mansoni*, *Leishmania donovani*, and *Toxoplasma gondii* (Wu et al., 2010; Jurado and Garcia, 2008; Lázár-Molnár et al., 2010; Liang et al., 2006; Bhadra et al., 2011). In mouse tumor models, similar improvements in exhausted CD8+ T cell functions have been demonstrated with loss of PD-1/PD-L signals. For example, knocking out or blocking PD-1 increases anti-tumor CD8+ T cell responses and improves survival in models of melanoma, renal cell carcinoma, and others (Blank et al., 2004, 2005; Okazaki et al., 2013). Thus, the PD-1/PD-L pathway is a central negative regulator of exhausted CD8+ T cells during

persisting infections and cancer. However, the mechanisms by which PD-1 regulates the development and maintenance of exhausted CD8+ T cells remain unclear. **Chapter 3** of this thesis will investigate the role of PD-1 in the induction of CD8+ T cell exhaustion.

Recent work has begun to elucidate the nuances of PD-1 regulation of exhausted CD8+ T cells (Blackburn et al., 2008; Paley et al., 2012; Kao et al., 2011). For example, $Tbet^{hi}$ and $Eomes^{hi}$ subsets of exhausted CD8+ T cells express different levels of PD-1 during chronic infection. $Tbet^{hi}$ progenitor cells express intermediate levels of PD-1. These $PD\text{-}1^{int}$ $Tbet^{hi}$ cells reside mainly in lymphoid tissues and maintain some ability to produce cytokines. While they possess low intrinsic turnover, $PD\text{-}1^{int}$ $Tbet^{hi}$ cells can robustly proliferate to give rise to a large population of CD8+ T cells that express high levels of PD-1 and the transcription factor Eomes. $PD\text{-}1^{hi}$ $Eomes^{hi}$ cells abundantly populate non-lymphoid organs, co-express several other inhibitory receptors and, while they are less efficient cytokine producers, maintain the ability to kill. The cooperative balance between $PD\text{-}1^{int}$ $Tbet^{hi}$ progenitor cells and $PD\text{-}1^{hi}$ $Eomes^{hi}$ progeny cells is critical for long-term maintenance of CD8+ T cell responses to chronic LCMV infection in mice (Paley et al., 2012). Importantly, while $PD\text{-}1^{int}$ $Tbet^{hi}$ cells are partially rescued by *in vivo* PD-1/L1 blockade, $PD\text{-}1^{hi}$ $Eomes^{hi}$ cells appear more terminally-differentiated and unresponsive to *in vivo* PD-1 pathway blockade (Blackburn and Wherry, 2008). Similar subsets of $Eomes^{hi}$ and $Tbet^{hi}$ CD8+ T cells have also been identified during chronic HCV and HIV infections (Paley et al., 2012; Buggert et al., 2014). How PD-1 regulates these different subsets of exhausted CD8+ T cells remains unclear. **Chapter 3** in this thesis will extend our understanding of the role of PD-1 on $Tbet^{hi}$ and $Eomes^{hi}$ cells.

1.5.4: Clinical Use of PD-1 Pathway Blockade

Recent clinical trials have extended many of these observations from mouse models to humans, demonstrating a potent ability of PD-1 pathway blockade to revitalize anti-tumor immunity in late-stage cancer patients. Phase 1 clinical trials with a fully human IgG4 anti-PD-1 antibody (nivolumab: Bristol-Myers Squibb) began in 2006. Tumor regression was observed by 18% of patients with non-small-cell lung cancer, 28% of patients with melanoma, and 27% of patients with rencal cell carcinoma, with very few immune-related toxicities observed (Topalian et al., 2012). Similar results were obtained around the same time in clinical trials using a fully human antibody against PD-L1 (Brahmer et al., 2012). Clinical responses were associated with infiltration of lymphocytes into metastatic tumors and with the expression of PD-L1 by tumors (Brahmer et al., 2012; Topalian et al., 2012; Pardoll, 2012). Importantly, these PD-1 pathway inhibitors demonstrated the greatest anti-tumor activity of any immunotherapeutic approach tested in the clinic for the past 30 years. Several antibodies targeting PD-1 and/or PD-L1 are currently in the clinical trial pipeline (ClinicalTrials.gov). Recently, Keytruda (pembrolizumab: Merck) became the first PD-1 inhibitor to be approved by the FDA for use in patients with advanced melanoma (FDA.gov).

1.6: The Lymphocytic Choriomeningitis Virus Model

The lymphocytic choriomeningitis virus, or LCMV, model of infection has been invaluable in the research community as a tool to study viral persistence, host-pathogen interactions, and anti-viral immunology. LCMV is an enveloped, ambisense RNA virus in the *Arenaviridae* family. The natural reservoir of LCMV is the common mouse, *Mus*

musculus. However, LCMV can cause disease in humans who are exposed to an infected rodent (Zhou et al., 2012; De La Torre, 2009). LCMV enters cells via binding of the GP-1 protein to its receptor, α-dystroglycan (Cao et al., 1998; Kunz, 2009). Due to the broad tissue expression of α-dystroglycan, LCMV can infect multiple cell types and, as a result, causes a systemic infection in mice. Despite widespread tissue distribution, LCMV is a non-cytopathic virus, which means that in naturally-infected mice there is minimal or no pathology associated with infection. In contrast, during experimental infection with LCMV strains, pathology is strictly immune-mediated (Zhou et al., 2012; Frebel et al., 2012; Borrow and Oldstone, 1999; Oldstone, 2006). There are multiple strains of LCMV that cause distinct courses of infection in mice. Here, the primary strains of interest are LCMV Armstrong (Arm), which causes an acutely-resolved infection in mice, and LCMV clone 13, which causes a systemic, chronic infection in mice (Matloubian and Ahmed, 2003; Ahmed et al., 1984; Dutko and Oldstone, 2011). Importantly, CD8+ T cell anti-viral activity is critical for the control of both of these strains of LCMV. For example, loss of IFN-γ or perforin results in lack of viral clearance and/or control during LCMV infection (Walsh et al., 1994; Dorman et al., 1999; Bartholdy et al., 2000). Dominant epitopes of LCMV that are recognized by CD8+ T cells are conserved between LCMV Armstrong and clone 13, allowing for analysis of CD8+ T cells with the same TCR specificity in these two different infection scenarios (Matloubian et al., 1990; Butz and Bevan, 1998; van der Most et al., 1998). Thus, LCMV infection of mice is an ideal model for the investigation of anti-viral CD8+ T cell responses.

1.6.1: LCMV Strains: Armstrong and clone 13

Infection with LCMV Arm causes an acutely-resolved infection in immunocompetent mice, with viral replication in the spleen, liver and kidney for approximately 10 days. Clearance of virus is associated with a potent anti-viral CD8+ T cell response that generates robust T cell memory (Salvato et al., 1988; Wherry et al., 2003a; Dutko and Oldstone, 2011). A variant of LCMV Arm, known as LCMV clone 13, leads to the establishment of a chronic infection in mice. LCMV clone 13 differs from LCMV Arm by only 2 amino acid changes, a single change in the polymerase protein and a single change in the GP-1 protein. Alterations in polymerase lead to enhanced replication of LCMV in dendritic cells, as well as increased viral titers in macrophages (Sevilla et al., 2000; Cao et al., 1998). In addition, the mutation in GP-1 results in higher affinity binding of LCMV to α-dystroglycan, which causes increased early infection of follicular dendritic cells and more severe destruction of lymphoid architecture (Mueller et al., 2007; Matloubian et al., 1990; Borrow and Oldstone, 1995). Infection of mice with LCMV clone 13 leads to systemic viral replication, with viremia lasting 2-3 months and viral reservoirs persisting in many tissues for the life of the mouse (Oldstone, 2009; Wherry et al., 2003a). CD8+ T cell responses to LCMV clone 13 become exhausted over time and are also diminished in magnitude compared to LCMV Arm (Zajac and Ahmed, 1998; Gallimore and Zinkernagel, 1998). Loss of CD4+ T cell help leads to life-long viremia during LCMV clone 13 and, as a result, more severe CD8+ T cell exhaustion (Matloubian et al., 1994; Blackburn et al., 2009). **Chapter 3** will utilize LCMV clone 13 as a model of chronic viral infection.

1.7: Influenza Virus Infection

Influenza A virus is a pathogen of significant human public health concern, as thousands of deaths occur from influenza infection each year (CDC, 2013). Influenza A belongs to the *Orthomyxoviridae* family and contains a negative-sense, segmented RNA genome. Subtyping of influenza strains is determined by properties of the two major influenza surface glycoproteins: haemagglutinin (HA) and neuraminadase (NA) (Bouvier and Lowen, 2010). Both proteins play important roles in the binding of influenza to its receptor, sialic acid, and entry into cells (Medina and García-Sastre, 2011; Mueller et al., 2010). Mice are not a natural host of influenza; however, several strains have been adapted by serial passage for use in mouse models. Intranasal infection of mice leads to infection of epithelial cells in the bronchi and alveoli. As influenza is a cytopathic virus, infection of epithelial cells leads to severe lung damage and pneumonitis (Rutigliano et al., 2014; Askovich et al., 2013; Bouvier and Lowen, 2010). Pathogenesis in mice can be monitored by weight loss, decreased blood oxygen saturation and, ultimately, survival (Blazejewska et al., 2011; Laidlaw et al., 2013; Askovich et al., 2013). Studies have shown that many arms of the immune response, including CD8+ T cells, CD4+ T cells, B cells, and NK cells, are important for viral clearance during primary infection, as well as for protection against secondary infection (Laidlaw et al., 2013). The focus of these studies is on CD8+ T cell responses to influenza infection. Following infection of lung epithelial cells, respiratory dendritic cells (DCs) carrying viral antigen drain to the mediastinal lymph node where $CD8^+$ T cell priming occurs (Kim and Braciale, 2009; Yoon et al., 2007). Some $CD8^+$ T cell priming also likely occurs in the spleen, mediated by DCs migrating from the lungs. Activated $CD8^+$ T cells then migrate back to the

infected lung where lung-resident DC populations sustain effector T cell responses that mediate viral clearance (McGill et al., 2008; Kim and Braciale, 2009).

1.7.1: Influenza Virus Mouse Model Strains: PR8 and X31

While many mouse adapted strains of influenza virus exist, these studies focus on the viruses PR8 and X31. PR8 (A/Puerto Rico/8/1934) is an H1N1 virus that was originally derived from a human influenza isolate. X31, in contrast, is an H3N2 virus with 6 of the internal genes of PR8 and the HA and NA genes derived from A/Aichi/2/1968 (Bouvier and Lowen, 2010; Medina and García-Sastre, 2011; Mueller et al., 2010). Intranasal infection with X31 virus leads to approximately 20-25% weight loss by day 4-5; however, all mice will recover and survive the infection. In contrast, PR8 infection typically leads to death of mice by day 5-6 post-infection. Consistent with these disease outcomes, PR8 virus infection was shown to result in 4x higher viral load in the lungs of mice at 18 hours post-infection compared to X31 virus infection (Askovich et al., 2013; Bouvier and Lowen, 2010; Laidlaw et al., 2013). In the following studies, recombinant PR8 and X31 viruses were utilized that contain the GP33-41 epitope of LCMV within the NA stalk region. These recombinant influenza viruses induce robust GP33-specific CD8+ T cell responses in mice (Mueller et al., 2010).

CHAPTER 2: PD-1 regulates the development and function of memory CD8+ T cells following respiratory viral infection

Equal contribution by Jernej Godec and Arlene Sharpe, Ph.D.

2.1: Introduction

The development of functional effector and memory CD8+ T cells following acute infection or vaccination requires the coordinated integration of key signals from antigen-TCR (signal 1), co-stimulation (signal 2) and inflammation (signal 3) (Curtsinger et al., 1999; Smith-Garvin et al., 2009; Acuto and Michel, 2003). The quantity and quality of signals 1, 2, and 3 can impact CD8+ T cell activation and the subsequent development of memory, but exactly how such signals regulate memory CD8+ T cell differentiation is unclear (Iezzi et al., 1998; Tubo et al., 2013; Obar and Lefrançois, 2010). In particular, signal 2 from co-stimulatory and/or co-inhibitory pathways has come under intense focus given the recent application of antibodies blocking co-inhibitory receptors, such as PD-1, for the treatment of cancer and chronic infections (Pardoll, 2012). Disrupting the PD-1:PD-L pathway during chronic infections and cancer can enhance T cell activity and improve outcomes (Barber et al., 2006; Topalian et al., 2012; Okazaki et al., 2013). In addition, clinical trials with monoclonal antibodies targeting this pathway have shown impressive 30–50% response rates in melanoma and multiple other cancers, including renal cell and non-small cell lung carcinoma (Brahmer et al., 2012; Topalian et al., 2012). Despite the clinical promise of PD-1 pathway blockade, how this pathway regulates the formation and/or differentiation of CD8+ T cell memory remains poorly understood.

In contrast to the well-established role for PD-1 inhibitory functions in mediating T cell exhaustion, there are conflicting data about the role of the PD-1 pathway in controlling effector and memory CD8+ T cell differentiation during the acute phase of infection. Lack of PD-1:PD-L signals during primary infection with rapidly disseminating strains of LCMV results in lethal T cell-mediated immunopathology during the first week of infection, consistent with an inhibitory role for this pathway during acute infection (Frebel et al., 2012; Barber et al., 2006). Absence of PD-1 signals was also reported to enhance the establishment of CD8+ T cell memory and/or skew T cells toward central memory in some cases (Allie et al., 2011). In addition, the secondary expansion of unhelped memory $CD8^{+}$ T cells during acute re-challenge could be improved by the blockade of PD-1:PD-L signals (Fuse et al., 2009). Other studies, in contrast, have reported that loss or blockade of the PD-1 pathway during acute infection can diminish T cell responses, suggesting a positive role for PD-1:PD-L interactions in other settings (Rowe and Sing Way, 2008; Talay et al., 2009; Yao et al., 2009). The reasons for these discrepancies and the precise role of the PD-1 pathway in the development of effector and memory CD8+ T cells following acute infections, therefore, remains incompletely understood.

One setting where the PD-1:PD-L pathway may be particularly important is in the respiratory tract. During virulent LCMV infection in PD-1 KO mice, damage to the pulmonary endothelium ultimately causes respiratory failure and contributes to mortality (Frebel et al., 2012). In addition, while human anti-PD-1 cancer immunotherapy has been generally well tolerated, several incidents of pneumonitis have raised concerns about respiratory complications of PD-1 blockade (Brahmer et al., 2012; Topalian et al., 2012). In mouse models of respiratory infections, PD-1:PD-L signals also have been implicated in regulating immune responses. PD-1 expression is upregulated on

responding CD8+ T cells during highly pathogenic influenza virus infection in mice and during human metapneumovirus (HMPV) infection. Furthermore, increased PD-L1 and PD-L2 expression in the respiratory tract during infection and inflammation may enhance signals through this pathway (Erickson et al., 2012; Keir et al., 2008). Thus, in the respiratory tract, PD-1:PD-L interactions have the potential to inhibit CD8+ T cell function, which may limit pulmonary tissue damage, but could also impair optimal pathogen control (Erickson et al., 2012). A better understanding of the consequences of PD-1 signals during acute respiratory infections is needed to determine how PD-1 controls the balance between the generation of protective immunity and immunopathology.

To address these issues we interrogated the role of the PD-1 pathway in effector and memory CD8+ T cell differentiation during influenza virus infection in the respiratory tract. Specifically, we investigated how disruption of PD-1:PD-L interactions impacted the induction, stability, and longevity of effector and memory CD8+ T cells following respiratory infection of mice lacking PD-1 (PD-1 KO) or both PD-L1 and PD-L2 (PD-L1/L2 DKO). Lack of PD-1:PD-L signals led to a striking compromise in $CD8^+$ T cell memory, including reduced memory CD8+ T cell numbers and impaired secondary responses. This defect in influenza-specific $CD8^+$ T cell memory in PD-1 KO and PD-L1/L2 DKO mice was associated with increased CD8+ T cell proliferation and expansion early during infection, as well as increased contraction and loss of developing memory CD8+ T cells during the effector to memory transition compared to WT mice. These effects were cell intrinsic because PD-1 KO CD8+ T cells adoptively transferred to WT mice had similar defects. These results are consistent with a role for PD-1:PD-L signals in limiting CD8+ T cell activation during acute infection. However, these data also indicate a crucial and previously unappreciated role for PD-1:PD-L signals in tempering

the strength of initial activation to promote or “tune” optimal CD8+ T cell memory formation. This tuning function of the PD-1 pathway during acute infection has implications for clinical use of PD-1/PD-L1 blockade, including important effects on the generation of CD8+ T cell memory following PD-1 cancer immunotherapy. This novel tuning function also suggests that it may be possible to exploit the PD-1:PD-L pathway to optimize immune memory following acute infection or vaccination

2.2: Methods

2.2.1: Mouse Strains

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice lacking PD-1 (PD-1 KO) or both PD-1 ligands, PD-L1 and PD-L2 (PD-L1/L2 DKO), have been described previously (Keir et al., 2007a, 2006). PD-1 KO P14 mice were generated by crossing germline PD-1 KO mice to P14 TCR transgenic mice (Pircher et al., 1989). PD-1 KO (Ly5.2+ or Ly5.2+Ly5.1+) and WT (Ly5.2+Ly5.1+ or Ly5.2+) P14 cells were isolated from peripheral blood and transferred i.v into C57BL/6 recipient mice at a 1:1 ratio (500 WT P14 cells:500 KO P14 cells). All mice were maintained in a pathogen-free facility and used according to institutional and National Institutes of Health guidelines. Harvard Medical School and the University of Pennsylvania School of Medicine are accredited by the American Association of Accreditation of Laboratory Animal Care.

2.2.2: Viral Infection

For primary infection, 6 to 12 week old mice were infected with recombinant influenza virus expressing the LCMV GP33-41 peptide (X31-GP33; $1.6x10^5$ $TCID_{50}$)

intranasally (i.n.). For re-challenge infection, mice were infected with PR8-GP33 ($10LD_{50}$ i.n) at least 35 days after primary infection. Recombinant influenza strains containing the LCMV GP_{33-41} epitope inserted into the neuraminidase protein stalk region were provided by Dr. Richard Webby (St. Jude Children's Research Hospital, Memphis, TN) (Decman et al., 2010; Mueller et al., 2010). These recombinant influenza strains replicate and exhibit pathogenicity similar to their non-recombinant counterparts. Prior to infections, mice were anesthetized by intraperitoneal (i.p.) injection of 2.5% Avertin (Sigma-Aldrich). Viral titers in lungs were determined by quantitative real-time PCR as described (Laidlaw et al., 2013).

2.2.3: Isolation of Cells from Tissues

Mouse lungs were mechanically disrupted in a GentleMACS Tissue Dissociator (Miltenyi Biotech) and incubated with 2 mg/ml collagenase D (Worthington Biochemical Corp.), and 3 μg/ml DNase I (Sigma) at 37°C for 30 min. Red blood cells (RBCs) were lysed using ACK lysis buffer (Lonza). The resulting single cell suspensions were filtered through a 70 mM cell strainer (BD Falcon). Spleen and lymph nodes were homogenized using a cell strainer and RBCs lysed with ACK lysing buffer. Single cell suspensions were stained, fixed with 2% paraformaldehyde (Electron Microscopy Sciences) and analyzed on an LSR II (BD Biosciences). For intracellular cytokine staining (IFN-γ and TNF-α), samples were stimulated with 0.5 μg/ml influenza A $NP_{366-374}$ peptide or GP_{33-41} peptide (both Genscript) or no peptide for 5 hours at 37°C in the presence of GolgiPlug (BD Biosciences), and then surface stained, fixed/permeabilized, and intracellularly stained using the Cytofix/Cytoperm kit (BD Biosciences), as directed by the manufacturer. For live/dead dye analysis, samples were washed in PBS (Gibco) and

stained with Live/Dead green fluorescent reactive dye (Invitrogen) in PBS at room temperature for 15 minutes, washed twice with PBS and then surface stained for analysis.

2.2.4: Antibodies, Tetramers, and Flow Cytometry

Antibodies to CD8 (53-6.7), CD44 (IM7), IFN-γ (XMG1.2), TNF-α (MP6-XT22), CD127 (SB/199), CD122 (TM-b1), CD62L (MEL-14) CD45.1 (A20), CD45.2 (104), CD103 (2E7) were purchased from Biolegend. Antibody to Ki-67 was purchased from BD Biosciences. The KLRG1 mAb (2F1) was purchased from Abcam. Poly-caspase analysis was performed with Flica Vybrant FAM Poly Caspases Assay Kit (Life Technologies). Dead cell exclusion was performed by live/dead fluorescent reactive dye (Invitrogen) staining. Tetramers for GP_{33-41}, $NP_{366-374}$, and $PA_{224-233}$ were prepared and used as described (Laidlaw et al., 2013). Flow cytometry data were acquired on BD LSR II flow cytometer and analyzed using FlowJo software (Tree Star).

2.2.5: BrdU Treatment and Detection

Animals were treated with 2mg of BrdU (Sigma-Aldrich) i.p. at the indicated time prior to tissue harvest and analysis. BrdU incorporation was assessed by the BrdU Flow Kit per manufacturer's instructions (BD Biosciences).

2.2.6: Statistical Analysis

Data were analyzed using Student's t-test for normally distributed data and p values <0.05 were considered significant.

2.3: Results

2.3.1: Virus-specific CD8+ T cell memory development is impaired in mice lacking PD-1 signals

To investigate the impact of PD-1 pathway deficiency on CD8+ T cell memory development following acute respiratory viral infection, we infected WT, PD-1 KO or PD-L1/L2 DKO mice with influenza virus X31-GP33 (H3N2) and examined virus-specific $CD8^+$ T cells 60-85 days later **(Figure 2.1A)**. The frequency and total number of virus-specific memory $CD8^+$ T cells were reduced in the lungs of PD-L1/L2 DKO **(Figure 2.1B)** and PD-1 KO mice **(Figure 2.1C)**. While true for all responses examined, the magnitude of the difference was greater for subdominant CD8+ T cell responses (PA_{224}-specific and GP_{33}-specific) than for immunodominant NP_{366}-specific responses **(Figure 2.1B and 2.1C)**. A similar decrease in CD8+ T cell responses was observed in the spleen, mediastinal (lung-draining) lymph node (dLN), and bone marrow (data not shown). While the expression of KLRG1, CD127 (IL-7R), CD103, and CD69 was similar between WT and PD-L1/L2 DKO mice **(Figure 2.2A)**, fewer memory CD8+ T cells from the DKO mice produced IFN-γ and TNF-α upon *ex vivo* re-stimulation with influenza peptides **(Figure 2.2B)**.

Figure 2.1

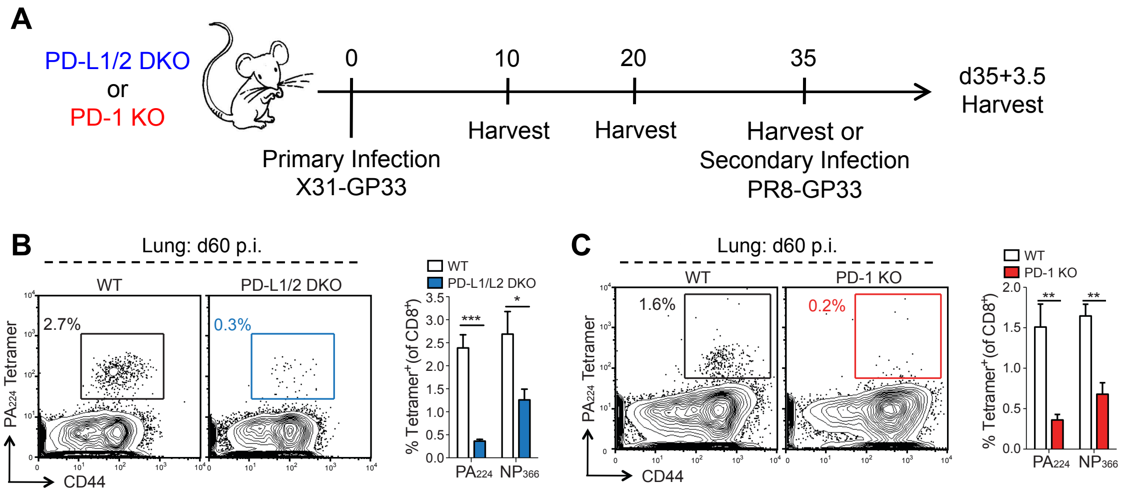


Figure 2.1: Magnitude of CD8+ T cell memory response is impaired in PD-1 pathway-deficient mice. (A) Schematic of experimental design. **(B)** Representative plots (left) show the frequency of H-2D^b PA224+ CD8+ T cells in the lungs of WT or PD-L1/L2 DKO mice on d60+ p.i. with X31-GP33. Numbers indicate percent CD8+ T cells positive for PA224+ tetramer. Summary of frequencies of CD8$^+$ T cells specific for other influenza epitopes is shown to the right. **(C)** Representative plots (left) show the frequency of H-2D^b PA224+ CD8+ T cells in the lungs of WT or PD-1 KO mice on d60+ p.i. with X31-GP33. Numbers indicate percent CD8$^+$ T cells positive for PA224+ tetramer. Summary of frequencies of CD8$^+$ T cells specific for other influenza epitopes is shown to the right.

Figure 2.2

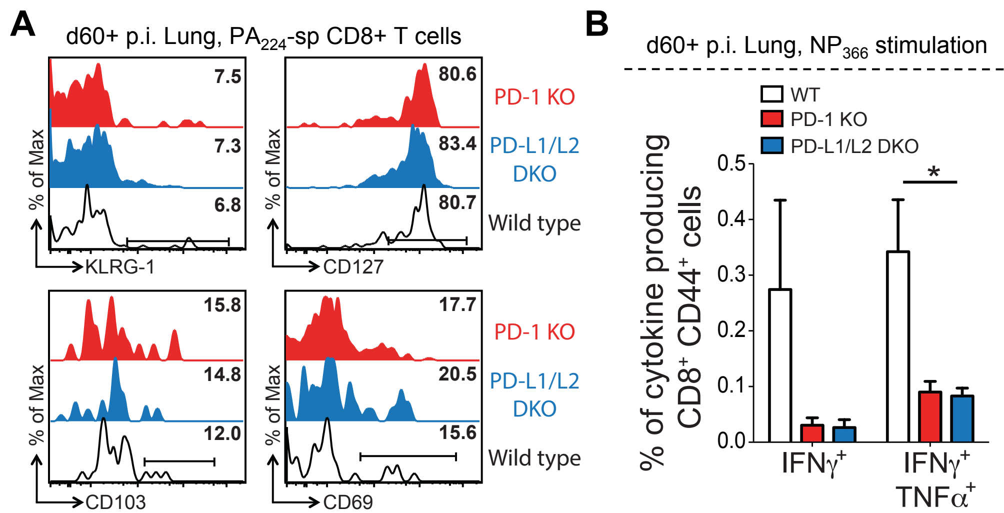


Figure 2.2: Quality of CD8+ T cell memory is impaired in PD-1 pathway-deficient mice. (A) Representative plots of flow-cytometric analyses of expression of memory and resident-memory markers on PA224+ CD8+ T cells in the lungs of WT or PD-L1/L2 DKO mice on d60+ p.i. with X31-GP33 (**B**) Summary of fraction of cytokine-producing CD8+ T cells from the lung following *ex vivo* stimulation with NP366 peptide on d60+ p.i.

A hallmark property of memory T cells is the ability to mount robust secondary responses upon re-infection. Recent studies suggest that disruption of PD-1:PD-L interactions during secondary challenge may improve CD8+ T cell recall responses (Erickson et al., 2012, 2014). However, it is unclear whether PD-1:PD-L interactions during primary viral infection promote or limit the formation of memory $CD8^+$ T cell populations capable of mounting potent secondary responses. To test the ability of memory CD8+ T cells that developed without the PD-1:PD-L pathway to mount secondary responses, we re-challenged X31-GP33 immune WT, PD-1 KO and PD-L1/L2 DKO mice with the heterotypic H1N1 strain of influenza virus, PR8-GP33. Since neutralizing antibodies do not cross-react between these viruses, memory CD8+ T cells play a central role in recall responses and protective immunity in this setting (Flynn et al., 1998; Mueller et al., 2010). PD-1 KO and PD-L1/L2 DKO mice had significantly increased viral titers in the lung compared to WT mice 3.5 days (d3.5) following re-challenge **(Figure 2.3A)**. This defect in viral control was accompanied by more severe weight loss in PD-1 KO and PD-L1/L2 DKO mice, while WT mice showed minimal morbidity **(Figure 2.3B)**. Failure to efficiently control re-challenge in PD-1 KO and PD-L1/L2 DKO mice was associated with lower frequencies and absolute numbers of influenza-specific CD8+ T cells in the lung at d3.5 post re-challenge **(Figure 2.3C and data not shown)**. Furthermore, fewer CD8+ T cells from PD-L1/L2 DKO mice produced cytokines upon influenza peptide stimulation at d3.5 post re-challenge **(Figure 2.3D)**. By d7 post re-challenge, however, the frequencies and numbers of virus-specific CD8+ T cells in the lungs were comparable in WT and PD-L1/L2 DKO mice and virus was cleared (data not shown). Thus, the defect in memory CD8+ T cell responses in PD-L1/L2 DKO mice manifests as a delay in the kinetics of viral clearance. This change in protective immunity is associated with reduced ability to mount a robust influenza-

specific secondary CD8+ T cell response. Taken together, these data indicate a key role for the PD-1 pathway in regulating the development of optimally functional CD8+ T cell memory.

Figure 2.3

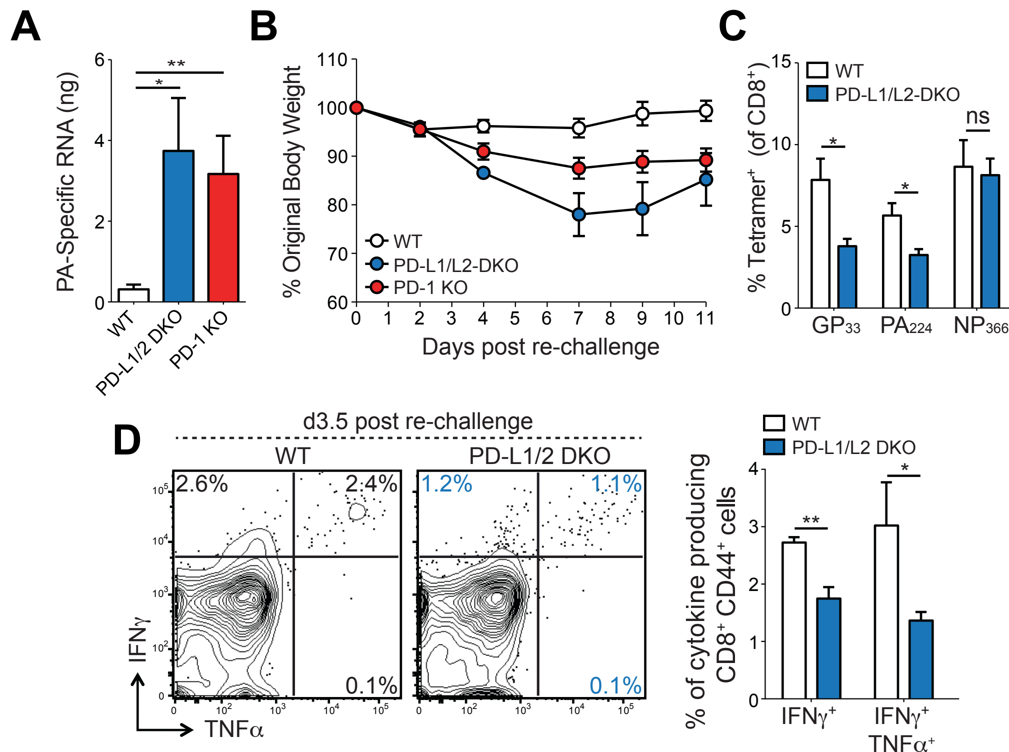


Figure 2.3: Defective recall capacity of memory CD8+ T cells in PD-1 pathway deficient mice. **(A)** Influenza viral titers in the lung at d3.5 p.i. and longitudinal weight loss (**B**) in X31-GP33 immune WT and PD-L1/L2 DKO mice following re-challenge with PR8-GP33. **(C)** Frequencies of indicated tetramer+ CD8+ T cells in lungs of X31-GP33 immune WT and PD-L1/L2 DKO mice at d3.5 following re-challenge. **(D)** Representative plots of intracellular cytokine staining (ICS) for IFN-γ and TNF-α production in lung CD8+ T cells from (D) stimulated ex vivo with NP366. Fraction of responding cells summarized to the right. Data are representative of 3-5 independent experiments with 4-5 mice per experiment.

2.3.2: The PD-1 pathway regulates the memory CD8+ T cell compartment by controlling initial $CD8^+$ T cell expansion and contraction.

Given the ability of PD-1 to modulate T cell activation, we hypothesized that alterations in CD8+ T cell memory may stem from early changes in T cell priming and effector differentiation. To test this possibility, we compared influenza-specific CD8+ T cell responses at the height of the primary response, d8-10 post-infection, in WT and PD-L1/L2 DKO mice infected with X31-GP33 virus. While all mice had similar frequencies of influenza-specific effector CD8+ T cells in the lung **(Figure 2.4A)**, there was a significant increase in the total number of CD8+ T cells in PD-L1/L2 DKO mice, consistent with a negative regulatory role for this pathway (**Figure 2.4B**). However, this enhanced expansion of CD8+ T cells in PD-L1/L2 DKO mice was associated with a subsequent decrease in the frequency of influenza-specific CD8+ T cells in these mice during the contraction phase, d15-20 p.i. (data not shown). While there was no significant difference in total numbers at d15-20 (data not shown), the ratio of DKO:WT virus-specific $CD8^+$ T cells began to dramatically decline after d10 p.i. **(Figure 2.4C)**. Of note, the immunodominant NP366-specific CD8+ T cell responses displayed slower kinetics in this deterioration **(Figure 2.4C)**. Thus, while PD-1 pathway deficiency led to an initial increase in virus-specific CD8+ T cells, there was an erosion of virus-specific CD8+ T cell responses in PD-L1/L2 DKO mice during the establishment of immunological memory. Analysis of CD8+ T cell functionality at d10 p.i. revealed no significant differences in the frequency of cytokine-producing cells in the lungs of WT, PD-1 KO or PD-L1/L2 DKO mice or the quality of the cytokine response on a per cell basis **(Figure 2.4D)**, suggesting that the major effect of removing PD-1 signals was enhanced numerical expansion. These findings suggest that PD-1 negatively regulates

CD8+ T cell effector differentiation predominantly by limiting overall CD8+ T cell expansion. Furthermore, these data also suggest suboptimal transition to memory without PD-1 or PD-L1/L2 signals.

Figure 2.4

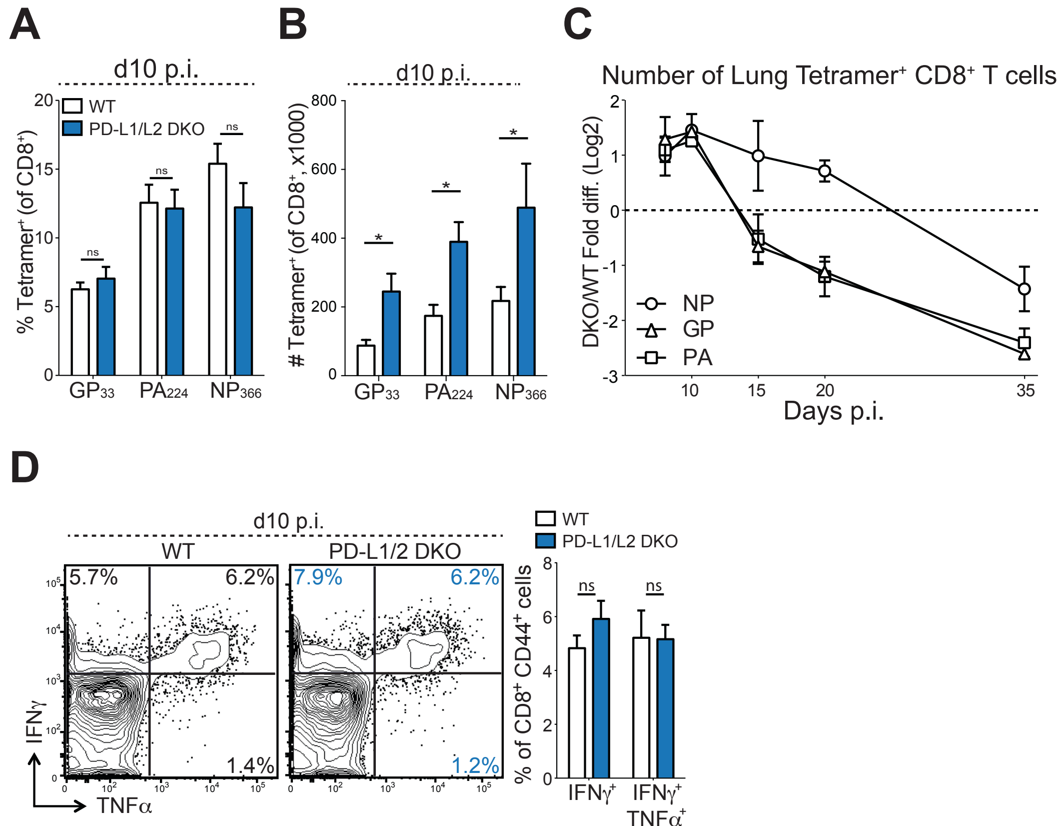


Figure 2.4: Altered effector CD8+ T cell expansion and contraction in the absence of PD-1 pathway signals. (A, B) Frequencies (A) and numbers (B) of indicated tetramer+ CD8+ T cells in the lungs of WT and PD-L1/L2 DKO mice at d10 after primary X31-GP33 infection. **(C)** Ratios of numbers of influenza-specific CD8+ T cells in PD-L1/L2 DKO vs. WT mice over time after primary X31-GP33 infection. (**D**) Representative plots of intracellular cytokine staining for IFN-γ and TNF-α production in lung CD8+ T cells following *ex vivo* stimulation with NP_{366}. Representative plots gated on CD8+

CD44+ T cells (left) and summary of fraction of percent responding cells (right) from 5 mice per group.

2.3.3: Increased turnover of CD8+ T cells in the absence of PD-1 signaling.

To better understand the differences in effector and memory CD8+ T cell development in the absence of PD-1 signals, we performed transcriptional profiling of influenza-specific CD8+ T cells from the lungs of WT and PD-L1/L2 DKO mice at d8 p.i. We focused these analyses on subdominant (PA244 and GP33) influenza-specific CD8+ T cells, since profound phenotypic and functional differences were observed in these populations at d8 p.i. Gene set enrichment analysis (GSEA) using Gene Ontology (GO) was used to elucidate pathways and biological processes uniquely regulated in the absence of PD-1 signals. Most of the significantly enriched pathways ($p<0.001$, $FDR<0.001$) were related to cell division **(Figure 2.5A)**. We further analyzed three representative pathways enriched in the WT cells – mitosis, spindle assembly, and DNA replication **(Figure 2.5B)**. Among those genes highly over-expressed in WT relative to PD-L1/L2 DKO CD8+ T cells, the majority were members of one or more of these enriched pathways **(Figure 2.5C)**. These data suggest a direct link between PD-1:PD-L signals and control of cell cycle during early activation and proliferation of virus-specific $CD8^+$ T cells during influenza infection.

Figure 2.5

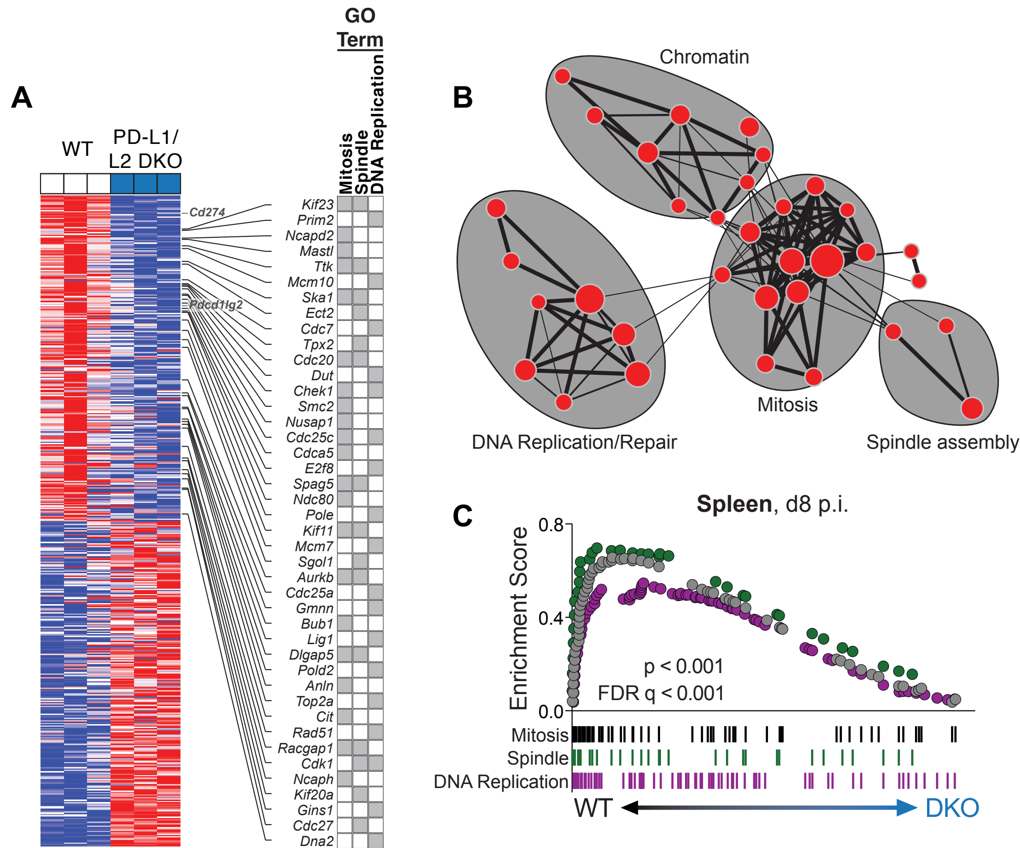


Figure 2.5: Altered expression of genes representing cell division pathways in WT versus PD-L1/L2 DKO virus-specific CD8+ T cells. (**A**) Top and bottom 250 differentially expressed genes in subdominant-epitope specific WT and DKO CD8+ T cells ranked by signal-to-noise values following filtration to top 10% of genes with highest mean absolute deviation across samples. Membership of genes in the three representative GO terms is shown on the right. **(B)** Significantly enriched (FDR<0.01) gene sets in WT CD8+ T cells clustered based on the extent of gene member overlap and annotated for the biological states/processes they represent. **(C)** Representative GO gene sets enriched in transcriptional profiles from virus-specific CD8^{+} T cells from the WT versus PD-L1/L2 DKO mice. Gene expression microarray was performed to compare subdominant influenza epitope-specific lung CD8+ T cells isolated from WT and PD-L1/L2 DKO mice at d8 following primary X31-GP33 infection.

Based on GSEA alone, it is challenging to determine whether enrichment of replication and cell division pathways in CD8+ T cells from WT versus PD-L1/L2 DKO mice reflects altered proliferation or if these gene expression changes could simply be due to differences in the contraction of WT versus DKO CD8+ T cell responses. To directly investigate the mechanism responsible for the quantitative differences in early WT and PD-1 pathway deficient CD8+ T cell responses, we compared proliferation and cell death of influenza-specific CD8+ T cells on d6-8 p.i. in the lung, spleen, and dLN of WT and PD-L1/L2 DKO mice. At d8 p.i., more influenza-specific CD8+ T cells in the spleens of PD-L1/L2 DKO mice expressed the cell cycle protein Ki-67 compared to WT mice, suggesting increased proliferation in the absence of PD-1 signaling **(Figure 2.6A**). These findings are consistent with the higher cell numbers observed in the lung of PD-L1/L2 DKO mice at d10 p.i. **(Figure 2.4B)**. However, significantly fewer virus-specific CD8+ T cells were Ki-67+ in the lungs of PD-L1/L2 DKO mice **(Figure 2.6A)**. These findings suggest that the increased number of CD8+ T cells in the lungs of PD-1 KO and PD-L1/L2 DKO mice at the early stages of infection is largely due to robust proliferation in lymphoid organs and homing/trafficking of these CD8+ T cells to the lung. Interestingly, PD-L1/L2 DKO mice had a higher frequency of influenza-specific CD8+ T cells that were positive for active caspase staining (FLICA) in the lung, but not in the spleen on day 8 p.i. **(Figure 2.6B and data not shown)** compared to WT mice, consistent with the increased contraction of the DKO populations. This increase in apoptosis of PD-L1/L2 was further confirmed by Annexin V staining in the lung (data not shown). Thus, the PD-1 pathway plays an important role in tempering early CD8+ T cell proliferation in lymphoid tissues, which is critical for CD8+ T cell survival at the site of infection and subsequent memory formation.

Figure 2.6

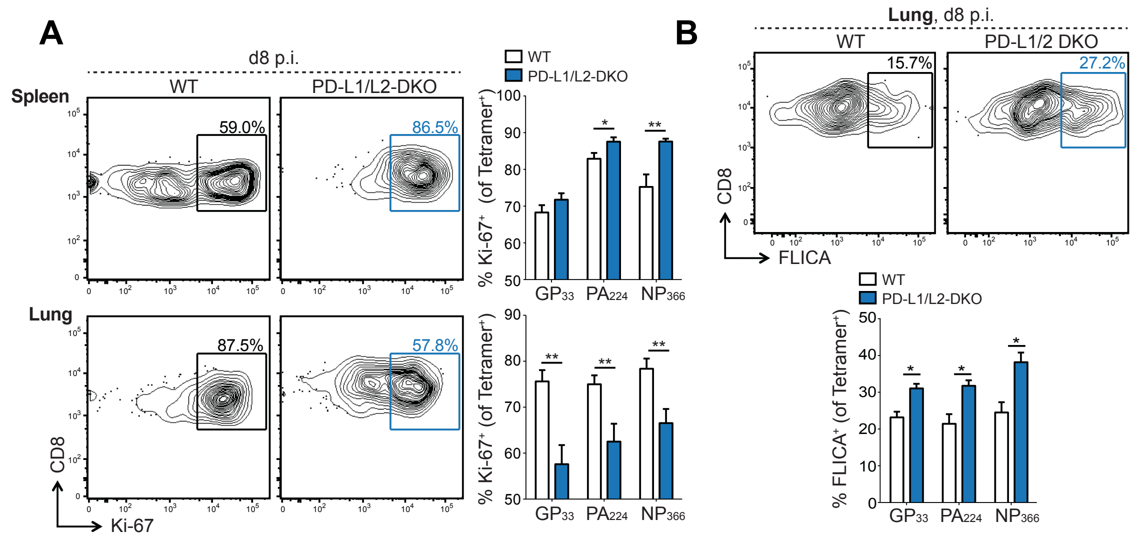


Figure 2.6: CD8+ T cells undergo robust proliferation but have reduced survival in the absence of PD-1 signaling in influenza-infected mice. **(A)** Flow cytometric analysis of Ki-67 expression by NP366-specific CD8+ T cells from WT and PD-L1/L2 DKO mice at d8 p.i. in the spleen (top) and lung (bottom). Numbers indicate fraction of Ki-67-expressing influenza-specific $CD8^+$ T cells. Representative plots shown to the left. Summary of Ki-67+ cells shown for indicated tetramers to the right. **(B)** Flow cytometric analysis of active caspase by FLICA staining of NP366-specific CD8+ T cells from WT and PD-L1/L2 DKO mice at d8 p.i. in the lung. Numbers indicate fraction of influenza-specific T cells positive for FLICA staining based on unstained controls. Representative plots shown on the left and summary of FLICA+ cells shown for indicated tetramers to the right.

2.3.4: The regulation of CD8+ T cell memory differentiation by PD-1 is cell-intrinsic.

One possible reason for altered proliferation and survival dynamics of the effector CD8+ T cell responses in PD-1 pathway deficient mice is that these mice clear a primary influenza infection faster than WT mice. Such an accelerated clearance of primary infection could result in truncated exposure of T cells to the optimal levels of antigen and inflammation needed for proper memory development. Indeed, PD-1 pathway deficient mice cleared influenza virus more rapidly than WT mice and had significantly decreased viral titers in the lungs on d3.5 after primary infection **(Figure 2.7A)**. Enhanced control of infection was associated with reduced weight loss and faster recovery of PD-1 pathway deficient mice **(Figure 2.7B)**. These observations suggest that early viral clearance may contribute to altered CD8+ T cell responses in PD-1 KO and PD-L1/L2 DKO mice. Thus, defective T cell memory in the absence of PD-1 signals could be due to accelerated control of infection and/or differences in T cell differentiation when cell-intrinsic PD-1 signals are absent.

Figure 2.7

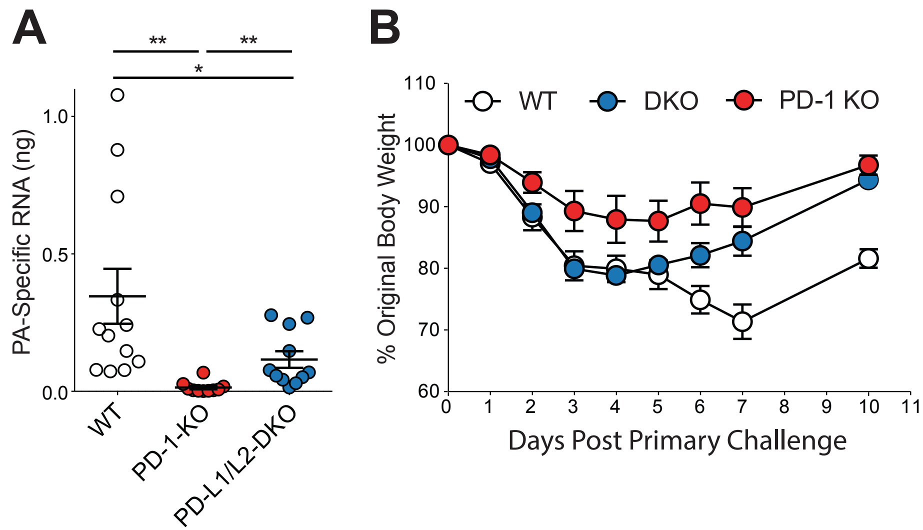


Figure 2.7: WT mice clear primary influenza infection more quickly than PD-1 pathway deficient mice. (A) Influenza viral titers in the lung at d3.5 p.i. and longitudinal weight loss (**B**) in WT and PD-L1/L2 DKO mice following primary infection with X31-GP33.

To directly test whether PD-1 signals had a cell-intrinsic role in regulating CD8+ T cell memory differentiation during influenza infection, we co-transferred equal numbers of WT and PD-1 KO P14 TCR transgenic CD8+ T cells (recognizing GP33-41 peptide presented by H-2D^{b}) into WT mice followed by infection with X31-GP33 virus **(Figure 2.8A)**. This approach allows for direct comparison of WT and PD-1 deficient CD8+ T cells in the same mice and excludes differences in precursor frequency, TCR repertoire, viral control, effects of PD-L1 and/or PD-L2 on APCs or influenza pathogenesis. Consistent with findings in PD-1 KO and PD-L1/L2 DKO mice, the frequency **(Figure 2.8B)** and total numbers **(Figure 2.8C)** of PD-1 KO P14 cells were significantly higher than WT P14 cells in the lung and spleen at d7 p.i., with similar trends in the blood and dLN. The increased frequency of PD-1 KO P14 cells was associated with increased BrdU incorporation in the spleen and dLN (Figure 2.9A) and increased Ki-67 expression in the spleen (data not shown), indicating that PD-1 regulates CD8+ T cell proliferation in a cell-intrinsic manner. Similar to virus-specific T cells in influenza-infected PD-1 and PD-L1/L2 DKO mice, PD-1 KO P14 cells and WT P14 cells incorporated BrdU to a similar extent in the lung at d7 p.i. **(Figure 2.9A)**. However, PD-1 KO P14 cells were more prone to cell death, as demonstrated by increased FLICA staining and surface Annexin V at d10 p.i. **(Figure 2.9B and data not shown)**.

Figure 2.8

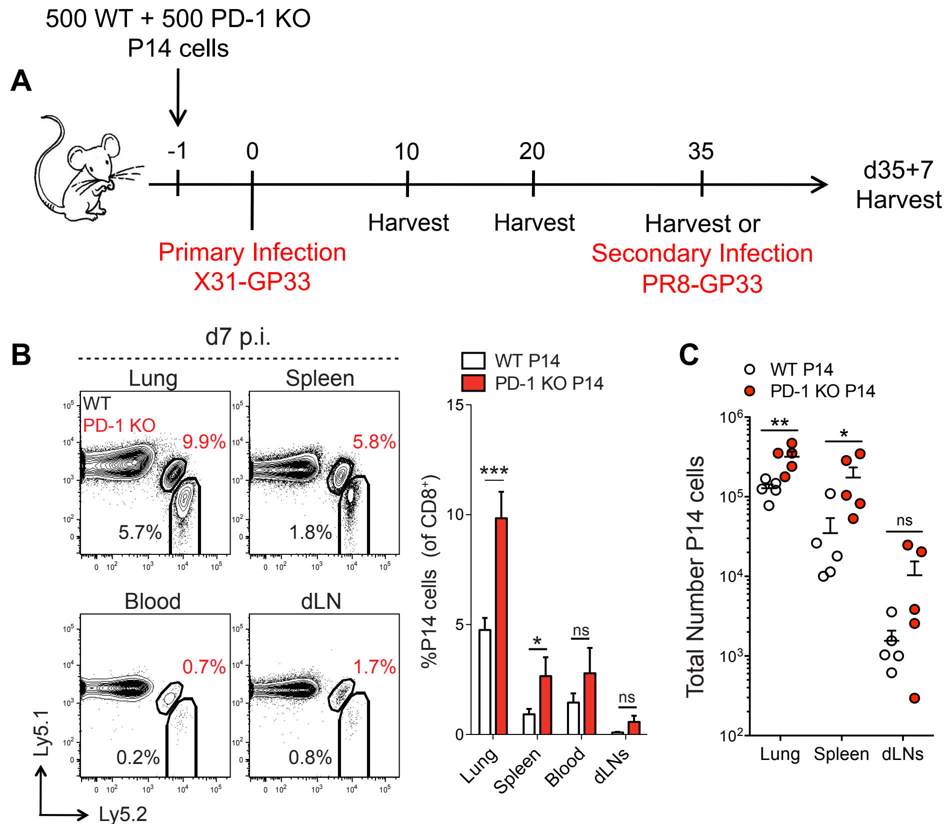


Figure 2.8: PD-1 controls the magnitude of the CD8+ T cell response in a cell-intrinsic manner. (A) Schematic of experimental design utilizing TCR transgenic P14 transfer **(B)** Representative plots (left) show the frequency of WT and PD-1 KO P14 cells in indicated organs at d7 p.i. with X31-GP33. Numbers indicate frequency of P14 cells as a percent of CD8+ T cells. Summary of frequencies of P14 cells is shown to the right. **(C)** Summary of absolute numbers of WT and PD-1 KO P14 cells at d7 p.i. with X31-GP33 in the spleen.

Figure 2.9

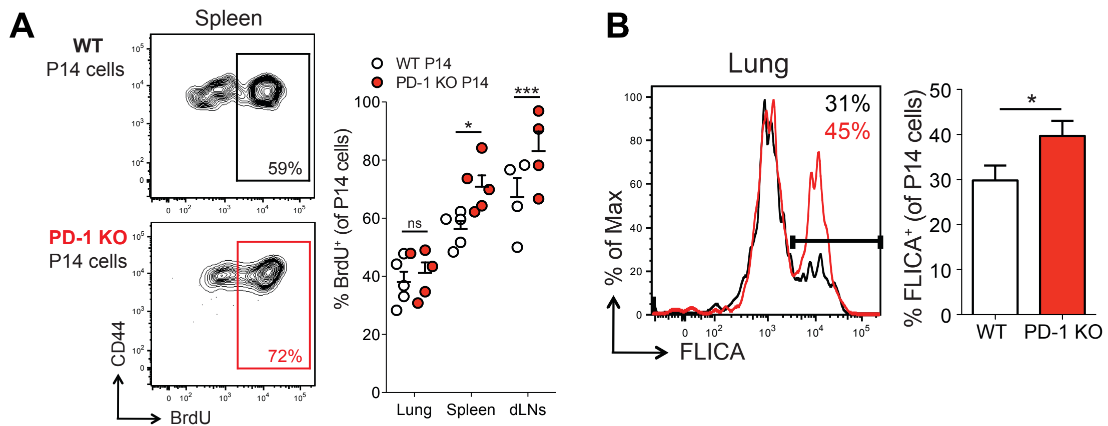


Figure 2.9: PD-1 regulates CD8+ T cell proliferation and cell death in a cell-intrinsic manner. (A) Flow cytometric analysis of BrdU incorporation by WT and PD-1 KO P14 cells in the spleen at d7 p.i. Numbers indicate fraction of BrdU+ P14 cells. Representative plots shown to the left. Summary of BrdU$^+$ P14 cells in different organs shown to the right. **(B)** Flow cytometric analysis of active caspase by FLICA staining of WT and PD-1 KO P14 T cells on d8 p.i. in the lung. Numbers indicate fraction of P14 cells positive for FLICA staining based on unstained controls. Representative plot shown to the left. Summary of FLICA$^+$ cells shown to the right.

We used the P14 co-transfer system to interrogate whether these early cell-intrinsic alterations in proliferation and cell death also resulted in aberrant memory development. We assessed the number, phenotype and functionality of WT and PD-1 KO P14 cells 35-100 days p.i. While WT P14 cells formed stable memory, PD-1 KO P14 cells underwent progressive contraction and were less abundant than WT P14 cells in the lung and spleen after 3 months **(Figure 2.10A)**, consistent with the memory attrition observed in the whole animal KO experiments described above. Although no differences in the differentiation state were observed based on expression of key memory markers (data not shown), significantly fewer PD-1 KO memory P14 cells from the lung or spleen were capable of producing IFN-γ and TNF-α upon re-stimulation compared to WT cells **(Figure 2.10B)**. To test whether memory recall capacity was compromised as a result of cell-intrinsic deficiency in PD-1 signals, influenza X31-GP33 immune mice containing WT and PD-1 KO P14 cells were re-challenged with PR8-GP33. Following re-challenge, PD-1 KO P14 memory cells displayed dramatically impaired secondary expansion compared to WT P14 cells in the same animals **(Figure 2.10C)**. These results point to a key function for PD-1 signals in regulating T cell proliferation and survival during the acute phase of infection and indicate that the proper regulation of these events during T cell priming and expansion has a major impact on subsequent $CD8^+$ T cell memory. Collectively, these data indicate that PD-1 regulates the differentiation and establishment of CD8+ T cell memory in a cell-intrinsic manner.

Figure 2.10

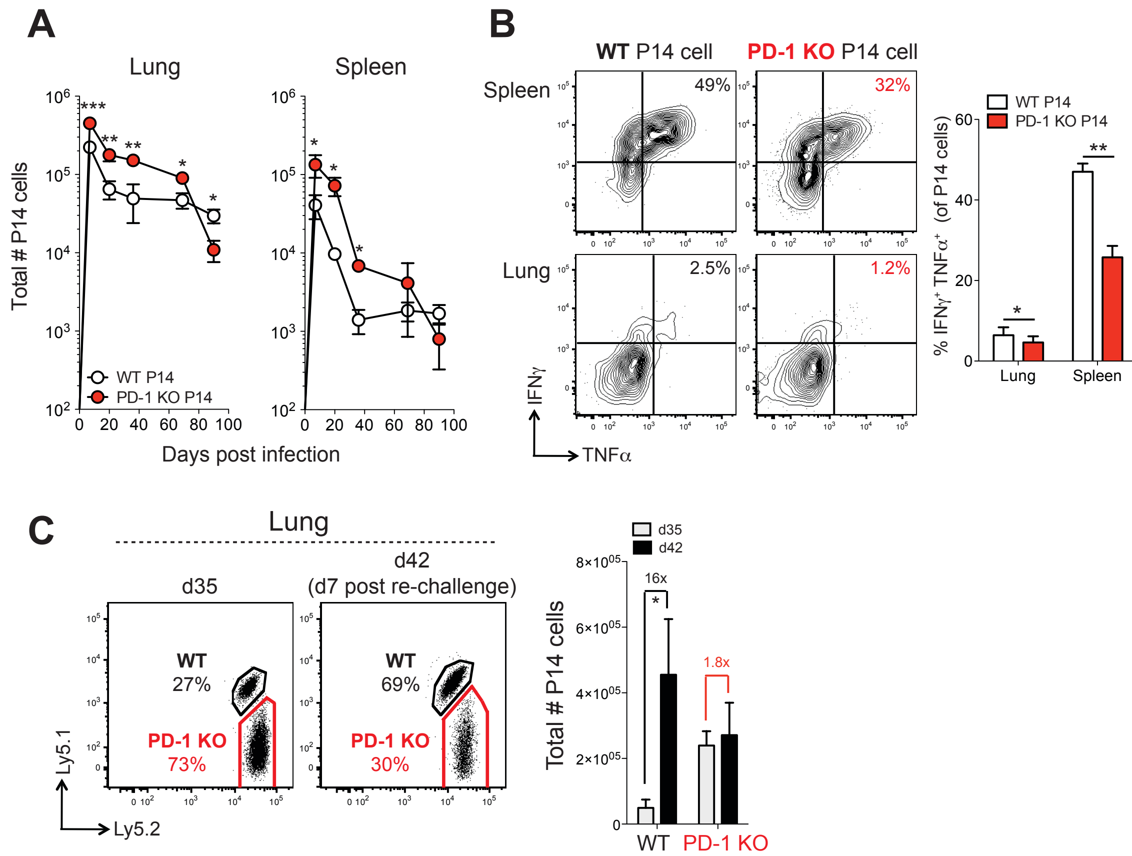


Figure 2.10: Cell-intrinsic defect in CD8+ T cell memory in the absence of PD-1. (A) Summary of WT and PD-1 KO P14 absolute cell numbers in the lung and spleen during primary X31-GP33 infection. **(B)** Representative plots of intracellular cytokine staining for IFN-γ and TNF-α production in WT and PD-1 P14 cells from spleen and lung (left) and summary data (right). **(C)** Representative plots of WT and PD-1 KO P14 cell percentages 35 days after primary infection with X31-GP33 (d35) and 7 days post re-challenge with PR8-GP33 (d42 after primary infection). Summary of fold change of numbers of P14 cells pre- and post- re-challenge shown to the right.

Figure 2.11

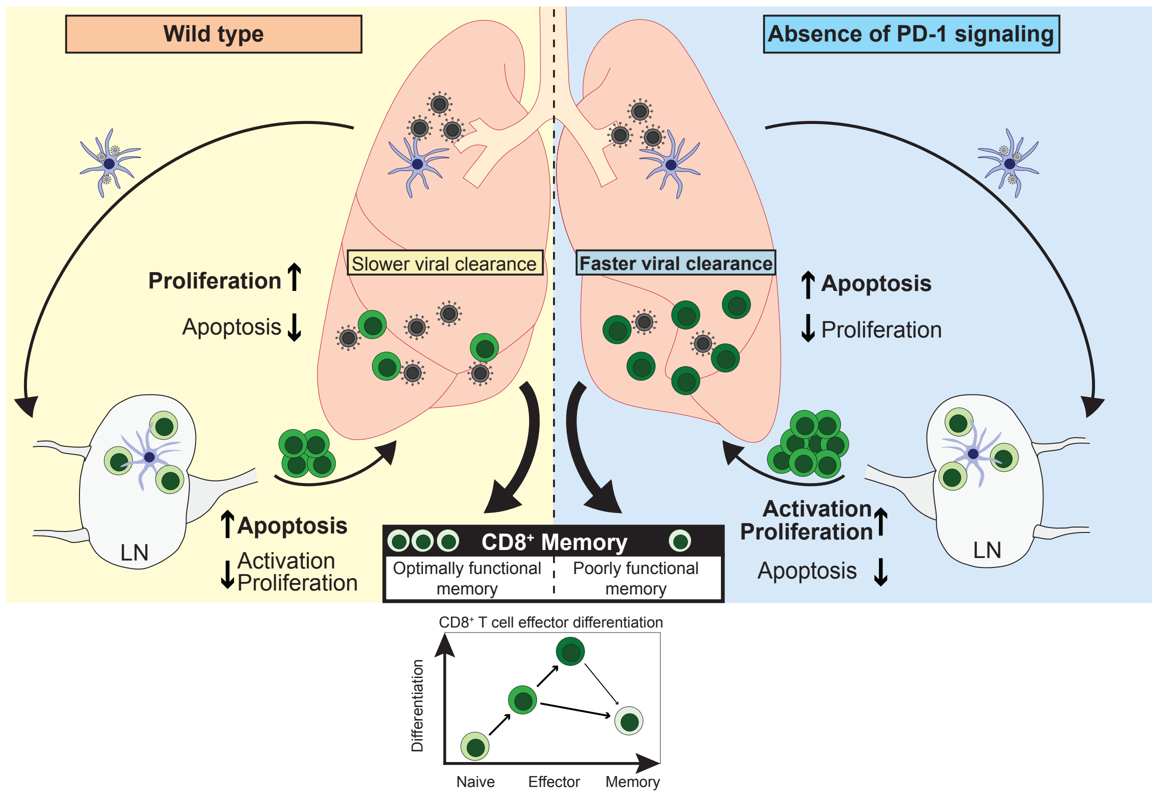


Figure 2.11: Model of the role for PD-1 signals in effector and memory CD8+ T cell responses to influenza virus infection. In WT mice (left), PD-1 pathway signals early during influenza virus infection restrict early activation and proliferation of virus-specific $CD8^+$ T cells in dLNs. These T cells then migrate into the infected lung, where inhibition by the PD-1 pathway promotes their survival and continued proliferation, allowing for clearance of influenza virus and the development of optimally-functional memory $CD8^+$ T cells. In settings of PD-1 pathway deficiency (right), virus-specific $CD8^+$ T cells undergo excessive proliferation in dLNs and migrate to the lungs in high numbers. While this leads to faster clearance of primary influenza infection, virus-specific $CD8^+$ T cells undergo increased apoptosis in the lung and, ultimately, develop into poorly functional and unstable memory cells.

2.4: Discussion

The PD-1 pathway has a key role in regulating T cell responses. This pathway has become an important immunotherapeutic target in cancer and has promise for the treatment of chronic infections. The rapid up-regulation of the PD-1 pathway during the effector phase of immune responses suggests that PD-1:PD-L signals also exert important immunoregulatory functions during effector and memory responses following acute infection or vaccination. However, the role of PD-1 signals in this context is poorly understood. Here we have investigated how the PD-1 pathway regulates the generation and maintenance of CD8+ T cell memory. We found that PD-1 KO and PD-L1/L2 DKO mice developed suboptimal memory CD8+ T cells that lack long-term stability and protect poorly against secondary re-challenge. This defect in memory was associated with early excessive proliferation of effector CD8+ T cells, coupled with increased cell death. Ultimately, without PD-1 signals, virus-specific CD8+ T cells lose or never develop the robust recall capacity and secondary proliferation that is characteristic of memory T cells. Taken together, our work reveals an important role for the PD-1 pathway in the optimal development of long-lived memory responses by acting to tune the strength of stimulation and temper early proliferation of CD8+ T cells (**Figure 2.11**).

During acute respiratory infections, the PD-1:PD-L pathway might have a particularly important role in controlling the balance between immunopathology and effective anti-pathogen immunity and memory development. For example, virulent LCMV infection in PD-1 KO mice leads to damage of the pulmonary endothelium that ultimately causes respiratory failure and contributes to mortality (Frebel et al., 2012). These studies suggest that PD-1 restricts T cell responses to acute stimulation in the lung, likely to prevent excessive immune-mediated damage. Studies of pathogenic respiratory

infections also suggest a role for PD-1:PD-L1 signals in limiting immunopathology (Erickson et al., 2012). Our findings now reveal that another key function of this negative regulation by PD-1 during acute stimulation is to moderate excessive T cell stimulation and thereby facilitate the development of optimal CD8+ T cell memory.

Several groups have demonstrated the potent ability of PD-1 to inhibit TCR and co-stimulatory signals, implicating the PD-1 pathway in effector and memory T cell responses (Butte et al., 2007; Freeman et al., 2000a; Parry and Riley, 2005; Yokosuka et al., 2012). However, there are conflicting data about how PD-1:PD-L signals impact effector and memory T cell responses during acute infection. In the respiratory tract, for example, antibody blockade of PD-L1 during human metapneumovirus (HMPV) and or genetic deletion of PD-1 during influenza virus infection enhanced CD8+ T cell functionality early in the immune response, consistent with our findings (Erickson et al., 2012, 2014). In addition, blockade of PD-L1 just prior to HMPV re-challenge leads to a more robust secondary CD8+ T cell response and enhanced viral clearance compared to control treated mice. However, other reports demonstrate that the lack of PD-1:PD-L signals can either enhance or inhibit primary effector CD8+ T cell responses following acute infection, depending on the model (Allie et al., 2011; Fuse et al., 2009; Rowe and Sing Way, 2008; Talay et al., 2009; Yao et al., 2009). While transient blockade of PD-1 signals can improve recall responses of memory CD8+ T cells, there is also evidence that genetic absence of PD-1 can skew memory CD8+ T cell differentiation towards either effector memory or central memory depending on the context. Thus, the precise role of PD-1:PD-L signals in the formation of long-term immunity and T cell memory has remained unclear.

Our results highlight the importance of PD-1 inhibitory signals in tempering initial CD8+ T priming. While DCs are a prime candidate for the specific APC involved in these

interactions, it is possible that PD-L1/L2 on other cell types could also negatively regulate T cells. The inhibition by the PD-1 pathway in lymphoid tissues likely plays a central role in early skewing of effector T cell differentiation. However, interactions between CD8+ T cells with PD-L1/L2 on resident DCs, infected epithelial cells and/or lung macrophage populations in the infected lung are also likely to be important. We find that CD8+ T cells have decreased survival in the lung between d10-20 p.i. when PD-1 signaling is absent. Taken together, these findings suggest that PD-1-mediated restraint during the early expansion of CD8+ T cells in draining lymphoid tissue is needed to promote the generation and survival of developing effector and memory CD8+ T cells.

The ability of the PD-1 pathway to modulate effector and memory CD8+ T cell differentiation makes it an attractive therapeutic target for infectious diseases and cancer, as well as for augmenting vaccination strategies. Blocking PD-1 pathway signals may prove beneficial in boosting early effector responses to promote pathogen or tumor clearance. The long-term effects of PD-1 pathway blockade, however, may improve memory T cell development in some settings, while preventing optimal memory formation in others. Better understanding of how the PD-1 pathway regulates CD8+ T cell responses in divergent settings of infectious disease and cancer will help inform how to administer PD-1 or PD-L1 blocking antibodies to enhance memory T cell responses.

In order to most effectively target the PD-1 pathway to improve T cell memory development, several key questions must be addressed. It is not yet clear when during the course of infection it is most beneficial to block the PD-1 pathway. The timing of PD-1 blockade appears to be key, and blockade at the time of initial CD8+ T cell priming may have different consequences from blockade of pre-existing memory T cells during a recall response. Indeed, our data suggest that very early removal of PD-1 signals during primary infection can prevent optimal memory differentiation, while other data

demonstrate that blockade of PD-1 just prior to re-challenge improves secondary expansion of memory $CD8^+$ T cells. It may be that targeting the PD-1 pathway during different stages of infection (i.e. priming, expansion, contraction, or memory phase) will result in distinct effects on CD8+ T cell differentiation, recall responses and/or memory maintenance. The answers to these questions will be critical for determining how to best modulate PD-1 to enhance responses to vaccines for infectious agents and cancer. Additionally, while we have focused primarily on the CD8+ T cell-intrinsic role of PD-1 on memory development, the PD-1 pathway regulates many other important cell types, including CD4+ T cells, B cells, NK cells, and APCs. It will be interesting to investigate how the loss of PD-1 pathway signals affects the behavior of these cell types during infection with influenza virus and infections.

In summary, we have identified a key role for the PD-1 pathway in controlling effector and memory CD8+ T cell differentiation during influenza virus infection. Negative regulation by PD-1 and its ligands is needed to promote optimal formation of long-lived, protective memory CD8+ T cells. Our studies implicate the PD-1 pathway as a prime target for altering development of effector and memory CD8+ T cell responses during therapeutic and vaccination strategies. We have identified control of cell cycle and apoptosis as one mechanism by which PD-1:PD-L signals influence CD8+ T cell memory. Future studies are needed to identify additional molecular pathways by which PD-1:PD-L signals regulate CD8+ T cell differentiation in different disease settings, as well as to further test the potential of combining PD-1 pathway manipulation with current vaccine strategies.

CHAPTER 3: Genetic Absence of PD-1 promotes accumulation of terminally-differentiated exhausted CD8+ T cells during chronic viral infection

3.1: Introduction

Chronic viral infections, such as HIV, HCV, and others, place a significant strain on anti-viral T cell responses, forcing continued proliferation, cytokine production, and killing of infected cells for months or years. As a result, anti-viral CD8+ T cell functions become sub-optimal over time, a phenomenon known as T cell exhaustion (Shankar et al., 2000; Zajac and Ahmed, 1998; Gallimore et al., 1998; Gruener et al., 2001a; Wherry, 2011). Two of the cardinal features of exhausted CD8+ T cells are the gradual loss of effector capabilities and the sustained high expression of multiple inhibitory receptors. Exhausted CD8+ T cells also have altered expression of key transcription factors, including Tbet, Eomesodermin (Eomes), and Blimp-1 (Wherry, 2011; Wherry et al., 2003a; Blackburn et al., 2009). Importantly, CD8+ T cell exhaustion contributes to failed immune control during chronic infection and cancer.

The inhibitory receptor Programmed Death-1 (PD-1) is considered to be a central regulator of CD8+ T cell exhaustion. Expression of PD-1 and its primary ligand, PD-L1, is highly upregulated during chronic infection and cancer (Barber et al., 2006; Freeman et al., 2006; Blank et al., 2005). The importance of this elevated PD-1 pathway expression has been demonstrated in several animal models where *in vivo* antibody-mediated blockade of PD-1 or PD-L1 re-invigorates exhausted CD8+ T cell responses and decreases viral load or tumor burden (Barber et al., 2006). Recent clinical trials have

extended these observations from mouse models to humans, demonstrating a potent ability of PD-1 pathway blockade to revitalize anti-tumor immunity in late-stage cancer patients (Brahmer et al., 2012; Topalian et al., 2012). The observations of reversibility of exhaustion by PD-1 pathway blockade demonstrate that exhausted CD8+ T cells, or at least a subset of the population, are not terminally dysfunctional. Furthermore, blockade of various other inhibitory receptors alone and in combination with PD-1/PD-L1 blockade suggests that PD-1 is the major inhibitory receptor controlling exhaustion (Blackburn and Wherry, 2008; Blackburn et al., 2009; Kassu et al., 2010; Wherry et al., 2007). While it is clear that PD-1 based therapies have exciting clinical potential and can dramatically improve exhausted T cell responses, the precise role of PD-1 in exhausted CD8+ T cells remains undefined.

A fundamental unresolved question is what role PD-1 signals play in initiating and/or establishing the program of T cell exhaustion. One possibility is that PD-1 directly causes the development of CD8+ T cell exhaustion. This question has previously been challenging to address since PD-1 pathway deficiency results in excessive CD8+ T cell-mediated immunopathology and altered viral pathogenesis, preventing analysis of T cell responses after the first week post-infection (Barber et al., 2006; Frebel et al., 2012). However, the robust functionality of CD8+ T cells in the absence of PD-1 at these early time-points suggests that T cell exhaustion may not develop without PD-1 signals. This outcome would implicate the PD-1 pathway as a major regulatory pathway inducing the development of exhausted T cell populations. Alternatively, PD-1 could inhibit CD8+ T cell function during chronic infection but may not play a direct role as the initiator of the program of exhaustion. In this scenario, CD8+ T cells could still become exhausted even in settings of PD-1 deficiency. The implications of these two distinct possibilities are

important for the design and implementation of therapies blocking or manipulating the PD-1 pathway in cancer, infections and other diseases.

Here we have used a dual adoptive transfer approach that allowed us to study the development of PD-1 deficient (PD-1 KO) anti-viral CD8+ T cell responses during chronic LCMV infection in the absence of lethal immune-mediated damage and altered viral pathogenesis. Surprisingly, genetic deletion of PD-1 on virus-specific CD8+ T cells was insufficient to prevent the development of exhaustion. In fact, PD-1 KO virus-specific CD8+ T cells were less functional and had higher expression of other inhibitory receptors compared to WT virus-specific CD8+ T cells. Early over-stimulation and robust proliferation in the absence of PD-1 led to disruption of a critical proliferative hierarchy between T-bet^{hi} and Eomes^{hi} subsets of exhausted CD8+ T cells that is necessary for sustained responses during chronic infections (Paley et al., 2012). As a result, long-term proliferation and stability of exhausted T cell responses was compromised without PD-1. However, improved cytotoxic ability and increased localization of exhausted CD8+ T cells to peripheral tissues without PD-1 suggests that the PD-1 pathway has previously unappreciated roles in shaping exhausted CD8+ T cell populations. These findings demonstrate that PD-1 signals do not instruct the development of CD8+ T cell exhaustion. PD-1 is, however, required to prevent terminal-differentiation of exhausted CD8+ T cells while simultaneously controlling important anatomical and functional aspects of these cells. These findings have important implications for understanding the long-term impact of PD-1 based therapies in humans.

3.2: Methods

3.2.1: Mice and Infections

PD-1 deficient (PD-1 KO) mice were crossed to C57BL/6 P14 mice to generate PD-1 KO P14 mice (Keir et al., 2007b; Pircher et al., 1989). Mice were infected with either 2 x 10^5 PFU of lymphocytic choriomeningitis virus (LCMV) Armstrong strain by i.p. injection to generate an acutely resolved infection or 4 x 10^6 PFU of LCMV clone 13 strain by i.v. injection to generate a chronic infection. In experiments with CD4 depletion, 200 mg of anti-CD4 antibody (GK1.5; BioXcell) was administered i.p. on d-1 and d1 of LCMV infection. Viral titers were measured as previously described (Kao et al., 2011). All animals were housed at the University of Pennsylvania (Philadelphia, PA). Experiments were performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

3.2.2: Adoptive Transfer and Lymphocyte Isolation

CD8+ T cells were isolated from peripheral blood or spleens of naïve WT or PD-1 KO P14 mice. For spleen isolation, P14 cells were purified by CD8 negative selection (Stem Cell Technologies). Depending on the experiment, the appropriate number of WT or PD-1 KO cells was transferred into separate naïve recipient mice or mixed at a 1:1 ratio at indicated cell numbers and transferred into naïve recipient mice. For proliferation assays, P14 cells were labeled with 10 mM CellTraceViolent (Life Technologies) before transfer. For flow cytometry after infection, major organs were removed on indicated days and single-cell suspensions were prepared as described.

3.2.3: Flow Cytometry and Cell Sorting

All cells were stained with LIVE/DEAD® Fixable Dead Cell Stain (Life Technologies) to discriminate live from dead cells. Surface staining was performed as described previously (Kao et al., 2011). For intracellular cytokine staining, single-cell suspensions were incubated for 5h at 37 °C with or without 1 μM gp33 peptide (KAVYNFATM) and were stained with a Cytofix/Cytoperm kit according to manufacturer's instructions (BD Biosciences). For intracellular staining of transcription factors, a Foxp3 staining kit was used according to the manufacturer's instruction (eBioscience). Antibodies used for flow cytometry were purchased from BD Biosciences (CD4, CD8, CD19, CD44, 2B4, Ki67), Biolegend (PD-1 (RMPI-30 clone), Tim-3, TNFa, IFNg, Tbet; San Diego, CA), R&D Systems (MIP-1a; Minneapolis, MN), or eBioscience (CD8, Lag3, CD160, 2B4, CD45.1, CD45.2, CD107a, Eomes). MHC class I peptide tetramers were made and used as described previously (Wherry et al., 2003a; Kao et al., 2011). Poly-caspase analysis was performed with Flica Vybrant FAM Poly Caspases Assay Kit (Life Technologies). Data were collected on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting was performed using a BD Aria II (BD Biosciences).

3.2.4: BrdU Treatment and Detection

Animals were treated with 2mg of BrdU (Sigma-Aldrich) i.p. daily for 1-5 days prior to tissue harvest and analysis. BrdU incorporation was assessed by the BrdU Flow Kit per manufacturer's instructions (BD Biosciences).

3.2.5: In vitro Stimulation

Naïve WT and PD-1 KO P14 cells were isolated from spleens by CD8 negative selection (Stem Cell Technologies) and cultured in RPMI-1640 medium in the presence of recombinant human IL-2 (100 U/mL; R&D Systems.) Naive P14 cells were stimulated for 4d by co-culturing with GP33 peptide-pulsed naïve splenocytes.

3.2.6: Statistical Analysis

Student's t-test (Paired and Unpaired) were performed using Prism software (Graphpad).

3.3: Results

3.3.1: Co-adoptive transfer strategy allows for examination of PD-1 deficient virus-specific CD8+ T cells during chronic LCMV infection

Direct infection of PD-1 or PD-L1 KO mice with LCMV clone 13 causes lethal immunopathology. Previous studies using adoptive transfer of LCMV-specific TCR-transgenic CD8+ T cells (P14 cells) also resulted in immunopathology when 10^4 PD-1 KO P14 cells were transferred (Frebel et al., 2012; Barber et al., 2006). We hypothesized that using lower numbers of P14 cells would avoid immune-mediated damage, allowing for analysis of PD-1 KO P14 cell exhaustion during chronic infection. Thus, a co-adoptive transfer strategy was employed in which equal numbers of WT and PD-1 KO P14 cells were transferred into congenically-disparate WT mice, followed by infection with LCMV clone 13 (**Figure 3.1A and 3.1B**). This strategy allows for direct comparison of WT and PD-1 KO P14 cell responses in the same recipient mice during chronic viral infection while controlling for precursor frequency, TCR repertoire, viral

load, inflammatory environment, and immunopathology. To determine if this co-adoptive transfer strategy mitigated alterations in viral load and/or morbidity that occur as a result of P14 transfer, viremia and adjusted survival were assessed in mice that received varying numbers of WT and PD-1 KO P14 cells (Pircher et al., 1989; Blattman et al., 2009; Frebel et al., 2012). Transfer of greater than 1000 total P14 cells (500 WT + 500 PD-1 KO) resulted in reduced viral load (**Figure 3.1C**) and significant morbidity (**Figure 3.1D**) in mice compared to no P14 transfer, consistent with previous reports (Frebel et al., 2012). However, transfer of 500 total P14 cells (250 WT + 250 PD-1 KO) did not alter significantly viremia or morbidity during chronic LCMV infection compared to mice that did not receive P14 cells (**Figure 3.1C and 3.1D**). These results demonstrate that a co-adoptive transfer approach with small numbers of WT and PD-1 KO P14 cells can be used to address the development of CD8+ T cell exhaustion in the absence of cell-intrinsic PD-1 signals.

Figure 3.1

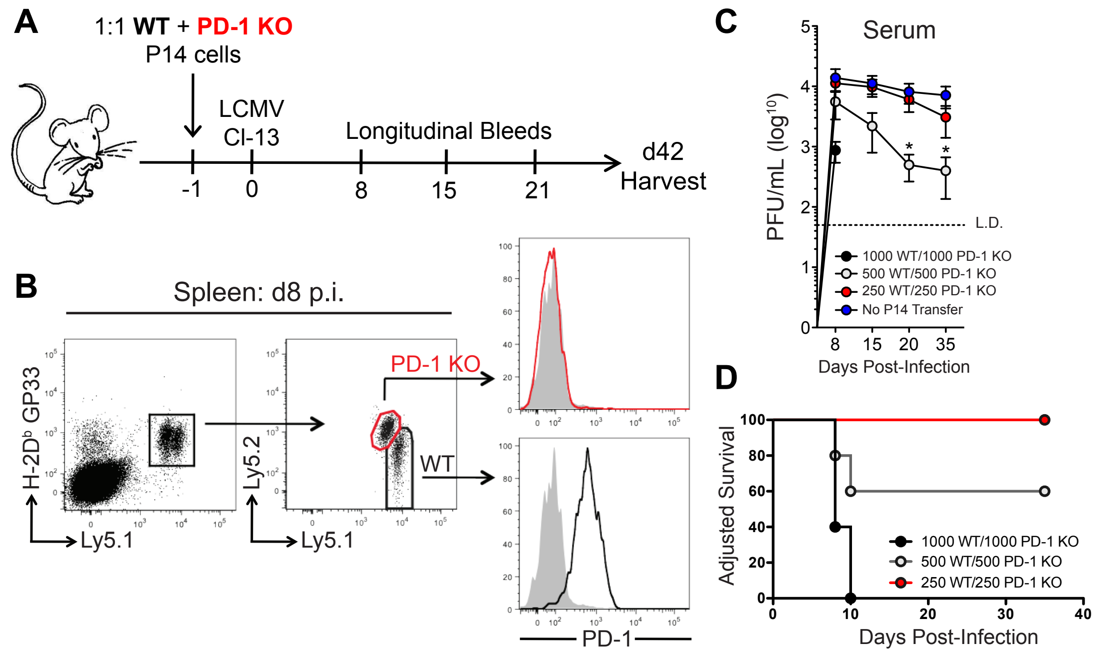


Figure 3.1: Adoptive transfer of WT and PD-1 KO P14 cells as a model to study T cell exhaustion in the absence of PD-1. (A) Schematic of co-adoptive transfer model. **(B)** Representative FACS plots of gating scheme for WT and PD-1 KO P14 cells. P14 cells are gated from total CD8+ T cells (far left) by expression of H-2D^b GP33 tetramer, congenic markers, and PD-1. **(C)** Longitudinal analysis of viral load in serum of mice that received indicated numbers of WT and PD-1 KO P14 cells followed by infection with LCMV clone 13 (+/- s.e.m.), *$p < 0.05$ (unpaired Student's t-test). **(D)** Survival curve of mice that received indicated numbers of WT and PD-1 KO P14 cells following LCMV clone 13 infection. "Adjusted Survival" indicates time to loss of >25% of total body weight and subsequent euthanasia.

3.3.2: CD8+ T cell exhaustion develops in the absence of PD-1

Previous studies demonstrating potent inhibition of CD8+ T cells by the PD-1 pathway suggest that PD-1 may be essential for the development of T cell exhaustion (Barber et al., 2006; Parry and Riley, 2005; Okazaki et al., 2013). Thus, we tested the hypothesis that PD-1 KO P14 cells would not become exhausted during chronic LCMV infection. First, we examined the ability of WT versus PD-1 KO P14 cells to produce cytokines at d42 p.i. following *ex vivo* peptide re-stimulation. At this time-point, WT P14 cells showed signs of classical exhaustion. Compared to control memory P14 cells generated following acute LCMV infection, these exhausted P14 cells less efficiently produced IFN-γ (average of 86% vs 51%) or co-produced IFN-γ and TNF-α (average of 76% vs 8%) (**Figure 3.2A**). Surprisingly, production of IFN-γ and TNF-α was even more severely reduced in the PD-1 KO P14 population, with an average of only 39% of cells producing IFN-γ and 1.6% of cells co-producing IFN-γ and TNF-α (**Figure 3.2A**). In addition, PD-1 KO P14 cells developed a significant reduction in IFN-γ production per cell, as evidenced by lower MFI of IFN-γ expression compared to WT P14 cells in the same mice (**data not shown**). PD-1 KO P14 cells also exhibited reduced poly-functionality compared to WT P14 cells, with minimal co-production of IFN-γ, TNF-α, and Mip1-α (**Figure 3.2B**). However, the ability to de-granulate, as measured by LAMP-1/CD107a expression, was retained (**Figure 3.2B**). Analysis of WT and PD-1 KO P14 cells in separate mice resulted in similar findings, confirming the CD8+ T cell intrinsic nature of this effect (**Figure 3.2C**).

Figure 3.2

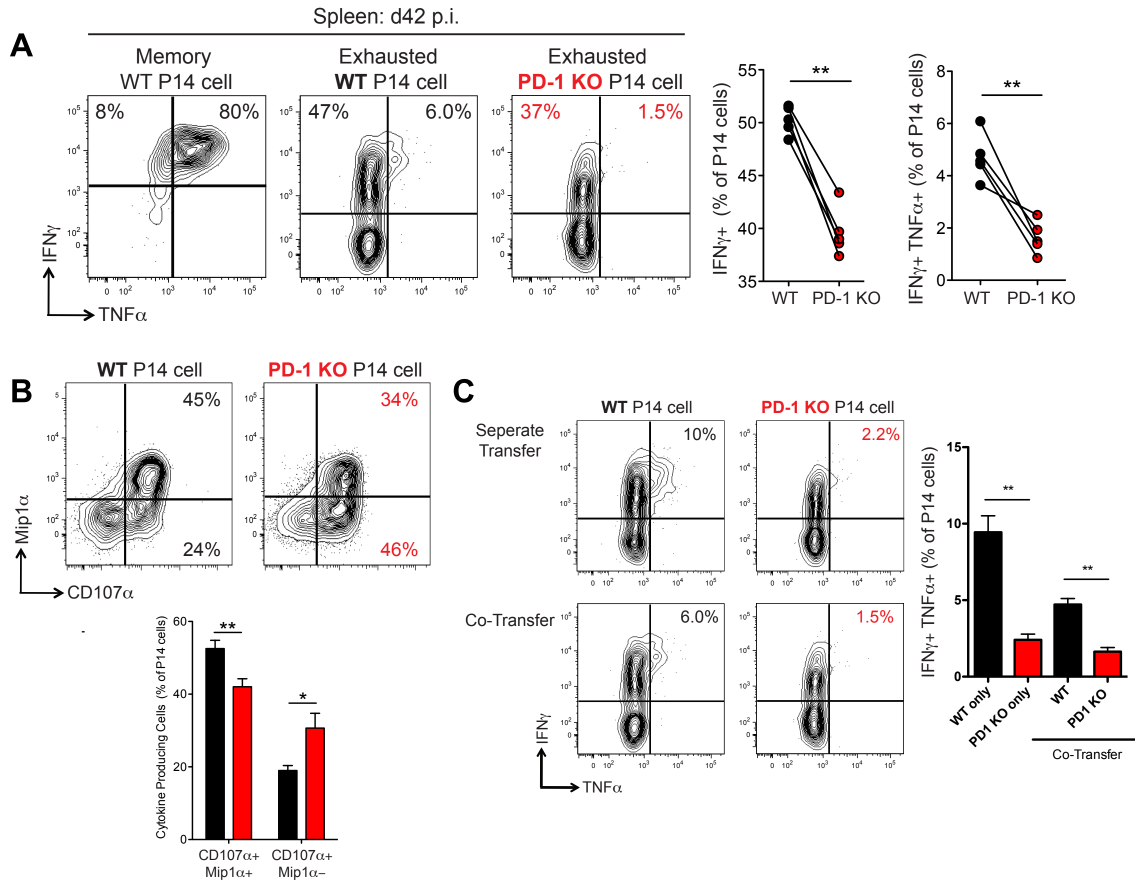


Figure 3.2: Severe loss of exhausted CD8+ T cell cytokine production in the absence of PD-1. (A) Intracellular cytokine staining for IFN-γ and TNF-α after stimulation with GP33 peptide (left). Values indicate frequency of P14 cells producing IFN-γ and/or TNF-α for individual (left) and multiple mice (right) at d42 p.i. in the spleen **(B)** Intracellular staining for Mip1-α and CD107a after stimulation with GP33 peptide (left). Values indicate frequency of P14 cells producing Mip1-α and/or CD107a for individual (left) and multiple mice (right) at d42 p.i. in the spleen. **(C)** Intracellular cytokine staining for IFN-γ and TNF-α after stimulation with GP33 peptide from mice with separate transfer of WT or PD-1 KO P14 cells compared to mice with co-transferred WT and PD-1 KO P14 cells (left). Values indicate frequency of P14 cells co-producing IFN-γ and TNF-α for individual (left) and multiple mice (right). (+/- s.e.m.) *$p < 0.05$ **$p < 0.01$ (paired Student's t-test)

Reduced cytokine production in the absence of PD-1 also corresponded with higher expression of multiple other inhibitory receptors, a second key feature of exhaustion. PD-1 KO P14 cells expressed higher Lag-3, 2B4, CD160, and Tigit than WT P14 cells at day 42 p.i. (**Figure 3.3A and 3.3B**). A higher frequency of PD-1 KO P14 cells also simultaneously co-expressed Lag-3, 2B4, Tim-3 and CD160 compared to WT (**Figure 3.3C**). These findings indicate that CD8+ T cell exhaustion can develop in the absence of PD-1. In fact, two defining features of exhausted CD8+ T cells, loss of cytokine poly-functionality and elevated inhibitory receptor co-expression, were more severe when CD8+ T cell intrinsic PD-1 signals were absent.

Figure 3.3

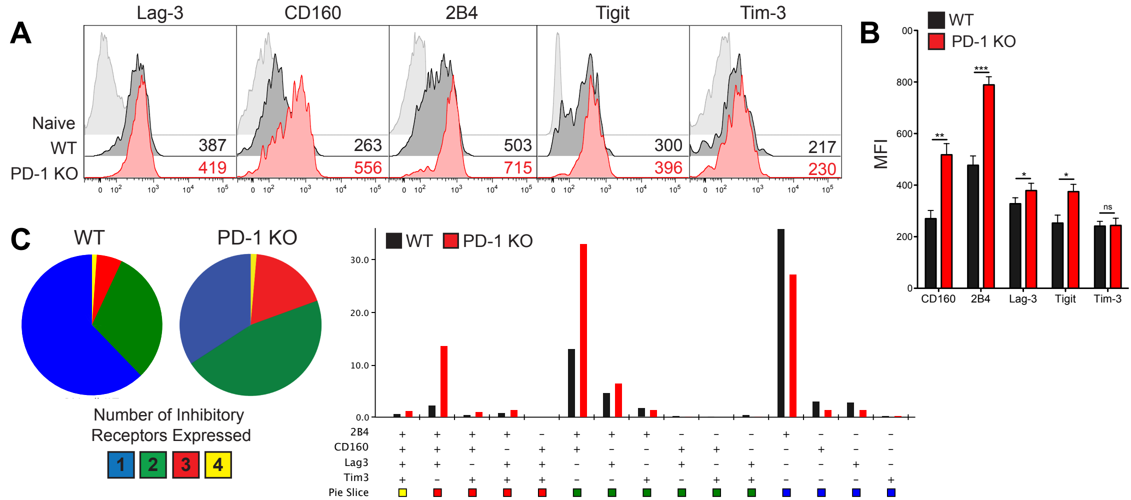


Figure 3.3: Increased inhibitory receptor expression on exhausted CD8+ T cells in the absence of PD-1. (A) Expression of indicated inhibitory receptors on naïve CD8+ T cells, WT P14 cells, and PD-1 KO P14 cells at d42 p.i. in the spleens of representative mice. Values indicate MFI of expression. **(B)** Summary of inhibitory receptor MFI on WT and PD-1 KO P4 cells at d42 p.i. in the spleen **(C)** Boolean gating analysis of the simultaneous expression of multiple inhibitory receptors (Lag-3, 2B4, CD160 and Tim-3) on WT and PD-1 KO P14 cells at d42 p.i. in the spleen. Pie charts display individual populations grouped according to total number of inhibitory receptors expressed (left). Bar graph displays relative abundance of each possible individual inhibitory receptor expression profile (right). (+/- s.e.m.) *p < 0.05 **p < 0.01 ***p < 0.001 (paired Student's t-test).

3.3.3: Despite early signs of dysfunction, PD-1 deficient CD8+ T cells proliferate robustly during the acute phase of infection

We next investigated if this increased exhaustion in the absence of PD-1 started early during chronic infection. To test this, the phenotype and function of WT and PD-1 KO P14 cells was analyzed at the peak of the effector response, d8 post-infection. Both WT and PD-1 KO P14 cells were highly capable of producing IFN-γ at d8 p.i. (**Figure 3.4A**), consistent with previous work (Parry and Riley, 2005; Frebel et al., 2012; Patsoukis et al., 2012; Yokosuka et al., 2012). However, PD-1 KO P14 cells displayed a subtle, but significant, reduction in production of IFN-γ and co-production of IFN-γ and TNF-α (**Figure 3.4A**). PD-1 KO P14 cells also had elevated expression and co-expression of several other inhibitory receptors at this time-point (**Figure 3.4B and data not shown**). Taken together, these data suggest that dysfunction of PD-1 KO P14 cells may begin early during the course of chronic infection. However, despite slight defects in cytokine production and elevated inhibitory receptor expression, PD-1 KO P14 cells greatly outnumbered WT P14 cells in the spleen, blood, and other organs at d8 p.i. **(Figure 3.5A and data not shown)**. Therefore, we hypothesized that loss of PD-1 signals might improve the activation and proliferation of virus-specific CD8+ T cells. In the first few days following T cell activation, PD-1 KO P14 cells had a trend towards a proliferative advantage from d2-3 p.i. in the spleen compared to WT P14 cells, but there was no difference in proliferation by d4 p.i. (**Figure 3.5B**). There was also no difference in the early proliferation of WT versus PD-1 KO P14 cells when co-cultured with GP33 peptide-pulsed splenocytes *in vitro* (**Figure 3.5B**). However, PD-1 KO P14 cells proliferated significantly more robustly than WT P14 cells between d7-8 p.i., as measured by Ki67 expression (**Figure 3.5C**) and BrdU incorporation (**Figure 3.5D**).

Despite this robust prolieration, WT and PD-1 KO P14 cells had similar expression of activation and differentiation markers, such as CD69, KLRG-1, CD27, CD44, CD62L, and CD25 during early activation (d1-4 p.i.) and during the effector phase (d8 p.i.) (data not shown). These findings suggest that PD-1 restricts virus-specific CD8+ T cell responses during chronic infection primarily by restraining proliferation during the effector phase. These data also suggest that control of proliferation during the effector phase corresponds with the degree of preservation of T cell function in the chronic phase of infection.

Figure 3.4

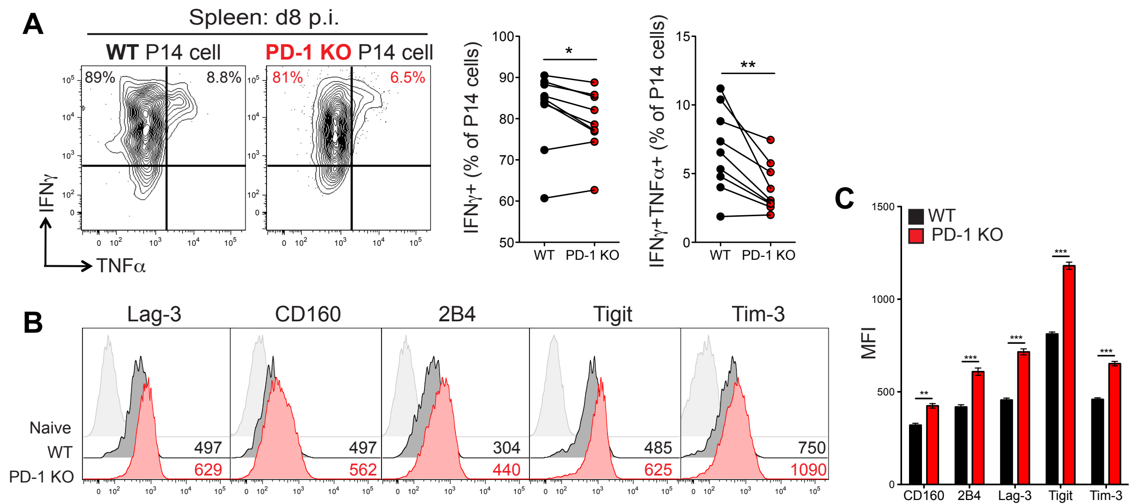


Figure 3.4: Early signs of CD8+ T cell exhaustion in PD-1 KO P14 cells. (A) Intracellular cytokine staining for IFN-γ and TNF-α after stimulation with GP33 peptide (left). Values indicate frequency of P14 cells producing IFN-γ and TNF-α for individual (left) and multiple mice (right) at d8 p.i. in the spleen. **(B)** Expression of indicated inhibitory receptors on naïve CD8+ T cells, WT P14 cells, and PD-1 KO P14 cells at d8 p.i. in the spleens of representative mice and multiple mice **(C)** Values indicate MFI of expression. Data are representative of 3 independent experiments with 5-8 mice per group. (+/- s.e.m.) *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ (paired Student's t-test).

Figure 3.5

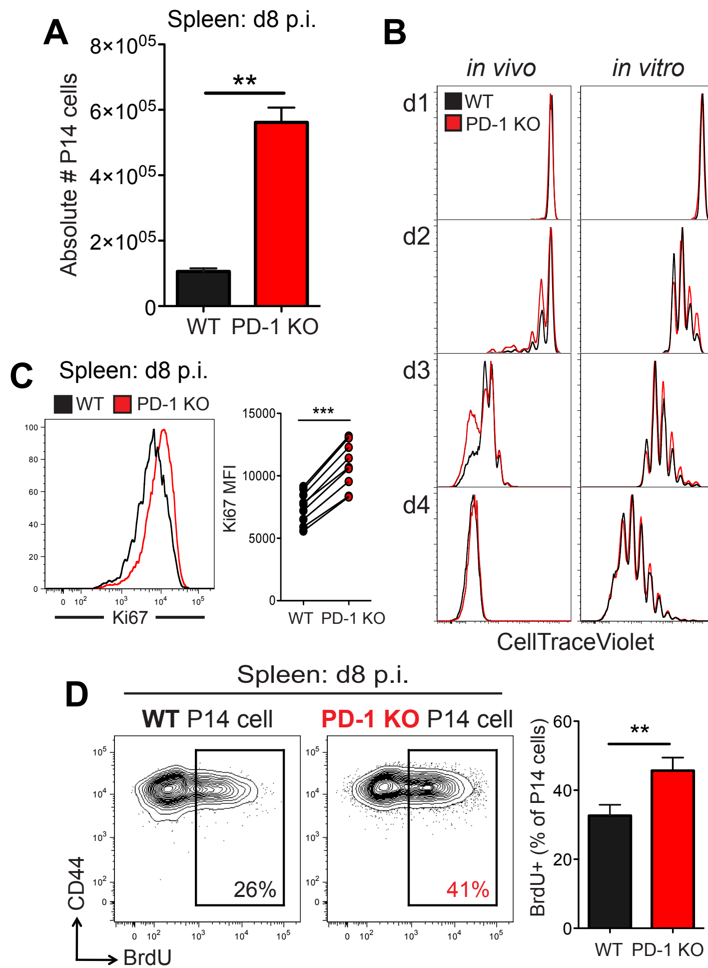


Figure 3.5: Increased expansion and proliferation of PD-1 KO P14 cells during effector phase (A) Absolute number of WT and PD-1 KO P14 cells at d8 p.i. in the spleen (+/- s.e.m.). **(B)** Expression of CellTraceViolet on WT and PD-1 KO P14 cells on indicated days post-infection with LCMV clone 13 in the spleen (left) or following *in vitro* stimulation with GP33-pulsed splenocytes (right). **(C)** Expression of Ki67 on WT and PD-1 KO P14 cells at d8 p.i. in the spleen **(D)** Expression of CD44 versus BrdU incorporation in WT and PD-1 KO P14 cells at d8 p.i. in the spleen following 24h BrdU pulse. Values indicate frequency of P14 cells positive for BrdU based on FMO staining control. (+/- s.e.m.), *p < 0.05 **p < 0.01 *** p < 0.001 (paired Student's t-test). All data are representative of 2-5 independent experiments with 5-8 mice per group.

3.3.4: Decreased survival of CD8+ T cells after the effector phase in the absence of PD-1

Because of the increased proliferation of PD-1 KO P14 during the effector phase, we next examined how the absence of PD-1 affected the transition to later stages of infection. After initial expansion, PD-1 KO P14 cells underwent a significantly greater contraction between days 15-42 p.i. (88% decrease) compared to WT P14 cells (75% decrease) (**Figure 3.6A**). We hypothesized that this more dramatic contraction was due to decreased survival in the absence of inhibitory signals from the PD-1 pathway. To test this, WT and PD-1 KO P14 cells were assessed for markers of cell death between days 14-20 p.i. in the spleen. A higher frequency of PD-1 KO P14 cells expressed Live/dead Aqua **(Figure 3.6B)** and Annexin V **(Figure 3.6C)**, markers of compromised cell membrane integrity that are associated with apoptosis and cell death. PD-1 KO P14 cells also displayed increased staining with fluorescent caspase substrates (FLICA) at d15 p.i. (**Figure 3.6D**). In addition, *ex vivo* survival of PD-1 KO P14 cells was decreased, as demonstrated by a significant reduction in the total frequency of live cells remaining after a 10-hour *in vitro* culture (data not shown). Thus, in the absence of PD-1, virus-specific CD8+ T cells experience considerably more cell death than WT CD8+ T cells of the same specificity in the same inflammatory environment. These findings suggest that PD-1 prevents over-stimulation of CD8+ T cells and subsequent excessive cell death during chronic viral infection.

Figure 3.6

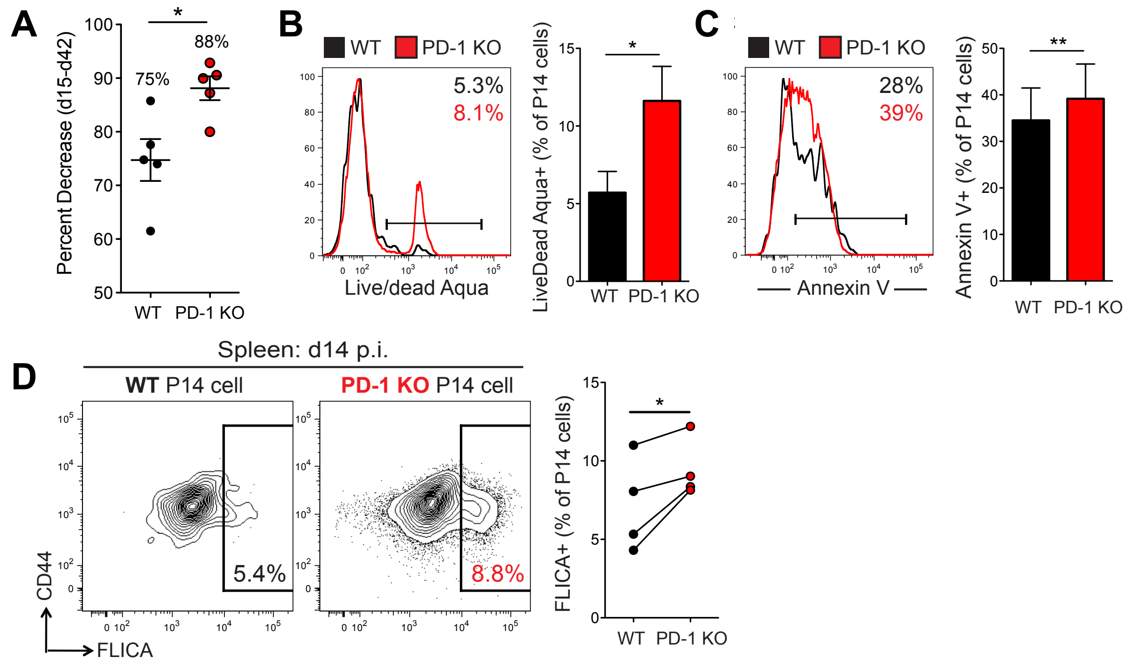


Figure 3.6: Reduced survival of PD-1 KO P14 cells during T cell contraction. **(A)** Fold decrease in frequency of WT and PD-1 KO P14 cells from peak of T cell response (d15 p.i.) to chronic phase of infection (d42 p.i.). Values indicate average fold decrease for each population (+/- s.e.m.), *p < 0.05 (unpaired Student's t-test). **(B)** Expression of Live/dead Aqua and **(C)** Annexin V in WT and PD-1 KO P14 cells at d18 p.i. in the spleen for individual (histograms) and multiple mice (bar graphs) (+/- s.e.m.). Values indicate frequency of P14 cells positive for staining based on FMO staining controls. **(D)** Expression of CD44 versus FLICA in WT and PD-1 KO P14 cells at d14 p.i. in the spleen for individual (left) and multiple mice (right). Values indicate frequency of P14 cells positive for FLICA dye based on FMO staining control, *p < 0.05 **p < 0.01 (paired Student's t-test). All data are representative of 2-4 independent experiments with at least 5 mice per group.

3.3.5: Decreased proliferation and long-term durability of PD-1 KO exhausted $CD8^+$ T cells

The increase in cell death of PD-1 KO P14 cells suggests that long-term durability of virus-specific CD8+ T cells may be compromised in the absence of PD-1. During LCMV clone 13 infection, the frequency and absolute number of WT P14 cells began to stabilize in the spleen and blood between d22-42 p.i. (**Figure 3.7A and data not shown**). PD-1 KO P14 cells, in contrast, declined in both frequency and absolute number until d42 p.i., suggesting that long-term durability of virus-specific CD8+ T cell responses may be compromised in the absence of PD-1 (**Figure 3.7A**). However, viremia during LCMV clone 13 infection of B6 mice gradually resolves by d40-60 p.i, complicating the interpretation of changes in exhaustion at late time-points. Therefore, we utilized a model of exhaustion where transient depletion of CD4+ T cells prior to infection with LCMV clone 13 leads to persistent, life-long high viremia (Matloubian et al., 1994). As observed in CD4+ T cell-sufficient clone 13 infection, WT P14 cell responses stabilized in frequency and absolute number after d48 p.i. in CD4+ T cell-depleted mice (**Figure 3.7B**). In contrast, while PD-1 KO P14 cell numbers were higher than WT P14 cells, the number of PD-1 KO P14 cells failed to stabilize and continued to decline for many months (**Figure 3.7B**). The inability of PD-1 KO P14 cells to form a stable, long-term population of exhausted CD8+ T cells in the spleen could be due, at least in part, to the increased cell death observed in this population. However, on-going antigen-driven proliferation is central to the durability of exhausted CD8+ T cell populations (Paley et al., 2012; Shin et al., 2007). Given the early robust expansion of virus-specific CD8+ T cells in the absence of PD-1, we asked if PD-1 KO P14 cells were able to sustain this proliferation into the chronic phase of infection. PD-1 KO P14 cells had dramatically

reduced expression of Ki67 at d42 p.i. in both CD4+ T cell-sufficient (clone 13) and CD4+ T cell-depleted (clone 13 + GK1.5) mice (**Figure 3.7C**). In addition, PD-1 KO P14 cells incorporated significantly less BrdU from days 35-42 p.i. than WT P14 cells (data not shown). Taken together, these findings suggest that increased cell death and loss of sustained proliferative ability contribute to the deceased stability of PD-1 KO P14 cells in chronic infection.

Figure 3.7

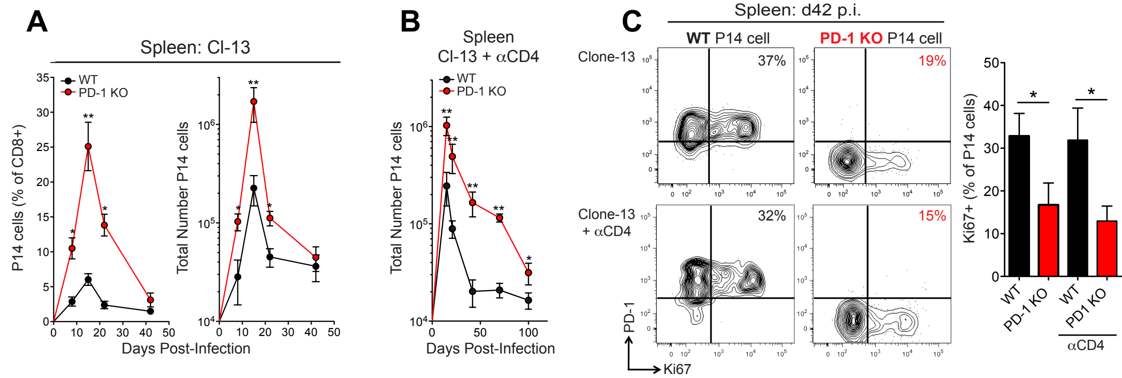


Figure 3.7: Diminished long-term proliferation and stability of CD8+ T cells in the absence of PD-1. (A) Longitudinal analysis of frequency (left) and absolute numbers (right) of WT and PD-1 KO P14 cells in the spleen during LCMV clone 13 infection. **(B)** Longitudinal analysis of absolute number of WT and PD-1 KO P14 cells in the spleen of mice treated with anti-CD4-antibody at d-1 and d1 of LCMV clone 13 infection. **(C)** Expression of PD-1 versus Ki67 in WT and PD-1 KO P14 cells at d42 p.i. in the spleen during infection with LCMV clone 13 with or without CD4 depletion. Values indicate frequency of P14 cells positive for Ki67 (+/- s.e.m.), *$p < 0.05$ **$p < 0.01$ (paired Student's t-test). All data are representative of 3 independent experiments with at least 5 mice per group.

3.3.6: PD-1 controls exhausted CD8+ T cell proliferative dynamics by regulating Tbet^{hi} and Eomes^{hi} subsets

Recent work has demonstrated that two subsets of exhausted CD8+ T cells, Tbet^{hi} progenitors and Eomes^{hi} progeny, exist in a proliferative hierarchy to sustain long-term CD8+ T cell responses during chronic viral infections (Paley et al., 2012). Based on these observations, we next investigated whether loss of PD-1 impacted the balance between these two subsets and if changes in these subsets could account for the altered proliferation and stability of the PD-1 KO P14 population. To test this idea, we assessed expression of Tbet and Eomes at days 8, 15 and 42 p.i. in WT and PD-1 KO P14 cells. At day 8 p.i., increased expression of both Tbet and Eomes was observed in PD-1 KO P14 cells, which is consistent with the increased activation of these cells early in infection (**Figure 3.8A**). However by day 15 p.i., PD-1 KO P14 cells had a significant decrease in Tbet MFI (**Figure 3.8A**) that was followed by a dramatic loss of Tbet^{hi} cells at day 42 p.i. (**Figure 3.8B** and **Figure 3.8C**). This loss of Tbet^{hi} cells corresponded with a skewing of the PD-1 KO P14 cell population towards the Eomes^{hi} subset, as demonstrated by an increase in both frequency of Eomes^{hi} cells and Eomes MFI at d15 (**Figure 3.8A**) and 42 p.i. (**Figure 3.8B** and **Figure 3.8C**). We next asked if this change in Tbet observed in exhausted PD-1 KO P14 cells truly represented a shift in subset dynamics to Eomes^{hi} progeny. To test this idea, PD-1 KO P14 cells were assessed for other cardinal features of Eomes^{hi} cells (Paley et al., 2012). PD-1 KO P14 cells, like WT Eomes^{hi} exhausted CD8+ T cells, were significantly increased in frequency in peripheral tissues, including the bone marrow and liver **(Figure 3.9A)**.

Figure 3.8

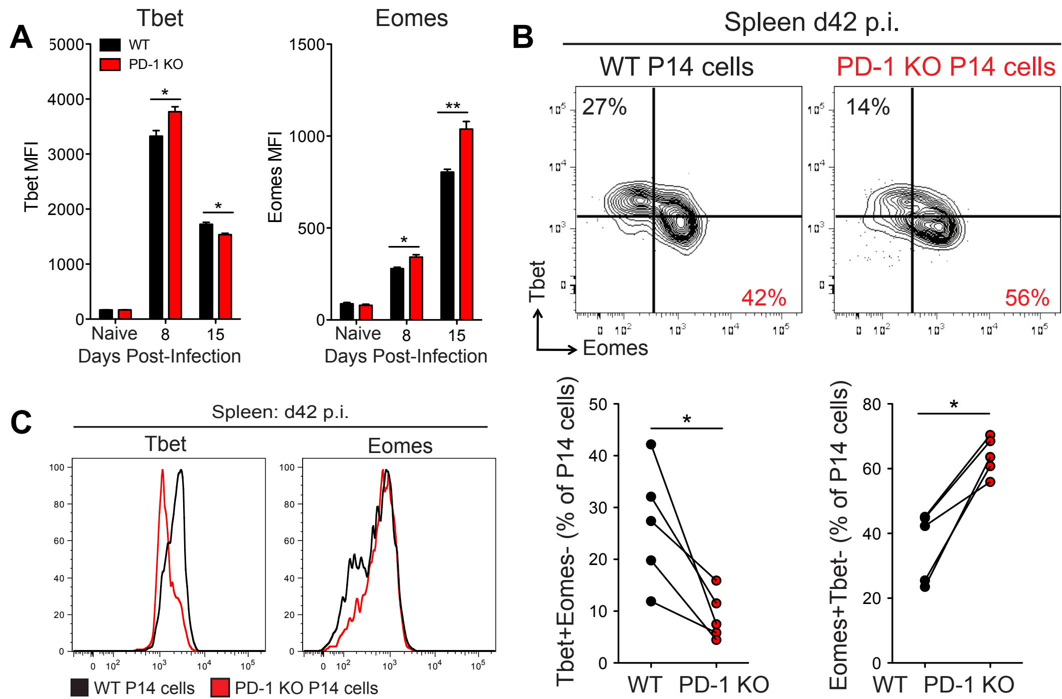


Figure 3.8: Altered dynamics of exhausted CD8+ T cell subsets in the absence of PD-1. (A) Expression of Tbet and Eomes in WT and PD-1 KO P14 cells in the spleen at d0 (naïve), d8 and d15 p.i. with LCMV clone 13 (+/- s.e.m.) **(B)** Expression of Tbet versus Eomes in WT and PD-1 KO P14 cells at d42 p.i. in the spleen of individual (top) and multiple mice (bottom). Values indicate frequency of P14 cells that are T-bet^{hi} Eomes^{lo} or Eomes^{hi} T-bet^{lo} **(C)** Representative plots of MFI of Tbet and Eomes expression in WT (black) and PD-1 KO (red) P14 cells at d42 p.i. in the spleen **$p < 0.01$ (paired Student's t-test). All data are representative of 2-5 independent experiments with at least 5 mice per group.

Key functional properties of $Eomes^{hi}$ cells were also observed in PD-1 KO P14 cells. Despite decreased cytokine production (**Figure 3.2A**) and proliferation (**Figure 3.7C**), PD-1 KO P14 cells possessed higher cytotoxicity than WT P14 cells **(Figure 3.9B)**, as well as increased expression of Granzyme B at d42 p.i. (**Figure 3.9C**). This is also consistent with the high expression of multiple inhibitory receptors observed on both PD-1 KO P14 cells and WT $Eomes^{hi}$ cells **(Figure 3.3A** and **Figure 3.3B)**. Thus, PD-1 KO P14 cells accumulate mainly as terminally-differentiated $Eomes^{hi}$ progeny. These findings suggest that PD-1 controls proliferation dynamics during chronic viral infection, at least in part, by preventing loss of $Tbet^{hi}$ progenitor cells. In addition to changes in proliferation, loss of PD-1 signals leads to an exhausted CD8+ T cell pool with more extensive tissue distribution and enhanced cytotoxicity, which may be an important consideration when using PD-1 blockade for chronic infections and cancer.

Figure 3.9

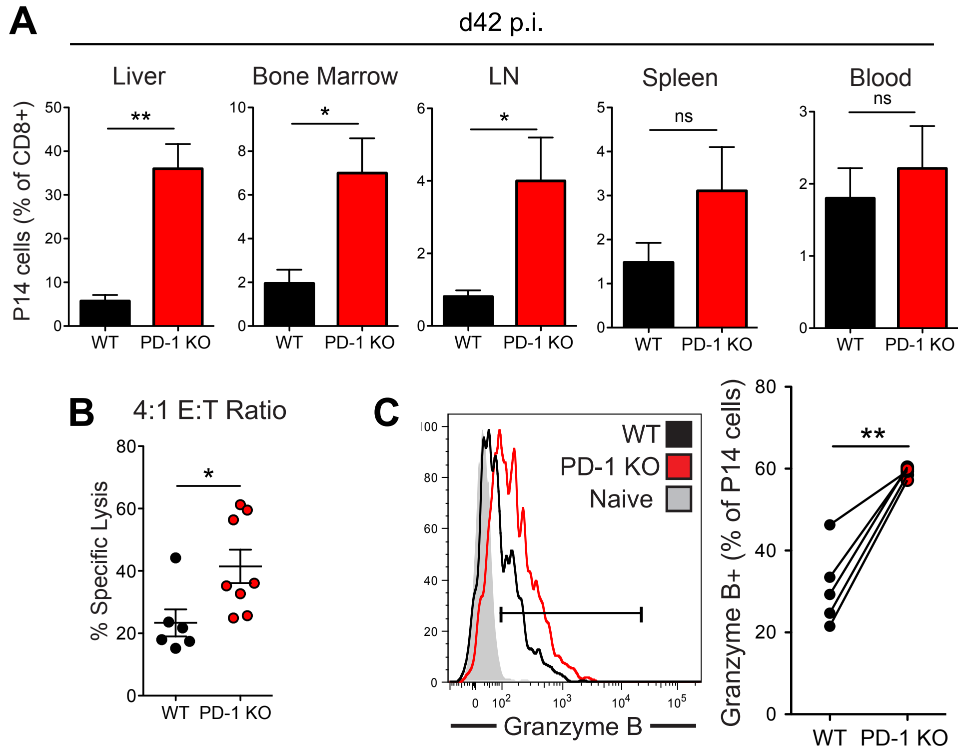


Figure 3.9: PD-1 KO P14 cells accumulate as Eomes^{hi} progeny with the same cardinal properties. (A) Frequency of P14 cells as a percentage of total CD8+ T cells in multiple organs at d42 p.i. with LCMV clone 13 (+/- s.e.m.). **(B)** Cytotoxicity of sorted WT and PD-1 KO P14 cells on d22 p.i. at E:T ratio of 4:1 following 18h incubation (+/- s.e.m.), *$p < 0.05$ (unpaired Student's t-test). **(C)** Expression of Granzyme B in naïve CD8+ T cells, WT P14 cells and PD-1 KO P14 cells in individual (left) and multiple mice (right), **$p < 0.01$ (paired Student's t-test). All data are representative of 2-5 independent experiments with at least 5 mice per group.

Figure 3.10

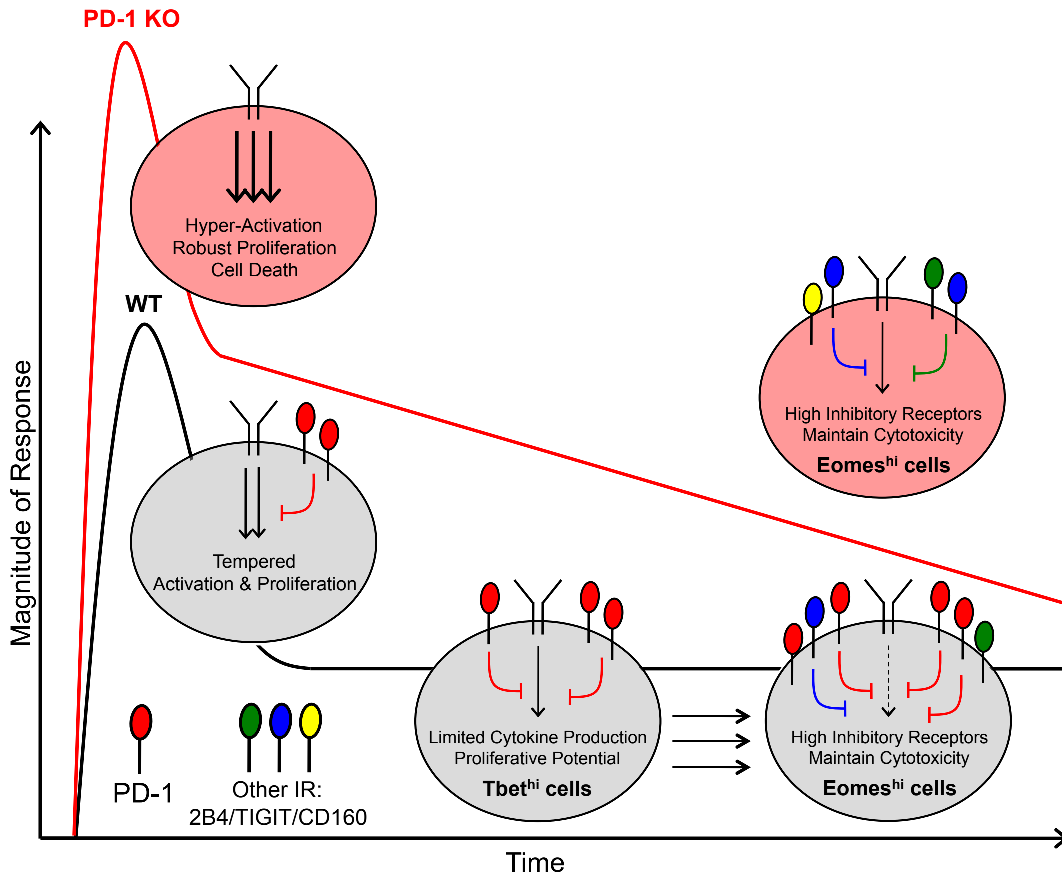


Figure 3.10: Model of the role for PD-1 in the development of CD8+ T cell exhaustion during chronic viral infection. In WT settings (black), PD-1 tempers the early activation and proliferation of CD8+ T cell responses to chronic infection, promoting survival of exhausted CD8+ T cells into the chronic phase of infection and the establishment of Tbet^{hi} and Eomes^{hi} subsets. In contrast, PD-1 deficiency (red) leads to the hyper-activation and excessive proliferation of early CD8+ T cell responses to chronic infection, resulting in increased cell death. This is associated with loss of the Tbet^{hi} subset of exhausted CD8+ T cells and the accumulation of terminally-differentiated Eomes^{hi} cells. As a result, PD-1 deficient exhausted CD8+ T cells exhibit unique functional properties and have largely lost their antigen-dependent proliferative ability, resulting in decreased population stability long-term.

3.3.7: Discussion

Expression of the inhibitory receptor PD-1 is strongly associated with CD8+ T cell exhaustion in chronic infection and cancer. The ability to partially restore T cell functions by PD-1/PD-L1 pathway blockade suggests a fundamental link between this pathway and the development of CD8+ T cell exhaustion (Barber et al., 2006; Freeman et al., 2006; Petrovas et al., 2006; Blank et al., 2005; Wherry, 2011). A key unanswered question, however, is whether or not PD-1 directly causes or induces T cell exhaustion. Here, we tested this idea using approaches that control for a number of confounding issues that previously obscured clear answers to this question. We found that genetic absence of PD-1 on antigen-specific CD8+ T cells did not prevent exhaustion during chronic LCMV infection. Consistent with the negative regulatory role of this pathway, CD8+ T cell expansion was greatly enhanced by the absence of PD-1 during the acute phase of infection (Barber et al., 2006; Parry and Riley, 2005; Patsoukis et al., 2012; Okazaki et al., 2013). However, PD-1 deficiency also led to increased apoptosis during the contraction phase. Moreover, permanent absence of PD-1 resulted in the dysregulation of a key proliferative hierarchy required to sustain exhausted $CD8^{+}$ T cell populations during chronic infection. Without PD-1, exhausted CD8+ T cells accumulated as $Eomes^{hi}$ cells, while the $T\text{-}bet^{hi}$ progenitor pool was depleted. In addition, this shift was accompanied by functional changes associated with an altered balance of these subsets. Cytokine production declined in the absence of PD-1 due to loss of the slightly more functional $T\text{-}bet^{hi}$ population, but cytotoxicity was improved since the $Eomes^{hi}$ subset has better killing capacity. These findings reveal an unexpected dual role for PD-1 signals during chronic viral infection. While PD-1 clearly negatively regulates CD8+ T cell responses during the chronic phase of infection (Barber et al.,

2006; Wherry, 2011), this pathway also plays a critical role in protecting exhausted CD8+ T cells from excessive stimulation, terminal exhaustion and erosion of the population over time. These findings have implications for prolonged clinical treatments with PD-1 pathway antagonists where the benefit of rejuvenating exhausted CD8 T cells may have to be balanced with the risk of over-stimulation and lack of long-term stability.

While complete genetic absence of PD-1 is unlikely to manifest clinically, several important genetic variants in and around the *PDCD1* gene have been described that are associated with autoimmunity, cancer prognosis or chronic viral infection (James et al., 2005; Zhang et al., 2012; Velázquez-Cruz et al., 2007; Muenst et al., 2010). While mechanistically it remains unclear how most of these genetic variants affect the expression or function of PD-1, these observations do suggest that genetic variation in the PD-1 pathway could be an important parameter affecting the development of T cell responses in humans. It will be interesting to determine whether there are any relationships between these human *PDCD1* polymorphisms and changes in the development and/or dynamics of exhausted T cells we have observed in mice. The potential impact of genetic variation in genes in the PD-1 pathway and outcomes of clinical PD-1 treatments remains to be defined. In addition to natural genetic variation, it is now possible to genetically engineer T cells for adoptive immunotherapeutic approaches (Restifo et al., 2012). PD-1 might be an interesting target to consider in such therapeutically modified T cell adoptive immunotherapy approaches. However, our data suggest that such approaches to modify or genetically ablate PD-1 should be considered with caution.

One interesting aspect of the PD-1 deficient CD8+ T cell response that develops during chronic LCMV infection is the elevated expression of many other inhibitory receptors. In the absence of PD-1, we observed higher expression of Lag-3,

2B4/CD244, CD160, and Tigit. Most, if not all, of these inhibitory receptors are upregulated following TCR signaling in acutely activated T cells and their elevated expression by PD-1 deficient CD8+ T cells may indicate a greater degree of ongoing TCR stimulation. These observations are consistent with the idea that TCR signaling is increased in the absence of PD-1 and that PD-1 is an essential rheostat or tuner of *in vivo* TCR signaling (Okazaki et al., 2013; Honda et al., 2014). In addition, the upregulation of other inhibitory receptors in the absence of PD-1 signals could partially explain the synergy observed during co-blockade of PD-1 and other inhibitory receptors and reveal opportunities for targeting other inhibitory receptors in conjunction with PD-1. Thus, these observations support use of co-blockade of multiple inhibitory receptors in cancer patients for optimal reversal of T cell exhaustion. This issue will be interesting to examine clinically as the timing and/or sequence with which different inhibitory receptor blockades are introduced may influence the therapeutic outcome.

Our data provide a cellular mechanism to explain the population-based effects of PD-1 deficiency during chronic infection. PD-1 appears critical for regulation of a proliferative hierarchy necessary to maintain exhausted CD8+ T cell populations. We have previously described a lineage relationship within the exhausted CD8+ T cell pool where exhausted PD-1^{int} T-bet^{hi} cells give rise to PD-1^{hi} $Eomes^{hi}$ cells. This transition is driven by persisting antigen and is accompanied by extensive division, downregulation of T-bet and upregulation of Eomes (Paley et al., 2012). These $Eomes^{hi}$ progeny are terminally differentiated and lack future proliferative capacity (Paley et al., 2012). Such exhausted CD8+ T cells subsets are found not only in chronic LCMV infection of mice, but also in human HCV and HIV infection. Our current data indicate a critical, but perhaps somewhat paradoxical, role for PD-1 in this proliferative hierarchy. Without PD-1, exhausted CD8+ T cells accumulate as $Eomes^{hi}$ terminally differentiated progeny.

This observation suggests that permanent loss of PD-1 signals may progressively erode exhausted CD8+ T cell populations. These data also indicate that PD-1 tempers the continual activation and differentiation of the T-bet^{hi} progenitor pool, in effect protecting this subset from chronic overstimulation and preserving these cells for future activation and generation of terminal progeny. Previous work demonstrated that T-bet directly represses expression of the *PDCD1* gene (Kao et al., 2011). In addition, persisting antigen stimulation can lead to loss of T-bet expression (Kao et al., 2011; Paley et al., 2012). Taken together, these observations suggest a model whereby PD-1 inhibits TCR signaling during chronic infection, which helps maintain T-bet expression. T-bet, in turn, prevents the highest level of PD-1 expression that may preserve cytokine production and proliferative potential in the Tbet^{hi} progenitor population. We would predict that variations in antigen and/or viral load would lead to an imbalance in this feedback loop. Settings of high antigen stimulation may favor the activation and proliferation of Tbet^{hi} progenitors followed by loss of T-bet expression, further upregulation of PD-1 and Eomes expression and, ultimately, terminal differentiation. Thus, our data suggest a novel dual role for PD-1 signals in negatively regulating CD8+ T cell proliferation and function during chronic infection, but also in sustaining Tbet^{hi} progenitor cells, a population critical for a proliferative hierarchy that contains chronic infection.

In summary, we have identified a novel role for the PD-1 pathway in regulating CD8+ T cell exhaustion during chronic viral infection. PD-1 signals are critical for preventing the early over-activation and proliferation of CD8+ T cells in response to chronic infection, thus promoting the establishment and stability of exhausted T cell responses. Additional work will be necessary to further characterize the molecular pathways regulated by PD-1 in exhausted CD8+ T cells and to compare genetic loss of PD-1 to the more clinically relevant setting of PD-1 pathway blockade.

Chapter 4: Concluding Remarks and Future Directions

4.1 Overview of Results

The PD-1 pathway regulates T cell activation and function by inhibiting TCR and co-stimulatory signals. A previously unresolved question, however, is how PD-1 pathway inhibition impacts the differentiation of CD8+ T cells in settings of acutely-resolved or chronic infection. In **chapter 2** of this thesis, the role of PD-1 signals in the development of effector and memory CD8+ T cells during acutely-resolved influenza infection was investigated. While loss of PD-1 or PD-1 ligands enhanced the expansion of effector CD8+ T cells, the development of long-lasting CD8+ T cell memory responses was impaired. Defects in the magnitude of the response, as well as in cytokine production and recall capacity of memory CD8+ T cells were observed in the absence of the PD-1 pathway. Thus, the inhibition of effector CD8+ T cell responses by PD-1 signals promotes the generation and longevity of memory responses in a setting of acutely-resolved infection (**Figure 2.11**).

The PD-1 pathway also plays a prominent role in settings of chronic antigen stimulation and CD8+ T cell exhaustion, including chronic infections and cancer. Previous work has demonstrated regulation of established exhausted CD8+ T cell responses by the PD-1 pathway; however, the role of PD-1 signals in the development of CD8+ T cell exhaustion is not well understood. In **chapter 3** of this thesis, PD-1 signals were shown to inhibit the early proliferation and expansion of virus-specific CD8+ T cells during chronic LCMV infection. Loss of PD-1 from the onset of infection, however, resulted in increased cell death during the contraction phase. Ultimately, PD-1 deficiency led to more severe dysfunction of cytokine production and proliferation in the chronic

phase of infection. However, PD-1 deficient CD8+ T cells retained cytotoxic ability and had enhanced infiltration into peripheral tissues. This PD-1-deficient exhaustion phenotype was associated with loss of Tbet^{hi} progenitor cells and the accumulation of terminally-differentiated Eomes^{hi} progeny (**Figure 3.10**).

Taken together, **chapters 2 and 3** of this thesis demonstrate a critical role of PD-1 signals in restricting the early activation, proliferation and expansion of CD8+ T cells during both acutely-resolved and chronic infections. Importantly, this early regulation of CD8+ T cell responses by the PD-1 pathway has profound long-term consequences on the development of memory or exhausted T cell responses. PD-1, therefore, acts to tune CD8+ T cell responses, allowing for a robust and effective anti-pathogen response but balancing this with the need generate long-lived memory or exhausted responses. This work has important implications for clinical targeting of the PD-1 pathway in both prophylactic vaccination and therapeutic treatment strategies. Given the recent FDA approval of PD-1 blocking monoclonal antibodies (FDA.gov), these novel roles for PD-1 should also be considered when designing therapy regimens.

4.2 Context dependent roles of PD-1 on CD8+ T cells

PD-1 has been demonstrated to modulate T cell responses in many areas of disease, including auto-immunity, cancer and infections (Francisco et al., 2010; Okazaki et al., 2013). In all of these settings, PD-1 plays a role in “tuning” the threshold of TCR activation; however, it has become apparent in recent years that PD-1 has unique, context-dependent functions, as well. **Chapters 2** and **3** of this thesis expand on this idea and identify novel similarities and differences in how PD-1 regulates the CD8+ T cell response to acutely-resolved vs. chronic viral infection. Specifically, the factors that seem to impact PD-1 mediated inhibition of CD8+ T cells include: the strength of TCR

stimulation, the duration of antigen exposure and the anatomical location of TCR:antigen signals. Below, these factors will be discussed in the context of the findings in this thesis.

4.2.1 PD-1 and TCR signal strength

The idea that strength of TCR:antigen stimulation impacts the extent of PD-1 mediated inhibition has been investigated in several *in vitro* settings (Bennett and Carreno, 2002; Yokosuka et al., 2012; Parry and Riley, 2005; Freeman et al., 2000b). Using a titration of CD3 and CD28 concentrations, Freeman et al. demonstrated that PD-1 inhibition of T cell proliferation was most robust at low or suboptimal levels of TCR engagement and CD28 signaling. However, higher or more optimal levels of TCR and CD28 signals seemed to circumvent the negative effects of PD-1 ligation (Freeman et al., 2000a). These studies were the first to suggest that the consequences of PD-1/L1 ligation depend on the strength of signaling through TCR and CD28. Recently, additional *in vitro* work has demonstrated that the level of PD-1 expression on the cell surface also impacts the amount of T cell inhibition, with higher levels of PD-1 corresponding with greater inhibition of T cell activation and functions (Wei et al., 2013). Importantly, it is the amount of PD-1 within the immunological synapse that appears to be most relevant (Yokosuka et al., 2012). These ideas are consistent with work presented in **chapter 2** of this thesis, where differences in PD-1 mediated inhibition *in vivo* seemed to correspond with TCR signal strength. For example, CD8+ T cells specific for sub-dominant epitopes of influenza seem to be most dramatically impacted by loss of PD-1 pathway signals. It may be that, within the same environment, CD8+ T cells with a TCR that receives lower levels of stimulation are more sensitive to PD-1-mediated inhibition, consistent with previous *in vitro* findings. Similar observations have been made anecdotally in our lab during LCMV clone 13 infection. Previous work has demonstrated that different epitopes

of LCMV receive different "levels" of epitope presentation (Wherry et al., 2003a). These varying amounts of TCR stimulation correlate with different outcomes of CD8+ T cell exhaustion. For example the NP396 epitope is displayed at high levels during LCMV clone 13, which results in rapid exhaustion and clonal deletion of NP396-specific CD8+ T cells. Other epitopes of LCMV, such as GP33 and G276, have lower MHC class 1 binding affinities and T cell stimulatory capacities. As a result, GP33-specific and GP276-specific CD8+ T cells become functionally exhausted but not clonally deleted (Wherry et al., 2003a). Unpublished work in our lab suggests that virus-specific CD8+ T cells that receive less TCR stimulation *in vivo* during chronic viral infection, particularly GP276-specific CD8+ T cells, are most responsive to PD-1 pathway blockade. This would be consistent with the idea that the TCR signal strength is a major contributing factor to the extent of PD-1 pathway inhibition *in vivo* during both acutely-resolved and chronic infections.

4.2.2: PD-1 and duration of antigen stimulation

The duration of antigen stimulation is another critical parameter that impacts PD-1 pathway inhibition. An example of this is demonstrated in **chapters 2** and **3** of this thesis, where absence of cell-intrinsic PD-1 results in different outcomes for CD8+ T cell responses to acutely-resolved influenza infection and chronic LCMV infection. Although there are many differences between influenza infection vs. LCMV clone-13 infection, PD-1 deficient P14 cells were used in both settings allowing for comparison of CD8+ T cells with same TCR specificity and affinity in the context of different durations of antigen exposure. Absence of PD-1 led to robust proliferation and expansion during the effector phase of acute influenza infection and chronic LCMV infection, a period when pathogen load is high in both settings. It should also be noted that this is a period when PD-1

expression is also normally elevated during acute or chronic infections. However, the role of PD-1 on the long-term CD8+ T cell responses is different following either the clearance or persistence of pathogen. PD-1 KO P14 cells upregulate properties of memory CD8+ T cells following acutely-resolved influenza infection, including cytokine production, recall capacity, and phenotypic memory markers. In contrast, during chronic LCMV infection, PD-1 KO P14 cells become severely exhausted, up-regulate multiple other inhibitory receptors and become terminally-differentiated. It may be that the continued exposure of virus-specific CD8+ T cells to antigen during chronic infection requires the counter-balance of inhibitory signals from PD-1. Thus, absence of PD-1 in settings of persistent antigen may be more detrimental to the long-term phenotype, function and survival of CD8+ T cells. Of note, despite these very different functional outcomes, in both settings of acutely-resolved and chronic infection loss of PD-1 pathway signals results in more defective memory or exhausted CD8+ T cell responses compared to wildtype CD8+ T cells.

4.2.3: PD-1 and anatomical location

The anatomical location of TCR:antigen and PD-1:PD-L1/2 interactions also seems to be an important factor in the amount of PD-1 mediated inhibition. Several studies support the idea that the “tuning” function of PD-1 protects the host from immune-mediated damage, particularly in sensitive and inflamed tissues (Frebel et al., 2012; Honda et al., 2014; Okazaki et al., 2013). For example, recent work has described that PD-1/PD-L1 signals are part of a regulatory mechanism that prevents prolonged T cell responses in inflamed tissues. Honda et al. show that PD-1 inhibits antigen-driven CD4+ T cell arrest and subsequent effector functions in a model of delayed hypersensitivity, essentially “desensitizing” CD4+ T cells at the site of inflammation. As a result, a system

is established where new effectors migrate into the inflamed tissue, perform their function and are subsequently desensitized. This pattern can then continue to effectively clear a pathogen while maximizing tissue viability (Honda et al., 2014). Similarly, in **chapter 2** of this thesis, the regulation of CD8+ T cell proliferation and survival by PD-1 was different in lymphoid tissues vs. the inflamed site of infection during influenza infection. PD-1 pathway deficiency led to increased CD8+ T cell proliferation in the spleen and dLN, but not in the lung. While this robust proliferation resulted in greater CD8+ T cell numbers in the lung, there was a subsequent increase in cell death of PD-1 pathway deficient CD8+ T cells at the site of infection. These findings are consistent with the idea that PD-1 can curb immune-mediated damage in inflamed sites by restricting the proliferation and effector functions of CD8+ T cells. It is interesting that, even in the absence of PD-1 signals, effector CD8+ T cells in the lung experienced more cell death. While one contributing factor is likely over-stimulation of PD-1 pathway deficient CD8+ T cells in lymphoid tissue, it may also be that other regulatory mechanisms in the lung are activated to prevent immunopathology.

Interestingly, during LCMV clone 13 infection, PD-1 KO P14 cells seem to preferentially localize to peripheral tissues at very high frequencies during the chronic phase of infection. It may also be that exhausted CD8+ T cells become "desensitized" at these sites; however, whether PD-1 is mediating this effect in exhausted CD8+ T cells is less clear. Overall, it seems that the anatomical location of TCR:antigen stimulation, as well as the context in which PD-1 engages PD-L1/2, can alter the effects and outcome of PD-1 inhibition on T cells. It may be that inflammatory cues or other signals at the site of infection collectively influence the role of PD-1 signals in areas of high pathogen load.

4.3: Temporal Regulation by the PD-1 pathway

The idea that strength, duration and location of antigen stimulation can impact PD-1 mediated inhibition also suggests that PD-1 will have different effects on T cells during different phases of an immune response. In other words, PD-1 may be playing distinct roles on naïve, effector, and memory or exhausted CD8+ T cells, which have very different exposures to TCR:antigen. For example, in an infection setting, PD-1 pathway signals will be present during initial naïve CD8+ T cell priming in lymphoid tissue. Previous work has demonstrated that on naïve T cells, PD-1 can impact TCR signaling cascades, T cell motility, cell cycle progression and, ultimately, effector functions (Parry and Riley, 2005; Patsoukis et al., 2012; Zinselmeyer et al., 2013; Yokosuka et al., 2012; Freeman et al., 2000a; Butte et al., 2007). Much of this work has been performed *in vitro* and it still remains unclear if identical mechanisms are in place during *in vivo* T cell priming. Given the robust expansion of PD-1 deficient CD8+ T cells during the effector phase (discussed below), it is likely that PD-1 regulates TCR signaling, metabolism, and cell-cycle progression during *in vivo* T cell priming.

The work presented in **chapters 2** and **3** extend our knowledge of the role of PD-1 during the effector phase of the immune response. In the absence of PD-1, CD8+ T cells undergo increased proliferation and expansion compared to wildtype CD8+ T cells during the effector phase of both acutely-resolved influenza infection and chronic LCMV infection. However, loss of PD-1 signals also resulted in decreased survival of CD8+ T cells during the contraction phase. These findings demonstrate that a central role for PD-1 is to prevent over-activation of CD8+ T cells during the effector phase and to promote survival CD8+ T cells long-term as they differentiate into memory or exhausted cells. This tempering of T cell activation during the effector phase is also likely a mechanism to

prevent immune-mediated damage in the host. Indeed, PD-L1 deficient mice die from LCMV clone-13 infection between days 7-9 from severe T cell-mediated immunopathology (Barber et al., 2006).

The data presented in **chapters 2** and **3** of this thesis also suggest that early PD-1 signals may not only quantitatively tune activation-induced signals, but also impact the differentiation pathway of CD8+ T cells. During acutely-resolved influenza infection, early PD-1 pathway signals seem to promote the development of optimally-functional CD8+ memory T cells. The defect in memory CD8+ T cells in the absence of PD-1 may be due to selective over-activation and loss of memory precursor cells. However, it could also be that PD-1 signals qualitatively change the gene expression pattern of CD8+ T cells early during priming or effector differentiation in a way that promotes memory development. Given the changes in gene expression observed in PD-L1/2 deficient mice during influenza infection, it may be that both mechanisms are in place. During LCMV clone 13 infection, excessive proliferation and cell death in the absence of PD-1 leads to loss of Tbet^{hi} progenitor cells and accumulation of Eomes^{hi} progeny, which corresponds with altered functionality and durability of exhausted CD8+ T cells. While this is certainly one mechanism that explains the observed phenotype, it is possible that the resulting Eomes^{hi} cells also have a different “program” of dysfunction in the absence of PD-1. Gene expression analysis of these cells should reveal any additional changes in differentiation status of these PD-1 deficient exhausted CD8+ T cells.

Overall, these findings suggest that early regulation of naïve and/or effector CD8+ T cells can dramatically impact long-term memory or exhausted T cell responses. Interestingly, previous work has demonstrated distinct regulation of established memory or exhausted CD8+ T cells by the PD-1 pathway during later stages of infection. Blockade of the PD-1 pathway on memory cells just prior to re-challenge has been

demonstrated to improve recall capacity of CD8+ T cells (Erickson et al., 2012, 2014). Similarly, PD-1/L1 blockade during the chronic phase of LCMV clone 13 infection improves CD8+ T cell functionality and lowers viral loads (Barber et al., 2006). One potential explanation for this discrepancy is that PD-1 signals differently in CD8+ T cells during later stages of infection. Exhausted and memory CD8+ T cells have distinct transcriptional profiles and changes in the expression of genes involved in signal transduction and metabolism (Wherry et al., 2007). Thus, making direct extrapolations of *in vitro* PD-1 signaling observations to *in vivo* generated exhausted and memory CD8+ T cells is not straightforward. In addition, the technical challenges of performing detailed biochemical analysis of signaling in exhausted CD8+ T cells generated *in vivo* have hampered direct interrogation of these questions. Furthermore, it is not known how different subsets of memory and exhausted cells, with different TCR affinities and levels of PD-1 expression, are regulated by the PD-1 pathway. Future work should help elucidate how early PD-1 signals alter the differentiation program of memory and exhausted CD8+ T cells. It will also be interesting to investigate the potentially different effects of PD-1 inhibition on naïve, effector, memory or exhausted CD8+ T cells. A better understanding of how PD-1 regulates CD8+ T cells at different stages of differentiation and in different phases of the immune responses will inform design of therapeutic strategies to target the PD-1 pathway.

4.4: Similarities between exhausted PD-1 deficient CD8+ T cells and exhausted PD-1^{hi} CD8+ T cells in chronic infection

A somewhat unexpected finding of the work presented in **chapter 3** of this thesis is that PD-1 deficiency leads to an exhausted CD8+ T cell pool with similar characteristics to PD-1^{hi} exhausted CD8+ T cells. Both PD-1 KO and PD-1^{hi} exhausted

cells are more terminally-differentiated, upregulate several other inhibitory receptors and have largely lost their ability to produce cytokines and proliferate. However, PD-1 KO and PD-1^{hi} cells retain cytotoxic ability and also preferentially localize to and accumulate in peripheral tissues. Transcriptional regulation of these cells seems to occur via high expression of Eomes and low expression of Tbet (Blackburn et al., 2008; Paley et al., 2012; Kao et al., 2011). So why do similar states of exhaustion develop in the complete absence of PD-1 and with the highest levels of PD-1 expression? One hypothesis is that in this state of terminal-exhaustion, PD-1 signals do not play a significant role in regulating the functionality or survival of these Eomes^{hi} cells. It may be that Eomes^{hi} cells do not get stimulated as frequently through their TCR and/or that PD-1 ligation is less frequent on this cells. The distinct localization of these cells could, in part, support this idea. Previous work in the lab has demonstrated that sites, such as the bone marrow, have similar viral clearance to the spleen and blood but lower expression levels of PD-L1. An idea that has emerged from this finding is that peripheral tissues can serve as a survival niche for more terminally-differentiated cells that are sensitive to cell death (Blackburn et al., 2010a). While the levels of PD-L1 may account for this, it could also be that other inflammatory or survival cues are present in these organs that provide a “safe” environment for Eomes^{hi} cells. Of note, it is still not clear what signals direct Eomes^{hi} cells to peripheral tissues and promote their accumulation there. The idea that PD-1 signals are not critical for regulation of Eomes^{hi} cells could also explain the observation that PD-1^{hi} cells are not responsive to PD-1 pathway blockade.

A complementary hypothesis to explain the similarities between PD-1 KO and PD-1^{hi} cells is that other inhibitory receptors play a more central role in regulating Eomes^{hi} cell functions. Both PD-1 KO and PD-1^{hi} cells upregulate the expression and co-expression of CD160, 2B4, and Lag-3 (Blackburn et al., 2009; Paley et al., 2012). It’s

possible that these or other inhibitory receptors “take over” as the central negative regulators of terminally-differentiated exhausted cells. It will be interesting to determine if co-blockade of PD-1 and other inhibitory receptors can potentially re-invigorate PD-1 KO or PD-1^{hi} cell functions.

Although there are many similarities between PD-1 KO and PD-1^{hi} exhausted CD8+ T cells, future work should begin to dissect differences between these cell populations. It may be that other transcription factors play important and distinct roles in these cell types, including BATF or Blimp-1. Transcriptional profiling of these cells will provide key insights into these differences and also may inform strategies to improve functionality of these terminally-differentiated cells.

4.5: How do you most effectively target the PD-1 pathway?

Despite the clear clinical promise and many early successes of PD-1/L1 blockade, the optimal therapeutic approach for targeting the PD-1 pathway remains to be determined. Transient blockade of PD-1 or PD-L1 can have profound effects on the re-invigoration of exhausted CD8+ T cell populations during chronic infections and cancer, including numerical expansion, improved effector function and reduced viral or tumor burden (Barber et al., 2006; Day et al., 2006; Freeman et al., 2006; Blank et al., 2005). However, permanent absence of PD-1 did not prevent the development of exhaustion during chronic LCMV infection, as described in **chapter 3**. In fact, CD8+ T cell dysfunction was increased with genetic PD-1 deficiency, resulting in terminal-exhaustion and decreased stability of the CD8+ T cell population. These findings have important implications for different therapeutic clinical approaches since long-term and/or “maintenance” PD-1 pathway blockade and genetically engineered T cells with altered PD-1 are being considered in human cancer patients. There are several key questions

that should be addressed to determine the most effective strategies for use of PD-1 pathway inhibitors in patients.

First, in the experimental model used here, PD-1 signaling is absent for both CD8+ T cell priming and throughout chronic stimulation. It remains unclear if PD-1 deficiency has the same effect in different stages of T cell differentiation, as previously discussed. It may be that loss of PD-1 signals during CD8+ T cell priming is detrimental, while removing PD-1 during later stages of infection will have more beneficial effects. The use of ER-Cre PD-1 floxed P14 mice will allow for selective deletion of PD-1 in CD8+ T cells during various stages of infection to begin to address this question.

Second, it is unclear whether genetic absence of PD-1 is equivalent to antibody-mediated blockade. Given the broad expression of PD-1 and PD-1 ligands, blockade of the PD-1 pathway will likely impact a variety of immune and non-immune cell types. In fact, unpublished work in our lab has demonstrated that PD-1/L1 blockade not only impacts CD8+ T cells, but also the frequency of B cells, regulatory T cells, neutrophils, and other key immune cells (Odorizzi, unpublished data). In addition, some preliminary studies in ER-Cre PD-1 flox mice have shown that genetic deletion of PD-1 during the chronic phase of LCMV infection improves CD8+ T cell effector functions to a different extent than antibody-mediated PD-1 blockade during the chronic phase of infection (Odorizzi, unpublished data). Therefore, the use of monoclonal antibodies with different binding affinities and *in vivo* half lives should be considered carefully, as the prolonged and complete loss of PD-1 signals may be significantly different than shorter term or incomplete blockade of the PD-1 pathway. In addition, it should be carefully considered how prolonged inhibition of PD-1 signals in cancer patients would subsequently impact their immune responses to vaccination or infection. The data presented here suggest

that pathogen-specific CD8+ T cell responses will be dysregulated without the PD-1 pathway.

Finally, the impact of prolonged inhibition of the PD-1 pathway may be considerably different in the presence versus absence of high-level antigen stimulation. In the current setting of chronic LCMV infection, where the antigen stimulation and “stress” on the CD8+ T cell compartment is high and constant, absence of a key negative regulatory pathway may result in T cell over-stimulation. In setting where antigen levels have been controlled to a low level, continued loss of PD-1 signals may be less detrimental to the stability to the T cell population, may promote CD8+ T cell functionality and may even be beneficial. Future studies will be necessary to dissect these important and clinically relevant possibilities.

4.6: Biomarkers for successful PD-1-based therapies

One interesting idea that arises from this work is that the effects of losing PD-1 signals can be monitored by changes in the phenotype of virus-specific CD8+ T cells. In **chapter 3** of this thesis, genetic absence of PD-1 leads to a dramatic loss of $Tbet^{hi}$ cells and accumulation of terminally-differentiated, $Eomes^{hi}$ exhausted CD8+ T cells. Although PD-1 blockade during the chronic phase of infection has different short-term functional consequences on CD8+ T cells, there is also a significant shift towards $Eomes^{hi}$ PD-1^{hi} progeny in this setting. In addition, this accumulation of $Eomes^{hi}$ PD-1^{hi} cells can be seen in the bulk CD8+ T cell population, along with increased Ki67 expression that accompanies the conversion of $Tbet^{hi}$ progenitors into $Eomes^{hi}$ cells. Of note, changes in Tbet expression seem more challenging to resolve in the bulk CD8+ T cell population (Odorizzi and Pauken, unpublished data). These findings suggest that expression of Eomes, PD-1 and perhaps Ki67 could be examined as a way to screen for successful

induction of immune responses following clinical use of PD-1 pathway blockade. Indeed, in collaboration with Bob Vonderhide and Andy Minn's lab, a mouse model of melanoma was used to demonstrate that successful radiation and checkpoint blockade correlated with induction of Ki67+ Granzyme B+ Eomes^{hi} PD-1^{hi} cells (Tywman and Rech et al. *Submitted*). Thus, the mechanisms of PD-1 mediated exhausted CD8+ T cell inhibition elucidated in this thesis may provide insight into biomarkers for clinical use.

4.7: Safety of PD-1-based therapies in patients

An additional consideration for the use of PD-1 pathway blockade is the safety of targeting a molecule with such profound and widespread immunoregulatory functions. Given the predisposition of *pdcd1* deficient mice for autoimmune disease, a major concern for targeting the PD-1 pathway is the potential for autoimmune side effects (James et al., 2005; Velázquez-Cruz et al., 2007; Francisco et al., 2010). This is especially concerning given the off-target effects of CTLA-4 blockade (Ipilimumab) in the clinic (Pardoll, 2012; Peggs and Allison, 2006). However, while some pulmonary and gastrointestinal adverse effects have been reported in PD-1/L1 blockade clinical trials, most patients tolerate PD-1 pathway blockade very well (Brahmer et al., 2012; Topalian et al., 2012). As we begin to better understand PD-1 pathway regulation of exhausted CD8+ T cells, it may even be possible to further decrease adverse effects by directly targeting molecules downstream of PD-1 signaling.

In addition to potential off-target effects, the work presented in **chapters 2** and **3** of this thesis also suggests that loss of PD-1 signals may alter CD8+ T cell responses to subsequent infections or vaccinations. With over 39 clinical trials currently underway with PD-1/L1 blockade, many patients will be armed with an immune system that has essentially lost the ability to signal through PD-1 (ClinicalTrials.gov). This may negatively

impact the formation of memory responses following vaccination, including CD8+ T cell memory but also potentially B cell and antibody responses (Good-Jacobson et al., 2010). In addition, exposure to infections may also lead to altered immune responses and, as a result, pathogen clearance. Future work should investigate these possibilities in patients who received PD-1 pathway blockade.

4.8 Summary

The work presented in this thesis provides novel insight into the role of PD-1 in regulating *in vivo* anti-viral CD8+ T cell responses. PD-1 plays a prominent role in regulating effector CD8+ T cell proliferation, expansion and survival, consistent with previous findings. However, studies in **chapters 2** and **3** of this thesis extend these observations to show that PD-1 also has a previously unappreciated role in shaping the development and maintenance of long-term memory or exhausted CD8+ T cell responses. This novel role for PD-1 signals highlights the complex regulation of CD8+ T cell responses by inhibitory receptors. Specifically, PD-1 balances the need for an effective anti-pathogen CD8+ T cell response with prevention of immunopathology and the generation of long-lived memory or exhausted T cell responses. Future studies will further dissect the molecular mechanisms responsible for the observed effects of PD-1 on effector, memory and exhausted CD8+ T cells. These findings also provide insight into targeting this clinically relevant pathway and stress the importance of further studies to better understand PD-1 mediated inhibition of CD8+ T cells and other immune cells.

Bibliography

Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* 3:939–951. doi:10.1038/nri1248.
Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int. Immunol.* 8:765–772. doi:10.1093/intimm/8.5.765.
Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science*. 272:54–60. doi:10.1126/science.272.5258.54.
Ahmed, R., A. Salmi, L.D. Butler, J.M. Chiller, and M.B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med.* 160:521–540.
Allie, S.R., W. Zhang, S. Fuse, and E.J. Usherwood. 2011. Programmed death 1 regulates development of central memory CD8 T cells after acute viral infection. *J. Immunol.* 186:6280–6286.
Angelosanto, J.M., and E.J. Wherry. 2010. Transcription factor regulation of CD8+ T-cell memory and exhaustion. *Immunol. Rev.* 236:167–175. doi:10.1111/j.1600-065X.2010.00927.x.
Appay, V., D.F. Nixon, S.M. Donahoe, G.M. Gillespie, T. Dong, A. King, G.S. Ogg, H.M. Spiegel, C. Conlon, C.A. Spina, D. V Havlir, D.D. Richman, A. Waters, P. Easterbrook, A.J. McMichael, and S.L. Rowland-Jones. 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* 192:63–75. doi:10.1084/jem.192.1.63.
Araki, K., A.P. Turner, V.O. Shaffer, S. Gangappa, S.A. Keller, M.F. Bachmann, C.P. Larsen, and R. Ahmed. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature*. 460:108–112. doi:10.1038/nature08155.
Askovich, P.S., C.J. Sanders, C.M. Rosenberger, A.H. Diercks, P. Dash, G. Navarro, P. Vogel, P.C. Doherty, P.G. Thomas, and A. Aderem. 2013. Differential Host Response, Rather Than Early Viral Replication Efficiency, Correlates with Pathogenicity Caused by Influenza Viruses. *PLoS One*. 8. doi:10.1371/journal.pone.0074863.
Backer, R.A., C. Helbig, R. Gentek, A. Kent, B.J. Laidlaw, C.X. Dominguez, Y.S. de Souza, S.E. van Trierum, R. van Beek, G.F. Rimmelzwaan, A. Ten Brinke, A.M. Willemsen, A.H.C. van Kampen, S.M. Kaech, J.M. Blander, K. van Gisbergen, and D. Amsen. 2014. A central role for Notch in effector CD8(+) T cell differentiation. *Nat. Immunol.* 15:1143–51. doi:10.1038/ni.3027.
Badovinac, V.P., J.S. Haring, and J.T. Harty. 2007. Initial T Cell Receptor Transgenic Cell Precursor Frequency Dictates Critical Aspects of the CD8+ T Cell Response to Infection. *Immunity*. 26:827–841. doi:10.1016/j.immuni.2007.04.013.
Badovinac, V.P., B.B. Porter, and J.T. Harty. 2002. Programmed contraction of CD8(+) T cells after infection. *Nat. Immunol.* 3:619–626.

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

Bartholdy, C., J.P. Christensen, D. Wodarz, and A.R. Thomsen. 2000. Persistent virus infection despite chronic cytotoxic T-lymphocyte activation in gamma interferon-deficient mice infected with lymphocytic choriomeningitis virus. *J. Virol.* 74:10304–10311. doi:10.1128/JVI.74.22.10304-10311.2000.

Bennett, F., and B. Carreno. 2002. Program Death-1 Engagement Upon TCR Activation Has Distinct Effects on Costimulation and Cytokine-Driven Proliferation: Attenuation of ICOS, IL-4, and IL-21, But Not CD28, IL-7, and IL-15 Responses. *J. Immunol.* 1–9.

Bennett, F., D. Luxenberg, V. Ling, I.-M. Wang, K. Marquette, D. Lowe, N. Khan, G. Veldman, K.A. Jacobs, V.E. Valge-Archer, M. Collins, and B.M. Carreno. 2003. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. *J. Immunol.* 170:711–718. doi:10.4049/jimmunol.170.2.711.

Bevan, M.J. 2011. Understand memory, design better vaccines. *Nat. Immunol.* 12:463–465.

Bhadra, R., J. Gigley, L. Weiss, and I. Khan. 2011. Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. *Proc. Natl. Acad. Sci.* 1–6.

Binder, G.K., and D.E. Griffin. 2001. Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons. *Science*. 293:303–306. doi:10.1126/science.1059742.

Blackburn, S., A. Crawford, H. Shin, A. Polley, G. Freeman, and E. Wherry. 2010a. Tissue-Specific Differences in PD-1 and PD-L1 Expression during Chronic Viral Infection: Implications for CD8 T-Cell Exhaustion. *J. Virol.* 84:2078–2089.

Blackburn, S., and J. Wherry. 2008. Selective expansion of a subset of exhausted CD8 T cells by PD-L1 blockade. *PNAS*. 1–6.

Blackburn, S.D., A. Crawford, H. Shin, A. Polley, G.J. Freeman, and E.J. Wherry. 2010b. Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: implications for CD8 T-cell exhaustion. *J. Virol.* 84:2078–2089. doi:10.1128/JVI.01579-09.

Blackburn, S.D., H. Shin, G.J. Freeman, and E.J. Wherry. 2008. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 105:15016–15021. doi:10.1073/pnas.0801497105.

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

Blank, C., I. Brown, A. Peterson, M. Spiotto, Y. Iwai, T. Honjo, and T. Gajewski. 2004. PD-L1/B7H-1 Inhibits Effector Phase of Tumor Rejection by T cell Receptor (TCR) Transgenic CD8+ T cells. *Cancer Res.* 1140–1145.

Blank, C., T.F. Gajewski, and A. Mackensen. 2005. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: Implications for tumor immunotherapy. *Cancer Immunol. Immunother.* 54:307–314. doi:10.1007/s00262-004-0593-x.

Blattman, J., E. Wherry, S. Ha, R. Van Der Most, and R. Ahmed. 2009. Impact of Epitope Escape on PD-1 Expression and CD8 T-Cell Exhaustion during Chronic Infection. *J. Virol.* 83:4386–4394.
Blazejewska, P., L. Koscinski, N. Viegas, D. Anhlan, S. Ludwig, and K. Schughart. 2011. Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. *Virology*. 412:36–45. doi:10.1016/j.virol.2010.12.047.
Boise, L.H., A.J. Minn, P.J. Noel, C.H. June, M.A. Accavitti, T. Lindsten, and C.B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity*. 3:87–98. doi:10.1016/1074-7613(95)90161-2.
Boni, C., P. Fisicaro, C. Valdatta, B. Amadei, P. Di Vincenzo, T. Giuberti, D. Laccabue, A. Zerbini, A. Cavalli, G. Missale, A. Bertoletti, and C. Ferrari. 2007. Characterization of Hepatitis B Virus (HBV)-Specific T-Cell Dysfunction in Chronic HBV Infection. *J. Virol.* 81:4215–4225.
Boomer, J.S., and J.M. Green. 2010. An enigmatic tail of CD28 signaling. *Cold Spring Harb. Perspect. Biol.* 2. doi:10.1101/cshperspect.a002436.
Borrow, P., and M. Oldstone. 1995. Virus-Induced Immunosuppression: Immune System-Mediated Destruction of Virus-Infected Dendritic Cells Results in Generalized Immune Suppression. *J. Virol.* 1–20.
Borrow, P., and M. Oldstone. 1999. Mechanism of Lymphocytic Choriomeningitis Virus Entry into Cells. *Virology*. 1–9.
Bouvier, N.M., and A.C. Lowen. 2010. Animal models for influenza virus pathogenesis and transmission. *Viruses*. 2:1530–1563. doi:10.3390/v20801530.
Brahmer, J.R., S.S. Tykodi, L.Q.M. Chow, W.-J. Hwu, S.L. Topalian, P. Hwu, C.G. Drake, L.H. Camacho, J. Kauh, K. Odunsi, H.C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T.M. Salay, S. Alaparthy, J.F. Grosso, A.J. Korman, S.M. Parker, S. Agrawal, S.M. Goldberg, D.M. Pardoll, A. Gupta, and J.M. Wigginton. 2012. Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *N. Engl. J. Med.* 366:2455–2465. doi:10.1056/NEJMoa1200694.
Brooks, D.G., M.J. Trifilo, K.H. Edelmann, L. Teyton, D.B. McGavern, and M.B. Oldstone. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat. Med.* 12:1301–1309.
Brown, K.E., G.J. Freeman, E.J. Wherry, and A.H. Sharpe. 2010. Role of PD-1 in regulating acute infections. *Curr. Opin. Immunol.* 22:397–401.
Buggert, M., J. Tauriainen, T. Yamamoto, J. Frederiksen, M.A. Ivarsson, J. Michaëlsson, O. Lund, B. Hejdeman, M. Jansson, A. Sönnerborg, R.A. Koup, M.R. Betts, and A.C. Karlsson. 2014. T-bet and Eomes Are Differentially Linked to the Exhausted Phenotype of CD8+ T Cells in HIV Infection. *PLoS Pathog.* 10. doi:10.1371/journal.ppat.1004251.
Butte, M.J., M.E. Keir, T.B. Phamduy, A.H. Sharpe, and G.J. Freeman. 2007. Programmed Death-1 Ligand 1 Interacts Specifically with the B7-1 Costimulatory Molecule to Inhibit T Cell Responses. *Immunity*. 27:111–122. doi:10.1016/j.immuni.2007.05.016.
Butz, E.A., and M.J. Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity*. 8:167–175.
Cao, W., M.D. Henry, P. Borrow, H. Yamada, J.H. Elder, E. V Ravkov, S.T. Nichol, R.W. Compans, K.P. Campbell, and M.B. Oldstone. 1998. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science*. 282:2079–81.

Carbone, F.R., L.K. Mackay, W.R. Heath, and T. Gebhardt. 2013. Distinct resident and recirculating memory T cell subsets in non-lymphoid tissues. *Curr. Opin. Immunol.* 25:329–333. doi:10.1016/j.coi.2013.05.007.

Center for Disease Control and Prevention (2010). Estimates of death associated with seasonal influenza - United States, 1976-2007. MMWR Morb Mortal Wkly Rep *59*, 1057-1062.

Center for Disease Control and Prevention (2011). Viral Hepatitis Statistics and Surveillance. http://www.cdc.gov/Hepatitis/Statistics/index.htm. Accessed November 1, 2014.

Chang, J.T., E.J. Wherry, and A.W. Goldrath. 2014. Molecular regulation of effector and memory T cell differentiation. *Nat. Immunol.* 15:1104–1115. doi:10.1038/ni.3031.

Charlton, J.J., I. Chatzidakis, D. Tsoukatou, D.T. Boumpas, G.A. Garinis, and C. Mamalaki. 2013. Programmed death-1 shapes memory phenotype CD8 T cell subsets in a cell-intrinsic manner. *J. Immunol.* 190:6104–14. doi:10.4049/jimmunol.1201617.

Chemnitz, J.M., R. V Parry, K.E. Nichols, C.H. June, and J.L. Riley. 2004. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol. (Baltimore, Md 1950)*. 173:945–954.

Chen, L., and D.B. Flies. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat. Rev. Immunol.* 13:227–42. doi:10.1038/nri3405.

Clinical Trials.gov (2014). PD-1 pathway clinical trials. https://clinicaltrials.gov/ct2/results?term=PD-1&Search=Search. Accessed November 10, 2014.

Cocchi, F., A.L. DeVico, A. Garzino-Demo, S.K. Arya, R.C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*. 270:1811–1815.

Crawford, A., and J. Wherry. 2009. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr. Opin. Immunol.* 1–8.

Cruz-Guilloty, F., M.E. Pipkin, I.M. Djuretic, D. Levanon, J. Lotem, M.G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J. Exp. Med.* 206:51–59. doi:10.1084/jem.20081242.

Curtsinger, J.M., D.C. Lins, and M.F. Mescher. 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J. Exp. Med.* 197:1141–1151. doi:10.1084/jem.20021910.

Curtsinger, J.M., and M.F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* 22:333–340. doi:10.1016/j.coi.2010.02.013.

Curtsinger, J.M., C.S. Schmidt, A. Mondino, D.C. Lins, R.M. Kedl, M.K. Jenkins, and M.F. Mescher. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* 162:3256–3262.

Curtsinger, J.M., J.O. Valenzuela, P. Agarwal, D. Lins, and M.F. Mescher. 2005. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J. Immunol.* 174:4465–4469. doi:10.4049/jimmunol.174.8.4465.

Day, C., D. Kaufmann, P. Kiepiela, J. Brown, E. Moodley, S. Reddy, E. Mackey, J. Miller, A. Leslie, C. Depierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. Wherry, H. Coovadia, P. Goulder, P. Klenerman, R. Ahmed, G.J.

Freeman, and B. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 443:350–354.
Decman, V., B.J. Laidlaw, L.J. Dimenna, S. Abdulla, K. Mozdzanowska, J. Erikson, H.C.J. Ertl, and E.J. Wherry. 2010. Cell-intrinsic defects in the proliferative response of antiviral memory CD8 T cells in aged mice upon secondary infection. *J. Immunol.* 184:5151–5159. doi:10.4049/jimmunol.0902063.
Dorman, S.E., G. Uzel, J. Roesler, J.S. Bradley, J. Bastian, G. Billman, S. King, A. Filie, J. Schermerhorn, and S.M. Holland. 1999. Viral infections in interferon-gamma receptor deficiency. 135.
Dutko, F., and M. Oldstone. 2011. Genomic and Biological Variation among Commonly Used Lymphocytic Choriomeningitis Virus Strains. *J. Gen. Virol.* 1–10.
Eppihimer, M.J., J. Gunn, G.J. Freeman, E.A. Greenfield, T. Chernova, J. Erickson, and J.P. Leonard. 2002. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation*. 9:133–145. doi:10.1038/sj/mn/7800123.
Erickson, J.J., P. Gilchuk, A.K. Hastings, S.J. Tollefson, M. Johnson, M.B. Downing, K.L. Boyd, J.E. Johnson, A.S. Kim, S. Joyce, and J. V Williams. 2012. Viral acute lower respiratory infections impair CD8+ T cells through PD-1. *J. Clin. Invest.* 122:2967–82. doi:10.1172/JCI62860.
Erickson, J.J., M.C. Rogers, A.K. Hastings, S.J. Tollefson, and J. V Williams. 2014. Programmed Death-1 Impairs Secondary Effector Lung CD8+ T Cells during Respiratory Virus Reinfection. *J. Immunol.* 193:5108–17. doi:10.4049/jimmunol.1302208.
U.S. Food and Drug Administration (FDA) (2014). FDA Press Release: FDA approves use of Keytruda for advanced melanoma. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm412802.htm. Accessed November 1, 2014.
Flynn, K.J., G.T. Belz, J.D. Altman, R. Ahmed, D.L. Woodland, and P.C. Doherty. 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity*. 8:683–691. doi:S1074-7613(00)80573-7 [pii].
Francisco, L., P. Sage, and A. Sharpe. 2010. The PD-1 pathway in tolerance and autoimmunity. *Immunol. Rev.* 1–24.
Frauwirth, K.A., J.L. Riley, M.H. Harris, R. V. Parry, J.C. Rathmell, D.R. Plas, R.L. Elstrom, C.H. June, and C.B. Thompson. 2002. The CD28 signaling pathway regulates glucose metabolism. *Immunity*. 16:769–777. doi:10.1016/S1074-7613(02)00323-0.
Frebel, H., V. Nindl, R.A. Schuepbach, T. Braunschweiler, K. Richter, J. Vogel, C.A. Wagner, D. Loffing-Cueni, M. Kurrer, B. Ludewig, and A. Oxenius. 2012. Programmed death 1 protects from fatal circulatory failure during systemic virus infection of mice. *J. Exp. Med.* 209:2485–99. doi:10.1084/jem.20121015.
Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000a. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* 192:1027–1034. doi:10.1084/jem.192.7.1027.
Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling,

M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000b. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* 192:1027–1034.

Freeman, G.J., E.J. Wherry, R. Ahmed, and A.H. Sharpe. 2006. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J. Exp. Med.* 203:2223–2227. doi:10.1084/jem.20061800.

Fuller, M.J., and A.J. Zajac. 2003. Ablation of CD8 and CD4 T cell responses by high viral loads. *J. Immunol. (Baltimore, Md 1950)*. 170:477–486.

Fuse, S., C.Y. Tsai, M.J. Molloy, S.R. Allie, W. Zhang, H. Yagita, and E.J. Usherwood. 2009. Recall responses by helpless memory CD8+ T cells are restricted by the up-regulation of PD-1. *J. Immunol. (Baltimore, Md 1950)*. 182:4244–4254.

Gallimore, A., A. Glithero, A. Godkin, A.C. Tissot, A. Plückthun, T. Elliott, H. Hengartner, R. Zinkernagel, and B.A. Gallimore. 1998. Induction and Exhaustion of Lymphocytic Choriomeningitis Virus – specific Cytotoxic T Lymphocytes Visualized Using Class I – Peptide Complexes. *J. Exp. Med.* 187:1383–1393. doi:10.1084/jem.187.9.1383.

Gallimore, A., and R. Zinkernagel. 1998. Induction and Exhaustion of Lymphocytic Choriomeningitis Virus–specific Cytotoxic T Lymphocytes Visualized Using Soluble Tetrameric Major Histocompatibility Complex Class I–Peptide Complexes. *J. Exp. Med.* 1–11.

Good-Jacobson, K.L., C.G. Szumilas, L. Chen, A.H. Sharpe, M.M. Tomayko, and M.J. Shlomchik. 2010. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat. Immunol.* 11:535–542. doi:10.1038/ni.1877.

Gruener, N.H., F. Lechner, M.C. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G.R. Pape, and P. Klenerman. 2001a. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J. Virol.* 75:5550–5558. doi:10.1128/JVI.75.12.5550-5558.2001.

Gruener, N.H., F. Lechner, M.C. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G.R. Pape, and P. Klenerman. 2001b. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J. Virol.* 75:5550–5558.

Guidotti, L.G., and F. V Chisari. 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* 19:65–91. doi:10.1146/annurev.immunol.19.1.65.

Hamann, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein, and R.A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J. Exp. Med.* 186:1407–1418. doi:10.1084/jem.186.9.1407.

Harty, J.T., and M.J. Bevan. 1999. Responses of CD8(+) T cells to intracellular bacteria. *Curr Opin Immunol*. 11:89–93.

Hess Michelini, R., A.L. Doedens, A.W. Goldrath, and S.M. Hedrick. 2013. Differentiation of CD8 memory T cells depends on Foxo1. *J. Exp. Med.* 210:1189–200. doi:10.1084/jem.20130392.

Heusel, J.W., R.L. Wesselschmidt, S. Shresta, J.H. Russell, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell*. 76:977–987. doi:10.1016/0092-8674(94)90376-X.

Honda, T., J.G. Egen, T. Lämmermann, W. Kastenmüller, P. Torabi-Parizi, and R.N. Germain. 2014. Tuning of Antigen Sensitivity by T Cell Receptor-Dependent

Negative Feedback Controls T Cell Effector Function in Inflamed Tissues. *Immunity*. 40:235–247. doi:10.1016/j.immuni.2013.11.017.
Huster, K.M., V. Busch, M. Schiemann, K. Linkemann, K.M. Kerksiek, H. Wagner, and D.H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc. Natl. Acad. Sci. U. S. A.* 101:5610–5615. doi:10.1073/pnas.0308054101.
Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity*. 8:89–95. doi:10.1016/S1074-7613(00)80461-6.
Intlekofer, A.M., N. Takemoto, E.J. Wherry, S.A. Longworth, J.T. Northrup, V.R. Palanivel, A.C. Mullen, C.R. Gasink, S.M. Kaech, J.D. Miller, L. Gapin, K. Ryan, A.P. Russ, T. Lindsten, J.S. Orange, A.W. Goldrath, R. Ahmed, and S.L. Reiner. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6:1236–1244. doi:10.1038/ni1268.
Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* 11:3887–3895.
Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 99:12293–12297. doi:10.1073/pnas.192461099.
Iwai, Y., S. Terawaki, M. Ikegawa, T. Okazaki, and T. Honjo. 2003. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J. Exp. Med.* 198:39–50.
James, E.S., S. Harney, B.P. Wordsworth, W.O.C.M. Cookson, S.J. Davis, and M.F. Moffatt. 2005. PDCD1: a tissue-specific susceptibility locus for inherited inflammatory disorders. *Genes Immun.* 6:430–437. doi:10.1038/sj.gene.6364223.
Jin, X., D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S. Perelson, and D.D. Ho. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* 189:991–998. doi:10.1084/jem.189.6.991.
Joshi, N.S., W. Cui, A. Chandele, H.K. Lee, D.R. Urso, J. Hagman, L. Gapin, and S.M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity*. 27:281–295.
June, C.H., J.A. Ledbetter, M.M. Gillespie, T. Lindsten, and C.B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472–4481. doi:10.1128/MCB.7.12.4472.Updated.
Jurado, J., and V. Garcia. 2008. Programmed death (PD)-1:PD-ligand 1/PD-ligand 2 pathway inhibits T cell effector functions during human tuberculosis. *J. Immunol.* 1–11.
Kaech, S.M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* 2:415–422. doi:10.1038/87720.
Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003a. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that

give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198. doi:10.1038/ni1009.
Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003b. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198.
Kaech, S.M., E.J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2:251–262.
Kägi, D., B. Ledermann, K. Bürki, R.M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu. Rev. Immunol.* 14:207–232. doi:10.1146/annurev.immunol.14.1.207.
Kägi, D., P. Seiler, J. Pavlovic, B. Ledermann, K. Bürki, R.M. Zinkernagel, and H. Hengartner. 1995. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur. J. Immunol.* 25:3256–3262. doi:10.1002/eji.1830251209.
Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science*. 265:528–530. doi:10.1126/science.7518614.
Kallies, A., A. Xin, G.T. Belz, and S.L. Nutt. 2009. Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8+ T Cells and Memory Responses. *Immunity*. 31:283–295. doi:10.1016/j.immuni.2009.06.021.
Kao, C., K.J. Oestreich, M.A. Paley, A. Crawford, J.M. Angelosanto, M.-A.A. Ali, A.M. Intlekofer, J.M. Boss, S.L. Reiner, A.S. Weinmann, and E.J. Wherry. 2011. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat. Immunol.* 12:663–671. doi:10.1038/ni.2046.
Kaplan, J.E., D. Hanson, M.S. Dworkin, T. Frederick, J. Bertolli, M.L. Lindegren, S. Holmberg, and J.L. Jones. 2000. Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. *Clin. Infect. Dis.* 30 Suppl 1:S5–S14. doi:10.1086/313843.
Kassu, A., R. Marcus, M. D'souza, E. Kelly-Mcknight, L. Golden-Mason, R. Akkina, A. Fontenot, C. Wilson, and B. Palmer. 2010. Regulation of Virus-Specific CD4+ T Cell Function by Multiple Costimulatory Receptors during Chronic HIV Infection. *J. Immunol.* 185:3007–3018.
Keir, M., M. Butte, G.J. Freeman, and A.H. Sharpe. 2008. PD-1 and Its Ligands in Tolerance and Immunity. *Annu. Rev. Immunol.* 26:677–704.
Keir, M.E., G.J. Freeman, and A.H. Sharpe. 2007a. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. *J. Immunol.* 179:5064–5070.
Keir, M.E., G.J. Freeman, and A.H. Sharpe. 2007b. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. *J. Immunol.* 179:5064–5070. doi:10.4049/jimmunol.179.8.5064.
Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203:883–895. doi:10.1084/jem.20051776.

Kim, M., W. Ouyang, W. Liao, M. Zhang, and M. Li. 2013. The transcription factor foxo1 controls central-memory CD8+ T cell responses to infection. *Immunity*. 39:286–297. doi:10.1016/j.immuni.2013.07.013.

Kim, T.S., and T.J. Braciale. 2009. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS One*. 4. doi:10.1371/journal.pone.0004204.

Kinter, A.L., E.J. Godbout, J.P. McNally, I. Sereti, G.A. Roby, M.A. O'Shea, and A.S. Fauci. 2008. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J. Immunol.* 181:6738–6746. doi:181/10/6738 [pii].

Kiser, J.J., and C. Flexner. 2013. Direct-acting antiviral agents for hepatitis C virus infection. *Annu. Rev. Pharmacol. Toxicol.* 53:427–49. doi:10.1146/annurev-pharmtox-011112-140254.

Kostense, S., K. Vandenberghe, J. Joling, D. Van Baarle, N. Nanlohy, E. Manting, and F. Miedema. 2002. Persistent numbers of tetramer+ CD8+ T cells, but loss of interferon-??+ HIV-specific T cells during progression to AIDS. *Blood*. 99:2505–2511. doi:10.1182/blood.V99.7.2505.

Kozako, T., M. Yoshimitsu, H. Fujiwara, I. Masamoto, S. Horai, Y. White, M. Akimoto, S. Suzuki, K. Matsushita, K. Uozumi, C. Tei, and N. Arima. 2009. PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, UK*. 23:375–382.

Kunz, S. 2009. Receptor binding and cell entry of Old World arenaviruses reveal novel aspects of virus–host interaction. *Virology*. 387:245–249.

Kurachi, M., R.A. Barnitz, N. Yosef, P.M. Odorizzi, M. a DiIorio, M.E. Lemieux, K. Yates, J. Godec, M.G. Klatt, A. Regev, E.J. Wherry, and W.N. Haining. 2014. The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. *Nat. Immunol.* 15:373–83. doi:10.1038/ni.2834.

De La Torre, J.C. 2009. Molecular and cell biology of the prototypic arenavirus LCMV: Implications for understanding and combating hemorrhagic fever arenaviruses. *Ann. N. Y. Acad. Sci.* 1171. doi:10.1111/j.1749-6632.2009.05048.x.

Lafon, M., and H. Wiendl. 2008. Detrimental Contribution of the Immuno-Inhibitor B7-H1 to Rabies Virus Encephalitis. *J. Immunol.* 1–11.

Laidlaw, B.J., V. Decman, M.A.A. Ali, M.C. Abt, A.I. Wolf, L.A. Monticelli, K. Mozdzanowska, J.M. Angelosanto, D. Artis, J. Erikson, and E.J. Wherry. 2013. Cooperativity Between CD8+ T Cells, Non-Neutralizing Antibodies, and Alveolar Macrophages Is Important for Heterosubtypic Influenza Virus Immunity. *PLoS Pathog.* 9. doi:10.1371/journal.ppat.1003207.

Latchman, Y., C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, and G.J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2:261–268. doi:10.1038/85330.

Lázár-Molnár, E., B. Chen, K.A. Sweeney, E.J. Wang, W. Liu, J. Lin, S.A. Porcelli, S.C. Almo, S.G. Nathenson, and W.R. Jacobs. 2010. Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* 107:13402–13407.

Lázár-Molnár, E., A. Gácser, G.J. Freeman, S.C. Almo, S.G. Nathenson, and J.D. Nosanchuk. 2008. The PD-1/PD-L costimulatory pathway critically affects host resistance to the pathogenic fungus Histoplasma capsulatum. *Proc. Natl. Acad. Sci. U. S. A.* 105:2658–2663.
Lee, S.J., B.C. Jang, S.W. Lee, Y. Il Yang, S. Il Suh, Y.M. Park, S. Oh, J.G. Shin, S. Yao, L. Chen, and I.H. Choi. 2006. Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-γ-induced upregulation of B7-H1 (CD274). *FEBS Lett.* 580:755–762. doi:10.1016/j.febslet.2005.12.093.
Liang, S.C., R.J. Greenwald, Y.E. Latchman, L. Rosas, A. Satoskar, G.J. Freeman, and A.H. Sharpe. 2006. PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *Eur. J. Immunol.* 36:58–64.
Liang, S.C., Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, and A.H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur. J. Immunol.* 33:2706–2716. doi:10.1002/eji.200324228.
Loke, P., and J. Allison. 2003. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc. Natl. Acad. Sci. U. S. A.* 100:5336–5341.
Maini, M.K., C. Boni, G.S. Ogg, A.S. King, S. Reignat, C.K. Lee, J.R. Larrubia, G.J. Webster, A.J. McMichael, C. Ferrari, R. Williams, D. Vergani, and A. Bertoletti. 1999. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. *Gastroenterology*. 117:1386–1396. doi:10.1016/S0016-5085(99)70289-1.
Matloubian, M., and R. Ahmed. 2003. Genetic Bass of Viral Persistence: Single Amino Acid Change in the Viral Glycoprotein Affects Ability of Lymphocytic Choriomeningitis Virus to Persist in Adult Mice. 1–6.
Matloubian, M., R.J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* 68:8056–8063. doi:56032.
Matloubian, M., T. Somasundaram, S.R. Kolhekar, R. Selvakumar, and R. Ahmed. 1990. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J. Exp. Med.* 172:1043–1048. doi:10.1084/jem.172.4.1043.
Matloubian, M., M. Suresh, A. Glass, M. Galvan, K. Chow, J.K. Whitmire, C.M. Walsh, W.R. Clark, and R. Ahmed. 1999. A role for perforin in downregulating T-cell responses during chronic viral infection. *J. Virol.* 73:2527–2536.
McAdam, A.J., A.N. Schweitzer, and A.H. Sharpe. 1998. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol. Rev.* 165:231–247. doi:10.1111/j.1600-065X.1998.tb01242.x.
McGill, J., N. Van Rooijen, and K.L. Legge. 2008. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *J. Exp. Med.* 205:1635–1646. doi:10.1084/jem.20080314.
Medina, R.A., and A. García-Sastre. 2011. Influenza A viruses: new research developments. *Nat. Rev. Microbiol.* 9:590–603. doi:10.1038/nrmicro2613.
Moskophidis, D., F. Lechner, H. Pircher, and R.M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*. 362:758–761. doi:10.1038/362758a0.
Van der Most, R.G., K. Murali-Krishna, J.L. Whitton, C. Oseroff, J. Alexander, S. Southwood, J. Sidney, R.W. Chesnut, A. Sette, and R. Ahmed. 1998. Identification

of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. *Virology*. 240:158–167. doi:10.1006/viro.1997.8934.

Mueller, M., S. Renzullo, R. Brooks, N. Ruggli, and M.A. Hofmann. 2010. Antigenic characterization of recombinant hemagglutinin proteins derived from different avian influenza virus subtypes. *PLoS One*. 5. doi:10.1371/journal.pone.0009097.

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

Muenst, S., S. Hoeller, N. Willi, S. Dirnhofer, and A. Tzankov. 2010. Diagnostic and prognostic utility of PD-1 in B cell lymphomas. *Dis. Markers*. 29:47–53. doi:10.3233/DMA-2010-0725.

Muenst, S., S.D. Soysal, F. Gao, E.C. Obermann, D. Oertli, and W.E. Gillanders. 2013. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. *Breast Cancer Res. Treat.* 139:667–676. doi:10.1007/s10549-013-2581-3.

Nishimura, H., Y. Agata, A. Kawasaki, M. Sato, S. Imamura, N. Minato, H. Yagita, T. Nakano, and T. Honjo. 1996. Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4-CD8-) thymocytes. *Int. Immunol.* 8:773–780.

Nishimura, H., and T. Honjo. 1998. Immunological studies of PD-1-deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int. Immunol.* 1–10.

Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 11:141–151. doi:10.1016/S1074-7613(00)80089-8.

Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 291:319–322. doi:10.1126/science.291.5502.319.

Obar, J.J., E.R. Jellison, B.S. Sheridan, D. a Blair, Q.-M. Pham, J.M. Zickovich, and L. Lefrançois. 2011. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. *J. Immunol.* 187:4967–78. doi:10.4049/jimmunol.1102335.

Obar, J.J., and L. Lefrançois. 2010. Early signals during CD8 T cell priming regulate the generation of central memory cells. *J. Immunol.* 185:263–272. doi:10.4049/jimmunol.1000492.

Okazaki, T., S. Chikuma, Y. Iwai, S. Fagarasan, and T. Honjo. 2013. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat. Immunol.* 14:1212–8. doi:10.1038/ni.2762.

Okazaki, T., A. Maeda, H. Nishimura, T. Kurosaki, and T. Honjo. 2001. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc. Natl. Acad. Sci. U. S. A.* 98:13866–13871. doi:10.1073/pnas.231486598.

Oldstone, M. 2009. Anatomy of Viral Persistence. *PLoS Pathog.* 5:e1000523.

Oldstone, M.B.A. 2006. Viral persistence: Parameters, mechanisms and future predictions. *Virology*. 344:111–118. doi:10.1016/j.virol.2005.09.028.

Paley, M.A., D.C. Kroy, P.M. Odorizzi, J.B. Johnnidis, D. V Dolfi, B.E. Barnett, E.K. Bikoff, E.J. Robertson, G.M. Lauer, S.L. Reiner, and E.J. Wherry. 2012. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science*. 338:1220–5. doi:10.1126/science.1229620.

Panhuis, W.G. Van, D. Ph, J. Grefenstette, S.Y. Jung, N.S. Chok, M. Sc, A. Cross, H. Eng, B.Y. Lee, V. Zadorozhny, S. Brown, D. Cummings, and D.S. Burke. 2013. Contagious Diseases in the United States from 1888 to the Present. *N. Engl. J. Med.*

Pardoll, D.M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer*. 12:252–264. doi:10.1038/nrc3239.

Parry, R., and J. Riley. 2005. CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms. *Mol. Cell. Biol.* 25:9543–9553.

Patsoukis, N., J. Brown, V. Petkova, F. Liu, L. Li, and V.A. Boussiotis. 2012. Selective Effects of PD-1 on Akt and Ras Pathways Regulate Molecular Components of the Cell Cycle and Inhibit T Cell Proliferation. *Sci. Signal.* 5:ra46–ra46. doi:10.1126/scisignal.2002796.

Pearce, E.L., A.C. Mullen, G.A. Martins, C.M. Krawczyk, A.S. Hutchins, V.P. Zediak, M. Banica, C.B. DiCioccio, D.A. Gross, C.A. Mao, H. Shen, N. Cereb, S.Y. Yang, T. Lindsten, J. Rossant, C.A. Hunter, and S.L. Reiner. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science (80-.).* 302:1041–1043.

Peggs, K., and J. Allison. 2006. Principles and use of anti-CTLA4 antibody in human cancer immunotherapy. *Curr. Opin. Immunol.* 1–8.

Petrovas, C., J. Casazza, J. Brenchley, D. Price, E. Gostick, W. Adams, M. Precopio, T. Schacker, M. Roederer, D. Douek, and R. Koup. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J. Exp. Med.* 203:2281–2292.

Piacenti, F.J. 2006. An update and review of antiretroviral therapy. *Pharmacotherapy*. 26:1111–1133.

Pircher, H., K. Bürki, R. Lang, H. Hengartner, and R.M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature*. 342:559–561. doi:10.1038/342559a0.

Plumlee, C., B. Sheridan, B. Cicek, and L. Lefrançois. 2013. Environmental cues dictate the fate of individual CD8+ T cells responding to infection. *Immunity*. 39:347–356. doi:10.1016/j.immuni.2013.07.014.

Quigley, M., F. Pereyra, B. Nilsson, F. Porichis, C. Fonseca, Q. Eichbaum, B. Julg, J.L. Jesneck, K. Brosnahan, S. Imam, K. Russell, I. Toth, A. Piechocka-Trocha, D. Dolfi, J. Angelosanto, A. Crawford, H. Shin, D.S. Kwon, J. Zupkosky, L. Francisco, G.J. Freeman, E.J. Wherry, D. Kaufmann, B. Walker, B. Ebert, and W. Haining. 2010. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat. Med.* 16:1147–1151.

Restifo, N.P., M.E. Dudley, and S.A. Rosenberg. 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat. Rev. Immunol.* 12:269–281. doi:10.1038/nri3191.

Riley, J.L. 2009. PD-1 signaling in primary T cells. *Immunol. Rev.* 229:114–125. doi:10.1111/j.1600-065X.2009.00767.x.

Rock, K.L., and A.L. Goldberg. 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* 17:739–79. doi:10.1146/annurev.immunol.17.1.739.

Rowe, J., and S. Sing Way. 2008. PDL-1 Blockade Impedes T Cell Expansion and Protective Immunity Primed by Attenuated Listeria monocytogenes. *J. Immunol.* 1–6.

Rutigliano, J. a, S. Sharma, M.Y. Morris, T.H. Oguin, J.L. McClaren, P.C. Doherty, and P.G. Thomas. 2014. Highly pathological influenza A virus infection is associated with augmented expression of PD-1 by functionally compromised virus-specific CD8+ T cells. *J. Virol.* 88:1636–51. doi:10.1128/JVI.02851-13.

Rutishauser, R.L., G.A. Martins, S. Kalachikov, A. Chandele, I.A. Parish, E. Meffre, J. Jacob, K. Calame, and S.M. Kaech. 2009. Transcriptional Repressor Blimp-1 Promotes CD8+ T Cell Terminal Differentiation and Represses the Acquisition of Central Memory T Cell Properties. *Immunity*. 31:296–308. doi:10.1016/j.immuni.2009.05.014.

Sallusto, F., D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401:708–712. doi:10.1038/44385.

Salvato, M., E. Shimomaye, P. Southern, and M.B. Oldstone. 1988. Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL+) small genomic segment and that of its variant, Clone 13 (CTL-). *Virology*. 164:517–522. doi:10.1016/0042-6822(88)90566-1.

Schmitz, J.E., M.J. Kuroda, S. Santra, V.G. Sasseville, M.A. Simon, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, J. Ghrayeb, M.A. Forman, D.C. Montefiori, E.P. Rieber, N.L. Letvin, and K.A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 283:857–860. doi:10.1126/science.283.5403.857.

Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K.P. Campbell, J.C. de La Torre, and M.B. Oldstone. 2000. Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells. *J. Exp. Med.* 192:1249–1260. doi:10.1084/jem.192.9.1249.

Shankar, P., M. Russo, B. Harnisch, M. Patterson, P. Skolnik, and J. Lieberman. 2000. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. *Blood*. 96:3094–3101.

Shin, H., S. Blackburn, J. Blattman, and E. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J. Exp. Med.* 204:941–949.

Shin, H., S.D. Blackburn, A.M. Intlekofer, C. Kao, J.M. Angelosanto, S.L. Reiner, and E.J. Wherry. 2009. A Role for the Transcriptional Repressor Blimp-1 in CD8+ T Cell Exhaustion during Chronic Viral Infection. *Immunity*. 31:309–320. doi:10.1016/j.immuni.2009.06.019.

Siegal, F.P., C. Lopez, G.S. Hammer, A.E. Brown, S.J. Kornfeld, J. Gold, J. Hassett, S.Z. Hirschman, C. Cunningham-Rundles, and B.R. Adelsberg. 1981. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. 305.

Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan. 2009. T cell activation. *Annu. Rev. Immunol.* 27:591–619. doi:10.1146/annurev.immunol.021908.132706.

Smolinski, M. S., Hamburg, M.A., & Lederberg, J. (eds). *Microbial threats to health: Emergence, detection, and response*. Washington, DC: Institute of Medicine, National Academies Press.

Stein, P.H., J.D. Fraser, and A. Weiss. 1994. The cytoplasmic domain of CD28 is both necessary and sufficient for costimulation of interleukin-2 secretion and association with phosphatidylinositol 3'-kinase. *Mol. Cell. Biol.* 14:3392–3402. doi:10.1128/MCB.14.5.3392.Updated.

Takemoto, N., A.M. Intlekofer, J.T. Northrup, E.J. Wherry, and S.L. Reiner. 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J. Immunol. (Baltimore, Md 1950)*. 177:7515–7519.

Talay, O., C.H. Shen, L. Chen, and J. Chen. 2009. B7-H1 (PD-L1) on T cells is required for T-cell-mediated conditioning of dendritic cell maturation. *Proc. Natl. Acad. Sci. U. S. A.* 106:2741–2746.

Telcian, A., V. Laza-Stanca, M. Edwards, J. Harker, H. Wang, N. Bartlett, P. Mallia, M. Zdrenghea, T. Kebadze, A. Coyle, P. Openshaw, L. Stanciu, and S. Johnston. 2011. RSV-Induced Bronchial Epithelial Cell PD-L1 Expression Inhibits CD8+ T Cell Nonspecific Antiviral Activity. *J. Infect. Dis.* 203:85–94.

Terawaki, S., S. Chikuma, S. Shibayama, T. Hayashi, T. Yoshida, T. Okazaki, and T. Honjo. 2011. IFN-α directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. *J. Immunol. (Baltimore, Md 1950)*. 186:2772–2779.

Tinoco, R., V. Alcalde, Y. Yang, K. Sauer, and E.I. Zuniga. 2009. Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity*. 31:145–157. doi:10.1016/j.immuni.2009.06.015.

Tivol, E., and A. and Sharpe. 2004. Loss of CTLA-4 Leads to Massive Lymphoproliferation and Fatal Multiorgan Tissue Destruction, Revealing a Critical Negative Regulatory Role of CTLA-4. *Immunity*. 1–7.

Topalian, S.L., F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, P.D. Leming, D.R. Spigel, S.J. Antonia, L. Horn, C.G. Drake, D.M. Pardoll, L. Chen, W.H. Sharfman, R.A. Anders, J.M. Taube, T.L. McMiller, H. Xu, A.J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G.D. Kollia, A. Gupta, J.M. Wigginton, and M. Sznol. 2012. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *N. Engl. J. Med.* 366:2443–2454. doi:10.1056/NEJMoa1200690.

Tubo, N.J., A.J. Pagán, J.J. Taylor, R.W. Nelson, J.L. Linehan, J.M. Ertelt, E.S. Huseby, S.S. Way, and M.K. Jenkins. 2013. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell*. 153:785–796. doi:10.1016/j.cell.2013.04.007.

Turtle, C.J., H.M. Swanson, N. Fujii, E.H. Estey, and S.R. Riddell. 2009. A Distinct Subset of Self-Renewing Human Memory CD8+ T Cells Survives Cytotoxic Chemotherapy. *Immunity*. 31:834–844. doi:10.1016/j.immuni.2009.09.015.

UNAIDS (2013). AIDS by the numbers. http://www.unaids.org/en/resources/documents/2013. Accessed November 1, 2014.

Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 Expression in Acute Hepatitis C Virus (HCV) Infection Is Associated with HCV-Specific CD8 Exhaustion. *J. Virol.* 80:11398–11403.

Valenzuela, J., C. Schmidt, and M. Mescher. 2002. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *J. Immunol.* 169:6842–6849.

Velázquez-Cruz, R., L. Orozco, F. Espinosa-Rosales, R. Carreño-Manjarrez, E. Solís-Vallejo, N.D. López-Lara, I.K. Ruiz-López, A.L. Rodríguez-Lozano, J.K. Estrada-Gil, G. Jiménez-Sánchez, and V. Baca. 2007. Association of PDCD1 polymorphisms with childhood-onset systemic lupus erythematosus. *Eur. J. Hum. Genet.* 15:336–341. doi:10.1038/sj.ejhg.5201767.

Velu, V., K. Titanji, B. Zhu, S. Husain, A. Pladevega, L. Lai, T. Vanderford, L. Chennareddi, G. Silvestri, G.J. Freeman, R. Ahmed, and R. Amara. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*. 458:206–210.

Virgin, H., J. Wherry, and R. Ahmed. 2009. Redefining Chronic Viral Infection. *Cell*. 1–21.

Walsh, C.M., M. Matloubian, C.C. Liu, R. Ueda, C.G. Kurahara, J.L. Christensen, M.T. Huang, J.D. Young, R. Ahmed, and W.R. Clark. 1994. Immune function in mice lacking the perforin gene. *Proc. Natl. Acad. Sci. U. S. A.* 91:10854–10858. doi:DOI 10.1073/pnas.91.23.10854.

Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K.P. Lee, C.B. Thompson, H. Griesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 270:985–988. doi:10.1126/science.270.5238.985.

Wei, F., S. Zhong, Z. Ma, H. Kong, A. Medvec, R. Ahmed, G.J. Freeman, M. Krogsgaard, and J.L. Riley. 2013. Strength of PD-1 signaling differentially affects T-cell effector functions. *Proc. Natl. Acad. Sci. U. S. A.* 110:E2480–9. doi:10.1073/pnas.1305394110.

Wherry, E.J. 2011. T cell exhaustion. *Nat. Immunol.* 131:492–499.

Wherry, E.J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* 78:5535–5545.

Wherry, E.J., D. Barber, S.M. Kaech, J.N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. U. S. A.* 101:16004–16009.

Wherry, E.J., J.N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003a. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J. Virol.* 77:4911–4927. doi:10.1128/JVI.77.8.4911-4927.2003.

Wherry, E.J., S.J. Ha, S.M. Kaech, W.N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J.N. Blattman, D.L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity*. 27:670–684. doi:10.1016/j.immuni.2007.09.006.

Wherry, E.J., V. Teichgräber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003b. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.

World Health Organization (2012). Immunization surveillance, assessment and monitoring. http://www.who.int/immunization/monitoring_surveillance/en/. Accessed November 1, 2014.

Williams, M.A., and M.J. Bevan. 2007. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 25:171–192. doi:10.1146/annurev.immunol.25.022106.141548.

Wong, P., and E.G. Pamer. 2003. CD8 T cell responses to infectious pathogens. *Annu. Rev. Immunol.* 21:29–70. doi:10.1146/annurev.immunol.21.120601.141114.

Wu, Y., C. Lin, K. Cheng, Y. Wang, I. Lin, Y. Chou, and P. Hsu. 2010. Increased programmed death-ligand-1 expression in human gastric epithelial cells in Helicobacter pylori infection. *Clin. Exp. Immunol.* 161:551–559.

Xiao, Y., S. Yu, B. Zhu, D. Bedoret, X. Bu, L.M. Francisco, P. Hua, J.S. Duke-Cohan, D.T. Umetsu, A.H. Sharpe, R.H. DeKruyff, and G.J. Freeman. 2014. RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. *J. Exp. Med.* 211:943–59. doi:10.1084/jem.20130790.

Yamazaki, T., and K. Okumura. 2005. Blockade of B7-H1 on Macrophages Suppresses CD4 + T Cell Proliferation by Augmenting IFN-γ -Induced Nitric Oxide Production. *J. Immunol.* 1–8.

Yamazaki, T., and H. Yagita. 2002. Expression of Programmed Death 1 Ligands by Murine T Cells and APC. *J. Immunol.* 1–9.

Yang, C.Y., J.A. Best, J. Knell, E. Yang, A.D. Sheridan, A.K. Jesionek, H.S. Li, R.R. Rivera, K.C. Lind, L.M. D'Cruz, S.S. Watowich, C. Murre, and A.W. Goldrath. 2011. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat. Immunol.* 12:1221–1229. doi:10.1038/ni.2158.

Yao, S., S. Wang, Y. Zhu, L. Luo, G. Zhu, S. Flies, H. Xu, W. Ruff, M. Broadwater, I. Choi, K. Tamada, and L. Chen. 2009. PD-1 on dendritic cells impedes innate immunity against bacterial infection. *Blood*. 113:5811–5818.

Yokosuka, T., M. Takamatsu, W. Kobayashi-Imanishi, A. Hashimoto-Tane, M. Azuma, and T. Saito. 2012. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J. Exp. Med.* 209:1201–1217. doi:10.1084/jem.20112741.

Yoon, H., K.L. Legge, S.J. Sung, and T.J. Braciale. 2007. Sequential activation of CD8+ T cells in the draining lymph nodes in response to pulmonary virus infection. *J. Immunol.* 179:391–399. doi:179/1/391 [pii].

Zajac, A., and R. Ahmed. 1998. Viral Immune Evasion Due to Persistence of Activated T Cells Without Effector Function. *J. Exp. Med*. 1–9.

Zhang, G., Q. Han, S. Duan, Z. Li, N. Li, Q. Zhu, J. Chen, Y. Lv, X. Zeng, Y. Chen, and Z. Liu. 2012. PDCD1 polymorphism amplifies the predisposing effect conferred by CTLA4 polymorphism in chronic hepatitis B virus infection. *Hum. Immunol.* 73:421–425. doi:10.1016/j.humimm.2012.01.013.

Zhang, N., and M. Bevan. 2011. CD8+ T Cells: Foot Soldiers of the Immune System. *Immunity*. 35:161–168.

Zhang, X., J.C.D. Schwartz, X. Guo, S. Bhatia, E. Cao, L. Chen, Z.Y. Zhang, M.A. Edidin, S.G. Nathenson, and S.C. Almo. 2004. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity*. 20:337–347. doi:10.1016/S1074-7613(04)00051-2.

Zhang, Y., G. Joe, E. Hexner, J. Zhu, and S.G. Emerson. 2005. Host-reactive CD8+ memory stem cells in graft-versus-host disease. *Nat. Med.* 11:1299–1305. doi:10.1038/nm1326.

Zhou, X., S. Ramachandran, M. Mann, and D.L. Popkin. 2012. Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future. *Viruses*. 4:2650–69. doi:10.3390/v4112650.

Zhou, X., S. Yu, D.-M. Zhao, J.T. Harty, V.P. Badovinac, and H.-H. Xue. 2010. Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. *Immunity*. 33:229–240. doi:10.1016/j.immuni.2010.08.002.

Zinkernagel, R.M., and P.C. Doherty. 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*. 251:547–548. doi:10.1038/251547a0.

Zinselmeyer, B.H., S. Heydari, C. Sacristan, D. Nayak, M. Cammer, J. Herz, X. Cheng, S.J. Davis, M.L. Dustin, and D.B. McGavern. 2013. PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. *J Exp Med*. 210:757–774. doi:10.1084/jem.20121416.