CD8 T cell Dysfunction during Chronic Toxoplasmosis

by Rajarshi Bhadra

B.S. in Microbiology, June 2005, Michigan State University

A Dissertation submitted to

The Faculty of
Columbian College of Arts and Sciences
of The George Washington University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

January 31st, 2012

Dissertation directed by

Imtiaz A. Khan
Professor of Microbiology, Immunology and Tropical Medicine

The Columbian College of Arts and Sciences of The George Washington University certifies that Rajarshi Bhadra has passed the Final Examination for the degree of Doctor of Philosophy as of December 2nd, 2011. This is the final and approved form of the dissertation.

CD8 T cell Dysfunction during Chronic Toxoplasmosis

Rajarshi Bhadra

Dissertation Research Committee:

Imtiaz A. Khan, Professor of Microbiology, Immunology and Tropical Medicine, Dissertation Director

David Leitenberg, Associate Professor of Microbiology, Immunology and Tropical Medicine, Committee Member

Sasa Radoja, Assistant Professor of Pediatrics and Microbiology, Immunology and Tropical Medicine, Committee Member

© Copyright 2011 by Rajarshi Bhadra
All rights reserved

Dedication

To my parents, younger brother and uncle who have always supported me through thick and thin.

Acknowledgements

The author wishes to thank Dr. Imtiaz A. Khan for taking me on as a graduate student and giving me the independence to pursue my own ideas. I would also like to thank current and former laboratory members for their support: Dr. Jason P. Gigley, Dr. Magali Moretto and Elizabeth Lawlor.

I would like to thank my committee members: Dr. David Leitenberg, Dr. Sasa Radoja and Dr. Stanislav Vukmanovic. I would also like to thank Dr. Peter J. Hotez for offering me insightful advice.

Abstract of Dissertation

CD8 T cell Dysfunction during Chronic Toxoplasmosis

Toxoplasmic encephalitis (TE) from reactivation of chronic Toxoplasma infection is major cause of morbidity and mortality in HIV infected Toxoplasma seropositive patients. Hence understanding the mechanisms responsible for parasite reactivation in chronically infected hosts is critical for evolving strategies for the development of immunotherapeutic agents against the parasite. Effective $CD8^+$ T cell responses, critical for control of chronic Toxoplasmosis in susceptible mice strains, paradoxically do not ensure their long-term survival. This suggests that $CD8^+$ T cell responses during late-chronic infection are incapable of preventing mortality in infected mice. However, the mechanism underlying this apparently ineffective $CD8^+$ T cell response during late-chronic infection has not been studied and it remains to be determined if this defect is responsible for loss of parasite control. We therefore tested whether $CD8^+$ T cells during late-chronic toxoplasmosis become dysfunctional, as reported in some viral infections.

Investigation of $CD8^+$ T cell response revealed that $CD8^+$ T cells during later phases of chronic toxoplasmosis are unable to mount a potent anamnestic response, a hallmark of robust adaptive immunity. Moreover $CD8^+$ T cells from these mice exhibit a progressive increase in expression of inhibitory molecule PD-1 with concomitant parasite reactivation. *In vivo* blockade of PD-1 interaction with its receptor PD-L1 not only augmented polyfunctional $CD8^+$ T cell response but it also controlled parasite recrudescence and prevented the mortality of chronically infected mice. To the best of our knowledge, this is the first report of parasite reactivation caused by PD-1 mediated $CD8^+$ T cell dysfunction.

Since immune exhaustion during chronic toxoplasmosis involves a loss of polyfunctional $CD8^+$ T cells, we next investigated how immune exhaustion specifically mediates preferential

attrition of this subset. Our study demonstrates that PD-1 is preferentially expressed on polyfunctional memory $CD8^+$ T cells which renders them susceptible to apoptosis. *In vitro* blockade of PD-1-PD-L1 pathway dramatically reduces apoptosis of polyfunctional and $IFN\gamma^+GranzymeB^-$ memory but not effector $CD8^+$ T cells. These findings, underscore the critical role of the PD-1-PD-L1 pathway in mediating attrition of this important $CD8^+$ T cell subset and addresses the mechanistic basis of how αPD-L1 therapy reinvigorates polyfunctional CD8 response during chronic infections. The conclusions of this study can have profound immunotherapeutic implications in combating recrudescent toxoplasmosis as well other chronic infections.

Apart from TCR signal and cytokine milieu, one of the most important factors governing CD8 response is the balance between positive signals from co-stimulatory receptors and the negative signals from inhibitory receptors. However, the role of co-stimulatory receptors during the rescue of this response has not been explored in any model of CD8 exhaustion. We demonstrate that one such co-stimulatory pathway, CD40-CD40L, plays a critical role during rescue of exhausted CD8 T cells. Blockade of this pathway abrogates the ameliorative effects of αPD-L1 treatment on CD8 T cells. Additionally, we demonstrate for the first time in an infectious disease model, that CD8 intrinsic CD40 signaling is important for optimal CD8 polyfunctionality, proliferation, T-bet upregulation and IL-21 signaling, albeit in the context of CD8 rescue. The critical role of CD40 during the rescue of exhausted CD8 T cells may provide a rational basis for designing novel therapeutic vaccination approaches. Cumulatively, these studies go a long way in understanding CD8 exhaustion in toxoplasma model and the insights afforded by them will help in designing improved vaccination strategies not only against this parasite but other persistent pathogens and tumors as well.

Table of Contents

Dedication iv

Acknowledgments v

Abstract of Dissertation vi

List of Figures ix

List of Abbreviations xi

Chapter 1: BACKGROUND 1

Chapter 2: SPECIFIC AIMS 24

Chapter 3: METHODS 37

Chapter 4: Control of Toxoplasma reactivation by rescue of dysfunctional CD8$^+$ T cell response via PD-1-PD-L1 blockade 37

Chapter 5: PD-1 mediated attrition of polyfunctional memory CD8$^+$ T cells in chronic Toxoplasma infection 75

Chapter 6: CD40-CD40L pathway plays a critical CD8 intrinsic and extrinsic role during rescue of exhausted CD8 T cells 95

Chapter 7: DISCUSSION 114

Chapter 8: BIBLIOGRAPHY 123

List of Figures

Figure 1. CD8 T cell effector mechanisms during acute and chronic toxoplasmosis. …..17

Figure 2. Vaccination strategy targeting CD8 T cell response against Toxoplasma. …....21

Figure 3. Parasite reactivation during late-chronic *T. gondii* infection. ..……..... 42

Figure 4. Validation of *in vivo* parasite stage specific gene transcription. ...…….44

Figure 5. Majority of *T. gondii* infected leukocytes exhibit a myeloid lineage phenotype.46

Figure 6. Increased parasitization of leukocytes in mice treated with anti-IFNγ during early-chronic infection. ….....……...48

Figure 7. Poor $CD8^+$ T cell response during late-chronic *T. gondii* infection. ……...50

Figure 8. PD-1 is expressed at high levels on $CD8^+$ T cells in both lymphoid and non-lymphoid tissue during late-chronic Toxoplasmosis. ...……..55

Figure 9. High PD-1 upregulation does not occur on newly generated $CD8^+$ T cells. ..…..57

Figure 10. Early initiation of drug treatment reduces $CD8^+$ T cell exhaustion. ...……...59

Figure 11. Blockade of PD-1-PDL-1 pathway reinvigorates $CD8^+$ T cell response in both lymphoid and non-lymphoid tissue in chronically infected mice. ……...……...63

Figure 12. Anti PDL-1 treatment reduces apoptosis of $PD1^{int}CD8^+$ T cells. ...……...65

Figure 13. Anti PDL-1 treatment preferentially rescues PD-1 expressing $CD8^+$ T cells. ...67

Figure 14. Anti-PDL-1 treatment controls parasite reactivation and prevents mortality in chronically infected mice. ...…….……...70

Figure 15. PD-1-PDL-1 blockade increases Eomes but not T-bet expression on cycling $CD8^+$ T cells. ...……...73

Figure 16. Majority of polyfunctional $CD8^+$ T cells during chronic toxoplasmosis exhibit a memory phenotype. ..…….....78

Figure 17. Frequency of memory phenotype $CD8^+$ T cells is not dramatically altered at the effector site. ……...…….....81

Figure 18. PD-1 is preferentially upregulated on CD43 expressing memory phenotype $CD8^+$ T cells. ……...……...85

Figure 19. PD-1 expression correlates with apoptosis of polyfunctional memory phenotype $CD8^+$ T cells. ……...……...89

Figure 20. *In vitro* anti-PDL-1 treatment prevents apoptosis of polyfunctional memory phenotype $CD8^+$ T cells. ……..……...93

Figure 21. High expression of CD40 on CD8 T cells in αPD-L1 treated mice. ...……...97

Figure 22. Blockade of CD40-CD40L pathway abrogates αPD-L1 mediated rescue of CD8 T cells. ……....……..101

Figure 23. CD40 plays a CD8 intrinsic role during rescue of exhausted CD8 T cells. ….105

Figure 24. CD40 plays a CD8 intrinsic role in mediating optimal upregulation of T-bet but not Eomes during rescue of exhausted CD8 T cells. ...……...……...107

Figure 25. CD40 plays a strictly T cell intrinsic role in mediating IL-21 and IL-21R expression on T cells. ……...110

Figure 26. CD4 intrinsic CD40 signaling is critical for upregulation of T follicular helper cell markers during rescue of exhausted CD8 T cells. ……..112

LIST OF ABBREVIATIONS

2B4	Cluster of Differentiation 244
AIDS	Acquired immunodeficiency syndrome
APC	antigen presenting cell
Bcl-6	B cell lymphoma 6 protein
BM	bone marrow
CD127	Cluster of Differentiation 127 (IL-7Rα)
CD40L	CD40 ligand
CTL	cytotoxic lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
E:T	Effector to Target Ratio
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Gzb	GranzymeB
h	hour
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hr	hour
HSV	Herpes Simplex Virus
i.g.	intra-gastric

i.p.	intra-peritoneal
i.v.	intra-veinous
ICOS	Inducible T cell co-stimulator
IFNγ	Interferon gamma
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-21R	IL-21 receptor
IL-7Rα	IL-7 receptor alpha chain
KLRG1	Killer cell lectin-like receptor subfamily G member 1
KO	knockout
LAG3	Lymphocyte-activation gene 3
LCMV	Lymphocytic Choriomeningitis Virus
MCMV	Mouse Cytomegalovirus
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	minute
MPEC	Memory precursor effector T cell
OVA	Ovalbumin
PD-1	Programmed death-1
PD-L1	Programmed cell death1 ligand1
PDL-1	Programmed cell death1 ligand1
PDL-2	Programmed cell death1 ligand2

pi	post-infection
SIV	Simian Immunodeficiency Virus
SLEC	Short lived effector T cell
Tcf1	T cell factor 1
Tcm	Central Memory T cells
TCR	T cell receptor
TE	Toxoplasmic encephalitis
Teff	Effector T cells
Tem	Effector Memory T cells
Tfh	T follicular helper cell
TIM3	T-cell immunoglobulin domain and mucin domain 3
TLA	Toxoplasma lysate antigen
TNFα	Tumor necrosis factor-alpha
VSV	Vesicular Stomatitis Virus
WT	wild-type
µg	microgram
µl	microliter

CHAPTER 1: BACKGROUND

1.1 Introduction

Toxoplasma gondii, an obligate intracellular parasite of the phylum apicomplexa infects a wide variety of warm blooded animals including humans (1). Besides complications during pregnancy, Toxoplasma infected immuno-competent adults remain asymptomatic in most cases (2). In the intermediate host, Toxoplasma undergoes stage conversion between the rapidly multiplying tachyzoite that is thought to be responsible for acute toxoplasmosis and the slowly replicating, relatively quiescent, primarily encysted bradyzoite stage that can persist possibly for life (3, 4). However, with loss of immune competence as in HIV/AIDS or immunosuppressive therapies, the parasite reactivates back to presumably tachyzoite stage and causes the development of toxoplasmic encephalitis and death of the host (5). Although the incidence of TE amongst AIDS patients declined considerably in US due to prophylactic treatment and HAART therapy, it still remains a significant problem in developing countries (6)**.**

Historically, *T. gondii* population structure has been divided into three clonal types based on genetic characteristics (7). However, recent studies have shown that this population structure is much more diverse in places such as rural Africa and South America where it has been associated with increased risk of primary and reactive ocular toxoplasmosis (7). Moreover, recent work has now correlated *T. gondii* infection with increased risk of dementia and Alzheimer's in the elderly (8). Therefore beyond TE in immuno-compromised individuals, immuno-competent adults may be at a higher risk for Toxoplasma related complications.

Apart from their toxicity, current drugs are only efficacious against the tachyzoite stage of this protozoan and not against the encysted bradyzoite stage (3). Although live attenuated strains of the parasite have been successfully used to vaccinate mice, they are not considered sufficiently safe for use in humans (9-12). Hence understanding the mechanisms responsible for immune control of parasites as well as parasite reactivation is critical for the development of immunotherapeutic agents against the pathogen.

Immune protection against many intracellular pathogens including viruses, bacteria and protozoa is provided by a robust cell mediated immunity (13, 14). T cells, macrophages, NK cells and cytokines such as IFNγ and TNFα and nitric oxide are the key effector components of this protective immune response (5, 15-17). However the development of this potent T cell immunity is critically dependent on appropriate interactions with APC (antigen presenting cells) such as dendritic cells both via cell surface receptor mediated interactions and a conducive cytokine milieu namely IL-12 and IL-15 (18-21). This activation leads to the proliferation, differentiation and acquisition of effector functions of the antigen specific T cells (22). The effector functions of activated antigen specific T cells include secretion of cytokines IFNγ and TNFα and cytotoxicity which promote further development of adaptive immunity and pathogen control.

As demonstrated by Frenkel *et al*. using a hamster model, humoral immunity provides little protection against acute toxoplasmosis (23). Adoptive transfer experiments by this group using injection of intact or lysed splenocytes from immunized animals to naïve recipients revealed that upon challenge only recipients that received intact splenocytes were protected. This suggested that protection against *T. gondii* is dependent on cell-mediated

immunity. Later using an antibody based depletion strategy it was demonstrated that both CD4 and CD8 T cells were important for control of infection with CD8 T cells playing the dominant role (24). Further studies by Suzuki *et al*, using IFNγ depletion and CD8 T cell adoptive transfer approach, identified IFNγ as a major mediator of resistance against toxoplasmosis (15). Subsequently, Khan *et al* developed the first antigen specific CD8 T cell clones capable of killing *T. gondii* tachyzoites *in vitro* via their cytotoxic activity (25). Combined, these studies suggest that the critical effector mechanisms involved in controlling *T. gondii* infection include, IFNγ production and cytotoxicity mediated by CD8 T cells. Subsequent studies have reinforced the paradigm that CD8 T cells are the dominant effector cell responsible for the control of long-term infection with CD4 T cells playing an important secondary role (26, 27). Since these seminal observations were made, the complex multi-factorial steps involved in CD8 activation, effector function acquisition and memory/effector differentiation during *T. gondii* infection have been elucidated with greater clarity. However, many questions regarding this process still remain to be addressed. In this chapter we present current knowledge of how this multi-step process leads to the activation of CD8 T cell response to *T. gondii* and how it modulates the potency of this infection. Additionally we discuss how this knowledge regarding *T. gondii* specific CD8 T cell response development can be exploited for development of immunotherapeutic agents.

1.2 Antigen presentation and priming of $CD8^+$ T cell immunity:

As a first step for activation of CD8 T cell response, cell-cell interaction between APC and CD8 T cell is needed (28). Subsequently, recognition of antigenic peptide presented by APC in context of MHC Class I by CD8 T cells is required (28). Early studies using in-bred

and out-bred mouse strains of mice showed that differences in the class I haplotype could influence the outcome of *T. gondii* infection, suggesting that beyond variations in inflammatory responses, a genetic component could influence the control of the parasite (29-31). Studies conducted using animals with deleted or mutated L^d allele demonstrated that mice such as Balb/c which possess this allele were protected while those that do not (C56BL/6, B10, CBA/J), exhibit susceptibility to infection (32-34). Experiments using mice expressing human MHC Class I transgenes showed similar allelic dependence for control of *T. gondii* (35). Hence, MHC Class I haplotype is an important determinant for the generation of protective CD8 T cells in response to *T. gondii* infection. Therefore identification of Class 1 restricted *T. gondii* specific epitopes would be helpful in generating vaccines that mount an effective CD8 response. Several studies have identified Toxoplasma surface antigens (SAG) and secreted proteins such as the rhoptry, dense granule and microneme proteins as T cell antigens (12, 36-40). However whether epitopes contained in such antigens indeed correspond to dominant *T. gondii* specific CD8 T cell response in Toxoplasma infected mice (i.e. without vaccination or immunization) has not been established till a short time back. Recent studies by Blanchard *et al* identified the decapeptide HPGSVNEFDF (HF10) from the GRA6 dense granule protein as the dominant naturally processed protein recognized by CD8 T cells during *T. gondii* infection in Balb/C ($H\text{-}2^d$) mice (41). This was followed by the discovery of two more H2-L^d restricted epitopes SPMNGGYYM and IPAAAGRFF from the dense granule protein GRA4 and rhoptry protein ROP7 respectively (42). Interestingly, while H-2Ld/GRA4 reactive CD8 T cell peaked during the acute phase, H-2Ld/ROP7 reactive CD8 T cells peaked during chronic phase, suggesting parasite-stage specific recognition of endogenous *T. gondii* derived CD8

T cell epitopes. Subsequently Wilson *et al* identified a novel H-2K^b restricted epitope SVLAFRRL derived from tgd057, a protein of unknown function (43). Hence the current challenge lies in identifying dominant human MHC Class I restricted epitopes for *T. gondii* antigens. Preliminary work from the laboratory of Rima McLeod has identified several epitopes from *T. gondii* antigens restricted by HLA-A02, a supertype family which is present in fifty percent of the human population (44). Using a pool of such peptides they showed that immunized HLA-A*0201 transgenic mice were protected against Toxoplasma challenge. However this protection was only partial and it remains to be established whether a similar pool of peptides can prevent TE in chronically infected individuals.

1.3 Role of costimulatory molecules in the development of CD8 T cell immunity against *T. gondii*

Apart from TCR-peptide-MHC interaction costimulatory molecules play a critical role in adequate CD8 T cell activation (45, 46). Co-stimulation occurs mainly via interaction of two families of proteins, the immunoglobulin (Ig) super-family members B7/CD28 and the TNFR/TNF super-family of proteins (47). Early investigations of co-stimulatory molecules in *T. gondii*, model using human peripheral blood mononuclear cells, demonstrated that blockade of both CD80 and CD86 reduced T cell activation (48). Subsequently, studies in mouse model demonstrated that CD28, a receptor for CD80 and CD86, was dispensable for protective immunity against the parasite despite reduced IFNγ production by T cells (49). Interestingly, when chronic infected mice were rechallenged with the RH strain of the parasite, CD28$^{-/-}$ mice as opposed to wild-type animals rapidly succumbed to the infection. Since a potent recall response is considered a hallmark of memory CD8 T cells, this data suggest that lack of CD28 impairs the development of memory T cells (50). Using an

antibody based approach, CD40 and CD40L were shown to play an important role in the *in vitro* activation of human T cells in response to *T. gondii.* (51). However *in vivo* studies using a mice model demonstrated that while this pathway affected macrophages it did not down-regulate T cell responses, suggesting that CD40-CD40L pathway is dispensable for robust T cell response (52). Since CD40-40L pathways were not found to be critical for the primary T cell response against Toxoplasma in mice, other pathways were investigated for their role in T cell activation against this pathogen. These studies focused on the CD28 homologue, inducible co-stimulator protein (ICOS) (53). Unlike CD28, however, ICOS is an inducible co-stimulatory molecule for T cell activation (53). Although $ICOS^{-/-}$ mice elicited a poor CD4 T cell response, the CD8 T cell immunity remained unaffected during acute toxoplasmosis (54). This suggests that neither ICOS-ICOSL signaling or differential CD4 T cell help affects primary CD8 response during acute toxoplasmosis. Interestingly over 90% of brain resident CD8 T cells expressed ICOS during the chronic phase of infection (55). In allograft rejection models, it has been shown that delayed but not early blockade of ICOS-ICOSL reduces effector CD8 development, suggesting a possible role of this pathway post-priming (56). However, in the Toxoplasma model it remains to be addressed whether ICOS-ICOSL pathway has a differential effect on CD8 T cells in lymphoid versus non-lymphoid tissue or during acute/chronic phase. The development of a potent CD8 T cell response with minimal immuno-pathology involves a delicate balance between positive signal from co-stimulatory receptors and negative signals from inhibitory receptors (57). However the role of the latter family of receptors on the CD8 T cell response against *T. gondii* has not been thoroughly investigated. Early work on these receptors focused on CTLA4, a receptor expressed on activated CD8 T cells that binds to CD80 and

CD86, albeit with much higher affinity than CD28 (58). Ex vivo treatment of splenocytes from Toxoplasma infected mice with anti-CD3 and CTLA4-Ig was found to significantly reduce IFNγ production in spleen but not in brain (49). However in our laboratory using a similar protocol for brain mononuclear cell preparation, we have noted that apart from leukocytes other cell types do not survive well *in vitro* [Bhadra and Khan, unpublished observations]. Hence for a more thorough understanding of the role of CTLA-4 on brain resident CD8 T cells in the endogenous micro-environment, *in vivo* blockade with anti-CTLA4 antibody needs to be performed. Apart from CTLA-4, PD-1, a negative regulator of activated T cells, has been shown to be up-regulated on CD8 T cells from toxoplasma infected C57BL/6 mice during the chronic phase (59). Although the significance of PD-1-PD-L1 pathway in mediating CD8 dysfunction in chronic viral models is well established, the role of this pathway in *T. gondii* model of infection remains to be extensively investigated (60, 61). Overall the mechanism related to the differential role of various co-stimulatory and inhibitory molecules in activating CD8 T cells and modulating their function is poorly understood. Hence a more through understanding of these receptors in *T. gondii* model is required for their potentially critical application as adjuvants during vaccination or other immunotherapeutic interventions. Using high-throughput approaches such as micro-array in combination with conditional knockouts and bone-marrow chimeras, we may be able to better indentify and characterize new targets of co-stimulation or co-inhibition on CD8 T cells and elucidate the CD8 intrinsic and extrinsic role of these molecules in mediating their activation, differentiation and effector function acquisition.

1.4 Role of cytokines in the development of CD8 T cell immunity against *T. gondii*

Once initial CD8 T cell activation takes place, their expansion and differentiation is modulated by the cytokine milieu (62). During *T. gondii* challenge, neutrophils (PMNs), macrophages, and dendritic cells and several inflammatory cell types infiltrate into the site of infection and exert their function by providing nitric oxide, cytokines and chemokines (20, 63-70). A critical cytokine produced by these cells during acute phase of infection is IL-12 which is necessary for optimal induction of a primary CD8 T cell response. (21, 71-73). The biologically active form of IL-12, IL-12p70 is composed of two subunits: the constitutively expressed IL-12p35 and the inducible IL-12p40 (74). IL-12p70 produced during *T. gondii* infection can induce CD8 T cells to produce IFNγ, differentiate, proliferate and expand (71, 72, 75, 76). The levels of IL-12p70 heterodimer and IL-12p40 production elicited by *T. gondii* is strain dependent and this can potentially cause differences in the robustness of CD8 responses (77). Despite the critical role of IL-12 in mediating a potent CD8 T cell immunity against this parasite, addition of IL-23, a cytokine structurally similar to IL-12p70, does not affect T cell mediated IFNγ production (78). Moreover CD8 T cells in toxoplasma infected $p19^{-/-}$ (IL-23 deficient) mice do not exhibit reduced activation vis-à-vis wild-type controls. Apart from IL-12, IL-27, an IL-12 related cytokine has also been recently shown to play a critical role in eliciting IFNγ production by CD8 T cells during acute toxoplasmosis (79). However whether IL-27 plays any role in the long-term CD8 response to *T. gondii* remains to be established. Interestingly, in the same study the authors found that CD8 intrinsic IFNγR signaling is not required for optimal IFNγ production by CD8 T cells *in vivo*.

Several cytokines, other than IL-12 related cytokines, are also important for CD8 T cell function in response to *T. gondii* infection. This includes members of the common-cytokine-receptor γ-chain cytokines like IL-2, IL-7 and IL-15 (80). Unlike other pathogens *T. gondii* does not induce a potent IL-2 response (81-83). Hence it is not surprising that in mice deficient in CD4 T cells which is considered to be the primary source of IL-2, primary CD8 response during acute toxoplasmosis remains unaffected (27). Interestingly, an independent study showed that *T. gondii* infected IL-$2^{-/-}$ mice exhibited poor CD8 response activation (84). While in the former study the animals were orally infected, in the latter, mice were challenged via intraperitoneal route. These apparently contradictory observations perhaps suggest a differential dependence on IL-2, based on the route of infection. Hence the effect of CD4 T cells or IL-2 on the maintenance of CD8 T cell memory as well as programming and differentiation of CD8 T cells effectors need to be investigated more thoroughly using both oral and intra-peritoneal routes of infection.

The role of γ_c family cytokine IL-15 in the maintenance of CD8 T cell memory response against Toxoplasma infection has been established by the studies conducted in our laboratory (18, 80, 85-87). Moreover during the acute response it plays a critical role in regulating CD8 burst size but not per cell function (80). Interestingly, IL-$15^{-/-}$ animals survive as well as wild type mice when infected with *T. gondii*, suggesting that there may be some redundancy in its functions (86, 88). Very little work has been done to investigate the role of IL-7, a related γ_c cytokine, in development of CD8 T cell responses against *T. gondii*. However, early studies from our group reported that exogenous treatment with the cytokine can augment cytotoxic CD8 T cell response against infection (81). Moreover, in a very

recent study we reported that in the absence of IL-15, endogenous IL-7 plays a critical role in the development of *T. gondii* specific CD8 T cell memory precursors via expression of anti-apoptotic protein Bcl-2 (80). As such, when IL-15$^{-/-}$ mice are depleted of IL-7, they exhibit extremely rapid mortality (80). Recently IL-21, another γ_c family member, has been shown to modulate the quality and potency of CD8 T cell response in both acute and chronic viral models of infection (89, 90). Thus it will be very important to address the role of this cytokine during Toxoplasma infection in susceptible and resistant mouse strains.

Apart from aforementioned cytokines, IL-10, a down-regulatory cytokine is induced during *T. gondii* infection (91). Interestingly the major source of this cytokine during acute toxoplasmosis is not Foxp3 expressing regulatory CD4 T cells but rather conventional T-bet^{+}Foxp3^{-} CD4 T cells (92). Recent studies have demonstrated that while IL-10 down-regulates CD8 response during chronic viral infection, it does not suppress CD8 immunity during acute viral infection (93). In contrast to these findings, Gazzinelli *et al* noted up-regulation of CD8 mediated TNFα but not IFNγ production in IL-10 deficient B6 mice during acute toxoplasmosis (91). Incidentally unlike viral models such as LCMV, CD8 response against Toxoplasma is dependent on IL-12 (94). As IL-10$^{-/-}$ mice overproduce IL-12p70, increased TNFα production by CD8 T cells in Toxoplasma infected IL-10$^{-/-}$ mice may be reflective of IL-12 mediated increase in number of antigen-specific CD8 T cells or augmented per cell functionality or both (91). Future studies with wild-type/IL-10R$^{-/-}$ mixed bone marrow chimerized mice are needed to address whether this augmented CD8 response in IL-10R$^{-/-}$ mice is indeed due to differential CD8 intrinsic IL-10 signaling. In agreement with observations in viral models, blockade of IL-10 in chronically infected Balb/c mice resulted in increased number of CD8 T cells in the brain with concurrent decrease in *T.*

gondii burden (95). However IL-10 blockade during acute or chronic toxoplasmosis in susceptible mice strains causes severe immuno-pathology and rapid mortality (92)[Bhadra and Khan , unpublished data]. Hence this potential risk must be taken into consideration while designing any immuno-therapeutic strategy utilizing IL-10 blocking regimen to boost CD8 response against *T. gondii*.

1.5 Differentiation of effector and memory CD8 T cell sub-populations:

After activation of naïve CD8 T cells during an infection, they expand over 10^4 fold and differentiate into a heterogeneous population of effector cells (96, 97). This population is composed of terminally differentiated effector cells termed short lived effector cells (SLEC) and cells that are destined to become long lived memory cells termed memory precursor effector cells (MPEC) (96, 98). Phenotypically SLECs can be discriminated from MPEC based on the expression of KLRG1 and lack of IL-7Rα expression on the former (98). Both SLEC and MPEC exhibit effector functions and low proliferative capacity (97, 98). However as MPEC further develop into mature memory CD8 T cells they become self-renewing and undergo homeostatic proliferation (97). After this initial expansion and differentiation, the majority of activated CD8 T cells undergoes a process termed contraction where the terminally differentiated SLECs undergo apoptosis leaving behind memory T cells (96). Many factors including, antigen avidity and affinity, co-stimulation and the level of inflammation can influence CD8 T cell differentiation (45, 99, 100). The mechanisms governing these processes are being investigated in viral and bacterial model systems and are only now beginning to be addressed in the field of *T. gondii* (98).

Seminal work from Joshi *et al* using *Listeria monocytogenes* reported that the master transcription factor T-bet acts as a rheostat whereby under highly inflammatory conditions

namely IL-12, T-bet up-regulation results in preferential CD8 differentiation to SLEC as opposed to MPEC (98). Similarly, a recent a study using the *T. gondii* model has demonstrated a critical role for IL-12 in mediating T-bet mediated generation of $KLRG1^+$ CD8 effector T cells (94). Although IL-12 is important for SLEC differentiation *in vivo*, a study using LCMV model demonstrated that their survival is critically dependent on IL-15 (98).

Memory CD8 T cells are critical for long term protection against many intracellular pathogens (97, 101). These long-lived cells can be further broadly categorized into two subsets: central memory and effector memory CD8 T cells (102). Effector memory CD8 T (Tem) cells are characterized by high cytotoxicity, lack of expression of lymphoid homing molecules such as CCR7 and CD62L and persistence primarily in non-lymphoid tissue (103, 104). Central memory T (Tcm) cells on the other hand, reside mainly in secondary lymphoid tissue, express high levels of IL-7Rα, CCR7 and CD62L and are less cytotoxic than effector memory T cells (102, 105). However these cells exhibit increased antigen sensitivity and self-renewal and mediate rapid and robust recall responses vis-à-vis the effector memory population. In various infectious disease models including HIV, development of a potent Tcm subset has been linked with improved outcome, suggesting that one of the goals of any vaccination regimen targeting CD8 T cell based immunity should be the generation of robust Tcm subset (106, 107). While the differential role of Tcm and Tem in the control of *T. gondii* infection remains to be thoroughly investigated, a recent study by Wilson *et al* suggests that high levels of IL-12, while beneficial for effector and effector memory CD8 population, has a detrimental effect on the number and function of tgd057-specific central memory CD8 T cells (43). As parasite virulence can alter the levels of IL-12 produced

during infection, one potential mechanism of immune escape may be via over production of IL-12, thereby inhibiting the development of Tcm subset which may result in deficient control of chronic infection (108).

1.6 Effector role of CD8 T cells during *T. gondii* infection

As mentioned earlier IFNγ plays a pivotal role in protective immunity against *T. gondii* infection. During chronic infection, CD8 T cells are major source of this cytokine, essential for controlling parasite reactivation (15, 26). IFNγ acts on surrounding cells, including macrophages, which in turn inhibit the proliferation of the parasite via induction of inducible nitric oxide synthetase (iNOS), reactive oxygen species (ROS) and Indoleamine 2,3-deoxygenase (IDO) (68, 109-117). Moreover IFNγ, via modulation of p47GTPases, regulates the survival of the parasite in activated macrophages (118). Thus IFNγ is likely to have two roles in controlling the parasite: initially during acute infection to reduce parasite numbers and then during chronic infection to exert immune pressure sufficient enough to maintain the parasites in the encysted bradyzoite stage (Figure 1). In agreement with this notion, IFNγ$^{-/-}$ mice or IFNγ depletion of acutely infected wild-type mice results in rapid mortality due to uncontrolled replication of the parasite (24, 119). Similarly anti-IFNγ treatment of chronically infected mice results in reactivation of encysted parasites (120, 121). CD8 T cells can also provide effector function via production of other cytokines including TNFα and through their cytolytic abilities (122). The role of TNFα in control of *T. gondii* infection was shown early on to be synergistic with IFNγ (123). A second study demonstrated that when infected per-orally, depletion of TNFα led to the death of susceptible C57Bl/6 mice (124) . An additional study investigating TE in toxoplasma

infected mice revealed that TNFα depletion of chronically infected mice caused reactivation of *T. gondii* as rapidly as neutralization of IFNγ, suggesting that TNFα played an equally important role in control of chronic *T. gondii* infection (5). Taken together, these studies reveal an important function of TNFα in the control of *T. gondii* parasites and development of TE in mice. In viral and bacterial model systems, CD8 T cells have been shown to produce copious amount of TNFα (125, 126). Although Gazzinelli *et al* demonstrated that CD8 T cells can indeed produce TNFα during acute toxoplasmosis, the role of CD8 produced TNFα during acute and chronic infection remains to be definitively addressed (91).

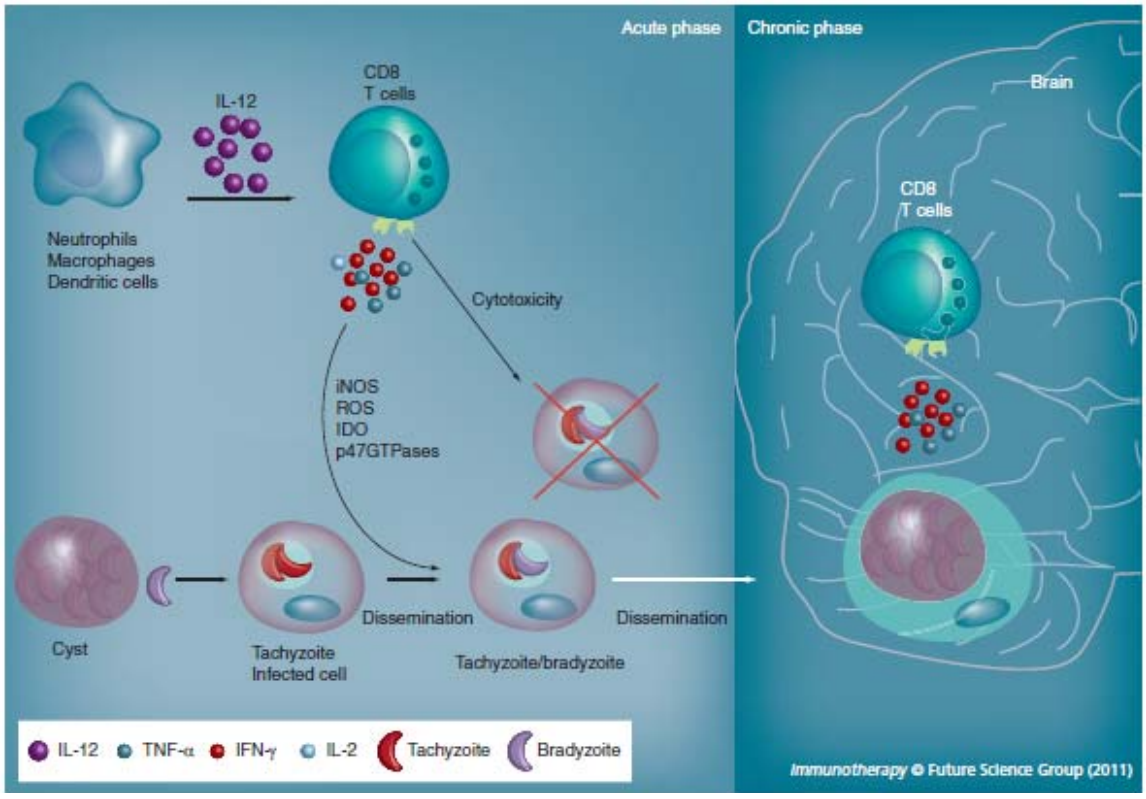
CD8 T cells have been shown to directly kill extracellular tachyzoites of *T. gondii* in culture (25). *In vitro* studies demonstrated that antigen-specific cytotoxic activity towards *T. gondii* infected cells was exhibited by human CD8 T cells (127, 128). Despite the findings from these studies, the role of this cytotoxic function of CD8 T cell in controlling *T. gondii* infection is not completely understood. Although perforin knock out (PKO) mice vaccinated with the attenuated mutant ts-4 displayed severely defective CTL activity against *T. gondii* infected target cells, these mice survived challenge with a virulent strain (129). A subsequent study using adoptive transfer of CD8 T cells from wild-type, IFNγ$^{-/-}$ or perforin$^{-/-}$ mice demonstrated that perforin mediated cytotoxicity was dispensable for prevention of TE in resistant mice strain (130). In contrast, another study using Toxoplasma susceptible mice demonstrated that adoptive transfer of immune CD8 T cells into infected SCID mice (treated with sulfadiazine to establish chronic infection) removed encysted parasites in the brain via a perforin dependent mechanism (131). However a recent paper by

Schaeffer *et al* demonstrating that CD8 T cells do not directly interact with intact toxoplasma cysts in brain, raises the question whether donor CD8 T cells are directly lysing intact cysts in drug treated SCID mice (132). This notion is further supported by observations in sulfadiazine treated IRF8$^{-/-}$ mice (IRF8$^{-/-}$ do not produce IL-12p40 and do not upregulate IFNγ) where an increase in number and size of parasitic foci in brain is noted over time (133, 134). This potentially suggests that drug treatment in immuno-deficient mice strains is unable to completely prevent cyst rupture and parasite release. Based on these two papers, it is likely that in drug treated SCID mice, cysts intermittently rupture and release parasites which are then killed by donor CD8 T cells before they can form new cysts. This would over time result in cyst reduction as observed by Suzuki *et al*. Hence in future studies it will be important to keep this possibility in mind. Apart from perforin mediated cytolysis, recent work by Jordan *et al* using cps1-1 strain of the parasite demonstrates that a perforin and Fas-FasL independent pathway may play a subsidiary role in CD8 mediated cytotoxicity (135). It will be critical to explore this pathway thoroughly in future studies.

Taken together, IFNγ seems to be the key effector cytokine during both acute and chronic phases of infection. However, the role of TNFα and cytotoxicity associated molecules like granzymeB and perforin in mediating CD8 effector functions during toxoplasmosis needs to be investigated further. Moreover how transcription factors other than T-bet such as Eomesodermin, BLIMP, Runx3 and Bcl-6 which have been shown to play a role in other model systems, regulate development, differentiation and function of CD8 T cells during *T. gondii* infection need to be addressed (136-140). Hitherto, a major hurdle in investigating these questions thoroughly was the lack of information regarding dominant CD8 epitopes. However advent of new tools such as MHC Class I tetramers, TCR

transgenic mice and OVA expressing transgenic parasites will permit dissection of effector mechanisms of various CD8 subsets and their correlation with immune protection during acute and chronic phases of infection with much greater clarity (41, 42, 59, 141, 142).

Figure 1. CD8 T cell effector mechanisms during acute and chronic toxoplasmosis. During the acute phase CD8 T cells primarily via the production of IFNγ and possibly TNFα and cytotoxicity associated molecules like perforin and granzymeB inhibit parasite replication and cause conversion of the parasite from tachyzoite stage to bradyzoite stage. IFNγ elicits the production of NO, ROS and IDO and activates p47GTPases which augments parasite clearance. During the chronic phase, CD8 produced IFNγ and possibly TNFα is vital for maintaining the parasites in the encysted stage.



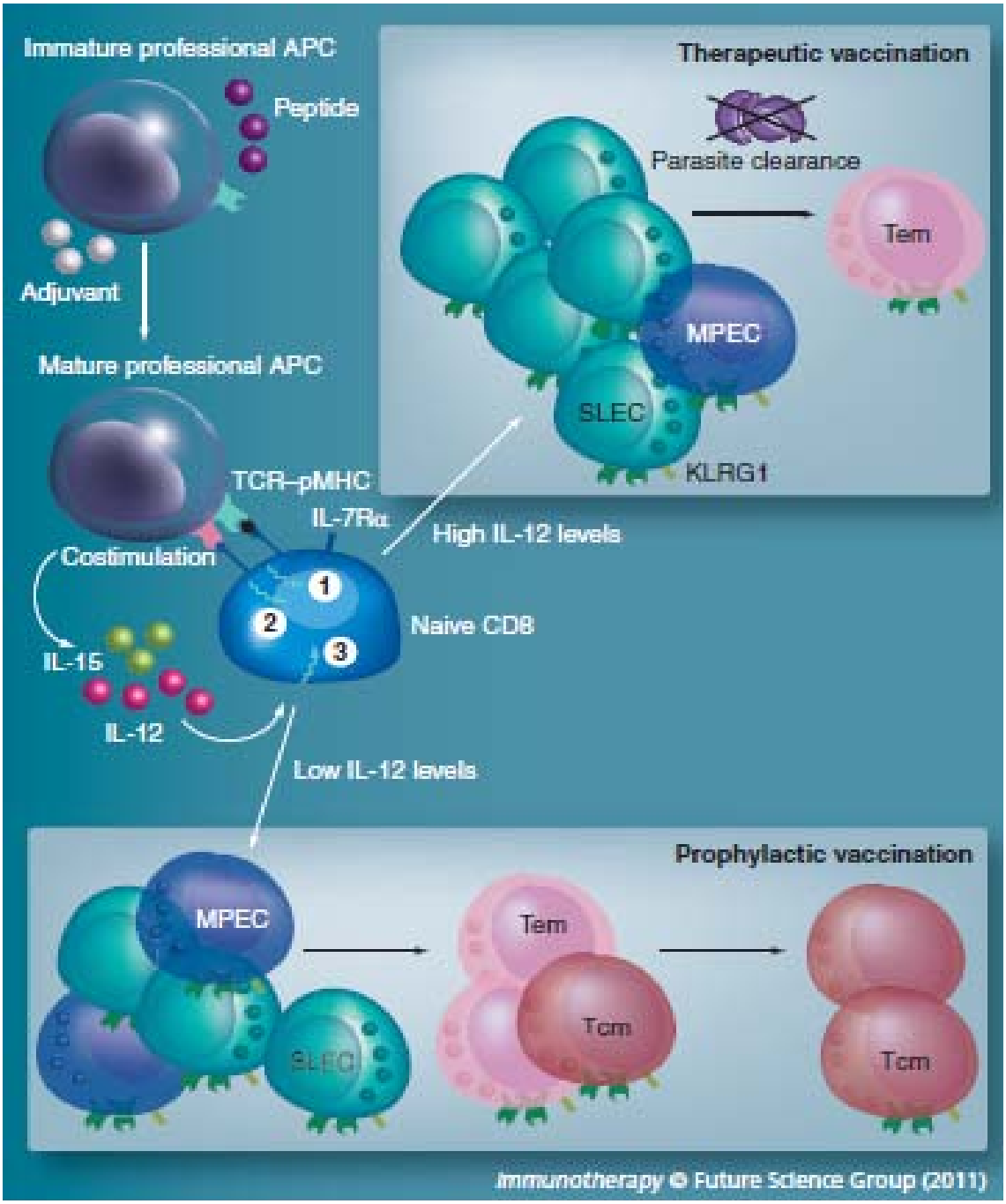
Adapted from (143)

1.7 Conclusion

There is little debate that CD8 T cells are critical for long-term control of toxoplasmosis. Hence for a vaccination strategy to be successful against this pathogen, a potent CD8 T cell response needs to be elicited. Considering the prevalence of toxoplasmosis in developing countries, any vaccine production strategy cannot utilize cost and labor intensive approaches like peptide-loaded dendritic cell or ex vivo CD8 manipulation for instance. Preliminary studies from Rima McLeod laboratory have shown partial efficacy of Toxoplasma peptide pool immunization in HLA-A*0201 transgenic mice (44). However a major obstacle that needs to be surmounted is the identification of dominant MHC Class I restricted epitopes for *T. gondii* recognized by CD8 T cells during acute and chronic toxoplasmosis in humans. Considering that Toxoplasma is a eukaryote with complex life-cycle, it is likely that for any vaccination regimen to be efficacious, multiple epitopes will need to be targeted (3, 144). Moreover depending on whether vaccination has prophylactic or therapeutic purpose, peptide pool and adjuvant selection may be different. For instance an adjuvant that induces high-levels of IL-12 or inflammation may be ideal for therapeutic vaccination strategies as it would elicit a potent effector response (Figure 2). On the other hand, use of such a regimen for a prophylactic vaccine will not be the best solution as it may not elicit a robust long-lived Tcm response. In viral infections including HIV, T cell polyfunctionality, i.e. the capacity to exhibit multiple effector mechanisms upon antigen encounter, has been identified as one of the most important immune correlates of protection (125, 145-147). While the role of CD8 polyfunctionality in Toxoplasma model has not been thoroughly investigated, any vaccine design targeting this parasite, must take this parameter into consideration. A recent study

using vaccination against SIV further reinforces this paradigm (148). In that report, frequency of polyfunctional CD8 T cells rather than antigen-specific CD8 T cell frequency correlated with immune protection. Incidentally the authors found that vaccination with TLR (Toll-like receptor) agonists along with IL-15 elicited the most potent polyfunctional CD8 responses. While TLR agonists (TLR2,3,9) enhance CD8 response by activating and maturing dendritic cells, IL-15 promotes the generation of high avidity long-lived CD8 T cells and increases homeostatic proliferation of memory CD8 T cells. The efficacy of a similar regimen needs to be experimented in vaccination protocols against *T. gondii*. Overall a better understanding of immune correlates of a robust long-lived CD8 response is critical for the development of successful vaccine against Toxoplasma.

Figure 2. Vaccination strategy targeting CD8 T cell response against Toxoplasma. Any vaccination strategy against Toxoplasma must involve appropriate selection of adjuvant and peptide pool such that there is optimal TCR-pMHC interaction (signal 1), costimulation (signal 2) and cytokine milieu (signal 3) during CD8 priming. In addition to peptide pool used, adjuvant selection may need to be altered depending on whether it is a prophylactic or therapeutic vaccine. Adjuvants that are highly inflammatory will result in preferential CD8 differentiation to effector lineage which may be ideal for therapeutic vaccination. Conversely, adjuvants that elicit comparatively lower inflammation will result in robust Tcm development which may be optimal for prophylactic vaccination regimens.



Adapted from (143)

1.8 Future perspectives

Understanding the generation of long-term CD8 T cell responses is central to the development of immunotherapeutic agents against *T. gondii* infection. However, at present a major limitation in this field is the lack of information regarding epitopes involved in eliciting this response in humans. In recent years, studies in this direction have already begun and we anticipate in the near future dominant human MHC restricted epitopes will be identified. This will enable us to determine and quantitate effector and memory CD8 T cell response generated during natural infection and various vaccination protocols. The information from these studies will provide an important breakthrough in the understanding the factors involved in the generation of long-term CD8 T cell immunity against complex intracellular parasites in humans and permit the development of improved immunotherapeutic regimens against this parasite.

CHAPTER 2: SPECIFIC AIMS

Toxoplasmic encephalitis (TE), a major cause of morbidity and mortality in Toxoplasma seropositive HIV infected population occurs primarily due to reactivation of chronic toxoplasmosis (149-151). Effective $CD8^+$ T cell response is critical for control of both acute and chronic Toxoplasmosis in susceptible mice strains (152). Although Toxoplasma elicits a robust $CD8^+$ T cell response during the acute phase (80), this response during the chronic phase paradoxically does not ensure long-term survival of susceptible mice strains (152). Taken together, these studies suggest that although critical for preventing parasite reactivation, endogenous $CD8^+$ T cell responses during late-chronic infection are incapable of preventing mortality in infected mice. However, the mechanism underlying this apparently ineffective $CD8^+$ T cell response during late-chronic infection has not been studied and it remains to be determined if this defect is responsible for loss of parasite control. We therefore hypothesize that $CD8^+$ T cells during late-chronic toxoplasmosis become dysfunctional leading to parasite reactivation and host mortality. We propose the following specific aims to address the proposal:

Specific Aim 1. To characterize how differential $CD8^+$ T cell functionality affects parasite control during early-chronic and late-chronic Toxoplasmosis. Our preliminary data show that during late-chronic toxoplasmosis $CD8^+$ T number and IFNγ production are reduced. This correlates with reduction in brain cyst and increased host mortality. Based on this, we **hypothesize** that $CD8^+$ T cells undergo exhaustion during chronic toxoplasmosis

which results in hierarchical loss of $CD8^+$ T cell functionality leading to parasite reactivation and consequent host mortality.

Specific Aim 2. To study the mechanism involved in $CD8^+$ T cell mediated dysfunction. Preliminary data demonstrate that PD-1, an inhibitory receptor of the B7-CD28 super-family that has been shown to be involved in chronic viral models of exhaustion (153) , is significantly upregulated on splenic and brain $CD8^+$ T cells during late-chronic Toxoplasmosis. Concomitant with this, increased expression of PD-1 receptor PDL-1 is noted on lymphocytes. Based on this, we hypothesize that high PD-1 expression induces $CD8^+$ T cell exhaustion during late chronic Toxoplasmosis and that blockade of PD-1-PDL-1 pathway will rescue exhausted $CD8^+$ T cells leading to control of parasite reactivation and prevention of host mortality.

Specific Aim 3. To study the role of co-stimulatory receptors during rescue of exhausted $CD8^+$ T cells. Preliminary data from our laboratory suggest that $CD8^+$ T cells from αPD-L1 treated chronically infected mice express high levels of the co-stimulatory molecule CD40 vis-à-vis isotype treated controls. Apart from TCR signal and cytokine milieu, one of the most important factors governing CD8 response is the balance between positive signals from co-stimulatory receptors and the negative signals from inhibitory receptors (143). It has been postulated that interplay between signals from co-stimulatory and inhibitory receptors may play an important role in T cell activation, differentiation, effector function and exhaustion (154). However, the role of co-stimulatory receptors during the rescue of this response has not been explored in any model of CD8 exhaustion.

Therefore we hypothesized that blockade of inhibitory PD-1-PD-L1 pathway in chronically infected mice would lead to upregulation of co-stimulatory receptors and such positive signals would be critical for the rescue of dysfunctional CD8 T cells

Chapter 3: METHODS

Mice

6 to 8 week old female C57BL/6 (CD45.2, CD90.2) and B6-Ly5.2 (CD45.1) mice were purchased from the National Cancer Institute. Similarly aged female B6.PL-Thy1^{a}/CyJ (CD90.1), CD40$^{-/-}$ and CD8$^{-/-}$ mice were purchased from the Jackson Laboratory. Animal studies were carried out in agreement with Institutional Animal Care and Use Committee approved guidelines at George Washington University Medical Center.

Bone marrow chimera generation

Mixed bone marrow chimeras were generated by injecting via i.v. route equal number ($5x10^6$ CD90.1 and $5x10^6$ CD40$^{-/-}$) of bone marrow cells from CD90.1 and CD40$^{-/-}$ mice into lethally irradiated (800 rads) CD45.1 recipients. During reconstitution the chimeras were maintained on medicated water (Sulfatrim; Hi-Tech Pharmacal) that was changed on weekly basis. After 8 weeks of bone marrow reconstitution chimeras were infected as discussed below.

Parasites, Infection and TLA preparation.

T. gondii cysts of ME49 strain were prepared from the brains of infected mice. For experimental infections, animals were infected (or rechallenged) with an average of 10 cysts/mouse via intra-gastric (i.g.) route. In some cases mice were infected intraperitoneally (i.p.) with $5x10^5$ parasites of RH strain (Type I). Infected mice were sacrificed by CO_2

inhalation at different time points pi. Toxoplasma Lysate Antigen (TLA) was extracted from RH strain of parasite and preparation was carried out as previously described (18).

Moribund mice

Mice were scored daily on a scale of 1 (asymptomatic)-5 (most severe) based on several aspects. Moribund mice were characterized by severe loss of mobility, hunched back, piloerection, ruffled fur and weight loss.

***In vivo* antibody treatment**

For *in vivo* IFNγ depletion, rat anti-IFNγ (4mg; clone XMG1.2; BioXCell) or rat IgG1 isotype-matched control antibody (4mg; BioXCell) were injected i.p. daily for 4 days starting at day 31 pi. Rat anti-PDL-1 (155) (200μg; MIH5; prepared 'in house') or rat IgG2a isotype-matched control antibody (200μg; BD Biosciences) were administered i.p. every third day for 2 weeks beginning at week 5 pi unless otherwise mentioned in the figure legends. MIH5 is a non-depleting antibody (156). For CD8 depletion, anti-PDL-1 treated mice were administered rat anti-CD8 (500μg; clone 2.43; BioXCell) i.p. every third day beginning at day 126 pi till termination of experiment. Control mice were administered rat IgG2b isotype-matched control antibody (200μg; BD Biosciences). For blockade of CD40L mice were injected with αCD40L (500μg; clone MR1; BioXCell) every third day i.p. starting at week 5 post infection (pi) for 2 weeks. Hamster IgG (500μg; BioXCell) was injected to control mice.

ELISA

Blood obtained via intra-cardiac puncture was allowed to coagulate for 30min. Then, it was centrifuged at 5000 rpm and serum was collected. The serum was stored at -20°C till further use. Serum IFNγ was assayed using ELISA Max Mouse IFNγ Kit (BioLegend) as per manufacturer's protocol.

Lymphocyte isolation and staining

Single cell suspension was prepared from spleen, liver, blood and brain using standard protocol (42, 43). Briefly, for spleen, single cell suspensions were made by mechanical disruption followed by red blood cell lysis. For hepatic lymphocyte enrichment, livers were perfused with 10 ml cold PBS, excised minced, and passed through a meshed screen. The cells were washed in cold PBS, and one liver equivalent was suspended in 15 ml of 30% Percoll (Sigma) solution in the presence of 100 IU/ml heparin (Sigma). The suspension was centrifuged for 10 min at 2000 rpm and the pellet was resuspended in cold PBS-2%FCS. For brain lymphocyte enrichment, brains were individually washed in HBSS-heparin (2 IU/ml) and then mashed through a 70 μm cell strainer, followed by gradient centrifuge (2000 rpm for 20 min) in 30% Percoll solution containing 100 IU/ml of heparin. Pellet was then resuspended in cold PBS-2% FCS. For peripheral blood, blood was initially collected in 2 ml of PBS containing 100U/ml of heparin. Partial red blood cell lysis was then carried out. For imnuo-phenotyping $1x10^6$ spleen or brain or liver cells were plated per well. For phenotyping peripheral blood cells, $3x10^6$ cells per well was used. For direct ex vivo assessment of apoptosis, BD AnnexinV Kit was used as per manufacturer's protocol.

The following antibodies were used in cell surface staining and intracellular staining of lymphocytes : anti-CD8β (clone H35-17.2; eBioscience), PD-1 (clone J43; eBioscience), PDL-1 (clone MIH-5; eBioscience), PDL-2 (clone TY25; eBioscience), Eomes (clone 21Mags8 for experiments in Chapter 4; clone Dan11Mag for experiments in Chapter 6; eBioscience), CD90.1 (clone OX-7; BioLegend), T-bet (clone 4B10; Santa Cruz Biotechnology), Granzyme B (clone GB11; Invitrogen), Ki-67 (clone B56; BD Biosciences), Active caspase-3 (clone C92-605; BD Biosciences), IFNγ (clone XMG1.2; BD Biosciences), NK1.1(clone PK136; eBioscience), CD11b (clone M1/70; eBioscience), GR1 (clone RB6-8C5; eBioscience), F4/80 (clone BM8; BioLegend), CD3 (clone 145-2C11; eBioscience), CD19 (clone 6D5; BioLegend), CD44 (clone IM7; BioLegend), CD127 (clone A7R34; eBioscience), CD43 (clone 1B11; BioLegend), IL-2 (clone JES6-5H4; eBioscience), TNFα (clone MP6-XT22; eBioscience), CD4 (clone GK1.5; eBioscience), Bcl-6 (clone mGl191E; eBioscience), KLRG1 (clone 2F1; eBioscience), IL-21 (clone mhalx21; eBioscience), IL-21R (clone 4A9; BioLegend), CD90.2 (clone 30-H12; BioLegend), CD45.1 (clone A20; BioLegend), CD40 (clone 1C10; eBioscience), CD40L (clone MR1; eBioscience) and CXCR5 (2G8; BD Biosciences). Appropriate isotype controls were used throughout. For sorting PD-1 expressing $CD8^+$ T cells, RMP1-30 (eBioscience) clone was utilized. This clone does not inhibit PD-1 interaction with its ligand (157).

Intracellular staining was performed after surface staining, using Cytofix/Cytoperm Kit (BD Biosciences) as per manufacturer's protocol. Cell fluorescence was measured with a BD FACS Calibur or BD FACS Aria or Cytek upgraded 8-color BD FACS Calibur

cytometer which accounts for differences in fluorescence scale. Data was analyzed using FlowJo (TreeStar) software. Live cells were lymphocyte gated based on forward-scatter and side-scatter. For Chapter 6 (Fig. 23-26), Fixable Live/Dead Aqua (Invitrogen) staining was performed prior to surface staining and live lymphocytes were gated based on Aqua negative stain, followed by lymphocyte gating based on forward-scatter and side scatter. For samples acquired using digital cytometers, aggregates were gated out based on scatter. Gating for positive and negative populations was set up based on FMO (fluorescence minus one) controls. For Chapter 6, following Flowjo analysis, computation for enumerating bifunctional and trifunctional cells was performed using PESTLE and SPICE programs, generously provided to us by Dr. Mario Roederer (NIH).

Intracellular cytokine detection

For cytokine detection in CD8 T cells, $5x10^5$ naïve splenocytes from congenic mice (Chapter4-5) or CD8-/- mice (Chapter 6) was mixed with equal number of brain or splenic cells from infected mice and restimulation was similarly carried out for 16h in presence of toxoplasma lysate antigen (TLA, 30 μg/ml). 0.65 μl/ml of monensin (BD Biosciences) and 0.65 μl/ml of brefeldin A (BD Biosciences) were added during the final 9h of stimulation. Cytokine detection in splenic CD4 T cells was similarly carried out without $CD8^{-/-}$ splenocyte addition.

***In vitro* agonistic CD40 treatment**

CD8 T cells were purified using biotinylated anti CD8α antibody (BioLegend) in conjunction with EasySep Biotin Selection Kit (STEMCELL Technologies). Over 90% purity was acheived as assessed by flow cytometry. Cells were plated into a 96 well plate at $5x10^5$ cells/well. Agonistic CD40 (FGK4.5, BioXCell) or isotype control (Rat IgG2A; BioXCell) was used *in vitro* at a final concentration of 10 μg/ml for 6 h at 37°C.

In vitro αPD-L1 treatment

Rat anti-PDL-1 (MIH5; prepared 'in house') or rat IgG2a isotype-matched control antibody (200μg; BD Biosciences) was added at final concentration of 30 μg/ml to splenocyte culture ($1x10^6$ cells/well). Unless otherwise mentioned, for detection of active caspase3, 5 h incubation was carried out at 37°C. For detection of active caspase3 expression in cytokine producing cells 16h incubation was carried out in presence of brefeldin A, monensin and TLA as mentioned earlier.

Flow cytometric detection of *T. gondii* infected cells

Single cell suspensions prepared as above were surface stained. Intracellular staining was performed as described above with some modifications. After permeabilization, staining with FITC labeled polyclonal anti Toxoplasma antibody (Abcam) was performed, followed by incubation with biotinylated anti-FITC antibody (FIT-22; BioLegend) and subsequent labeling with Streptavidin FITC (eBioscience). Cells were analyzed on mononuclear leukocyte gate based on forward-scatter and side-scatter.

BrdU injection and staining

BrdU (BD Biosciences) was injected at 1mg/mouse i.p. on alternate days between week 4 and week 5 pi. Splenocytes and brain cells were analyzed for BrdU incorporation at week 5 and week 7 pi. Staining was performed using FITC BrdU Flow Kit (BD Biosciences) as per manufacturer's protocol.

Cytotoxic T lymphocyte (CTL) Assay

CTL assay was performed as previously described (81). Briefly $CD8^+$ T cells stimulated with TLA were incubated with infected ^{51}Cr labeled macrophages at various effector-target ratios in 96 well U-bottomed plates. After 4h incubation the supernatants were measured for radioactive release and percentage of cytotoxic response calculated.

Real-time RT-PCR

Total RNA was harvested from flash frozen infected mouse brains by homogenization in Trizol reagent (Invitrogen), followed by extraction using phenol chloroform, digestion with DNAse-1 (Roche) and then further purification with RNeasy spin columns (Qiagen). cDNAs were generated using M-MLV reverse transcriptase (Invitrogen) and semi-quantitative real-time PCR was performed using iQ SYBR Green Supermix (BioRad) with primers for ENO-1, ENO-2, SAG-1, BAG-1 and Toxoplasma specific actin (TgACT-1; IDT) on an iCycler iQ thermal cycler (BioRad). Samples used for RT-PCR were first normalized for constitutively expressed Tg-ACT1. PCR reactions were carried out using 1 cycle at 95° C for 10 minutes followed by a two step cycle, one at 95° C for 15 seconds then another at 60° C for 1 minute repeated 40X. PCR products were then analyzed for

quality by melt curve analysis. Quantitation of transcripts for ENO-1,ENO-2,SAG-1 and BAG-1 relative to day 10 post infection (day 10 post infection relative transcript level = 1.0) were calculated according to the Pfaffl method of quantitation (158). Primer sequences are as follows:

5'TgACT-1 TCCCGTCTATCGTCGGAAAG

3'TgACT-1 CCATTCCGACCATGATACCC

5'SAG-1 ATCGCCTGAGAAGCATCACTG

3'SAG-1 CGAAAATGGAAACGTGACTGG

5'BAG-1 GACGTGGAGTTCGACAGCAAA

3'BAG-1 ATGGCTCCGTTGTCGACTTCT

5'ENO-1 GGTATTGATATGCTTATGGTGGAG

3'ENO-1 GCGATGTATTTGTATAGTGGTAGG

5'ENO-2 CCGTGACAAGGACCAAAC

3'ENO-2 ACTCGTTCTTAGTTCCATCG

Quantification of Parasite Burden by Real-time PCR

Animals were euthanized via CO_2 overdose and blood was harvested via intra-cardiac puncture and flash frozen in liquid nitrogen. DNA was extracted from entire organs using a Qiagen DNeasy Tissue Kit (Qiagen). Amplification of parasite DNA from 400 ng of purified tissue DNA was performed using primers specific for the *T. gondii* B1 gene (159) at 10 pMol each per reaction (IDT) and amplified by real-time fluorogenic PCR using iQ SYBR Green Supermix (Biorad) on an iCycler iQ thermal cycler (BioRad). Parasite DNA equivalents were used as a standard curve to which infected mouse tissue DNA samples

were compared. Parasite genome equivalents were calculated via extrapolation from the standard curve.

In vitro bradyzoite induction and imaging

As previously published parasites (Type II) were cultured in human fibrolasts for 3 days at pH8 and 2 days at pH7 for bradyzoite and tachzyzoite differentiation respectively (160). IFA was done as previously described (160). Briefly, samples were fixed for 30 min with 2% buffered formalin, washed 3 times with PBS, permeablized with 0.2% Triton X100 for 20 min, washed 3 times with PBS, blocked with1% BSA at 4°C, washed 3 times with PBS and then incubated with the primary antiserum (mAB 74.18: BAG-1 at 1:100 dilution and mAB DG52 SAG-1 at 1:500 dilution) for 1 hr at 37° C. Following incubation with the primary antiserum the slides were then washed 3 times with PBS and then incubated with secondary antiserum (fluorescein or rhodamine anti mouse IgG at 1:100) for 1 hr at 37°C, washed 3 times with PBS and then examined under a Nikon Diaphot fluorescent microscope utilizing the correct filters for the secondary antibody label (i.e. either rhodamine and fluorescein).

Drug treatment

Toxoplasma infected mice were administered sulfamethoxazole and trimethoprim oral suspension (Hi-Tech Pharmacal) via drinking water. A previous study has shown that this regimen has similar clinical efficacy to sulfadiazine-pyrimethamine treatment and produces fewer adverse effects (161). 5ml of the suspension containing 200 mg of sulfamethoxazole

and 40 mg of trimethoprim was added to 245 ml of drinking water. Medicated water was replaced every third day.

Statistical analysis

Unless otherwise mentioned statistical analysis was performed using unpaired two-tailed Student's t test with $P<0.05$ taken as statistically significant. For comparing wild-type and $CD40^{-/-}$ T cell data from chimeras within the same treatment group, paired two-tailed t test was used while for comparing WT or KO data between different treatment groups (i.e. isotype vs. αPD-L1 treated chimera) unpaired two-tailed t test was utilized. Comparison of survival curves was performed using Log-rank (Mantel-Cox) Test. Error bars in graphs represent standard deviation of values of individual mice in the group from one experiment. All statistical analysis and bar graphs were prepared using GraphPad Prism Software. Pie charts were prepared using Microsoft Excel.

Chapter 4: Control of Toxoplasma reactivation by rescue of dysfunctional $CD8^+$ T cell response via PD-1-PD-L1 blockade

4.1 Introduction

In the intermediate host, Toxoplasma undergoes stage conversion between the rapidly replicating tachyzoite that is thought to be responsible for acute toxoplasmosis and the slowly replicating, relatively quiescent, primarily encysted bradyzoite stage that can persist possibly for life (3). Effective $CD8^+$ T cell response, critical for control of both acute and chronic Toxoplasmosis in susceptible mice strains, paradoxically do not ensure their long-term survival (162). Poor long-term survival of Toxoplasma infected B10 mice is associated with reduced number of intra-cerebral T cells and TE development (31). Additionally, depletion of $CD8^+$ T cells rather than $CD4^+$ T cells has a greater effect on mortality of chronically infected animals (120). Taken together, these studies suggest that although critical for preventing parasite reactivation, $CD8^+$ T cell responses during late-chronic infection are incapable of preventing mortality in infected mice. However, the mechanism underlying this apparently ineffective $CD8^+$ T cell response during late-chronic infection has not been studied and it remains to be determined if this defect is responsible for loss of parasite control. We therefore tested whether $CD8^+$ T cells during late-chronic toxoplasmosis become dysfunctional, as reported in some viral infections (163).

4.2 RESULTS

4.2.1 Toxoplasma infected mice show increased tachyzoite-specific gene expression with concomitant decrease in $CD8^+$ T cell response during chronic toxoplasmosis

Humans are most commonly infected orally with Type II strains of Toxoplasma (164). To establish the loss of immune control during chronic infection we first wanted to indentify the kinetics of long-term survival in B6 mice infected orally with type II strain (ME49) cysts. Most of these animals succumb to infection after 7 weeks of challenge (Fig. 3*A*). Host innate and adaptive immunity are thought to cause differentiation of tachyzoites into chronic slow growing bradyzoites (165). Since encysted parasites can persist for life and are not believed to be associated with disease pathology, several studies have postulated that rather than the incidence of parasites which remain primarily encysted in chronically infected brains, the degree of bradyzoite-tachyzoite inter-conversion is a better hallmark of disease reactivation (3, 7, 166). Therefore we assayed parasite reactivation by changes in parasite stage specific gene expression. We measured the transcription of the tachyzoite stage-specific Enolase-II (ENO-2) and the bradyzoite stage-specific Enolase-I (ENO-1), two genes that have been previously shown to change in very high levels (167-169). Concomitant with poor survival of *T. gondii* infected mice during the late-chronic phase, increased ENO-2 expression and decreased ENO-1 expression was noted at week 7 pi, suggesting parasite reactivation (Fig. 3*B*). Interestingly, we observed that expression of both ENO-2 and ENO-1 occurs at week 10, indicating that despite limited tachyzoite to bradyzoite differentiation, the parasites are still undergoing reactivation i.e. a bradyzoite to tachyzoite conversion. (Fig 3*B*). To verify that ENO-2 and ENO-1 corresponded to the tachyzoite and bradyzoite stage of *T. gondii*, the gene expression patterns of the parasite

were examined again *in vivo* using established models of acute and chronic infection. Mice were infected with Type I RH strain (lethal acute infection) or Type II ME49 strain (chronic) (161, 170). Some of the ME49 infected animals were treated with drugs known to control reactivation of chronic infection (161, 170). As expected, RH infected mice exhibited high tachyzoite-specific gene expression (SAG-1, ENO-2) and minimal bradyzoite-specific gene expression (BAG-1, ENO-1) (Fig. 4*A*). In contrast, drug treated mice showed reduced SAG-1 and ENO-2 transcript levels and increased BAG-1 and ENO-1 expression vis-à-vis untreated ME49 infected mice. Next we further confirmed the stage-specificity of the above markers using Type II parasites maintained *in vitro* under tachyzoite or bradyzoite inducing conditions (160). Immunofluorescent microscopic analysis showed that stage-specific protein expression corresponded with our stage-specific transcript profile (Fig. 4*B,C*)

Tissue cysts in brain occur almost exclusively in neurons and not in leukocytes during the chronic phase (171). Since a recent study has shown that leukocytes can be used for parasite dissemination during the acute phase (172), we examined these cells for parasitization as a further readout of reactivation. To characterize, whether parasite reactivation increased the frequency of Toxoplasma infected leukocytes, mononuclear leukocyte-gated blood and brain cells were examined for parasites by flow cytometry. While minimal infection of blood and brain leukocytes was noted during the early-chronic phase, high infection rates were observed during the late-chronic period particularly in moribund mice (Fig. 5*A*). Similar to observations noted by Courret *et al* (172), the level of parasite infection was much higher in circulating leukocytes vis-à-vis brain leukocytes assayed at the same time. It has been speculated that this may be due to multiple

populations of non-leukocytic brain lineages that are differentially permissive to *T. gondii* in the brain (172). Next we assessed if reactivated parasites preferentially infected specific leukocyte subsets in a site dependent manner. Irrespective of tissue, the majority of parasitized leukocytes corresponded to a myeloid phenotype ($Lineage^{-}F4/80^{+}GR1^{+}$, Fig. 5*B*-*E*). Since IFNγ depletion during chronic Toxoplasmosis has been reported to induce parasite reactivation and host mortality , we determined if chronically (early phase) infected anti-IFNγ treated mice showed increased leukocyte parasitization (120). In agreement with the parasite kinetic data presented above, anti-IFNγ treatment resulted in a marked increase in percentage of infected leukocytes (Fig. 6*A*,*B*). Taken together, our data suggests that during late-chronic phase, parasite stage conversion results in reactivation of disease.

As Toxoplasma infection induces a robust $CD8^{+}$ T cell immunity that is known to be critical for long-term protection, it is unclear why this potent immune response failed to prevent recrudescence of infection (120). By measuring the kinetics of $CD8^{+}$ T cell immunity we demonstrate that $CD8^{+}$ T cell response peaked at week 3-5 pi followed by gradual contraction (Fig. 7*A*). Concomitant with this, reduced frequency of splenic and brain $IFN\gamma^{+}$Granzyme B^{+} and $IFN\gamma^{-}$Granzyme B^{+} $CD8^{+}$ T cells was noted (Fig. 7*B*).

One of the quintessential features of robust long-term $CD8^{+}$ T cell immunity is the generation of a rapid response upon secondary challenge (50). To verify the potency of secondary response, Toxoplasma infected animals were rechallenged during the early-chronic and late-chronic phase and assessed for cycling $CD8^{+}$ T cells (Fig. 7*C*). Mice rechallenged during the early-chronic phase demonstrated a significant increase in absolute

number of proliferating splenic $CD8^+$ T cells over unchallenged mice (Fig. 7*C*). In contrast, no significant change in cycling $CD8^+$ T cells was noted in rechallenged animals during the late-chronic phase. The data clearly demonstrates that a potent recall response, a hallmark of robust $CD8^+$ T cell immunity is compromised during later stages of chronic toxoplasmosis.

Figure 3. Parasite reactivation during late-chronic *T. gondii* infection. (A) C57BL/6 mice were infected with 10 ME49 cysts orally and survival was monitored on a daily basis. (B) Relative expression of ENO-1 and ENO-2 at weeks 3,5,7 and 10 pi in brain was computed for each sample. Transcript levels at day 10 pi was taken as 1. The data represent two experiments with at least 4 mice per group. Error bars represent standard deviation throughout.

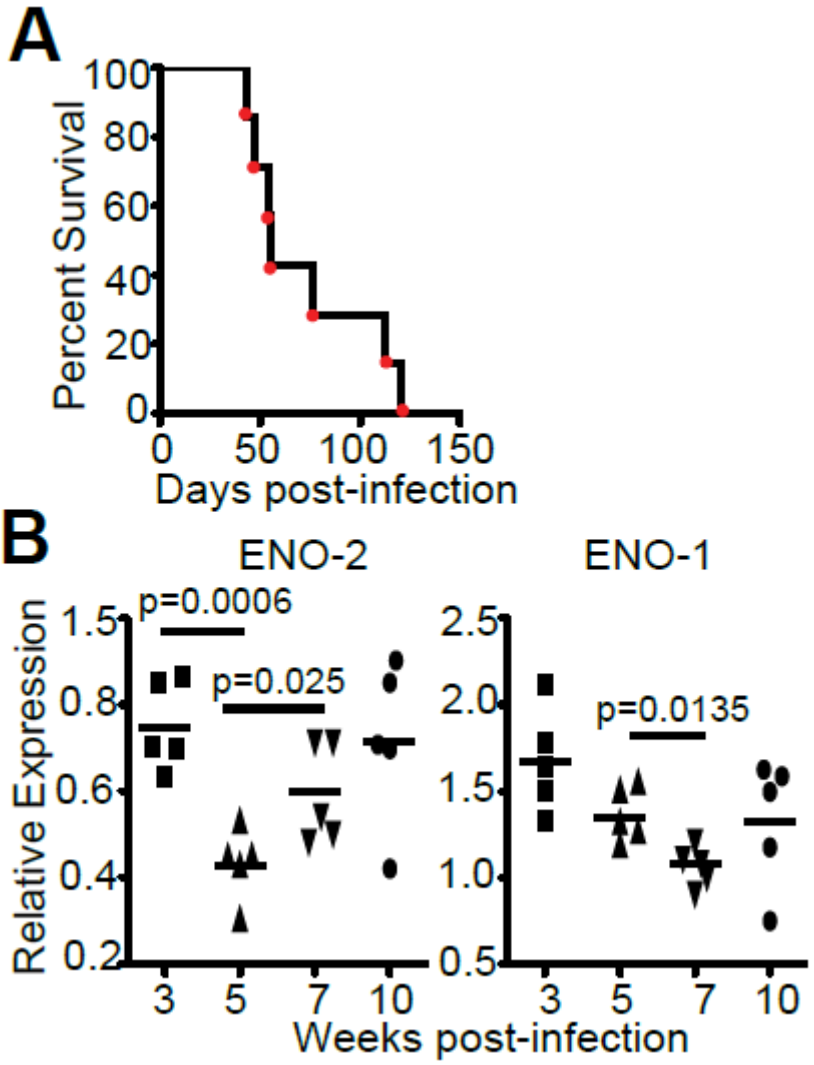

A
Percent Survival
100
80
60
40
20
0
0
50
100
150
Days post-infection
B
ENO-2
ENO-1
p=0.0006
p=0.025
p=0.0135
Relative Expression
1.5
0.8
0.6
0.4
0.2
2.5
2.0
1.5
1.0
0.5
3
5
7
10
3
5
7
10
Weeks post-infection

Figure 4. Validation of *in vivo* parasite stage specific gene transcription. (A) Relative expression of SAG-1, BAG-1, ENO-2 and ENO-1 was computed in ME49 (type II strain) and RH (type I strain) infected mice brains. Transcript levels at day 10 pi (ME49 infected) was taken as 1. The data represent two experiments with 3-5 mice per group. (B) Left panel depicts SAG-1 staining of ME49 parasites cultured *in vitro* under tachyzoite skewing conditions (pH 7). The right panel depicts BAG-1 staining of ME49 parasite under bradyzoite skewing conditions (pH 8). (C) Relative expression of SAG-1, BAG-1, ENO-2 and ENO-1 was calculated for *in vitro* culture parasites. Transcript levels for parasites cultured *in vitro* under tachyzoite skewing conditions was taken as 1. The data represent at least 2 experiments with 3 replicates per condition

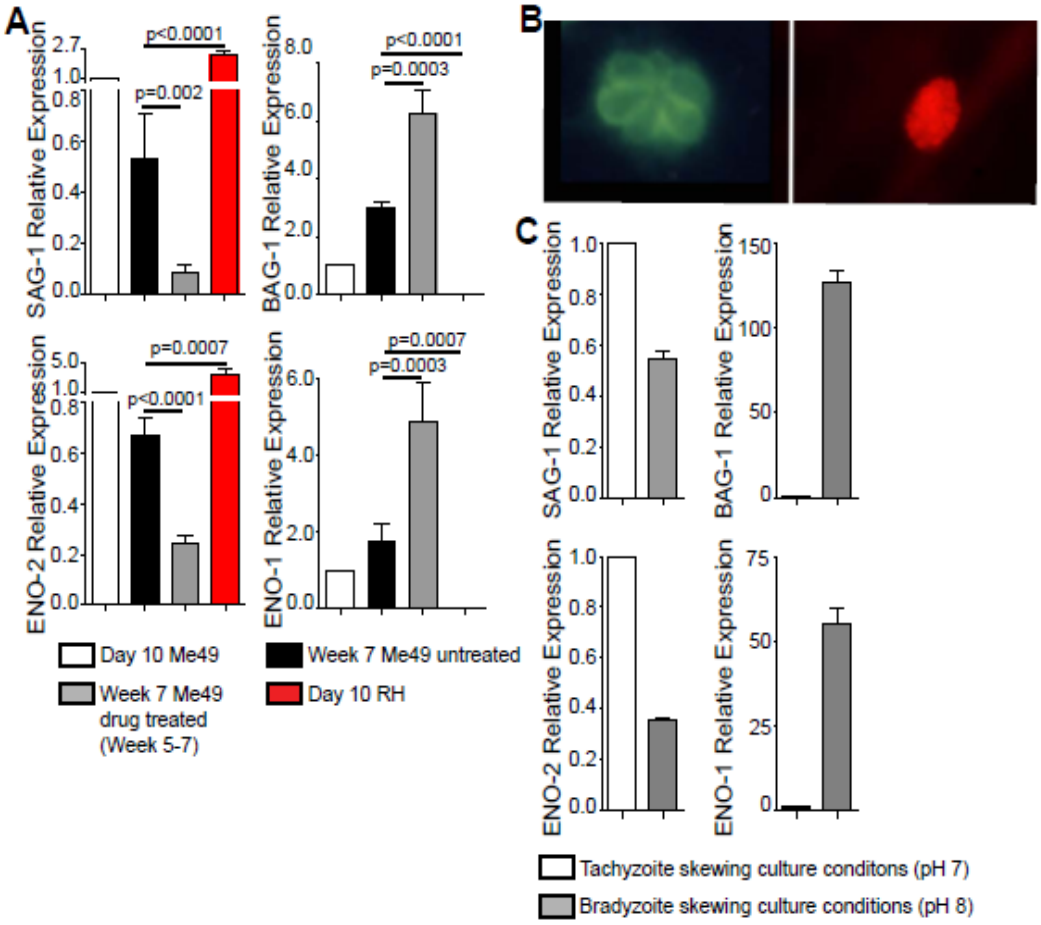

A
SAG-1 Relative Expression
p<0.0001
p=0.002
BAG-1 Relative Expression
p<0.0001
p=0.0003
ENO-2 Relative Expression
p=0.0007
p<0.0001
ENO-1 Relative Expression
p=0.0007
p=0.0003
Day 10 Me49
Week 7 Me49 drug treated (Week 5-7)
Week 7 Me49 untreated
Day 10 RH
B
C
SAG-1 Relative Expression
BAG-1 Relative Expression
ENO-2 Relative Expression
ENO-1 Relative Expression
Tachyzoite skewing culture conditons (pH 7)
Bradyzoite skewing culture conditions (pH 8)

Figure 5. Majority of *T. gondii* infected leukocytes exhibit a myeloid lineage phenotype. (A) *T. gondii* infected cells in brain and blood leukocyte gated samples were assayed by flow cytometry at various time points pi. (B) represents the frequency of *T. gondii* infected cells in different tissues (spleen, blood, liver and brain) at week 7 pi. (C) depicts the mononuclear leukocyte composition in the above samples. (D) *T. gondii* infected mononuclear cells were analyzed for various leukocyte subsets. (E) depicts expression of GR1 and F4/80 on *T. gondii* infected $Lin^{-}CD11b^{hi}$ cells. Lin denotes any of the following lineage markers: CD3 or CD19 or NK1.1. The data represent two experiments with 4 mice per group.

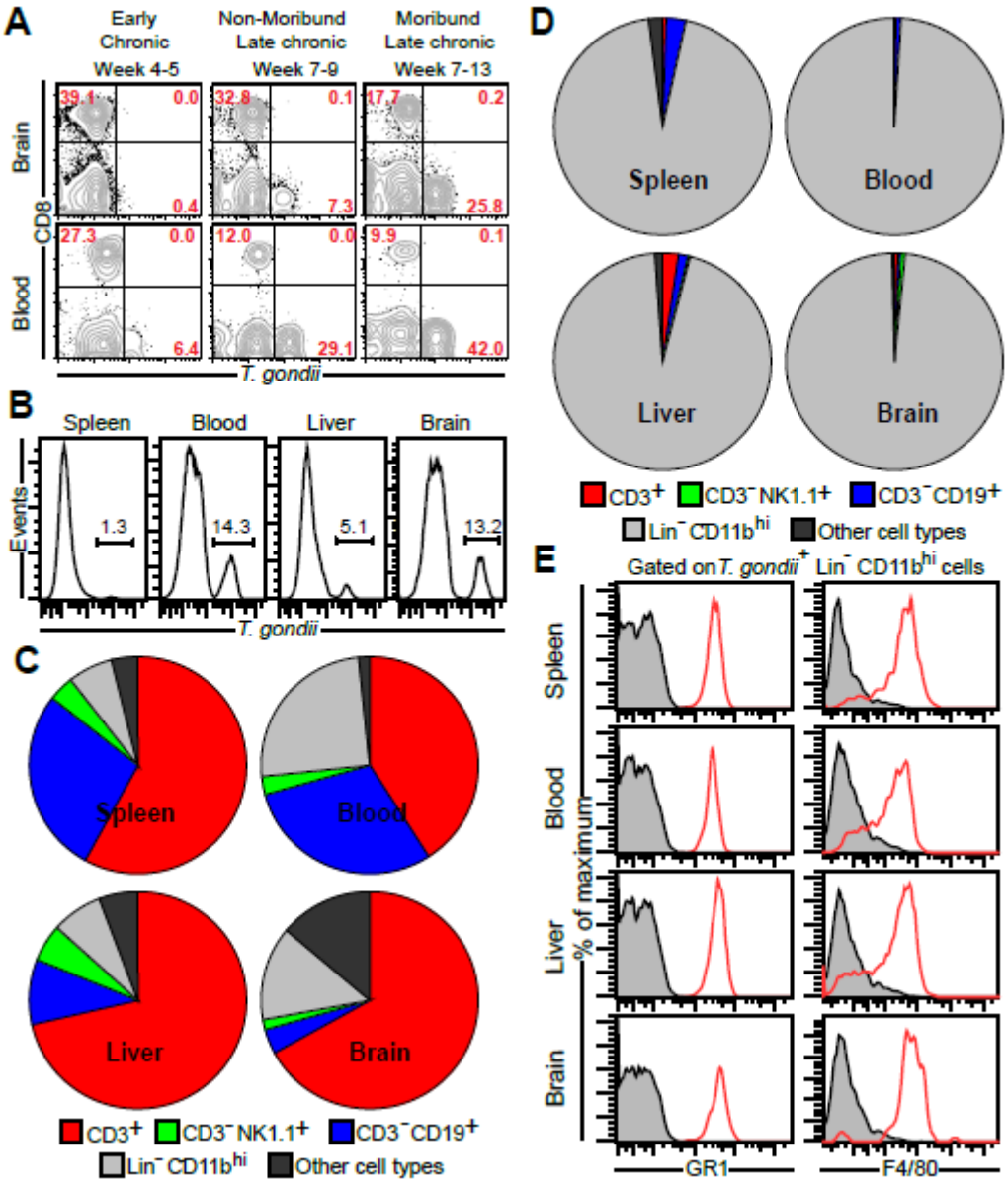

A
Early Chronic Week 4-5
Non-Moribund Late chronic Week 7-9
Moribund Late chronic Week 7-13
Brain
Blood
CD8
39.1
0.0
0.4
32.8
0.1
7.3
17.7
0.2
25.8
27.3
0.0
6.4
12.0
0.0
29.1
9.9
0.1
42.0
T. gondii
B
Spleen
Blood
Liver
Brain
Events
1.3
14.3
5.1
13.2
T. gondii
C
Spleen
Blood
Liver
Brain
CD3+ CD3-NK1.1+ CD3-CD19+
Lin-CD11bhi Other cell types
D
Spleen
Blood
Liver
Brain
CD3+ CD3-NK1.1+ CD3-CD19+
Lin-CD11bhi Other cell types
E
Gated on T. gondii+ Lin-CD11bhi cells
Spleen
Blood
Liver
Brain
% of maximum
GR1
F4/80

Figure 6. Increased parasitization of leukocytes in mice treated with anti-IFNγ during early-chronic infection. (A) Anti-IFNγ antibody was injected daily, starting at day 31. Animals were sacrificed 4 days after initiation of treatment. (B) *T. gondii* infected cells in brain leukocyte gated samples were assayed by flow cytometry**.** Data is representative of two experiments with 5 mice per group.

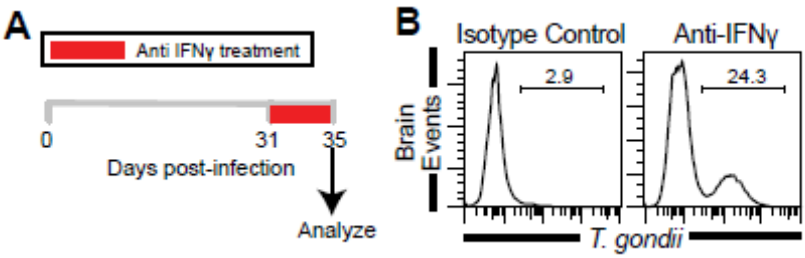
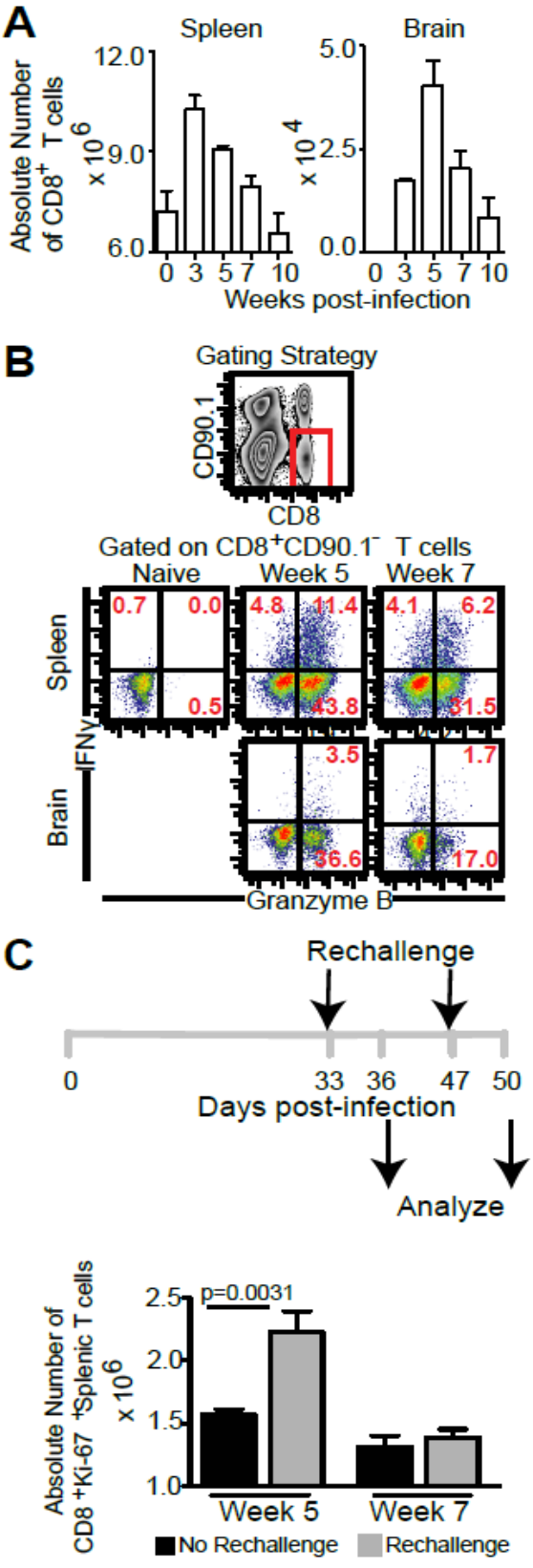

A
Anti IFNγ treatment
0
31
35
Days post-infection
Analyze
B
Isotype Control
Anti-IFNγ
2.9
24.3
Brain
Events
T. gondii

Figure 7. Poor $CD8^+$ T cell response during late-chronic *T. gondii* infection. (A) shows the absolute number of $CD8^+$ T cells at weeks 3,5,7 and 10 pi. (B) IFNγ and Granzyme B production by $CD8^+$ T cells was evaluated in splenocytes or brain cells from infected mice in presence Toxoplasma lysate antigen (TLA). (C) At week 5 and 7 post primary infection, mice were rechallenged orally with 10 cysts and animals were sacrificed 4 days later. Absolute number of cycling $CD8^+$ T cells was evaluated in spleen during early-chronic and late-chronic infection with or without rechallenge. The data represent two experiments with at least 4 mice per group. Error bars represent standard deviation throughout.


A
Spleen
Brain
Absolute Number of CD8+ T cells
x 10^6
x 10^4
12.0
9.0
6.0
5.0
2.5
0.0
0 3 5 7 10
0 3 5 7 10
Weeks post-infection
B
Gating Strategy
CD90.1
CD8
Gated on CD8+CD90.1- T cells
Naive
Week 5
Week 7
Spleen
Brain
IFNγ
0.7
0.0
0.5
4.8
11.4
43.8
4.1
6.2
31.5
3.5
36.6
1.7
17.0
Granzyme B
C
Rechallenge
0
33
36
47
50
Days post-infection
Analyze
Absolute Number of CD8+Ki-67+Splenic T cells
x 10^6
p=0.0031
2.5
2.0
1.5
1.0
Week 5
Week 7
No Rechallenge
Rechallenge

4.2.2 $CD8^+$ T cells upregulate PD-1 during chronic toxoplasmosis

The role of PD-1 in mediating dysfunction in chronic parasitic models has not been extensively addressed (173, 174). Although PD-1 expression during toxoplasmosis has been recently reported (59) its role in mediating $CD8^+$ T cell dysfunction or pathogenesis as shown in chronic viral models like LCMV, HBV, HCV and HIV (153), is currently unknown. Analysis of $CD8^+$ T cells revealed a progressive increase of PD-1 expression and frequency during the chronic stage of parasite infection (Fig. 8*A*). Of the two PD-1 ligands, PDL-1 and PDL-2, the MFI of the former but not the latter increased during the course of chronic infection (Fig. 8*B*,*C*).

Since increased PD-1 MFI correlated with decreased frequency of splenic $IFN\gamma^+CD8^+$ T cells (Fig. 7*B*, 8*A*)., we hypothesized that $PD\text{-}1^{hi}CD8^+$ T cells would have reduced capacity to release IFNγ. To address this, we measured the proportion of IFNγ producing cells within $PD\text{-}1^{hi}CD8^+$ and $PD\text{-}1^{int}CD8^+$ T cell population. No significant difference in cytokine positive cells between the two populations was noted (Fig. 8*D*,*E*). Although this suggests that PD-1 expression does not directly regulate IFNγ production in $CD8^+$ T cells, the role of other factors such as extent and duration of PD-1 cross-linking as well as frequency of PD-1-PDL-1 engagement in regulating $CD8^+$ T cell mediated IFNγ production cannot be ruled out. Irrespective of the time point, very few $PD\text{-}1^{lo}$ cells produced IFNγ, indicating that an overwhelming majority of $CD8^+$ T cells in this subset are naïve. Although we did not observe a significant difference in frequency of $IFN\gamma^+CD8^+$ T cells between the $PD\text{-}1^{hi}$ and $PD\text{-}1^{int}$ $CD8^+$ T cell subsets, we hypothesized that the reduced frequency of splenic $IFN\gamma^+CD8^+$ T cells during late-chronic infection could be due to

apoptosis of PD-1^{hi} cells. As recent studies have suggested that apoptosis can be coupled to cell cycle progression (175, 176), we incubated splenocytes from week 7 infected mice for 5h at 37°C and stained for CD8, PD-1, Ki-67 and active caspase-3, the active form of the apoptosis related cysteine peptidase. While cycling PD-1^{hi}CD8^{+} T cells exhibited a higher expression of active caspase-3 than non-cycling PD-1^{hi}CD8^{+} T cells, PD-1$^{int/lo}$ cells had a much lower MFI than cycling or non-cycling PD-1^{hi} cells (Fig. 8*F*,*G*), suggesting that cycling PD-1^{hi}CD8^{+} T cells exhibit poor survival.

Since PD-1 has been reported to be transiently expressed in acute viral infections (153), increased PD-1 MFI during later stages of chronic toxoplasmosis could be due to generation of new PD-1 expressing effectors in response to parasite recrudescence. To address this we used a BrdU pulse-chase approach and then measured PD-1 expression on BrdU^{+} and Brdu^{-} CD8^{+} T cells during early-chronic and late-chronic phase (Fig. 9*A-D*). Dramatic increase in PD-1 MFI and frequency of PD-1^{hi} cells was noted only in BrdU labeled fraction. This suggests that PD-1 up-regulation is a graded process and majority of PD-1^{hi}CD8^{+} T cells at week 7 pi are not new effector cells generated in response to parasite recrudescence.

Considering that majority of PD-1^{hi}CD8^{+} T cells during the late-chronic phase are not newly generated effectors, we wanted to address if control of infection during the early or later phases of infection would result in reduced CD8 exhaustion. To evaluate this, we treated mice with sulfamethoxazole and trimethoprim at various time-points pi as show in Figure 10*A* (161). Since reduced antigen burden in drug treated mice can result in increased naïve T cell numbers which can potentially confound interpretation of data, we analyzed

brain, a tissue known to recruit $CD8^+$ T cells in a strictly antigen-specific manner (177). Only early initiation of drug treatment resulted in decreased PD-1 MFI on $CD8^+$ T cells during the late-chronic phase (Fig. 10*B*). Moreover, $CD8^+$ T cells from these mice exhibited higher proportion of $IFN\gamma^+GranzymeB^-$ and $IFN\gamma^+GranzymeB^+$ $CD8^+$ T cells (Fig. 10*C*). However, $IFN\gamma^-GranzymeB^+$ $CD8^+$ T cells underwent a moderate decrease in these animals (Fig. 10*C*). This may be attributed to attenuated inflammation or antigen load in drug treated mice which can result in sub-optimal development or maintenance of granzyme B expressing short-lived effector subset (98, 178). Combined, this suggests that control of infection during the early phases of infection may be critical for minimizing induction of CD8 dysfunction during the chronic phase.

Figure 8. PD-1 is expressed at high levels on $CD8^+$ T cells in both lymphoid and non-lymphoid tissue during late-chronic Toxoplasmosis. (A) PD-1 expression on $CD8^+$ T cells from tissues (spleen, brain and liver) was measured at week 3, 5, 7 and 10 pi. MFI denotes mean fluorescence intensity. (B,C) PDL-1 and PDL-2 expression was evaluated on mononuclear cells in these tissues. (D,E) Splenocytes from chronically infected mice were restimulated with TLA and frequency of IFNγ expressing $CD8^+$ T cells in PD-1^{lo}, PD-1^{int} and PD-1^{hi} subsets was evaluated. (F,G) Splenocytes from chronically infected animals were incubated at 37°C for 5h and active caspase 3, Ki-67 and PD-1 was detected on $CD8^+$ T cells by flow cytometry. Similar results were observed in the presence of TLA (data not shown). The data represent 3 experiments with at least 4 mice per group.

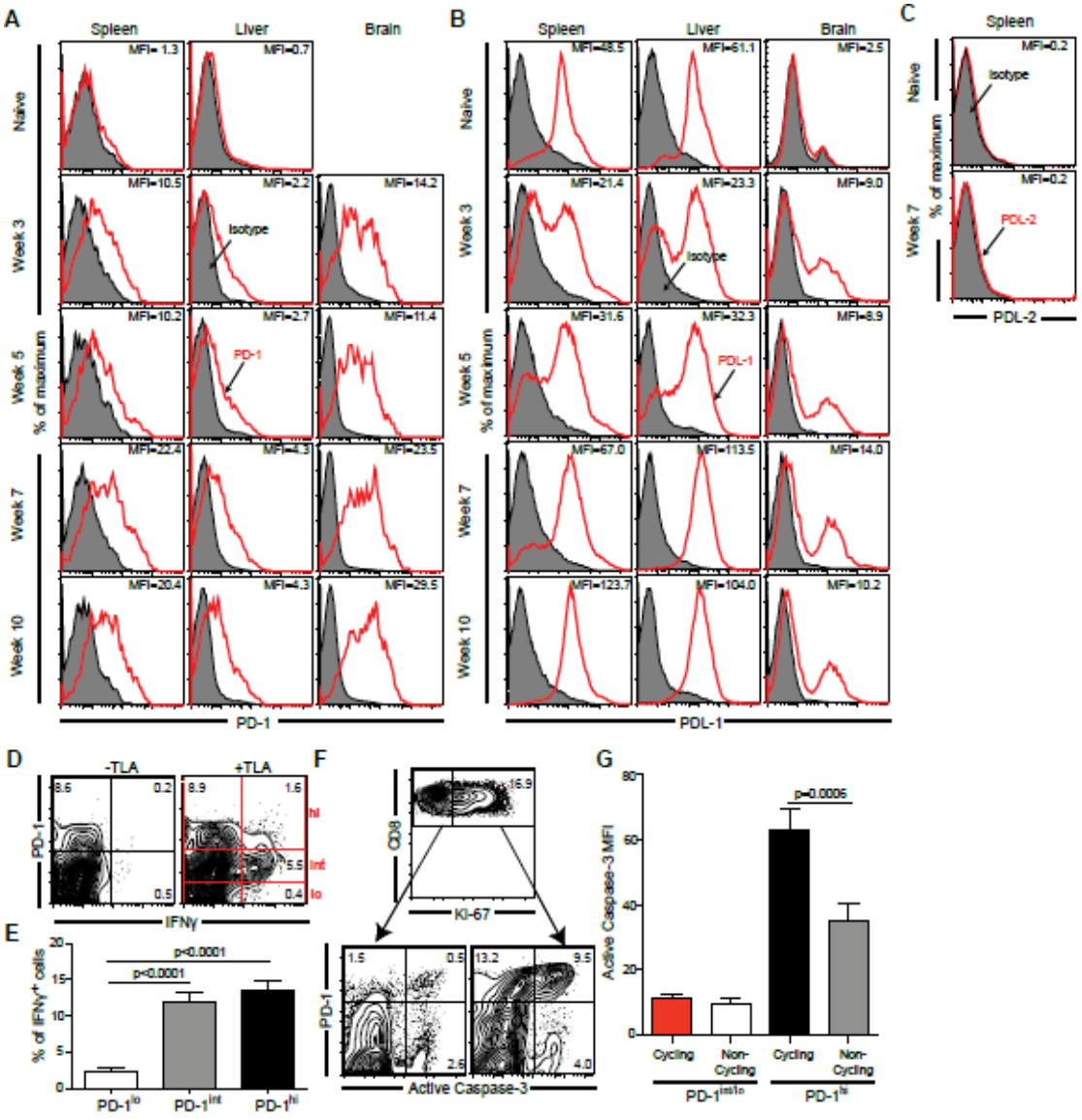

A
Spleen
Liver
Brain
Naive
Week 3
Week 5
Week 7
Week 10
% of maximum
MFI= 1.3
MFI=0.7
MFI=10.5
MFI=2.2
MFI=14.2
Isotype
MFI=10.2
MFI=2.7
MFI=11.4
PD-1
MFI=22.4
MFI=4.3
MFI=23.5
MFI=20.4
MFI=4.3
MFI=29.5
PD-1
B
Spleen
Liver
Brain
MFI=48.5
MFI=61.1
MFI=2.5
MFI=21.4
MFI=23.3
MFI=9.0
Isotype
MFI=31.6
MFI=32.3
MFI=8.9
PDL-1
MFI=67.0
MFI=113.5
MFI=14.0
MFI=123.7
MFI=104.0
MFI=10.2
PDL-1
C
Spleen
Naive
Week 7
% of maximum
MFI=0.2
Isotype
MFI=0.2
PDL-2
PDL-2
D
-TLA
+TLA
PD-1
IFNγ
8.6
0.2
0.5
8.9
1.6
5.5
0.4
hi
Int
lo
E
% of IFNγ+ cells
p<0.0001
p<0.0001
PD-1lo
PD-1Int
PD-1hi
F
CD8
KI-67
16.9
PD-1
Active Caspase-3
1.5
0.5
2.6
13.2
9.5
4.0
G
Active Caspase-3 MFI
p=0.0005
Cycling
Non-Cycling
Cycling
Non-Cycling
PD-1Int/lo
PD-1hi

Figure 9. High PD-1 upregulation does not occur on newly generated $CD8^+$ T cells. (A) Using a pulse-chase approach, BrdU was injected into infected mice starting at week 5 pi. Treatment was performed on alternate days for 1 week and animals were sacrificed at week 5 and 7 pi. (B) Splenic $CD8^+$ T cells were evaluated for BrdU incorporation by flow cytometry. (C) Frequency of PD-1^{hi} cells was assessed on splenic $BrdU^+$ and $BrdU^-$ $CD8^+$ T cells. (D) PD-1 expression was also assessed in the above subsets. Fold difference was computed as (Difference in mean PD-1 MFI between the two time points)/(mean PD-1 MFI at week 5). Data represent two experiments with at least 4 mice per group.

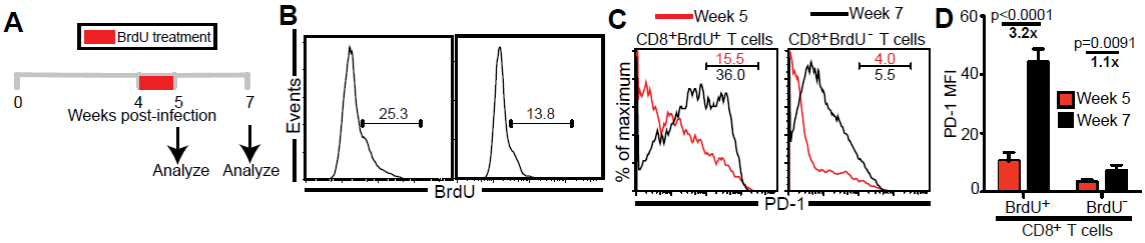
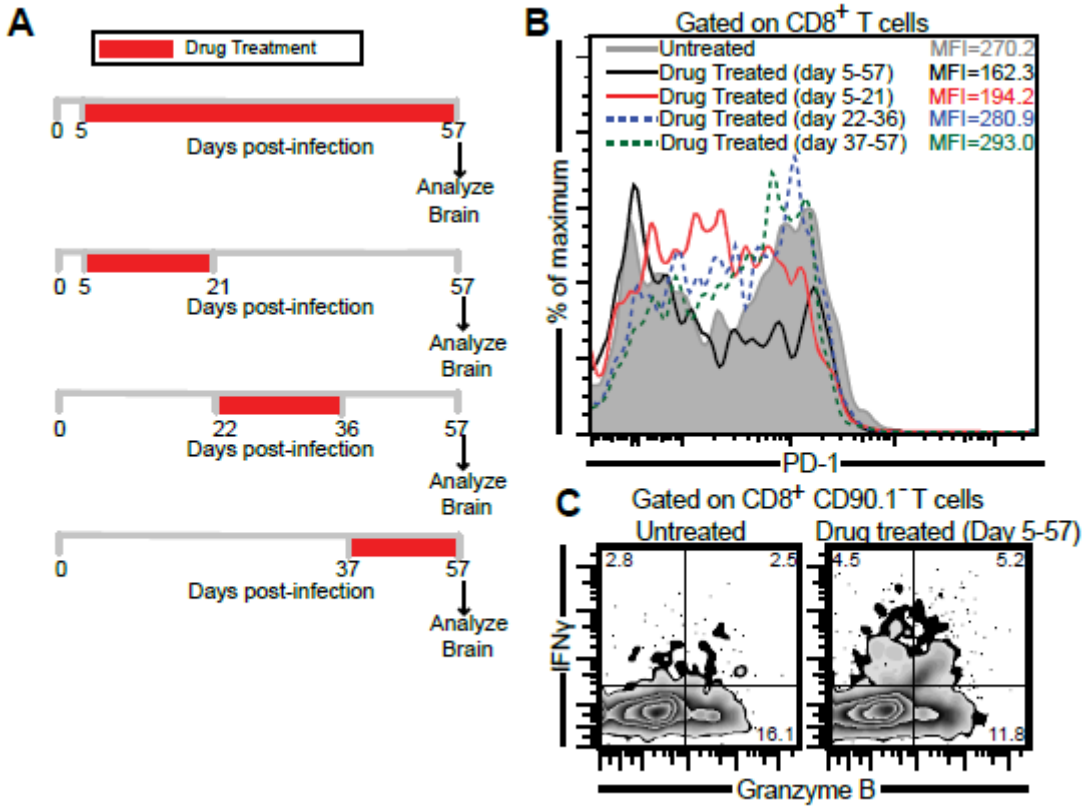

A
BrdU treatment
0
4
5
7
Weeks post-infection
Analyze
Analyze
B
Events
25.3
13.8
BrdU
C
Week 5
Week 7
CD8+BrdU+ T cells
CD8+BrdU- T cells
15.5
36.0
4.0
5.5
% of maximum
PD-1
D
p<0.0001
3.2x
p=0.0091
1.1x
Week 5
Week 7
PD-1 MFI
60
40
20
0
BrdU+
BrdU-
CD8+ T cells

Figure 10. Early initiation of drug treatment reduces $CD8^+$ T cell exhaustion. (A) Toxoplasma infected mice were treated with sulfamethoxazole and trimethoprim as shown in the figure. Brains from untreated and various drug treated animals were analyzed at day 57 pi. (B) PD-1 expression was assessed on $CD8^+$ T cells in brain by flow cytometry. (C) IFNγ and Granzyme B production by $CD8^+$ T cells was evaluated in brain cells from infected mice in presence of TLA. Data represent two experiments with at least 4 mice per group.


A
Drug Treatment
0 5
57
Days post-infection
Analyze Brain
0 5
21
57
Days post-infection
Analyze Brain
0
22
36
57
Days post-infection
Analyze Brain
0
37
57
Days post-infection
Analyze Brain
B
Gated on CD8+ T cells
Untreated
MFI=270.2
Drug Treated (day 5-57)
MFI=162.3
Drug Treated (day 5-21)
MFI=194.2
Drug Treated (day 22-36)
MFI=280.9
Drug Treated (day 37-57)
MFI=293.0
% of maximum
PD-1
C
Gated on CD8+ CD90.1- T cells
Untreated
Drug treated (Day 5-57)
2.8
2.5
16.1
4.5
5.2
11.8
IFNγ
Granzyme B

4.2.3 Blockade of PD-1-PDL-1 pathway reinvigorates $CD8^+$ T cell response

Next, we addressed if the dysfunctional $CD8^+$ T cell response could be revived by blocking PD-1-PDL-1 pathway. To address this, animals were administered a non-depleting anti-PDL-1 antibody starting at week 5 pi for a period of 2 weeks and thereafter $CD8^+$ T cell response in spleen, liver and brain quantified (156). Although a significant increase in the absolute number of $CD8^+$ T cells was noted in all the tissues examined, the most dramatic increase occurred in brain (Fig. 11*A*). To determine if this increase was due to enhanced proliferation, we measured Ki-67 expression in the $CD8^+$ T cells. Unlike spleen and liver, we were unable to detect increased frequency of cycling $CD8^+$ T cells in anti-PDL-1 treated groups in brain (Fig. 11*B*). Taken together, this suggests that one of the mechanisms for increased brain $CD8^+$ T cells in anti-PDL-1 treated mice is not *in situ* proliferation but possibly increased recruitment of these cells to the tissue.

As IFNγ production (15) and cytotoxicity (179) are known to be critical for control of toxoplasmosis, we examined if blockade with anti-PDL-1 augmented these functions within the $CD8^+$ T cell population. Anti-PDL-1 treatment increased the frequency of $IFN\gamma^+$Granzyme B^+ and $IFN\gamma^-$Granzyme B^+ $CD8^+$ T cells in spleen and brain as well as *ex vivo* cytotoxicity of splenic $CD8^+$ T cells (Fig. 11*C*,*D*). We then measured, if anti-PDL-1 treatment decreased the frequency of PD-1^{hi} cells. Surprisingly, PD-1-PDL-1 blockade caused a further increase in the frequency of PD-1 expressing $CD8^+$ T cells (Fig. 11*E*). Since we demonstrated that high PD-1 expression was associated with apoptosis, we determined if rescue of $CD8^+$ T cell response by PD-1-PDL-1 blockade was due to reduced apoptosis. To address this under steady state conditions without confounding effects of

proliferation and graded PD-1 increase, we evaluated apoptotic $CD8^+$ T cells in splenocytes from chronically infected mice incubated for 5h with anti-PDL-1 or control antibody (Fig. 12*A*-*C*). Anti-PDL-1 treatment mediated reduction in apoptosis occurred primarily in PD-1^{int} subset and only moderate decrease in apoptosis was noted in PD-1^{hi} subset (Fig. 12*A*-*C*). To further address if anti-PDL-1 treatment was capable of rescuing pre-existing PD-1 expressing $CD8^+$ T cells *in vivo*, we adoptively transferred sorted PD-1^+CD8^+ T cells from chronically infected CD45.1 mice (week 5-6) to similarly infected CD45.2 mice and then treated recipients with anti-PDL-1 antibody (Fig. 13*A*). Anti-PDL-1 treatment increased the absolute number of donor cells in both spleen and brain (Fig. 13*B*-*D*). However, while the recipient $CD8^+$ T cell population (comprising of both PD-1^+ and PD-1^-) expanded 6 fold at the effector site (vis-à-vis isotype treated) , the donor population underwent an over 20 fold expansion (Fig. 13*B*-*D*). This suggests that anti-PDL-1 treatment preferentially rescues pre-existing PD-1 expressing cells.

Figure 11. Blockade of PD-1-PDL-1 pathway reinvigorates $CD8^+$ T cell response in both lymphoid and non-lymphoid tissue in chronically infected mice. (A) Chronically infected mice were treated with anti PDL-1 for 2 weeks and then sacrificed at week 7 pi. Absolute number of $CD8^+$ T cells was evaluated in spleen, liver and brain. (B) The frequency of proliferating $CD8^+$ T cells was assessed by intracellular staining for Ki-67. (C) IFNγ and Granzyme B production by $CD8^+$ T cells was evaluated in splenocytes or brain cells from infected mice in presence of TLA. (D) Antigen-specific cytotoxicity of splenic $CD8^+$T cell was measured by ^{51}Cr-release assay. (E) PD-1 expression on splenic $CD8^+$ T cells from anti PDL-1 or isotype control antibody treated mice was evaluated by flow cytometry. The data represent 3 experiments with at least 4 mice per group.

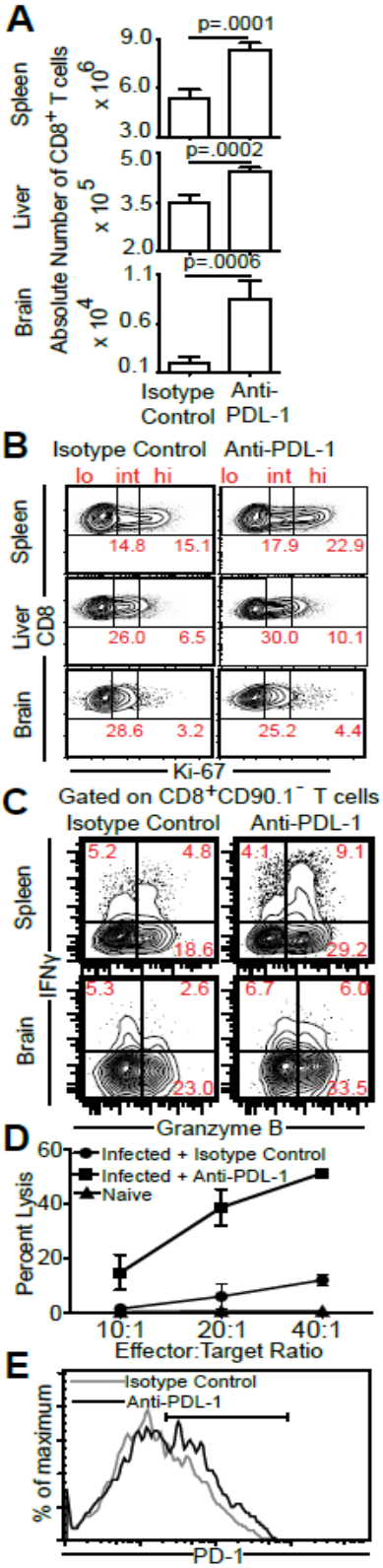

A
p=.0001
p=.0002
p=.0006
Spleen
Liver
Brain
Absolute Number of CD8+ T cells
x 10^6
x 10^5
x 10^4
9.0
6.0
3.0
5.0
3.5
2.0
1.1
0.6
0.1
Isotype Control
Anti-PDL-1
B
Isotype Control
Anti-PDL-1
lo int hi
lo int hi
Spleen
Liver
Brain
CD8
14.8 15.1
17.9 22.9
26.0 6.5
30.0 10.1
28.6 3.2
25.2 4.4
Ki-67
C
Gated on CD8+CD90.1- T cells
Isotype Control
Anti-PDL-1
Spleen
Brain
IFNγ
5.2 4.8
18.6
4.1 9.1
29.2
5.3 2.6
23.0
6.7 6.0
33.5
Granzyme B
D
Infected + Isotype Control
Infected + Anti-PDL-1
Naive
Percent Lysis
60
40
20
0
10:1 20:1 40:1
Effector:Target Ratio
E
Isotype Control
Anti-PDL-1
% of maximum
PD-1

Figure 12. Anti PDL-1 treatment reduces apoptosis of $PD1^{int}CD8^{+}$ T cells. (A,B) Splenocytes from chronically infected mice (week 7 pi) were incubated *in vitro* for 5h at 37° C with isotype control or anti-PDL-1 antibody. This was followed by evaluation of PD-1 expressing $CD8^{+}$ T cell subsets for active caspase-3 expression. (C) depicts reduction in active caspase-3 expression in anti PDL-1 treated splenocytes in $PD\text{-}1^{hi}$, $PD\text{-}1^{int}$ and $PD\text{-}1^{lo}$ $CD8^{+}$ T cells. Data represent 3 experiments with at least 4 mice per group.

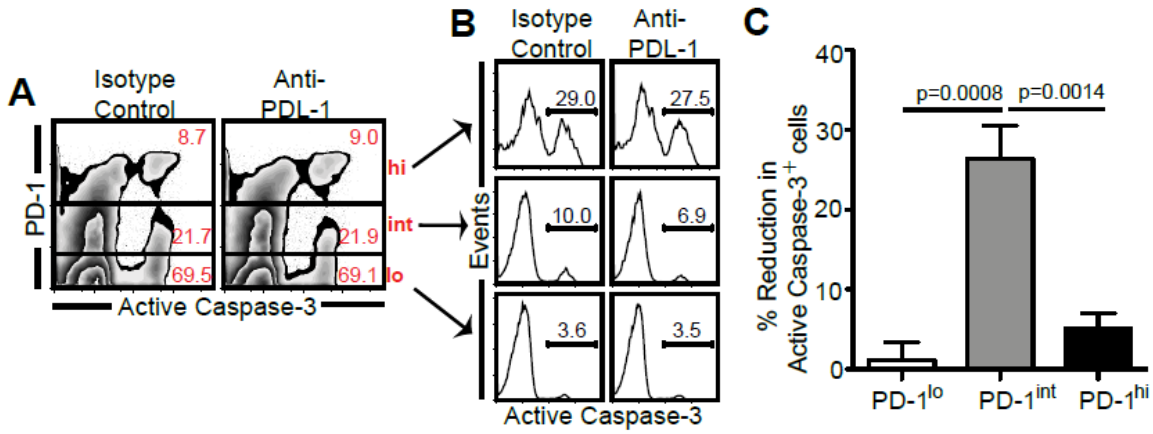
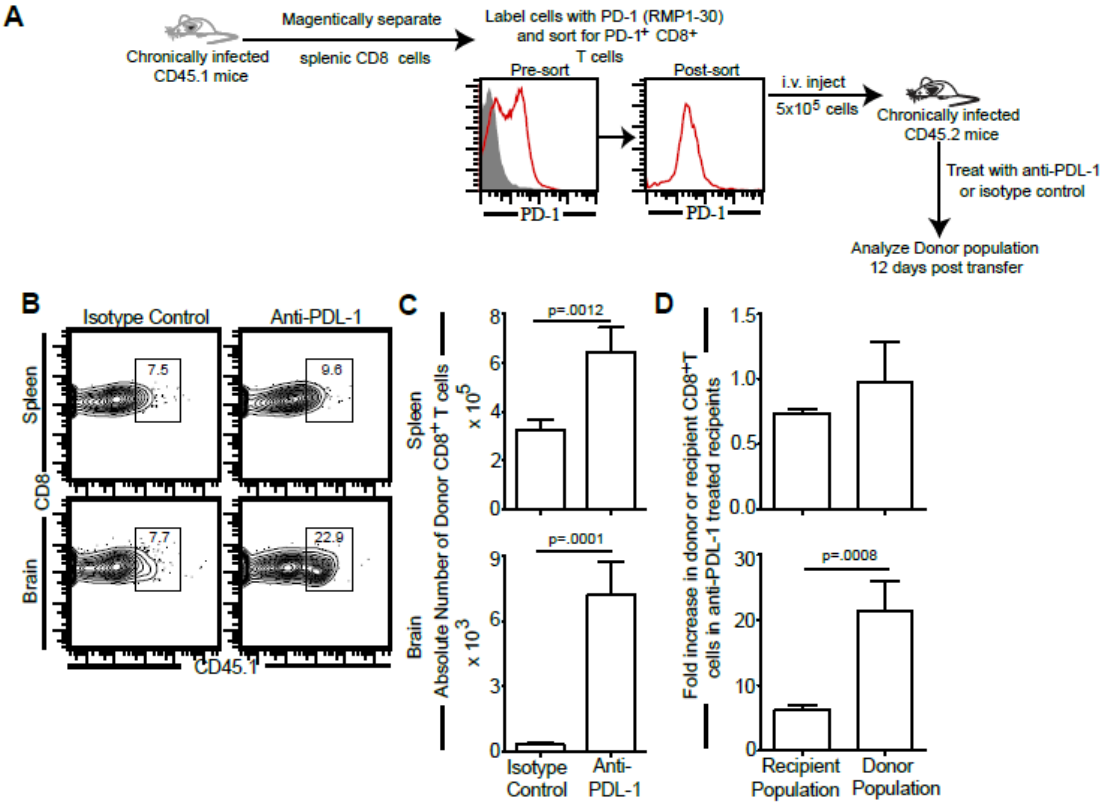

A
Isotype Control
Anti-PDL-1
PD-1
8.7
9.0
21.7
21.9
69.5
69.1
hi
int
lo
Active Caspase-3
B
Isotype Control
Anti-PDL-1
29.0
27.5
10.0
6.9
3.6
3.5
Events
Active Caspase-3
C
% Reduction in Active Caspase-3+ cells
40
30
20
10
0
p=0.0008
p=0.0014
PD-1lo
PD-1int
PD-1hi

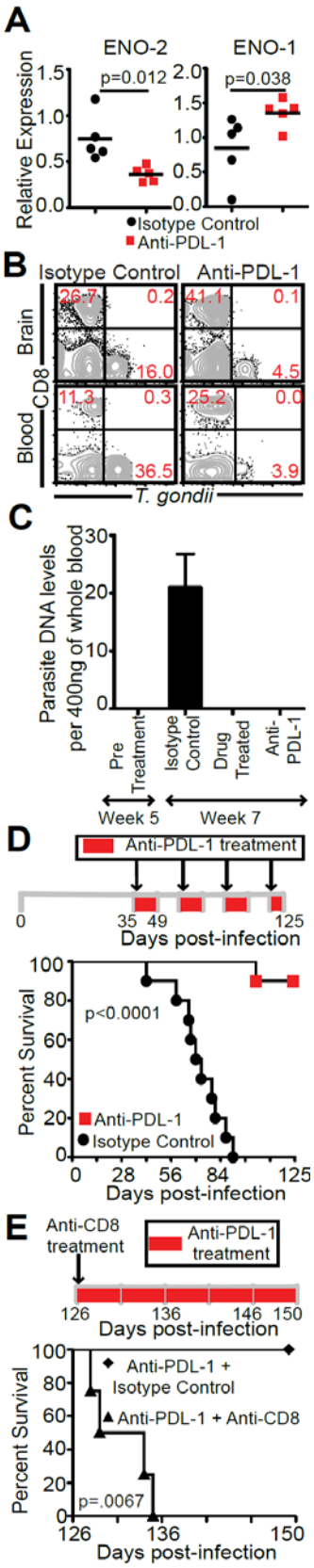
Figure 13. Anti PDL-1 treatment preferentially rescues PD-1 expressing $CD8^+$ T cells. (A) $5x10^5$ PD-1 expressing splenic $CD8^+$ T cells isolated from chronically infected (week 5-6) CD45.1 mice were adoptively transferred to infected (week 5-6) CD45.2 mice. Recipients were then treated with isotype control or anti-PDL-1 antibody. 12 days post-transfer recipients were sacrificed. Filled histogram represents staining with isotype-control antibody. (B,C) Frequency and absolute number of donor $CD8^+$ T cells was assessed in spleen and brain of recipient mice. (D) Fold increase in donor or recipient population on anti-PDL-1 treatment in recipient mice was computed as (Absolute Number of donor or recipient $CD8^+$ T cells in anti-PDL-1 treated mice – Arithmetic Mean of absolute number of donor or recipient $CD8^+$ T cells in isotype control antibody treated mice)/(Arithmetic Mean of absolute number of donor or recipient $CD8^+$ T cells in isotype control antibody treated mice). Data represent two experiments with at least 4 mice per group.


A
Chronically infected CD45.1 mice
Magentically separate
splenic CD8 cells
Label cells with PD-1 (RMP1-30) and sort for PD-1+ CD8+ T cells
Pre-sort
Post-sort
PD-1
PD-1
i.v. inject 5x10^5 cells
Chronically infected CD45.2 mice
Treat with anti-PDL-1 or isotype control
Analyze Donor population 12 days post transfer
B
Isotype Control
Anti-PDL-1
Spleen
Brain
CD8
CD45.1
7.5
9.6
7.7
22.9
C
Spleen
Brain
Absolute Number of Donor CD8+ T cells
x 10^5
x 10^3
p=.0012
p=.0001
Isotype Control
Anti-PDL-1
D
Fold increase in donor or recipient CD8+T cells in anti-PDL-1 treated recipeints
p=.0008
Recipient Population
Donor Population

4.2.4 Blockade of PD-1-PDL-1 pathway controls Toxoplasma recrudescence

Finally, we wanted to address if this augmented $CD8^+$ T cell response was able to control recrudescence by examining the gene expression of Toxoplasma parasites in brains of anti-PDL-1 or control antibody treated mice. Anti-PDL-1 treated mice revealed more of a bradyzoite-specific than tachyzoite-specific gene expression (Fig. 14*A*). Moreover, anti-PDL-1 treated mice exhibited much lower frequency of Toxoplasma infected leukocytes in brain and blood than control group (Fig. 14*B*). As previously stated, since encysted parasites can persist for life in immuno-competent host brains without causing pathology, we examined parasite genomic DNA in blood (3). Detection of parasite DNA in blood by PCR has been used to diagnose cerebral toxoplasmosis in patents with AIDS (180). As shown in Fig. 14*C*, parasite genomic DNA was only detectable in control antibody treated and not in drug or anti-PDL-1 treated blood. Next, we monitored long-term survival of anti-PDL-1 treated mice. Surprisingly, while 100% of control mice died by day 91 pi, 90% of anti-PDL-1 treated mice survived till the termination (day 125 pi) of experiment (Fig. 14*D*). Further, to demonstrate that increased survival of anti-PDL-1 treated mice was CD8 dependent, the surviving mice were treated with anti-CD8 antibody. As shown in Fig. 14*E*, CD8 depletion abrogated the survival of anti-PDL-1 treated animals. Taken together, the above data suggests that anti-PDL-1 treatment augments sub-optimal $CD8^+$ T cell response in chronically infected mice which prevents reactivation of infection leading to host survival.

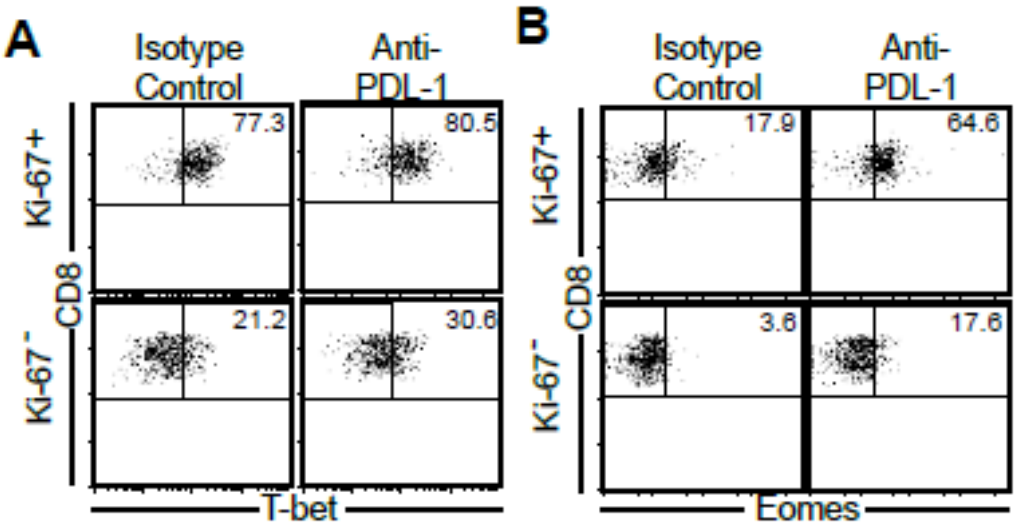
Figure 14. Anti-PDL-1 treatment controls parasite reactivation and prevents mortality in chronically infected mice. (A) Relative expression of ENO-1 and ENO-2 was computed for each sample. Transcript levels at day 10 pi was taken as 1. (B) *T. gondii* infected cells in brain and blood leukocyte gated samples (n=5) were assayed by flow cytometry. (C) Parasite genomic DNA level was computed in whole blood prior to initiation of treatment (week 5 pi) or 2 weeks after initiation of treatment (week 7 pi) (n=4-5) (D) Anti-PDL-1 antibody was injected every third day, starting at week 5 pi (n=10). In order to minimize the development of an allogeneic response, the antibody was administered with 2 week interruptions till termination of experiment. (E) Surviving anti PDL-1 treated mice from the previous experiment were administered anti PDL-1 along with anti CD8 (n=4) or anti PDL-1 and control antibody (n=4) every third day till the conclusion of experiment. Survival was monitored on a daily basis. Data represent two experiments.


A
ENO-2
ENO-1
p=0.012
p=0.038
Relative Expression
Isotype Control
Anti-PDL-1
B
Isotype Control
Anti-PDL-1
Brain
Blood
CD8
26.7
0.2
41.1
0.1
16.0
4.5
11.3
0.3
25.2
0.0
36.5
3.9
T. gondii
C
Parasite DNA levels per 400ng of whole blood
Pre Treatment
Isotype Control
Drug Treated
Anti-PDL-1
Week 5
Week 7
D
Anti-PDL-1 treatment
Days post-infection
Percent Survival
p<0.0001
Anti-PDL-1
Isotype Control
E
Anti-CD8 treatment
Anti-PDL-1 treatment
Anti-PDL-1 + Isotype Control
Anti-PDL-1 + Anti-CD8
p=.0067

4.2.5 Anti PDL-1 treatment upregulates Eomes in $CD8^+$ T cells from chronically infected mice

T-box factors, T-bet and Eomesodermin, play a critical role in development, survival and function of $CD8^+$ T cells (136, 181). As shown in Fig. 15*A*-*B*, anti-PDL-1 treatment dramatically augments expression of Eomes but not T-bet (unaffected vis-à-vis acutely infected mice) in cycling $CD8^+$ T cells (Fig. 15*A*,*B*). Incidentally, the critical role of Eomes in mediating $CD8^+$ T cell responsiveness to IL-15 (136), a cytokine crucial for survival of memory as well as effector $CD8^+$ T cells, and granzyme B (138, 182, 183) expression in T cells has been recently elucidated. Significantly, our data shows that $CD8^+$ T cell dysfunction affects granzyme B expression more than IFNγ production (Fig. 7*B*). The significance of Eomes expression in our current model will have to await further investigation.

Figure 15. PD-1-PDL-1 blockade increases Eomes but not T-bet expression on cycling CD8$^+$ T cells. (A,B) T-bet and Eomes expression was evaluated in cycling and non-cycling splenic CD8$^+$ T cells from isotype or anti-PDL-1 antibody treated chronically infected mice (week 7 pi) by flow cytometry. The data represents 2 experiments with at least 4 mice per group.


A
Isotype Control
Anti-PDL-1
77.3
80.5
21.2
30.6
Ki-67+
Ki-67-
CD8
T-bet
B
Isotype Control
Anti-PDL-1
17.9
64.6
3.6
17.6
Ki-67+
Ki-67-
CD8
Eomes

Chapter 5: PD-1 mediated attrition of polyfunctional memory $CD8^+$ T cells in chronic Toxoplasma infection

5.1 Introduction

In the previous chapter, we demonstrated that $CD8^+$ T cells from chronically infected mice exhibit progressive functional exhaustion, concomitant with increased expression of PD-1, an inhibitory receptor on their surface (184). This dysfunction results in reactivation of latent infection which eventually leads to death of the infected animals. *In vivo* blockade of PD-1 interaction with its receptor PDL-1, reinvigorated polyfunctional (i.e. the capacity for a single T cell to exhibit multiple functions) $CD8^+$ T cell response and prevented their mortality. Incidentally, the observation that HIV infected non-progressors are superior at maintaining virus-specific polyfunctional $CD8^+$ T cells than progressors (185), suggests that this polyfunctional population constitutes an important part of protective immune response against intracellular pathogens. However, how immune exhaustion causes attrition of polyfunctional $CD8^+$ T cells during chronic infections has not been addressed in parasitic or even in viral models of persistent infection. Considering the prevalence of TE in Toxoplasma sero-positive AIDS patients (186), approaches that boost CD8 polyfunctionality can have profound implications against both HIV and *T. gondii* infection. Despite wide Toxoplasma seropositivity in the global population, current initiatives for vaccine development against this parasite are prophylactic in nature (36). Since majority of the mortality caused by Toxoplasma is due to parasite reactivation in immuno-compromised patients (187), there is an urgent need for developing immunotherapeutic vaccination approaches to combat this infection

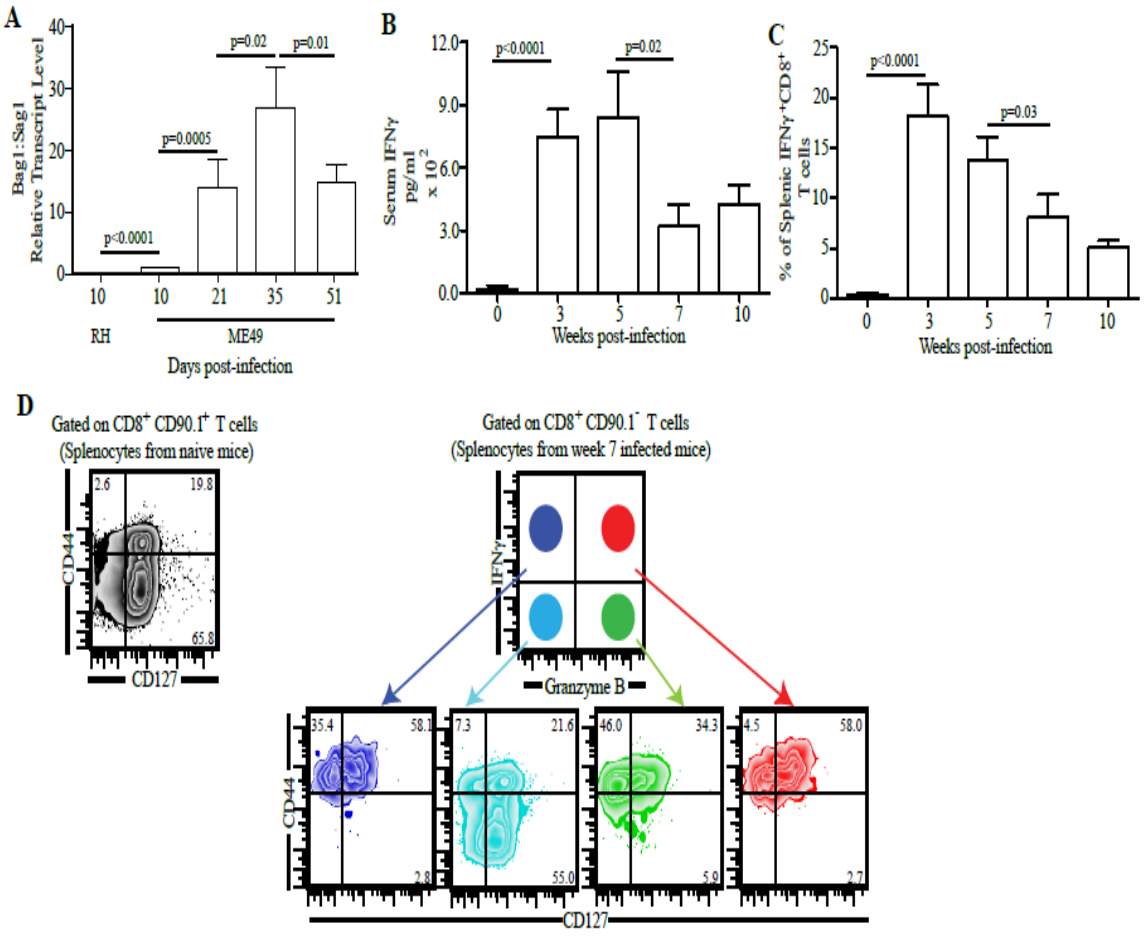
5.2 Results

5.2.1 Majority of polyfunctional $CD8^+$ T cells during chronic toxoplasmosis exhibit a memory phenotype

Toxoplasma infection induces a robust $CD8^+$ T cell response, which has been reported to be critical for protection against host infection (129). However during the late-chronic phase, the parasite undergoes reactivation i.e. conversion from chronic stage associated bradyzoite (slowly replicating, primarily encysted, relatively quiescent) to acute stage associated tachyzoite (rapidly replicating) (3). This was previously assessed by evaluating parasite stage specific markers, ENO1 (bradyzoite) and ENO2 (tachyzoite) (184). To further verify the kinetics of parasite reactivation we utilized two additional stage specific markers in the current study: Sag1, a marker of the tachyzoite stage and Bag1, a marker of the bradyzoite stage (3). Similar to ENO1 and ENO2, analysis of Bag1:Sag1 ratio of relative transcript level revealed a decrease at week 7, suggesting that parasites indeed undergo reactivation during the late-chronic stage (Fig 16*A*). Earlier studies from our laboratory have reported increased susceptibility of these animals around this time (184). Since IFNγ has been shown to be critical for control of Toxoplasma (15), we measured serum levels of IFNγ as well production of this cytokine by splenic $CD8^+$ T cells. Concomitant with parasite reactivation, decreased serum IFNγ levels (Fig. 16*B*) and reduced IFNγ production by $CD8^+$ T cells (Fig, 16*C*) was noted during the late-chronic phase. This is in agreement with our previously published data which shows a decrease in polyfunctional ($IFN\gamma^+GranzymeB^+$) $CD8^+$ T cell response in spleen and brain at week 7 post-infection (184). Since recent studies in viral models have established polyfunctionality as one of the most critical correlates of viral clearance or vaccine efficacy (188, 189), we

next investigated whether the polyfunctional $CD8^+$ T cells during the chronic phase of infection belonged to the effector or memory subset. To address this we performed 5 color flow cytometry on restimulated splenocytes, utilizing well established naïve/effector/memory markers CD44 and CD127 (190) to discriminate various CD8 subsets. Irrespective of the time-point examined, during the chronic phase, the majority of $CD8^+IFN\gamma^+GranzymeB^-$ and $CD8^+IFN\gamma^+GranzymeB^+$ exhibited a memory phenotype ($CD44^{hi}CD127^{hi}$) while $CD8^+IFN\gamma^-GrnazymeB^+$ T cells showed an effector subset phenotype (Fig. 16*D*). In contrast, the overwhelming majority of $CD8^+IFN\gamma^-GranzymeB^-$ T cells were $CD44^{lo}CD127^{hi}$, akin to phenotype of naïve splenocytes (Fig. 16*D*).

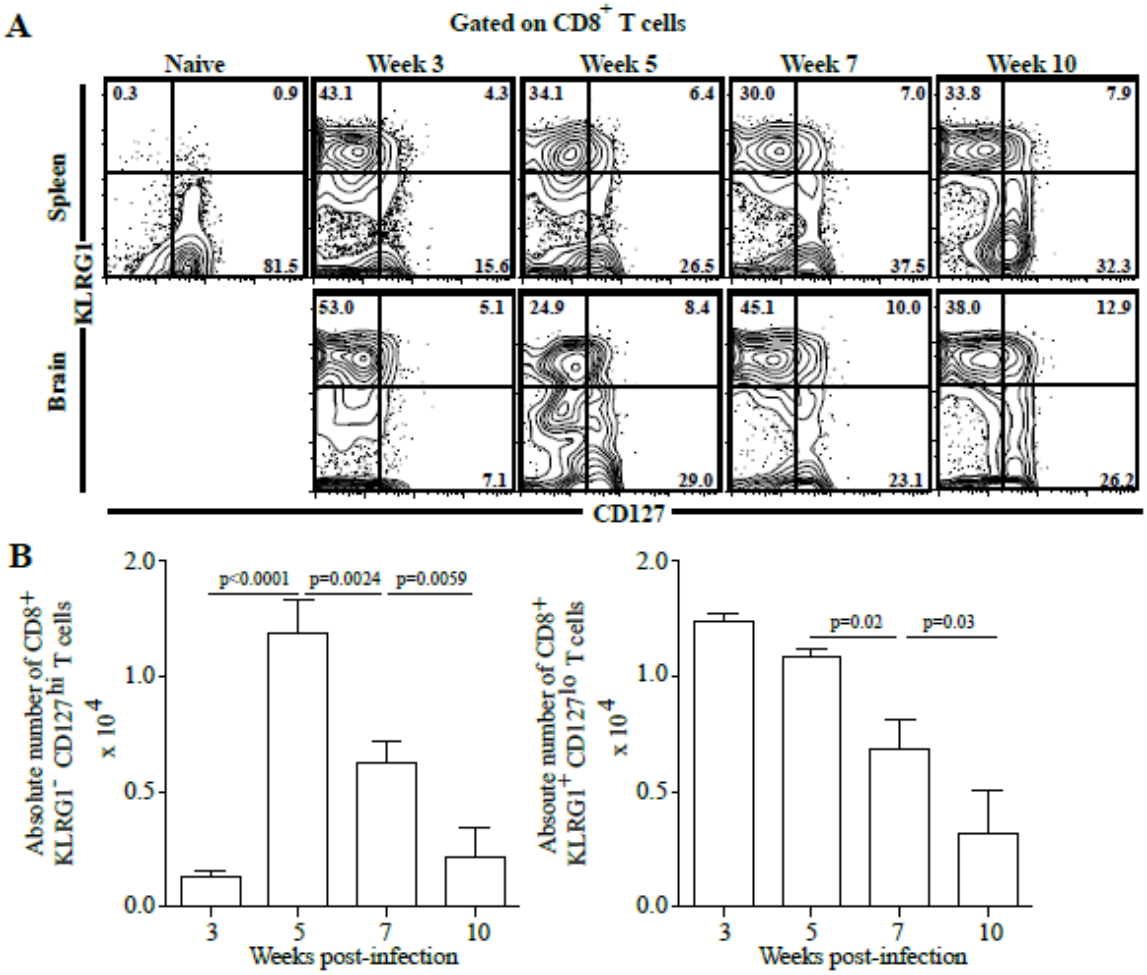
Figure 16. Majority of polyfunctional $CD8^+$ T cells during chronic toxoplasmosis exhibit a memory phenotype. A, C57BL/6 mice were infected with 10 ME49 cysts orally or RH intra-peritoneally. Ratio of relative expression Bag1 to Sag1 in brain was computed for each sample. Transcript levels at day 10 post-infection (ME49 infected) was taken as 1. B, Serum from mice at week 3, 5, 7 and 10 post-infection was evaluated for IFNγ by ELISA. C, Splenic $CD8^+$ T cells at the above time points were assessed for IFNγ production by restimulation of splenocytes with TLA. D, $IFN\gamma^+$Granzyme B^-, $IFN\gamma^+$Granzyme B^+, $IFN\gamma^-$Granzyme B^+ and $IFN\gamma^-$Granzyme B^- splenic $CD8^+$ T cell subsets form chronically infected (week 7) mice were evaluated for expression of CD44 and CD127. Due to low number of IFNγ or Granzyme B expressing $CD8^+$ T cells in naïve spleens , total $CD8^+$ T cell population was assessed for CD44 and CD127 expression The data represent 2 experiments with at least 4 mice per group. Error bars represent standard deviation throughout.


A
Bag1:Sag1 Relative Transcript Level
p<0.0001
p=0.0005
p=0.02
p=0.01
10
10
21
35
51
RH
ME49
Days post-infection
B
Serum IFNγ pg/ml x 10^2
p<0.0001
p=0.02
Weeks post-infection
C
% of Splenic IFNγ+CD8+ T cells
p<0.0001
p=0.03
Weeks post-infection
D
Gated on CD8+ CD90.1+ T cells
(Splenocytes from naive mice)
CD44
CD127
2.6
19.8
65.8
Gated on CD8+ CD90.1- T cells
(Splenocytes from week 7 infected mice)
IFNγ
Granzyme B
CD44
CD127
35.4
58.1
2.8
7.3
21.6
55.0
46.0
34.3
5.9
4.5
58.0
2.7

5.2.2 Normal $CD8^+$ T cell development during *T. gondii* infection

Since majority of polyfunctional ($IFN\gamma^+GranzymeB^+$) and $IFN\gamma^+GranzymeB^-$ $CD8^+$ T cells exhibit a memory phenotype and there is a loss of polyfunctional $CD8^+$ T cell response during late-chronic Toxoplasmosis, we hypothesized a poor development or maintenance of memory $CD8^+$ T cells during later phases of chronic Toxoplasmosis. To address this we evaluated the expression of KLRG1 and CD127 on $CD8^+$ T cells. Previous studies have shown that effector cells express KLRG1 but minimal CD127 while memory CD8 T cells express high levels of CD127 but minimal KLRG1 (98). Since naïve non-specific $CD8^+$ T cells also express CD127, we examined the phenotype in brain, a site that specifically recruits $CD8^+$ T cells in a strictly antigen-specific manner (177). Contrary to our expectations based on the degree of attrition of polyfunctionality, we only noted moderate differences in frequency of $CD127^{hi}$ cells (Fig. 17*A*). Although dramatic changes in frequency of $CD8^+CD127^{hi}$ T cells were not noted in brain, the absolute numbers of both effector and memory phenotype $CD8^+$ T cells were reduced in this tissue during the later phases of infection (Fig. 17*B*).

Figure 17. Frequency of memory phenotype $CD8^+$ T cells is not dramatically altered at the effector site. A, KLRG1 and CD127 expression on $CD8^+$ T cells from spleen and brain was assessed at week 3, 5, 7 and 10 post-infection. B, depicts the absolute number of $CD8^+KLRG1^-CD127^{hi}$ (left panel) and $CD8^+KLRG1^+CD127^{lo}$ (right panel) T cells in brain at weeks 3, 5, 7 and 10 post-infection. The data represent 2 experiments with at least 4 mice per group.

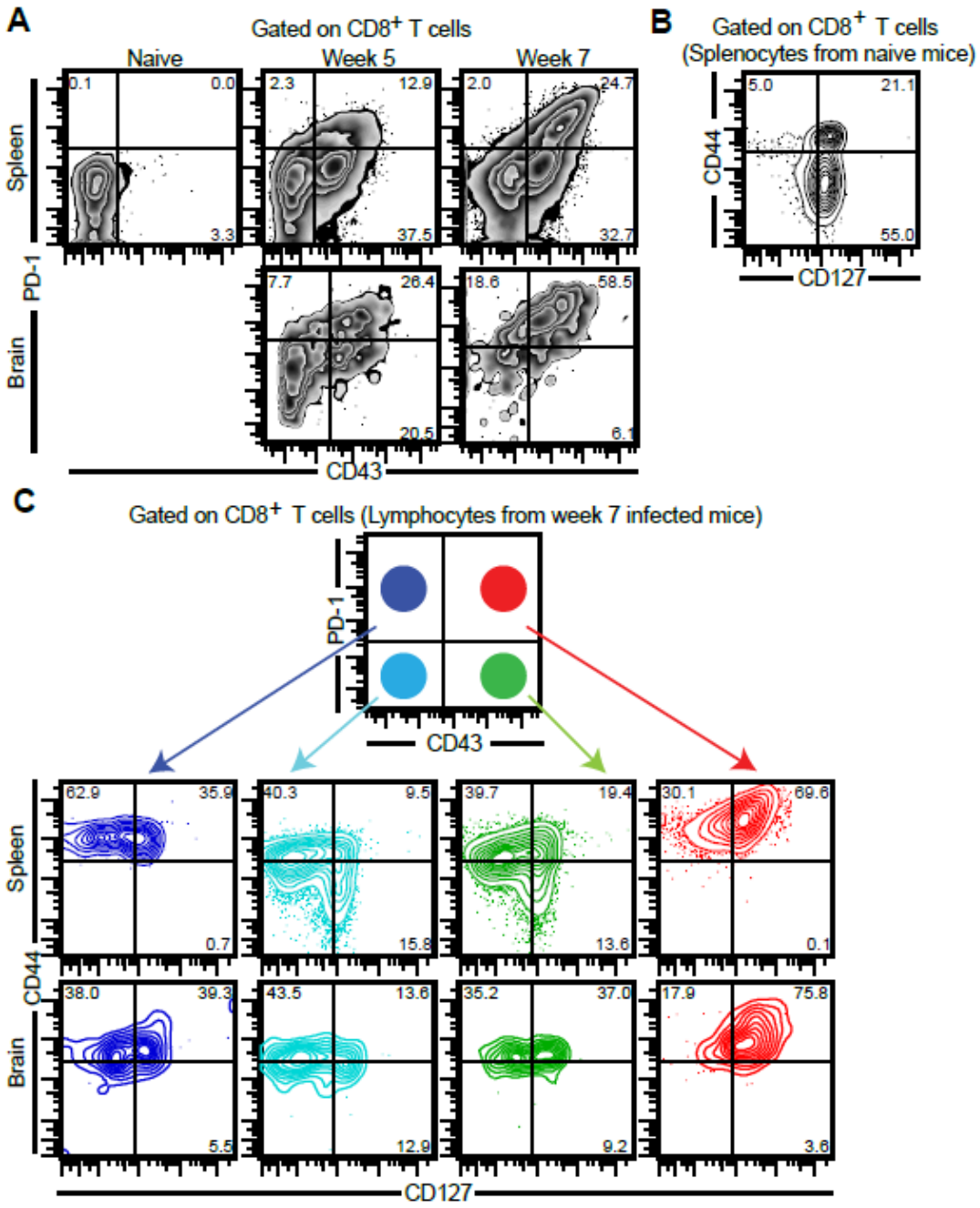

A
Gated on CD8+ T cells
Naive
Week 3
Week 5
Week 7
Week 10
Spleen
Brain
KLRG1
CD127
0.3
0.9
81.5
43.1
4.3
15.6
34.1
6.4
26.5
30.0
7.0
37.5
33.8
7.9
32.3
53.0
5.1
7.1
24.9
8.4
29.0
45.1
10.0
23.1
38.0
12.9
26.2
B
Absolute number of CD8+ KLRG1- CD127hi T cells x 10^4
p<0.0001
p=0.0024
p=0.0059
Weeks post-infection
Absoute number of CD8+ KLRG1+ CD127lo T cells x 10^4
p=0.02
p=0.03
Weeks post-infection

5.2.3 Preferential expression of PD-1 on memory $CD8^+$ T cells

In the previous chapter (Chapter 4) we demonstrated that PD-$1^{hi}CD8^+$ T cells were preferentially apoptotic as evaluated by elevated active caspase3 expression (184). Based on this report and the finding that the frequency of memory phenotype $CD8^+$ T cells were only moderately affected at the effector site during late-chronic toxoplasmosis, we hypothesized that PD-1 was expressed both on memory and effector $CD8^+$ T cells. Hence, we next assessed expression of the PD-1 molecule on effector or memory $CD8^+$ T cells population during *T. gondii* infection. Since a previous study using the acute-infection causing LCMV Armstrong strain had shown that CD43 (activation associated isoform) expression can be used to discriminate effector ($CD43^{hi}$) from memory ($CD43^{lo}$) $CD8^+$ T cells, we examined the expression of this molecule on $CD8^+$ T cells in spleen and brain (191). Polychromatic flow cytometry revealed that the majority of PD-1^{hi} cells in both tissues co-expressed CD43 and that an increase in PD-1 expression correlated with an increase in CD43 expression (Fig. 18*A*). To further verify if PD-$1^{hi}CD43^+$, PD-$1^{hi}CD43^-$, PD-$1^{int/lo}CD43^{hi}$ and PD-$1^{int/lo}CD43^-$ $CD8^+$ T cells indeed belonged to memory or effector subsets, we examined them for expression of canonical memory markers CD44 and IL-7Rα (CD127) (190) . Surprisingly, despite elevated CD43 expression, the majority of PD-$1^{hi}CD43^{hi}$ cells in both tissues co-expressed high levels of CD44 and CD127 (Fig. 18*B*, *C*). Notwithstanding the preponderance of memory phenotype cells in PD-$1^{hi}CD43^{hi}$ subset, a direct correlation, akin to PD-1 and CD43 expression was not evident between PD-1 and CD127 expression (data not shown). In contrast, most cells in the other three subsets, irrespective of CD43 expression exhibited lower frequency of $CD127^{hi}$ cells (Fig. 18*C*)

These findings suggest that PD-1^{hi} memory CD8^{+} T cell population represents a phenotype distinct from memory CD8^{+} T cells generated in acute infection models (191).

Figure 18. PD-1 is preferentially upregulated on CD43 expressing memory phenotype CD8$^+$ T cells. A, Splenic and brain CD8$^+$ T cells were evaluated for PD-1 and CD43 expression at week 5 and week 7 pi. B and C, PD-1^{hi}CD43$^+$, PD-1^{hi}CD43$^-$, PD-1$^{int/lo}$CD43$^+$ and PD-1$^{int/lo}$CD43$^-$ CD8$^+$ T cell subsets from spleen and brain of chronically infected mice (week 7) were further evaluated for CD44 and CD127 expression. Due to low number of PD-1^{hi} or CD43 expressing CD8$^+$ T cells in naïve spleens, total CD8$^+$ T cell population was assessed for CD44 and CD127 expression. Data represent 3 experiments with at least 4 mice per group.

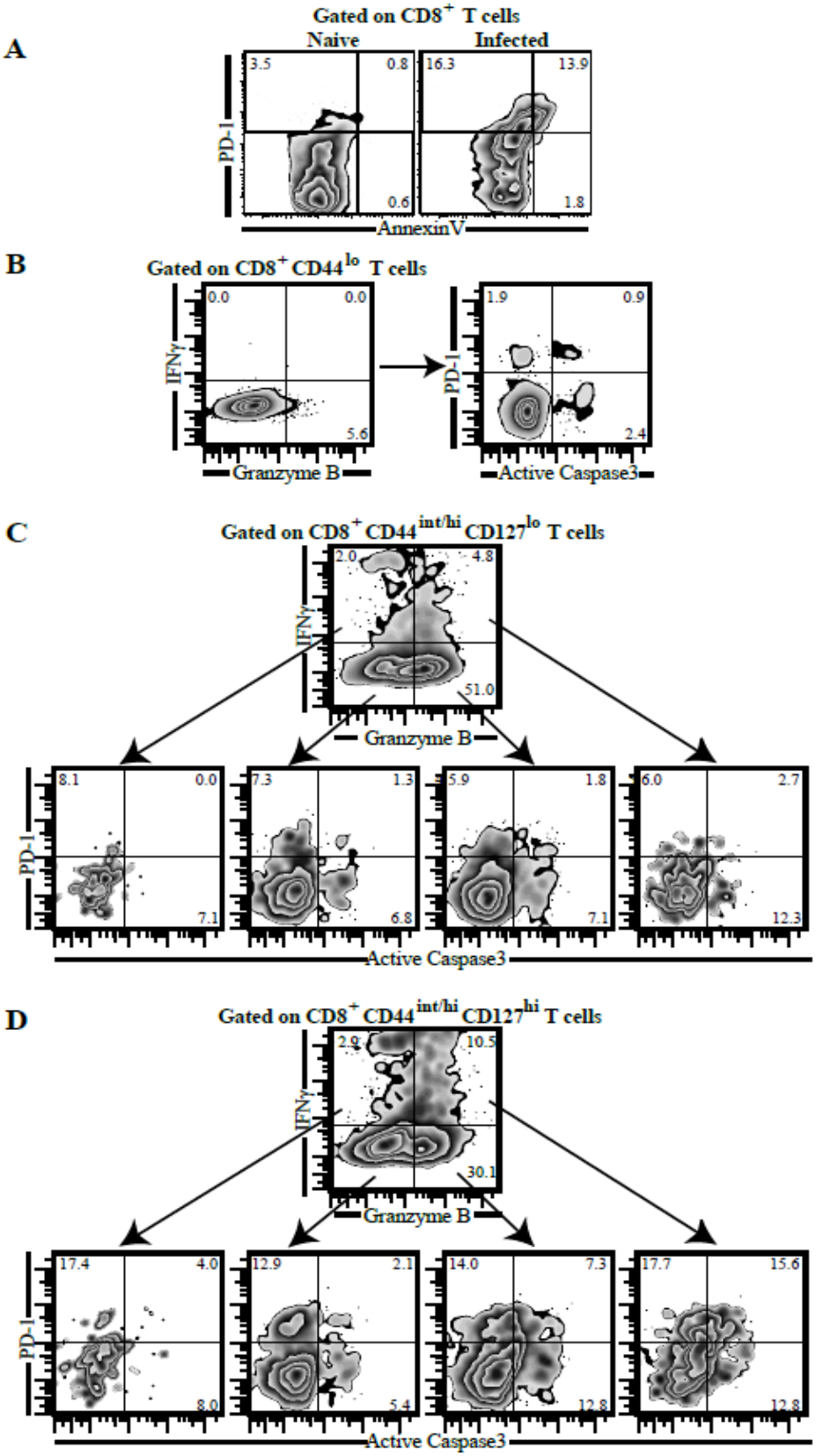

A
Gated on CD8+ T cells
Naive
Week 5
Week 7
Spleen
Brain
PD-1
CD43
0.1
0.0
3.3
2.3
12.9
37.5
2.0
24.7
32.7
7.7
26.4
20.5
18.6
58.5
6.1
B
Gated on CD8+ T cells
(Splenocytes from naive mice)
CD44
CD127
5.0
21.1
55.0
C
Gated on CD8+ T cells (Lymphocytes from week 7 infected mice)
PD-1
CD43
Spleen
Brain
CD44
CD127
62.9
35.9
0.7
40.3
9.5
15.8
39.7
19.4
13.6
30.1
69.6
0.1
38.0
39.3
5.5
43.5
13.6
12.9
35.2
37.0
9.2
17.9
75.8
3.6

5.2.4 Increased expression of PD-1 is associated with apoptosis of polyfunctional memory CD8$^+$ T cells

As mentioned earlier, PD-1^{hi}CD8$^+$ T cells during chronic toxoplasmosis express high levels of active caspase3 (184). Since a previous study demonstrated that despite elevated active caspase3 expression anergic T cells do not undergo apoptosis (192), we wanted to revalidate our earlier findings by assessing the distribution of membrane phosphatidly-serine using fluorochome-conjugated AnnexinV which is considered a hallmark of apoptosis (193). As shown in Fig 19*A*, PD-1 expressing CD8$^+$ T cells were primarily AnnexinV$^+$. This suggests that active caspase3 expression in PD-1^{hi}CD8$^+$T cells indeed correlates with their apoptotic potential. As demonstrated earlier, PD-1 is preferentially expressed on memory phenotype CD8$^+$ T cells and polyfunctional CD8$^+$ T cells primarily exhibited a memory phenotype. Thus, we hypothesized PD-1-PDL-1 pathway causes preferential apoptosis of PD-1 expressing polyfunctional memory CD8$^+$ T cells. To address this we measured active caspase3 expression on the relevant subsets in restimulated splenocytes. As expected naïve phenotype (CD8$^+$CD44^{lo}) T cells exhibited minimal IFNγ and GranzymeB production and low levels of PD-1 and active caspase3 expression (Fig. 19*B*). Next we analyzed, polyfunctional populations in the effector CD8$^+$ T cell subset (CD8$^+$CD44$^{int/hi}$CD127^{lo}) for apoptotic potential (Fig. 19*C*). No dramatic difference in frequency of PD-1$^+$active caspase3$^+$ expressing cells was noted between CD8$^+$CD44$^{int/hi}$CD127^{lo}IFNγ$^+$GranzymeB$^-$, CD8$^+$CD44$^{int/hi}$CD127^{lo}IFNγ$^+$GranzymeB$^+$ and CD8$^+$CD44$^{int/hi}$CD127^{lo}IFNγ$^-$GranzymeB$^+$ subsets. However, PD-1$^-$ polyfunctional effector CD8$^+$ T cells were more apoptotic than the other subsets. This potentially suggests that PD-1 independent apoptotic mechanisms may be involved in mediating preferential attrition of

polyfunctional effector CD8 T cells. Finally, we examined the memory subset by gating on $CD8^{+}CD44^{int/hi}CD127^{hi}$ cells (Fig. 19*D*). In agreement with our previous observations with direct *ex vivo* staining, memory phenotype $CD8^{+}$ T cells had substantially higher frequency of PD-1 expressing CD8 T cells than naïve or effector phenotype $CD8^{+}$ T cells. In concurrence with our hypothesis, the highest levels of $PD1^{+}ActiveCaspase^{+}$ memory $CD8^{+}$ T cells was noted in polyfunctional memory population, followed by $CD8^{+}CD44^{int/hi}CD127^{hi}IFN\gamma^{-}GranzymeB^{+}$, $CD8^{+}CD44^{int/hi}CD127^{hi}IFN\gamma^{+}GranzymeB^{-}$ and $CD8^{+}CD44^{int/hi}CD127^{hi}IFN\gamma^{-}GranzymeB^{-}$ subsets. Combined, our data strongly suggests a strong correlation between PD-1 expression and apoptosis of polyfunctional memory $CD8^{+}$ T cells.

Figure 19. PD-1 expression correlates with apoptosis of polyfunctional memory phenotype CD8$^+$ T cells. A, Splenic CD8$^+$ T cells from chronically infected (week 7) mice were evaluated for PD-1 expression and AnnexinV reactivity. B, C and D, PD-1 and active caspase3 expression was assessed in IFNγ$^+$GranzymeB$^-$, IFNγ$^-$GranzymeB$^-$, IFNγ$^+$GranzymeB$^+$ and IFNγ$^-$GranzymeB$^+$ naïve/effector/memory phenotype CD8$^+$ T cells by 7 color flow cytometry. The data represent 2 experiments with at least 4 mice per group.

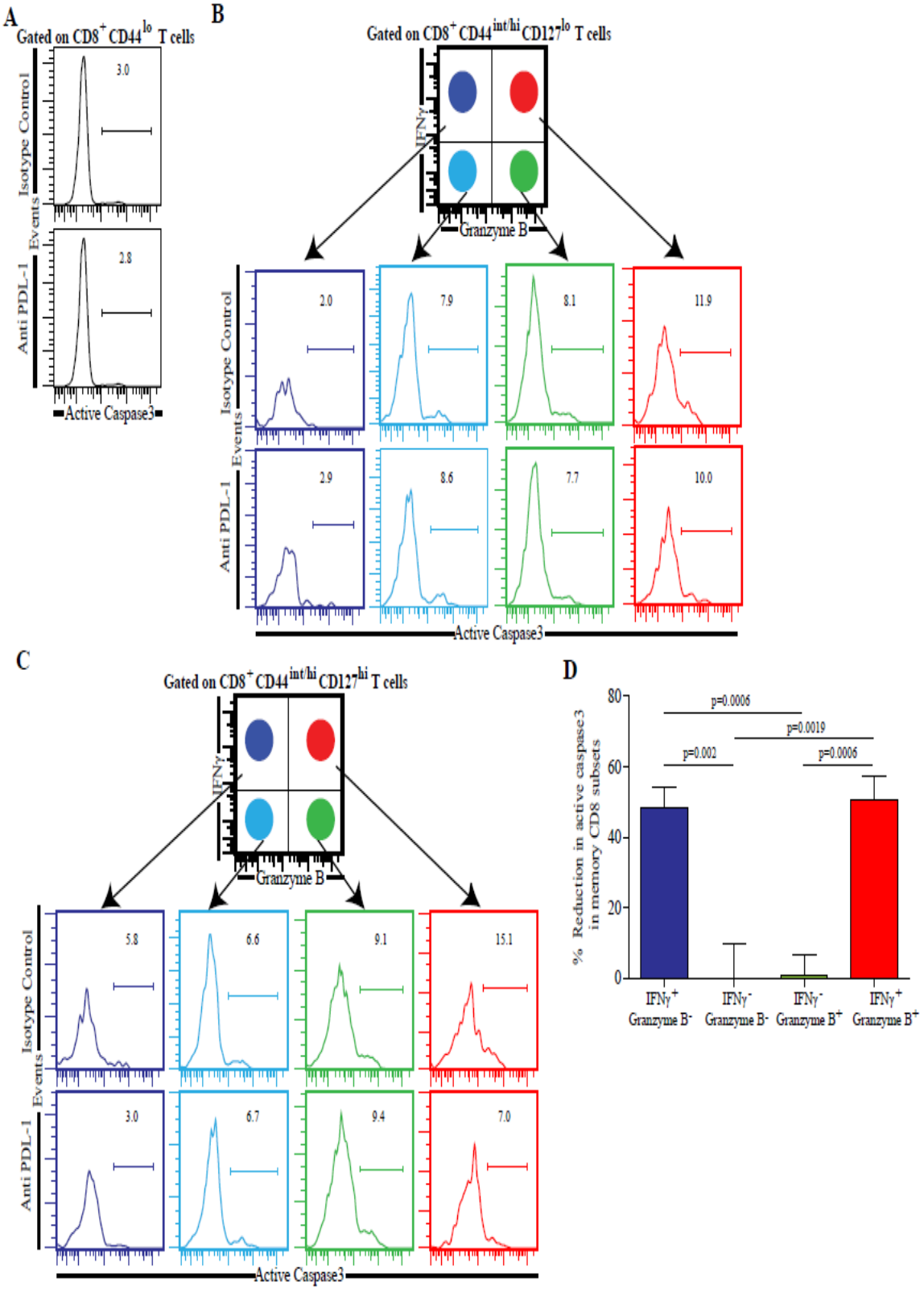

A
Gated on CD8+ T cells
Naive
Infected
3.5
0.8
0.6
16.3
13.9
1.8
PD-1
AnnexinV
B
Gated on CD8+ CD44lo T cells
0.0
0.0
5.6
IFNγ
Granzyme B
1.9
0.9
2.4
PD-1
Active Caspase3
C
Gated on CD8+ CD44int/hi CD127lo T cells
2.0
4.8
51.0
IFNγ
Granzyme B
8.1
0.0
7.1
7.3
1.3
6.8
5.9
1.8
7.1
6.0
2.7
12.3
PD-1
Active Caspase3
D
Gated on CD8+ CD44int/hi CD127hi T cells
2.9
10.5
30.1
IFNγ
Granzyme B
17.4
4.0
8.0
12.9
2.1
5.4
14.0
7.3
12.8
17.7
15.6
12.8
PD-1
Active Caspase3

5.2.5 PD-1-PDL-1 blockade reduces apoptosis of polyfunctional and IFNγ$^+$CD8$^+$ T cells

To directly link the role of increased PD-1 expression on polyfunctional memory CD8$^+$ T cells with their apoptosis, we performed *in vitro* treatment of splenocytes with isotype control or anti PDL-1 antibody (MIH5, blocking but non-depleting) (156). The rationale for performing *in vitro* studies is that apoptotic cells get rapidly eliminated *in vivo* and thus an accurate assessment of apoptosis cannot be made. *In vitro* incubation of splenocytes for 16 hr with anti PDL-1 (and TLA) did not alter CD8$^+$ T cell functionality irrespective of the subset examined (data not shown). It is possible that with longer multi-day incubation we may observe an increase in polyfunctionality in anti-PDL-1 treated cells, akin to the *in vivo* scenario (194) . However with longer incubations, CD8$^+$ T cells in anti PDL-1 treated groups may undergo rapid expansion and as a result, it will not be possible to discriminate the differential effects of apoptosis and expansion on the various CD8 subsets (194). To additionally verify that 16 hr incubation with anti PDL-1 did not cause proliferation of CD8$^+$ T cells, Ki-67 expression was assessed. However, no change in Ki-67 levels was noted between isotype and anti PDL-1 treated CD8$^+$ T cells (data not shown). As expected, anti PDL-1 treatment had no effect on apoptosis of naïve phenotype CD8$^+$ T cells (Fig. 20*A*). Surprisingly, blockade of PD-1-PDL-1 pathway failed to reduce apoptosis of effector CD8$^+$ T cells, irrespective of IFNγ or GranzymeB production (Fig. 20*B*). Similar to its effect on naïve and effector phenotype CD8$^+$ T cells, anti PDL-1 treatment did not affect the apoptosis of memory phenotype CD8$^+$CD44$^{int/hi}$CD127^{hi}IFNγ$^-$ GranzymeB$^-$ cells or CD8$^+$CD44$^{int/hi}$CD127^{hi}IFNγ$^-$GranzymeB$^+$ cells (Fig. 20*C*). However, anti PDL-1 treatment was equally efficacious in reducing the apoptosis of both the

polyfunctional and $CD8^{+}CD44^{int/hi}CD127^{hi}IFN\gamma^{+}GranzymeB^{-}$ memory phenotype cells (Fig. 20*C,D*). Considering that IFNγ is considered to be critical in mediating protection against Toxoplasma as well other pathogens (195, 196), the mechanistic insight offered by the current study can have important ramifications in incorporating anti PDL-1 antibody in immunotherapeutic applications.

Figure 20. *In vitro* anti-PDL-1 treatment prevents apoptosis of polyfunctional memory phenotype CD8$^+$ T cells. Splenocytes from chronically infected (week 7) mice were treated with anti-PDL-1 and stimulated overnight with TLA. A, depicts active caspase3 expression in isotype and anti-PDL-1 treated naïve phenotype CD8$^+$ T cells. B, represents active caspase3 expression in IFNγ$^+$GranzymeB$^-$, IFNγ$^-$GranzymeB$^-$, IFNγ$^+$GranzymeB$^+$ and IFNγ$^-$GranzymeB$^+$ effector phenotype CD8$^+$ T cells treated with anti-PDL-1 or control antibody. C, IFNγ or GranzymeB producing memory phenotype CD8$^+$ T cells in isotype or anti-PDL-1 treated splenocytes were evaluated for active-caspase3 expression by polychromatic flow cytometry. D, depicts anti-PDL-1 mediated reduction in active caspase3 in various memory CD8 subsets. The data represent two experiments with at least 4 mice per group.


A
Gated on CD8+ CD44lo T cells
Isotype Control
Anti PDL-1
Events
3.0
2.8
Active Caspase3
B
Gated on CD8+ CD44int/hi CD127lo T cells
IFNγ
Granzyme B
Isotype Control
Anti PDL-1
Events
2.0
7.9
8.1
11.9
2.9
8.6
7.7
10.0
Active Caspase3
C
Gated on CD8+ CD44int/hi CD127hi T cells
IFNγ
Granzyme B
Isotype Control
Anti PDL-1
Events
5.8
6.6
9.1
15.1
3.0
6.7
9.4
7.0
Active Caspase3
D
% Reduction in active caspase3 in memory CD8 subsets
p=0.0006
p=0.0019
p=0.002
p=0.0006
IFNγ+ Granzyme B-
IFNγ- Granzyme B-
IFNγ- Granzyme B+
IFNγ+ Granzyme B+

Chapter 6: CD40-CD40L pathway plays a critical CD8 intrinsic and extrinsic role during rescue of exhausted CD8 T cells

6.1 Introduction

CD8 exhaustion as reported in chronic viral and tumor models involves a hierarchical loss of functions (cytokine, proliferation, cytotoxicity) and in extreme cases CD8 T cells can be physically deleted in a PD-1 dependent manner (197). As shown in Chapter 4, CD8 T cells from chronically infected mice exhibit progressive functional exhaustion, concomitant with increased PD-1 expression, in the Toxoplasma model (184). This dysfunction results in reactivation of latent toxoplasmosis leading to host mortality. *In vivo* blockade of PD-1 interaction with its receptor PD-L1, preferentially reinvigorates pre-existing PD-1 expressing CD8 T cells, thereby preventing disease recrudescence and host mortality (184). Apart from TCR signal and cytokine milieu, one of the most important factors governing CD8 response is the balance between positive signals from co-stimulatory receptors and the negative signals from inhibitory receptors (143). It has been postulated that the interplay between signals from co-stimulatory and inhibitory receptors may play an important role in T cell activation, differentiation, effector function and exhaustion (154). However, the role of co-stimulatory receptors during the rescue of this response has not been explored in any model of CD8 exhaustion. Therefore we hypothesized that blockade of inhibitory PD-1-PD-L1 pathway in chronically infected mice would lead to upregulation of co-stimulatory receptors and such positive signals would be critical for the rescue of dysfunctional CD8 T cells. We identified CD40 as one of the molecules highly upregulated on CD8 T cells in αPD-L1 treated chronically infected mice.

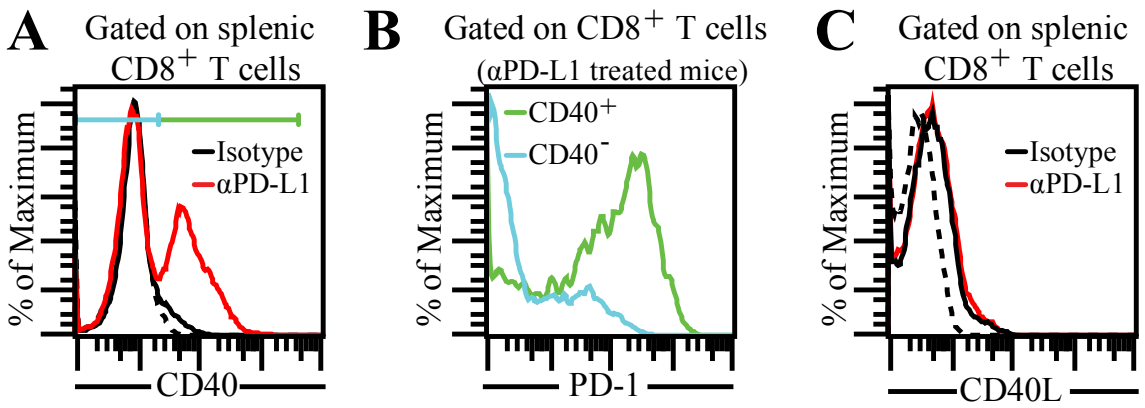
A previous report using HY-TCR transgenic model demonstrated that CD8 intrinsic CD40 signaling is critical for memory generation (198). However, this notion has been challenged by multiple studies in infectious disease models where CD40 deficient memory CD8 T cells has been shown to elicit a normal immune response in a CD40 sufficient environment (199). Similarly in the Toxoplasma model, CD40-CD40L pathway has been shown to play a minimal role in T cell response development (52). This discrepancy in CD40 dependence has been attributed to the nature of antigen and the highly inflammatory conditions associated with infectious disease (199). However, the role of CD40-CD40L pathway during rescue of exhausted CD8 T cells has not been addressed in any model.

6.2 Results

6.2.1 αPD-L1 treatment upregulates CD40 but not CD40L on CD8 T cells

To identify co-stimulatory molecules involved in the rescue of exhausted CD8 T cells, we examined a panel of these receptors on CD8 T cells in chronically infected mice treated with αPD-L1 or control antibody (data not shown). CD40 was identified as one of the co-stimulatory molecules showing high levels of differential expression. Minimal CD40 levels were noted on splenic CD8 T cells from control mice while high expression of this molecule occurred on CD8 T cells from αPD-L1 treated animals (Fig. 21*A*). Furthermore CD40 was preferentially expressed on PD-1^{+} subset (Fig. 21*B*) in αPD-L1 treated mice. Despite high CD40 expression on CD8 T cells from αPD-L1 treated mice, no differential expression of its receptor, CD40L was noted between CD8 T cells from control and antibody treated animals (Fig. 21*C*). Combined, the above data points towards potential involvement of CD40-CD40L pathway during rescue of exhausted CD8 T cells.

Figure 21. High expression of CD40 on CD8 T cells in αPD-L1 treated mice. *A*, Chronically infected mice were treated with αPD-L1 for 2 weeks and then sacrificed at week 7 pi. CD40 expression was analyzed on splenic CD8 T cells. Dotted line represents background fluorescence throughout. *B*, represents PD-1 expression on $CD40^+$ and $CD40^-$ splenic CD8 T cells in αPD-L1 treated mice. *C*, Splenic CD8 T cells from control and αPD-L1 treated mice were evaluated for CD40L expression. The data represent 3 experiments with at least 4 mice per group.

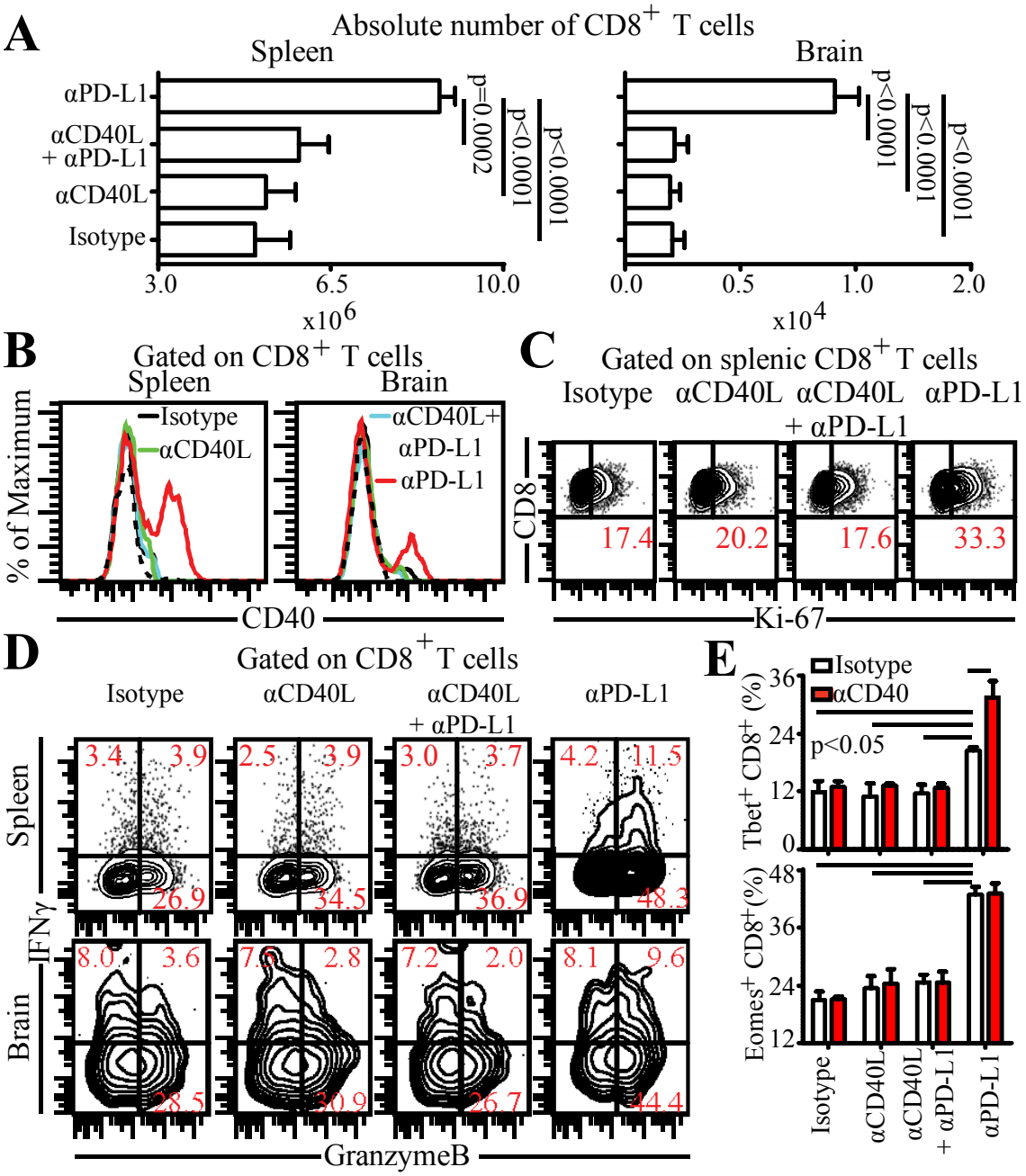

A
Gated on splenic
CD8+ T cells
% of Maximum
Isotype
αPD-L1
CD40
B
Gated on CD8+ T cells
(αPD-L1 treated mice)
% of Maximum
CD40+
CD40-
PD-1
C
Gated on splenic
CD8+ T cells
% of Maximum
Isotype
αPD-L1
CD40L

6.2.2 CD40-CD40L signaling plays a critical role during rescue of exhausted CD8 T cells

Since CD40 is differentially expressed on reinvigorated CD8 T cells, we next investigated if CD40-CD40L signaling played any role during the rescue process. To address this, chronically infected mice were administered control antibody or αPD-L1 or αCD40L or both (αPD-L1 and αCD40L) for 2 weeks (weeks 5-7) and then analyzed at week 7 pi. Blockade of CD40-CD40L pathway alone had no profound effect on T cells-absolute number, CD40 expression, proliferation and functionality of CD8 T cells remained unaltered in both spleen and brain (Fig. 22 *A,B,C* and *D*) (52). While αPD-L1 treatment by itself was able to augment the above parameters (absolute number, CD40 expression, proliferation and effector functions), surprisingly, co-administration of αCD40L and αPD-L1 abrogated the rescue effect (Fig. 22*A,B,C* and *D*) (184). This suggests that the CD40-CD40L pathway plays a critical role during rescue of dysfunctional CD8 T cells. T-box family transcription factors, T-bet and Eomes, have been shown to be important for proliferation, effector functions and survival of CD8 T cells (136). To further investigate the role of CD40 signaling on CD8 transcriptional control, purified splenic CD8 T cells from the above mice were treated *in vitro* with agonistic CD40 or control antibody. The expression of the above transcription factors was then evaluated by intracellular staining. *In vivo* co-administration of αPD-L1 and αCD40L abrogated the increase in T-bet and Eomes expression and *in vitro* treatment with agonistic CD40 failed to augment these molecules (Fig. 22*E*). Surprisingly, agonistic CD40 treatment of CD8 T cells from αPD-L1 treated mice resulted in further increase in T-bet but not in Eomes (Fig. 22*E*). This suggests that

while Eomes expression in CD8 T cells is independent of CD8 intrinsic CD40 signaling, T-bet expression is at least partially dependent on CD40 signaling directly on CD8 T cells.

Figure 22. Blockade of CD40-CD40L pathway abrogates αPD-L1 mediated rescue of CD8 T cells. *A*, αPD-L1 or αCD40L or both were administered to chronically infected mice for 2 weeks and then sacrificed at week 7 pi. Absolute number of CD8 T cells was evaluated in spleen and brain. CD40 (*B*) and Ki-67 (*C*) expression was assessed in these mice by flow cytometry. *D*, IFNγ and Gzb production by CD8 T cells was evaluated in splenocytes or brain cells from infected mice in presence of TLA. *E*, Splenic CD8 T cells purified from *in vivo* antibody treated animals were evaluated for T-bet (top panel) and Eomes (bottom panel) after 6h *in vitro* stimulation with agonistic CD40 or control antibody. The data represent 2 experiments with at least 4 mice per group. Error bars represent standard deviation throughout.


A
Absolute number of CD8+ T cells
Spleen
Brain
αPD-L1
αCD40L + αPD-L1
αCD40L
Isotype
p=0.0002
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p<0.0001
3.0
6.5
10.0
x10^6
0.0
0.5
1.0
2.0
x10^4
B
Gated on CD8+ T cells
Spleen
Brain
Isotype
αCD40L
αCD40L+ αPD-L1
αPD-L1
% of Maximum
CD40
C
Gated on splenic CD8+ T cells
Isotype
αCD40L
αCD40L + αPD-L1
αPD-L1
CD8
17.4
20.2
17.6
33.3
Ki-67
D
Gated on CD8+ T cells
Isotype
αCD40L
αCD40L + αPD-L1
αPD-L1
Spleen
Brain
IFNγ
3.4
3.9
26.9
2.5
3.9
34.5
3.0
3.7
36.9
4.2
11.5
48.3
8.0
3.6
28.5
7.6
2.8
30.9
7.2
2.0
26.7
8.1
9.6
44.4
GranzymeB
E
Isotype
αCD40
p<0.05
Tbet+ CD8+ (%)
36
24
12
0
Eomes+ CD8+(%)
48
36
24
12
Isotype
αCD40L
αCD40L + αPD-L1
αPD-L1

6.2.3 CD8 intrinsic CD40 signaling is important for optimal rescue of exhausted CD8 T cells

The above data suggest that CD40 signaling directly on CD8 T cells is capable of modulating T-bet expression. However, it does not address if T cell intrinsic CD40-CD40L interaction *in vivo* can elicit a similar effect. Moreover, it does not rule out the possibility of CD40 expression on other cell types via changes in micro-environment such as cytokines or antigen burden, affecting CD8 T cells. To address the CD8 intrinsic role of CD40 during rescue of exhausted CD8 T cells *in vivo*, we generated bone marrow (BM) chimeras by injecting similar number of bone marrow cells from CD90.1 (WT) and CD90.2$^{+/+}$CD40$^{-/-}$ (KO) into lethally irradiated CD45.1 WT recipients (Fig. 23A). After reconstitution, the mixed bone BM chimeras were infected with *T. gondii*, treated with αPD-L1 for 2 weeks (weeks 5-7) and then analyzed at week 7 pi (Fig. 23*A*). In control antibody treated animals, WT and KO CD8 T cells were present in ~ 1:1 ratio and no difference in PD-1 expression was noted (Fig. 23*B,C* and *D*). However, after anti PD-L1 treatment this ratio was altered to 2:1 in both spleen and brain, suggesting that CD40 plays a CD8 intrinsic role in mediating their expansion during the rescue process (Fig. 3*B,C*). To further investigate if differential expansion of WT CD8 T cells in αPD-L1 treated chimeras was due to increased proliferation of these cells, we performed intracellular staining for Ki-67. As shown in Fig. 23*E* and *F*, irrespective of CD40 expression αPD-L1 treated chimeras showed greater CD8 T cell proliferation than controls. However, while CD40 deficiency minimally affected proliferation of CD8 T cells in control chimeras, it had a more pronounced effect in αPD-L1 treated chimeras. Next, we measured apoptosis, by examining active caspase3 expression. Regardless of CD40 deficiency, αPD-L1 treatment reduced CD8 apoptosis

(Fig. 23*G*,*H*). Nevertheless, WT CD8 T cells were moderately more apoptotic than KO cells in both control and treated chimeras (Fig. 3*G*,*H*). Combined, this suggests that proliferation is the major mechanism responsible for preponderance of WT CD8 T cells in αPD-L1 treated chimeras. Next we examined if CD40 played a CD8 intrinsic role on effector functions of reinvigorated CD8 T cells. Irrespective of αPD-L1 treatment or CD40 deficiency, minimal IL-2 production was noted in CD8 T cells (data not shown). WT or KO CD8 T cells produced similar levels of IFNγ, Gzb and TNFα in control chimeras in both spleen and brain (Fig. 23*I*). While αPD-L1 treatment augmented these molecules in both WT and KO CD8 T cells, the latter exhibited far lower levels of Gzb and especially IFNγ and TNFα (Fig. 23*I*). Moreover, the percentage of both bifunctional and trifunctional CD8 T cells was highly depressed in KO CD8 T cells in treated animals (Fig. 23*J*). Finally we examined the transcriptional profile of CD8 T cells. Irrespective of CD40 deficiency similar levels of Eomes were noted in CD8 T cells in αPD-L1 treated chimeras (Fig. 24*A*,*B*). In contrast, αPD-L1 treatment augmented T-bet preferentially in WT CD8 T cells (Fig. 24*C*,*D*). Considering that Eomes has a critical role in mediating cytotoxicity, this may explain why KO CD8 T cells in αPD-L1 treated chimeras have unaffected single Gzb^+ CD8 T cells but yet have attenuated IFNγ and TNFα production (136). Together, the data suggest that during rescue of exhausted CD8 T cells, CD40 plays a CD8 intrinsic role in mediating proliferation and effector functions of CD8 T cells. However unlike αCD40L treatment, CD40 deficiency on CD8 T cells in a CD40 sufficient environment does not entirely abrogate the ameliorative effects of αPD-L1, suggesting that that CD40 has an important CD8 extrinsic role as well.

Figure 23. CD40 plays a CD8 intrinsic role during rescue of exhausted CD8 T cells. *A*, Chronically infected mixed BM chimeras were treated with αPD-L1 for 2 weeks and analyzed at week 7 pi. *B* and *C*, Relative abundance of WT (CD90.1) and KO (CD90.2) CD8 T cells evaluated in spleen and brain by flow cytometry is presented as representative flow plots (*B*) or bar graphs (*C*). *D*, represents frequency of WT and KO splenic CD8 T cells in control chimeras expressing PD-1. *E* and *F*, The percentage of proliferating congenic splenic CD8 T cells was assessed by intracellular staining for Ki-67. Flow plots in red denote KO CD8 T cells throughout. Data is presented as histograms (*E*) or bar graphs (*F*). *G* and *H*, Histograms (*G*) and bar graphs (*H*) depict active caspase expressing CD8 T cells, evaluated after incubating splenocytes from chimeras at 37°C for 5h. *I*, IFNγ, Gzb and TNFα production by congenic CD8 T cells were evaluated in splenocytes or brain cells from infected mice in presence of TLA. J, represents the percentage of bifunctional (top panel) and trifunctional (bottom panel) CD8 T cells in spleen and brain. Results are representative of 2 experiments with at least 3 mice per group.

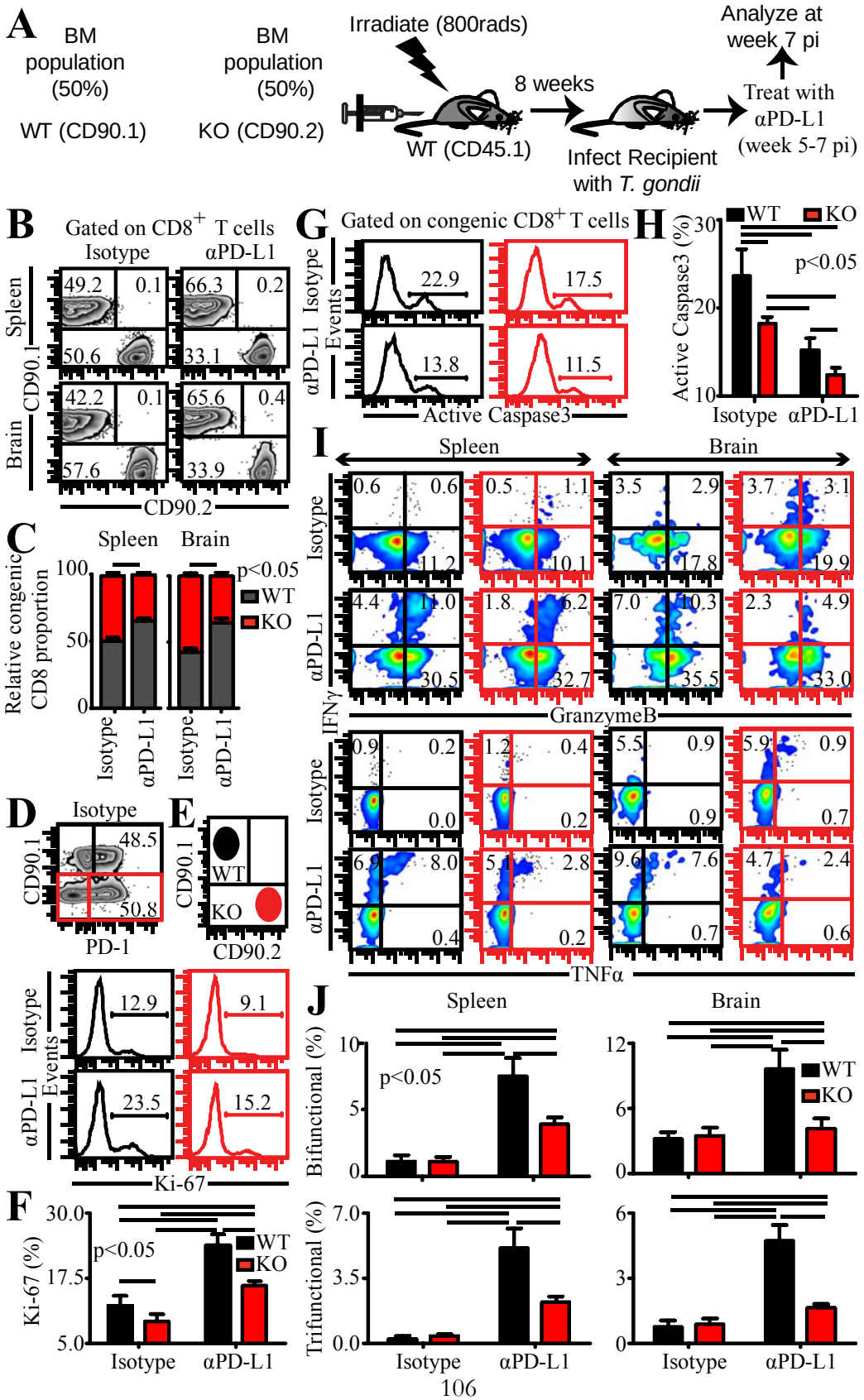
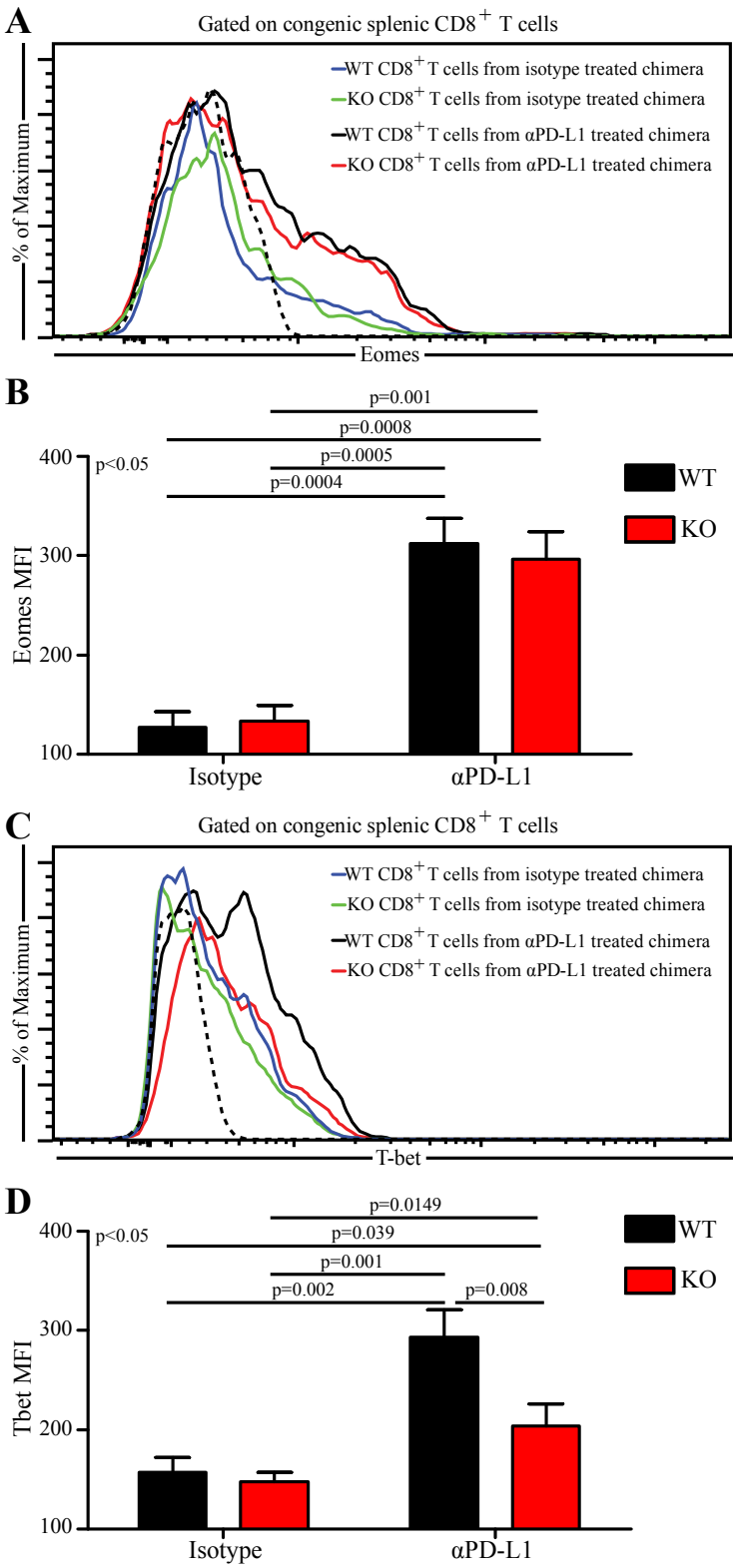

A
BM population (50%)
WT (CD90.1)
BM population (50%)
KO (CD90.2)
Irradiate (800rads)
WT (CD45.1)
8 weeks
Infect Recipient with T. gondii
Treat with αPD-L1 (week 5-7 pi)
Analyze at week 7 pi
B
Gated on CD8+ T cells
Isotype
αPD-L1
Spleen
Brain
CD90.1
CD90.2
49.2
0.1
50.6
66.3
0.2
33.1
42.2
0.1
57.6
65.6
0.4
33.9
C
Spleen
Brain
Relative congenic CD8 proportion
p<0.05
WT
KO
100
50
0
Isotype
αPD-L1
D
Isotype
CD90.1
PD-1
48.5
50.8
E
CD90.1
CD90.2
WT
KO
Isotype
αPD-L1
Events
12.9
9.1
23.5
15.2
Ki-67
F
Ki-67 (%)
30.0
17.5
5.0
p<0.05
WT
KO
Isotype
αPD-L1
G
Gated on congenic CD8+ T cells
Isotype
αPD-L1
Events
22.9
17.5
13.8
11.5
Active Caspase3
H
WT
KO
Active Caspase3 (%)
30
20
10
p<0.05
Isotype
αPD-L1
I
Spleen
Brain
Isotype
αPD-L1
IFNγ
GranzymeB
TNFα
0.6
0.6
11.2
0.5
1.1
10.1
3.5
2.9
17.8
3.7
3.1
19.9
4.4
11.0
30.5
1.8
6.2
32.7
7.0
10.3
35.5
2.3
4.9
33.0
0.9
0.2
0.0
1.2
0.4
0.2
5.5
0.9
0.9
5.9
0.9
0.7
6.9
8.0
0.4
5.1
2.8
0.2
9.6
7.6
0.7
4.7
2.4
0.6
J
Spleen
Brain
Bifunctional (%)
10
5
0
p<0.05
12
6
0
WT
KO
Trifunctional (%)
7.0
3.5
0.0
6
3
0
Isotype
αPD-L1

Figure 24. CD40 plays a CD8 intrinsic role in mediating optimal upregulation of T-bet but not Eomes during rescue of exhausted CD8 T cells. Eomes (A,B) and T-bet (C,D) expression evaluated by direct ex vivo intracellular staining of congenic splenic CD8 T cells from mixed marrow chimeras is presented as histograms (A,C) or bar graphs (B,D). The data represent 2 experiments with at least 3 mice per group.


A
Gated on congenic splenic CD8+ T cells
WT CD8+ T cells from isotype treated chimera
KO CD8+ T cells from isotype treated chimera
WT CD8+ T cells from αPD-L1 treated chimera
KO CD8+ T cells from αPD-L1 treated chimera
% of Maximum
Eomes
B
p=0.001
p=0.0008
p=0.0005
p=0.0004
p<0.05
400
300
200
100
Eomes MFI
WT
KO
Isotype
αPD-L1
C
Gated on congenic splenic CD8+ T cells
WT CD8+ T cells from isotype treated chimera
KO CD8+ T cells from isotype treated chimera
WT CD8+ T cells from αPD-L1 treated chimera
KO CD8+ T cells from αPD-L1 treated chimera
% of Maximum
T-bet
D
p=0.0149
p=0.039
p=0.001
p=0.002
p=0.008
p<0.05
400
300
200
100
Tbet MFI
WT
KO
Isotype
αPD-L1

6.2.4 CD40 plays an important role in mediating IL-21 and IL-21R expression.

A previous study has demonstrated that CD40 stimulation upregulates IL-21R expression on chronic lymphocytic leukemia B cells (200). Considering that IL-21-IL-21R pathway has been shown to be important for alleviating CD8 exhaustion in chronic viral models, we hypothesized that increased CD40 expression on CD8 T cells in αPD-L1 treated mice would result in upregulation of IL-21R on CD8 T cells (89). In agreement with our hypothesis, CD40 expressing splenic and brain CD8 T cells in αPD-L1 treated WT animals, expressed higher IL-21R levels than $CD40^-$ population (Fig. 25*A*). To further examine, if augmented IL-21R expression was due to CD8 intrinsic CD40 signaling, splenic CD8 T cells were evaluated in antibody treated mixed BM (WT:KO) chimeras. As expected, αPD-L1 treatment substantially increased IL-21R levels only on WT CD8 T cells in the chimera (Fig. 25*B*,*C*). Irrespective of αPD-L1 treatment, minimal IL-21R expression was noted in KO CD8 T cells in both groups of chimera (Fig. 25*B*,*C*). Similar trends were also noted for CD4 T cells (Fig. 25*D*,*E*). This suggests that CD40 plays a strictly T cell intrinsic role in mediating IL-21R expression. Since IL-21 can also act in an autocrine fashion, we postulated that reduced IL-21R expression would lead to lower IL-21 production (201). As shown in Fig. 25*F* and *G*, WT CD4 T cells from αPD-L1 treated chimeras produced substantially more IL-21 than control chimeras. However, KO CD4 T cells irrespective of αPD-L1 treatment produced minimal IL-21. Examination T follicular helper (Tfh) cells (as defined by the following markers: CXCR5, PD-1 and Bcl-6), which are considered to be the major producers of IL-21, revealed similar trends (Fig. 26*A*-*C*) (202). Combined, this suggests that CD40 signaling plays a critical T cell intrinsic role in mediating IL-21-IL-21R signaling.

Figure 25. CD40 plays a strictly T cell intrinsic role in mediating IL-21 and IL-21R expression on T cells. *A*, IL-21R expression was measured in $CD40^+$ and $CD40^-$ splenic and brain CD8 T cells from αPD-L1 treated C57BL/6 mice. Congenic splenic CD8 (*B* and *C*) and CD4 (*D* and *E*) T cells in control and αPD-L1 treated BM chimeras were assessed for IL-21R expression. Data is presented as histograms (*B* and *D*) or bar graphs (*C* and *E*). *F* and *G*, Flow plots (*F*) and bar graphs (*G*) depict the percentage of IL-21 producing splenic CD4 T cells in these chimeras. The data represent 2 experiments with 3 mice per group.

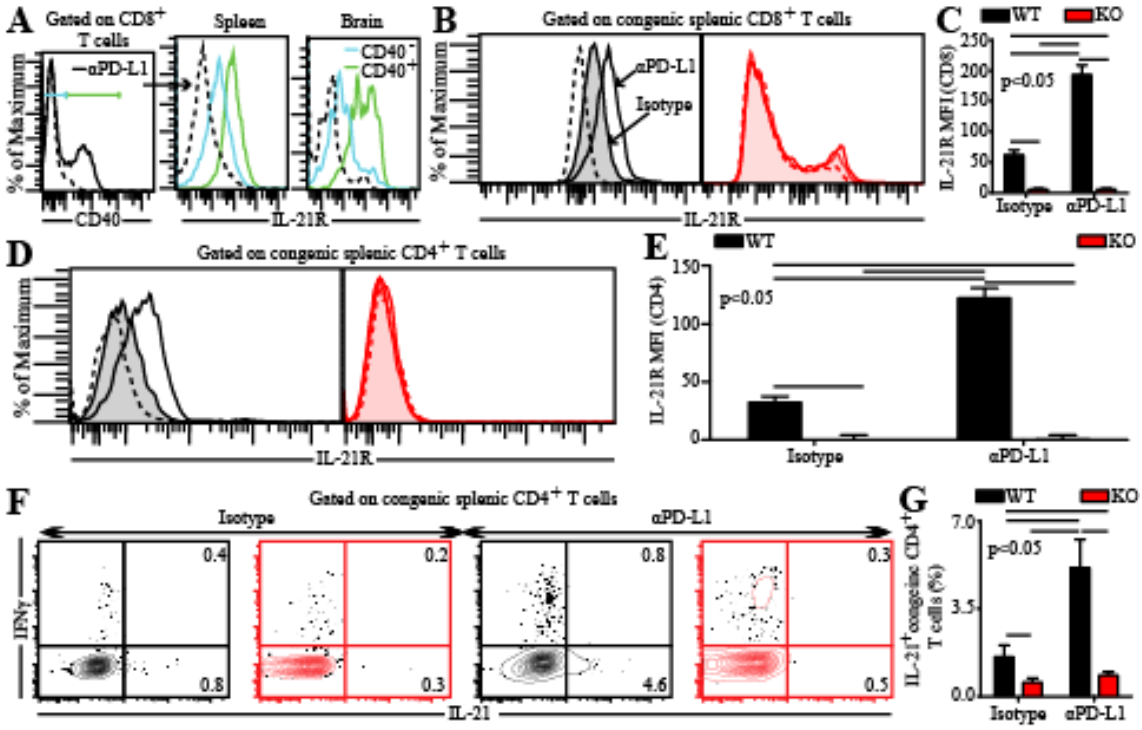
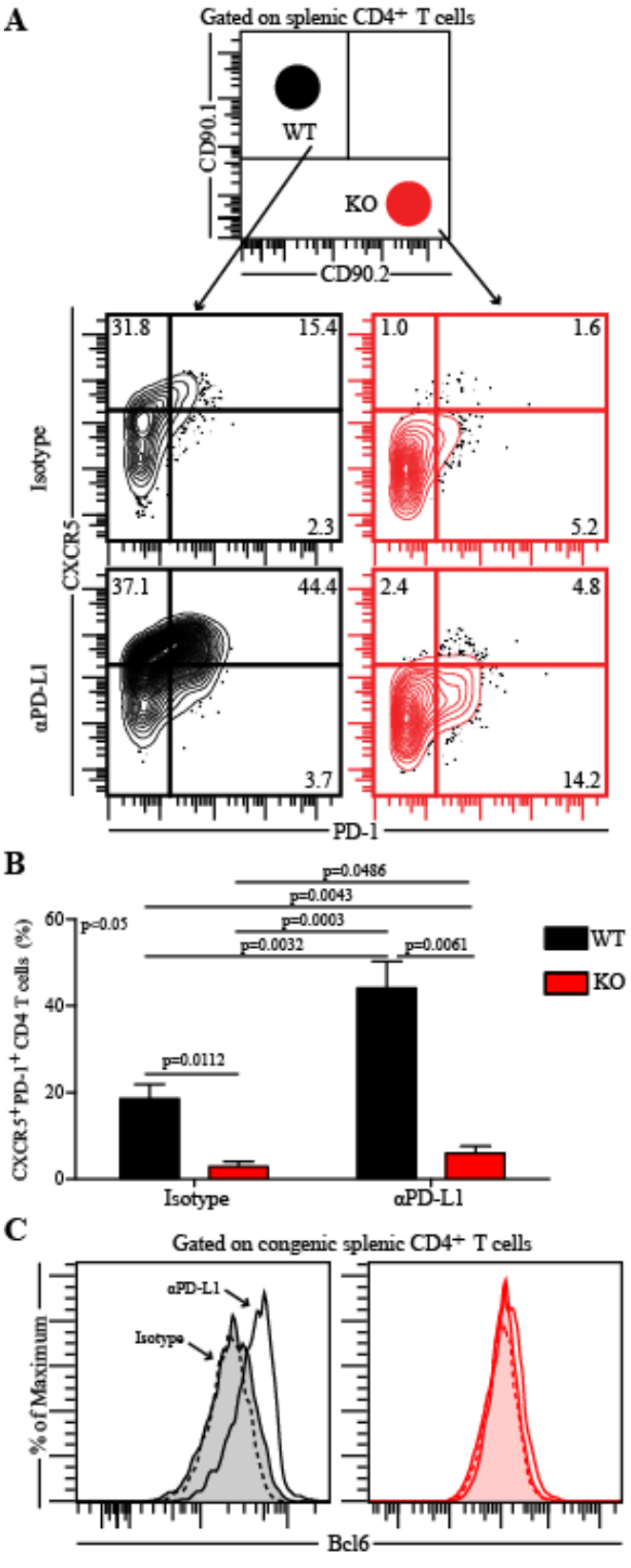

A
Gated on CD8+ T cells
Spleen
Brain
αPD-L1
CD40-
CD40+
% of Maximum
CD40
IL-21R
B
Gated on congenic splenic CD8+ T cells
αPD-L1
Isotype
% of Maximum
IL-21R
C
WT
KO
IL-21R MFI (CD8)
p<0.05
Isotype
αPD-L1
D
Gated on congenic splenic CD4+ T cells
% of Maximum
IL-21R
E
WT
KO
IL-21R MFI (CD4)
p<0.05
Isotype
αPD-L1
F
Gated on congenic splenic CD4+ T cells
Isotype
αPD-L1
IFNγ
0.4
0.8
0.2
0.3
0.8
4.6
0.3
0.5
IL-21
G
WT
KO
IL-21+ congenic CD4+ T cells (%)
p<0.05
Isotype
αPD-L1

Figure 26. **CD4 intrinsic CD40 signaling is critical for upregulation of T follicular helper cell markers during rescue of exhausted CD8 T cells.** Tfh markers CXCR5 and PD-1 (A,B) and Bcl-6 (C) were evaluated in congenic splenic CD4 T cells from BM chimeras by flow cytometry. Data are presented as flow plots (A,C) or bar graphs (B). Results are representative of 2 experiments with 3 mice per group.


A
Gated on splenic CD4+ T cells
CD90.1
WT
KO
CD90.2
31.8
15.4
2.3
1.0
1.6
5.2
37.1
44.4
3.7
2.4
4.8
14.2
Isotype
αPD-L1
CXCR5
PD-1
B
p=0.0486
p=0.0043
p=0.0003
p=0.0032
p=0.0061
p<0.05
p=0.0112
WT
KO
CXCR5+PD-1+ CD4 T cells (%)
60
40
20
0
Isotype
αPD-L1
C
Gated on congenic splenic CD4+ T cells
αPD-L1
Isotype
% of Maximum
Bcl6

Chapter 7: DISCUSSION

$CD8^+$ T cell exhaustion has been reported in several chronic viral infections like LCMV, HBV, HCV, HIV and SIV which are characterized by high levels of persisting viremia (203). This exhaustion is manifested by gradual loss of $CD8^+$ T cell effector functions (cytokine production, cytotoxicity, proliferation and recall responses) and in extreme situations $CD8^+$ T cells can be physically deleted (204). In contrast, in infection models like MCMV characterized by low levels of persistent viremia, antigen-specific T cells remain functional and respond vigorously to viral challenge (203, 205). In models of reactivation like HSV that induce latent infection punctuated by periods of reactivation, functional long lived $CD8^+$ T cell response is generated (206). Based on these findings, it has been suggested that $CD8^+$ T cell exhaustion occurs only in presence of uncontrolled persistent viremia (105, 203, 207, 208). The data presented here documents that $CD8^+$ T cell dysfunction is not restricted to viral infections alone but can be extended to intracellular protozoan parasite like *T. gondii*. Although $CD8^+$ T cell dysfunction observed in the current study is similar in certain facets to certain viral pathogens, in its dependence on PD-1- PDL-1 interaction, it discounts the conventional paradigm that $CD8^+$ T cell exhaustion occurs only in infectious disease models with uncontrolled persistent pathogen load. Moreover, notwithstanding the fact that the role of PD-1-PDL-1 pathway has been addressed in chronic viral models of $CD8^+$ T cell exhaustion, its significance in bacterial or parasitic models of $CD8^+$ T cell dysfunction has not been extensively investigated.

Our studies demonstrate that despite a potent development of $CD8^+$ T cell immunity during the early stages of chronic infection, the endogenous $CD8^+$ T cell response is unable

to control parasite reactivation at the later time points. This is in agreement with a previous study by Gazzinelli *et al* which demonstrated that parasite reactivation in B6 mice during late-chronic phases is associated with increased expression of tachyzoite specific genes in brain (5). Additionally, there is increase in parasite levels in the periphery. A recent study has demonstrated the $CD11b^+$ leukocytes can act as shuttles for parasite migration during the acute infection phase (172). Hence it would be interesting to investigate in future studies whether during parasite reactivation, $CD11b^+$ leukocytes which are preferentially parasitized during the late-chronic phase potentially serve a similar function and its role in mediating disease pathogenesis. Cumulatively, our results show that long-term exposure to a persistent pathogen despite initial control of parasitemia can lead to $CD8^+$ T cell dysfunction and parasite reactivation. This CD8 exhaustion with concomitant PD-1 upregulation is characterized by reduced effector cell number, decreased polyfunctionality, increased apoptosis and poor recall response and expansion. Preliminary studies using drug treatment suggest that control of infection during the early but not later phases is critical for minimizing PD-1 upregulation and CD8 dysfunction. This suggests that high antigen burden or inflammation (including anti-inflammatory cytokines) or both that occurs during acute phase of infection may be primarily responsible for defective development of a long-lived robust CD8 response during the chronic phase (203, 209). Based on a recent study by Chang *et al* demonstrating that T cell fate imprinting can occur as early as the first cell division, it is hardly surprising that T cell microenvironment (cytokine, antigen burden etc.) during the early phase of Toxoplasma infection can have such a profound effect during the later phases (178). Our findings are further supported by a study in LCMV model where antigen burden has been shown to be the key determinant for development of CD8

dysfunction (203). However any control of infection or antigen burden can potentially affect inflammation. Hence in future studies it will be critical to discriminate the differential role of inflammatory milieu and antigen burden in mediating CD8 dysfunction. Recent reports have identified role of IL-10 in mediating dysfunctional $CD8^+$ T cell response during LCMV infection (210). However, anti-IL10R treatment of mice carrying chronic Toxoplasma infection led to their increased mortality due to strong inflammatory response (92). This suggests that any strategy for preventing Toxoplasma reactivation must involve an appropriate balance between enhanced pro-inflammatory mediators without excessive suppression of anti-inflammatory factors. Interestingly, anti-PDL-1 treatment not only augmented the $CD8^+$ T cell response but also prevented mortality in chronically infected mice by controlling parasite reactivation.

How blockade of PD-1-PDL-1 pathway modulates $CD8^+$ T cell response at the molecular level is poorly understood in infectious disease models. Recent studies have shown that differential TCR signal strength can affect not only memory CD8 programming but also development of a potent polyfunctional CD8 response (145, 211). Considering that PD-1 ligation has been shown to inhibit TCR signaling via downregulation of ZAP-70 and PKCθ *in vitro*, it is possible that anti-PDL-1 treatment restores robust CD8 response during chronic Toxoplasma infection via increased TCR sensitivity (212). Although the mechanistic basis of CD8 dysfunction and PD-1 up-regulation need to be investigated more thoroughly in future studies, the data presented in this study provide significant insights into the role of PD-1-PDL-1 pathway in mediating $CD8^+$ T cell dysfunction and concomitant parasite reactivation. These results are especially significant in view of a minimal role played by CTLA-4, a related inhibitory molecule in regulating T cell response

during TE (49). Our findings have important implications in the use of PD-1-PDL-1 blockade in combination with drug regimen for controlling TE in immuno-compromised patients as well as use of similar therapy in other dysfunction induced reactivation type diseases.

Recent studies, primarily in viral models have established T cell polyfunctionality as a critical predictor of efficacious antiviral immune response and vaccine potency (213, 214). However, role of polyfunctional $CD8^+$ T cells in parasitic or bacterial infection models has not been thoroughly investigated. Unlike chronic viral models, in Mycobacterial infection, disease progression is not associated with contraction of polyfunctional T cells (215). This makes a pathogen specific study of polyfunctionality and host morbidity and mortality critical for determining whether gain or loss of polyfunctionality is linked to pathogen clearance. In contrast, in Toxoplasma model loss of polyfunctional $CD8^+$ T cells is strongly associated with PD-1 mediated $CD8^+$ T cell exhaustion and recrudescence of disease. Moreover anti PDL-1 treatment not only upregulated polyfunctional $CD8^+$ T cell response but also prevented parasite reactivation. Combined, this creates a strong basis for investigating how PD-1 mediates preferential attrition of polyfunctional $CD8^+$ T cells during chronic Toxoplasmosis.

We demonstrate for the first time that during chronic toxoplasmosis majority of the polyfunctional $CD8^+$ T cells exhibit a memory phenotype. Although PD-1 expressing memory phenotype polyfunctional $CD8^+$ T cells were preferentially apoptotic, paradoxically, the proportion of memory $CD8^+$ T cells at the effector site (brain) was not dramatically altered. Incidentally, in chronic infection models there is a continuous recruitment of naïve T cells which contributes to the pathogen specific T cell response

(216). Based on our observation in *T. gondii* model that frequency of memory $CD8^+$ T cell is not dramatically affected yet they are preferentially apoptotic, it is tempting to speculate that naïve T cells recruited during the chronic phase preferentially differentiate to memory precursor effector cells. This hypothesis is further supported by the observation that inflammatory cytokines are reduced during chronic Toxoplasmosis. Incidentally reduced inflammation, as shown by Kaech lab (217), results in preferential differentiation to memory precursor effector $CD8^+$ T cells. Hence in future studies it will be important to address this issue using adoptive T cells transfer approaches and partial bone-marrow chimeras.

However, the above hypothesis does not explain why the absolute numbers of both effector and memory $CD8^+$ T cells are drastically reduced during chronic Toxoplasmosis. One explanation of this could be reduced thymic output during chronic toxoplasmosis. While thymic atrophy has been reported in the Toxoplasma model, its role on $CD8^+$ T cell response development and disease pathogensis has not be extensively studied (218). Apart from thymic atrophy, decreased effector or memory T cell number could be a consequence of attenuated naïve T cell priming. Since, PDL-1, the receptor of PD-1 is also highly expressed during late-chronic Toxoplasmosis (184), another explanation is PDL-1 expression on antigen presenting cells (APC) downregulating their priming ability (costimulation, cytokines). Apart from aforementioned effects on recent thymic emigrants, the possibility of anti-apoptotic cytokines like IL-15 (primarily produced by APC) (219) directly affecting base-line survival of pre-existing memory and effector cells independent of CD8 intrinsic PD-1 expression need to be considered in future studies

One of the hallmarks of robust $CD8^+$ T cell immunity is the capacity to mount a potent recall response (50). As demonstrated in this work, $CD8^+$ T cells during the later stages of chronic infection are deficient in this regard (184). Interestingly, $CD43^{hi}$ memory $CD8^+$ T cells which constitute the dominant memory phenotype $CD8^+$ T cell population during late-chronic Toxoplasmosis, has been shown to elicit poor recall response in Sendai virus model (220). Incidentally CD43 has been reported to decrease expression of Bcl-2, an anti-apoptotic molecule, on $CD8^+$ T cells during LCMV infection (221). During chronic toxoplasmosis, since majority of PD-1 expressing memory phenotype $CD8^+$ T cells co-express high levels of CD43, the contribution of this molecule towards apoptosis of exhausted $CD8^+$ T cells needs to be addressed in future investigations.

Since PD-1 was preferentially expressed on memory phenotype $CD8^+$ T cells during chronic Toxoplasmosis, we next investigated if PD-1 expressing cells in this subset were polyfunctional or monofunctional. While PD-1 expression on memory $CD8^+$ T cells did not have a one to one correlation with levels of IFNγ and GranzymeB expression, these cells (PD-1 expressing memory $CD8^+$ T cells) were indeed more apoptotic. Although high levels of apoptosis were also noted for polyfunctional effector $CD8^+$ T cells, this subset exhibited minimal PD-1 expression. This suggests that apart from PD-1, other PD-1 independent apoptosis inducing mechanisms are at play here. Considering that exhaustion in viral models has been associated with upregulation of multiple inhibitory receptors, the role of molecules such Tim-3, 2B4, CTLA-4, Lag3, CD160 and others need to be investigated for their role in inducing apoptosis of polyfunctional $CD8^+$ T cells (61). Although $CD8^+CD44^{int/hi}CD127^{hi}IFN\gamma^+GranzymeB^-$ memory phenotype cells exhibited comparatively lower proportions of $PD\text{-}1^+ActiveCaspase3^+$ cells, *in vitro* anti PDL-1

treatment resulted in decrease of apoptosis in this subset similar to polyfunctional memory $CD8^+$T cells. Paradoxically, despite considerable expression of both PD-1 and active caspase-3, $CD8^+CD44^{int/hi}CD127^{hi}IFN\gamma^-GranzymeB^+$ subset remained refractory to the anti-apoptotic effects of *in vitro* anti PDL-1 treatment. As mentioned earlier, this may be due to co-expression of other inhibitory receptors which may dampen the ameliorative effects of anti-PDL-1. Alternatively, this subset may require prolonged anti-PDL-1 treatment for reducing its apoptotic potential. These possibilities may not be mutually exclusive.

Reinvigoration of $CD8^+$ T cell response via anti PDL-1 administration has profound significance for treatment of tumors and chronic infections such as HBV, HCV, HIV (197, 222, 223) . Development of a robust polyfunctional T cell response is considered of one the hallmarks of potent anti-pathogen immunity. However few reports have addressed the mechanistic basis for the development of polyfunctional response. A recent study has demonstrated that strong antigen stimulation results in development of polyfunctional T cells response (224). Yet, in chronic infections where not only high antigen burden is present but also elevated levels of $CD8^+$ T cell expressed PD-1, polyfunctionality is lost. However how PD-1-PDL-1 pathway causes attrition of this polyfunctional response in chronic infections has not been investigated before. The current study strongly suggests that not withstanding the per cell decrease in function, PD-1 expression on polyfunctional memory $CD8^+$ T cells strongly pre-disposes them to apoptosis. Significantly, transient *in vitro* blockade of PD-1-PDL-1 pathway is potent enough to reduce apoptosis of this important subset. In summary, the conclusions of this present report have profound

implications for immunotherapeutic regimens targeting polyfunctional CD8 T cell response against chronic infections.

One of the other highlights of the current work is the critical role of positive signals from a co-stimulatory molecule in mediating rescue of exhausted CD8 T cells. We show for the first time in an infectious disease model that CD40 plays an important CD8 intrinsic role in mediating CD8 T cell proliferation and poly-functionality, albeit in the context of reinvigoration of exhausted CD8 T cells. As mentioned earlier αPD-L1 therapy preferentially rescues pre-existing PD-1 expressing CD8 T cells in chronically infected animals, suggesting that differential recruitment of naïve T cells into antigen-specific pool plays at best a minor role during the rescue process (184). Based on this, it is tempting to speculate that CD40-CD40L pathway is especially important for this subset. However, further investigation is needed to definitively discriminate the effects of CD40-CD40L signaling on pre-existing CD8 T cells vs. new naïve cells entering the antigen specific pool during chronic toxoplasmosis. Another important question that needs to be addressed in future studies is, how CD8 intrinsic CD40 signaling affects CD8 T cell function. While preliminary data suggests that T-bet may be involved in this process, it is likely that other transcription factors and cytokines such as IL-21 may be involved as well. Moreover the role of molecules directly involved in CD40 signal transduction such as TRAF6, which has been shown to be critical for memory CD8 development in the Listeria model (an acute infection model), needs to be investigated in future studies of CD8 exhaustion (225). Another important and novel finding of the current work is the demonstration that CD40 plays a strictly T cell intrinsic role in mediating up-regulation of IL-21 and its receptor. As mentioned earlier, IL-21-IL21R signaling has been shown to play an important role in

preventing CD8 exhaustion (89). In future studies, it will be important to discriminate between IL-21 dependent and independent role of CD8 intrinsic CD40 signaling during the rescue of exhausted CD8 T cells. The current findings complemented with a better understanding of the CD40-IL-21 axis in mediating dysfunctional CD8 T cell rescue will lead to more efficacious therapeutic vaccination strategies against tumors and chronic infections.

REFERENCES

1. Dubey, J. P. 1998. Advances in the life cycle of Toxoplasma gondii. *Int J Parasitol* 28:1019-1024.

2. Wallon, M., C. Liou, P. Garner, and F. Peyron. 1999. Congenital toxoplasmosis: systematic review of evidence of efficacy of treatment in pregnancy. *Bmj* 318:1511-1514.

3. Lyons, R. E., R. McLeod, and C. W. Roberts. 2002. Toxoplasma gondii tachyzoite-bradyzoite interconversion. *Trends Parasitol* 18:198-201.

4. Matrajt, M., R. G. Donald, U. Singh, and D. S. Roos. 2002. Identification and characterization of differentiation mutants in the protozoan parasite Toxoplasma gondii. *Mol Microbiol* 44:735-747.

5. Gazzinelli, R. T., I. Eltoum, T. A. Wynn, and A. Sher. 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol* 151:3672-3681.

6. Vidal, J. E., A. V. Hernandez, A. C. de Oliveira, R. F. Dauar, S. P. Barbosa, Jr., and R. Focaccia. 2005. Cerebral toxoplasmosis in HIV-positive patients in Brazil: clinical features and predictors of treatment response in the HAART era. *AIDS Patient Care STDS* 19:626-634.

7. Sibley, L. D., A. Khan, J. W. Ajioka, and B. M. Rosenthal. 2009. Genetic diversity of Toxoplasma gondii in animals and humans. *Philos Trans R Soc Lond B Biol Sci* 364:2749-2761.

8. Carruthers, V. B., and Y. Suzuki. 2007. Effects of Toxoplasma gondii infection on the brain. *Schizophr Bull* 33:745-751.

9. Gigley, J. P., B. A. Fox, and D. J. Bzik. 2009. Cell-mediated immunity to Toxoplasma gondii develops primarily by local Th1 host immune responses in the absence of parasite replication. *J Immunol* 182:1069-1078.

10. Tan, T. G., E. Mui, H. Cong, W. H. Witola, A. Montpetit, S. P. Muench, J. Sidney, J. Alexander, A. Sette, M. E. Grigg, A. Maewal, and R. McLeod. 2010. Identification of T. gondii epitopes, adjuvants, and host genetic factors that influence protection of mice and humans. *Vaccine* 28:3977-3989.

11. Escajadillo, A., and J. K. Frenkel. 1991. Experimental toxoplasmosis and vaccine tests in Aotus monkeys. *Am J Trop Med Hyg* 44:382-389.

12. Jongert, E., C. W. Roberts, N. Gargano, E. Forster-Waldl, and E. Petersen. 2009. Vaccines against Toxoplasma gondii: challenges and opportunities. *Mem Inst Oswaldo Cruz* 104:252-266.

13. Kaufmann, S. H. 1999. Cell-mediated immunity: dealing a direct blow to pathogens. *Curr Biol* 9:R97-99.

14. Thorne, K. J., and J. M. Blackwell. 1983. Cell-mediated killing of protozoa. *Adv Parasitol* 22:43-151.

15. Suzuki, Y., M. A. Orellana, R. D. Schreiber, and J. S. Remington. 1988. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science* 240:516-518.

16. Khan, I. A., J. D. Schwartzman, T. Matsuura, and L. H. Kasper. 1997. A dichotomous role for nitric oxide during acute Toxoplasma gondii infection in mice. *Proc Natl Acad Sci U S A* 94:13955-13960.

17. Hunter, C. A., L. Ellis-Neyer, K. E. Gabriel, M. K. Kennedy, K. H. Grabstein, P. S. Linsley, and J. S. Remington. 1997. The role of the CD28/B7 interaction in the regulation of NK cell responses during infection with Toxoplasma gondii. *J Immunol* 158:2285-2293.

18. Khan, I. A., and L. H. Kasper. 1996. IL-15 augments CD8+ T cell-mediated immunity against Toxoplasma gondii infection in mice. *J Immunol* 157:2103-2108.

19. Aliberti, J., D. Jankovic, and A. Sher. 2004. Turning it on and off: regulation of dendritic cell function in Toxoplasma gondii infection. *Immunol Rev* 201:26-34.

20. Liu, C. H., Y. T. Fan, A. Dias, L. Esper, R. A. Corn, A. Bafica, F. S. Machado, and J. Aliberti. 2006. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice. *J Immunol* 177:31-35.

21. Khan, I. A., T. Matsuura, and L. H. Kasper. 1994. Interleukin-12 enhances murine survival against acute toxoplasmosis. *Infect Immun* 62:1639-1642.

22. van Lier, R. A., I. J. ten Berge, and L. E. Gamadia. 2003. Human CD8(+) T-cell differentiation in response to viruses. *Nat Rev Immunol* 3:931-939.

23. Frenkel, J. K. 1967. Adoptive immunity to intracellular infection. *J Immunol* 98:1309-1319.

24. Suzuki, Y., and J. S. Remington. 1988. Dual regulation of resistance against Toxoplasma gondii infection by Lyt-2+ and Lyt-1+, L3T4+ T cells in mice. *J Immunol* 140:3943-3946.

25. Khan, I. A., K. A. Smith, and L. H. Kasper. 1988. Induction of antigen-specific parasiticidal cytotoxic T cell splenocytes by a major membrane protein (P30) of Toxoplasma gondii. *J Immunol* 141:3600-3605.

26. Suzuki, Y., and J. S. Remington. 1990. The effect of anti-IFN-gamma antibody on the protective effect of Lyt-2+ immune T cells against toxoplasmosis in mice. *J Immunol* 144:1954-1956.

27. Casciotti, L., K. H. Ely, M. E. Williams, and I. A. Khan. 2002. CD8(+)-T-cell immunity against Toxoplasma gondii can be induced but not maintained in mice lacking conventional CD4(+) T cells. *Infect Immun* 70:434-443.

28. John, B., T. H. Harris, E. D. Tait, E. H. Wilson, B. Gregg, L. G. Ng, P. Mrass, D. S. Roos, F. Dzierszinski, W. Weninger, and C. A. Hunter. 2009. Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. *PLoS Pathog* 5:e1000505.

29. Araujo, F. G., D. M. Williams, F. C. Grumet, and J. S. Remington. 1976. Strain-dependent differences in murine susceptibility to toxoplasma. *Infect Immun* 13:1528-1530.

30. McLeod, R., P. Eisenhauer, D. Mack, C. Brown, G. Filice, and G. Spitalny. 1989. Immune responses associated with early survival after peroral infection with Toxoplasma gondii. *J Immunol* 142:3247-3255.

31. Deckert-Schluter, M., D. Schluter, D. Schmidt, G. Schwendemann, O. D. Wiestler, and H. Hof. 1994. Toxoplasma encephalitis in congenic B10 and BALB mice: impact of genetic factors on the immune response. *Infect Immun* 62:221-228.

32. Brown, C. R., and R. McLeod. 1990. Class I MHC genes and CD8+ T cells determine cyst number in Toxoplasma gondii infection. *J Immunol* 145:3438-3441.

33. Suzuki, Y., K. Joh, O. C. Kwon, Q. Yang, F. K. Conley, and J. S. Remington. 1994. MHC class I gene(s) in the D/L region but not the TNF-alpha gene determines development of toxoplasmic encephalitis in mice. *J Immunol* 153:4649-4654.

34. Brown, C. R., C. A. Hunter, R. G. Estes, E. Beckmann, J. Forman, C. David, J. S. Remington, and R. McLeod. 1995. Definitive identification of a gene that confers resistance against Toxoplasma cyst burden and encephalitis. *Immunology* 85:419-428.

35. Brown, C. R., C. S. David, S. J. Khare, and R. McLeod. 1994. Effects of human class I transgenes on Toxoplasma gondii cyst formation. *J Immunol* 152:4537-4541.

36. Kur, J., L. Holec-Gasior, and E. Hiszczynska-Sawicka. 2009. Current status of toxoplasmosis vaccine development. *Expert Rev Vaccines* 8:791-808.

37. Beghetto, E., H. V. Nielsen, P. Del Porto, W. Buffolano, S. Guglietta, F. Felici, E. Petersen, and N. Gargano. 2005. A combination of antigenic regions of Toxoplasma gondii microneme proteins induces protective immunity against oral infection with parasite cysts. *J Infect Dis* 191:637-645.

38. Khan, I. A., K. H. Ely, and L. H. Kasper. 1994. Antigen-specific CD8+ T cell clone protects against acute Toxoplasma gondii infection in mice. *J Immunol* 152:1856-1860.

39. Reichmann, G., H. Dlugonska, and H. G. Fischer. 2002. Characterization of TgROP9 (p36), a novel rhoptry protein of Toxoplasma gondii tachyzoites identified by T cell clone. *Mol Biochem Parasitol* 119:43-54.

40. Scorza, T., S. D'Souza, M. Laloup, J. Dewit, J. De Braekeleer, H. Verschueren, M. Vercammen, K. Huygen, and E. Jongert. 2003. A GRA1 DNA vaccine primes cytolytic CD8(+) T cells to control acute Toxoplasma gondii infection. *Infect Immun* 71:309-316.

41. Blanchard, N., F. Gonzalez, M. Schaeffer, N. T. Joncker, T. Cheng, A. J. Shastri, E. A. Robey, and N. Shastri. 2008. Immunodominant, protective response to the parasite Toxoplasma gondii requires antigen processing in the endoplasmic reticulum. *Nat Immunol* 9:937-944.

42. Frickel, E. M., N. Sahoo, J. Hopp, M. J. Gubbels, M. P. Craver, L. J. Knoll, H. L. Ploegh, and G. M. Grotenbreg. 2008. Parasite stage-specific recognition of endogenous Toxoplasma gondii-derived CD8+ T cell epitopes. *J Infect Dis* 198:1625-1633.

43. Wilson, D. C., G. M. Grotenbreg, K. Liu, Y. Zhao, E. M. Frickel, M. J. Gubbels, H. L. Ploegh, and G. S. Yap. 2010. Differential regulation of effector- and central-memory responses to Toxoplasma gondii Infection by IL-12 revealed by tracking of Tgd057-specific CD8+ T cells. *PLoS Pathog* 6:e1000815.

44. Cong, H., E. J. Mui, W. H. Witola, J. Sidney, J. Alexander, A. Sette, A. Maewal, and R. McLeod. 2010. Towards an immunosense vaccine to prevent toxoplasmosis: protective Toxoplasma gondii epitopes restricted by HLA-A*0201. *Vaccine* 29:754-762.

45. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu Rev Immunol* 25:171-192.

46. Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* 211:81-92.

47. Sharpe, A. H. 2009. Mechanisms of costimulation. *Immunol Rev* 229:5-11.

48. Subauste, C. S., R. de Waal Malefyt, and F. Fuh. 1998. Role of CD80 (B7.1) and CD86 (B7.2) in the immune response to an intracellular pathogen. *J Immunol* 160:1831-1840.

49. Reichmann, G., E. N. Villegas, L. Craig, R. Peach, and C. A. Hunter. 1999. The CD28/B7 interaction is not required for resistance to Toxoplasma gondii in the brain but contributes to the development of immunopathology. *J Immunol* 163:3354-3362.

50. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22:561-570.

51. Subauste, C. S., M. Wessendarp, R. U. Sorensen, and L. E. Leiva. 1999. CD40-CD40 ligand interaction is central to cell-mediated immunity against Toxoplasma gondii: patients with hyper IgM syndrome have a defective type 1 immune response that can be restored by soluble CD40 ligand trimer. *J Immunol* 162:6690-6700.

52. Reichmann, G., W. Walker, E. N. Villegas, L. Craig, G. Cai, J. Alexander, and C. A. Hunter. 2000. The CD40/CD40 ligand interaction is required for resistance to toxoplasmic encephalitis. *Infect Immun* 68:1312-1318.

53. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402:827-832.

54. Wilson, E. H., C. Zaph, M. Mohrs, A. Welcher, J. Siu, D. Artis, and C. A. Hunter. 2006. B7RP-1-ICOS interactions are required for optimal infection-induced expansion of CD4+ Th1 and Th2 responses. *J Immunol* 177:2365-2372.

55. Villegas, E. N., L. A. Lieberman, N. Mason, S. L. Blass, V. P. Zediak, R. Peach, T. Horan, S. Yoshinaga, and C. A. Hunter. 2002. A role for inducible costimulator protein in the CD28- independent mechanism of resistance to Toxoplasma gondii. *J Immunol* 169:937-943.

56. Harada, H., A. D. Salama, M. Sho, A. Izawa, S. E. Sandner, T. Ito, H. Akiba, H. Yagita, A. H. Sharpe, G. J. Freeman, and M. H. Sayegh. 2003. The role of the ICOS-B7h T cell costimulatory pathway in transplantation immunity. *J Clin Invest* 112:234-243.

57. Croft, M. 2005. The evolving crosstalk between co-stimulatory and co-inhibitory receptors: HVEM-BTLA. *Trends Immunol* 26:292-294.

58. Alegre, M. L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1:220-228.

59. Wilson, E. H., T. H. Harris, P. Mrass, B. John, E. D. Tait, G. F. Wu, M. Pepper, E. J. Wherry, F. Dzierzinski, D. Roos, P. G. Haydon, T. M. Laufer, W. Weninger, and C. A. Hunter. 2009. Behavior of parasite-specific effector CD8+ T cells in the brain and visualization of a kinesis-associated system of reticular fibers. *Immunity* 30:300-311.

60. Blattman, J. N., and P. D. Greenberg. 2006. PD-1 blockade: rescue from a near-death experience. *Nat Immunol* 7:227-228.

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

62. Kalia, V., S. Sarkar, and R. Ahmed. 2010. CD8 T-cell memory differentiation during acute and chronic viral infections. *Adv Exp Med Biol* 684:79-95.

63. Bliss, S. K., B. A. Butcher, and E. Y. Denkers. 2000. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *J Immunol* 165:4515-4521.

64. Yap, G. S., and A. Sher. 1999. Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-gamma- and tumor necrosis factor (TNF)-alpha-dependent host resistance to the intracellular pathogen, Toxoplasma gondii. *J Exp Med* 189:1083-1092.

65. Aliberti, J., C. Reis e Sousa, M. Schito, S. Hieny, T. Wells, G. B. Huffnagle, and A. Sher. 2000. CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells. *Nat Immunol* 1:83-87.

66. Bliss, S. K., L. C. Gavrilescu, A. Alcaraz, and E. Y. Denkers. 2001. Neutrophil depletion during Toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. *Infect Immun* 69:4898-4905.

67. Del Rio, L., S. Bennouna, J. Salinas, and E. Y. Denkers. 2001. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during Toxoplasma gondii infection. *J Immunol* 167:6503-6509.

68. Borges, J. S., and W. D. Johnson, Jr. 1975. Inhibition of multiplication of Toxoplasma gondii by human monocytes exposed to T-lymphocyte products. *J Exp Med* 141:483-496.

69. Remington, J. S., J. L. Krahenbuhl, and J. W. Mendenhall. 1972. A role for activated macrophages in resistance to infection with Toxoplasma. *Infect Immun* 6:829-834.

70. Robben, P. M., M. LaRegina, W. A. Kuziel, and L. D. Sibley. 2005. Recruitment of Gr-1+ monocytes is essential for control of acute toxoplasmosis. *J Exp Med* 201:1761-1769.

71. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher. 1993. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci U S A* 90:6115-6119.

72. Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. *J Immunol* 153:2533-2543.

73. Yap, G., M. Pesin, and A. Sher. 2000. Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. *J Immunol* 165:628-631.

74. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3:133-146.

75. Cooper, A. M., and S. A. Khader. 2007. IL-12p40: an inherently agonistic cytokine. *Trends Immunol* 28:33-38.

76. Scharton-Kersten, T., P. Caspar, A. Sher, and E. Y. Denkers. 1996. Toxoplasma gondii: evidence for interleukin-12-dependent and-independent pathways of interferon-gamma production induced by an attenuated parasite strain. *Exp Parasitol* 84:102-114.

77. Robben, P. M., D. G. Mordue, S. M. Truscott, K. Takeda, S. Akira, and L. D. Sibley. 2004. Production of IL-12 by macrophages infected with Toxoplasma gondii depends on the parasite genotype. *J Immunol* 172:3686-3694.

78. Lieberman, L. A., F. Cardillo, A. M. Owyang, D. M. Rennick, D. J. Cua, R. A. Kastelein, and C. A. Hunter. 2004. IL-23 provides a limited mechanism of resistance to acute toxoplasmosis in the absence of IL-12. *J Immunol* 173:1887-1893.

79. Mayer, K. D., K. Mohrs, W. Reiley, S. Wittmer, J. E. Kohlmeier, J. E. Pearl, A. M. Cooper, L. L. Johnson, D. L. Woodland, and M. Mohrs. 2008. Cutting edge: T-bet and IL-27R are critical for in vivo IFN-gamma production by CD8 T cells during infection. *J Immunol* 180:693-697.

80. Bhadra, R., H. Guan, and I. A. Khan. 2010. Absence of both IL-7 and IL-15 severely impairs the development of CD8 T cell response against Toxoplasma gondii. *PLoS One* 5:e10842.

81. Kasper, L. H., T. Matsuura, and I. A. Khan. 1995. IL-7 stimulates protective immunity in mice against the intracellular pathogen, Toxoplasma gondii. *J Immunol* 155:4798-4804.

82. Haque, S., I. Khan, A. Haque, and L. Kasper. 1994. Impairment of the cellular immune response in acute murine toxoplasmosis: regulation of interleukin 2 production and macrophage-mediated inhibitory effects. *Infect Immun* 62:2908-2916.

83. Candolfi, E., C. A. Hunter, and J. S. Remington. 1994. Mitogen- and antigen-specific proliferation of T cells in murine toxoplasmosis is inhibited by reactive nitrogen intermediates. *Infect Immun* 62:1995-2001.

84. Villegas, E. N., L. A. Lieberman, S. R. Carding, and C. A. Hunter. 2002. Susceptibility of interleukin-2-deficient mice to Toxoplasma gondii is associated with a defect in the production of gamma interferon. *Infect Immun* 70:4757-4761.

85. Khan, I. A., and L. Casciotti. 1999. IL-15 prolongs the duration of CD8+ T cell-mediated immunity in mice infected with a vaccine strain of Toxoplasma gondii. *J Immunol* 163:4503-4509.

86. Combe, C. L., M. M. Moretto, J. D. Schwartzman, J. P. Gigley, D. J. Bzik, and I. A. Khan. 2006. Lack of IL-15 results in the suboptimal priming of CD4+ T cell response against an intracellular parasite. *Proc Natl Acad Sci U S A* 103:6635-6640.

87. Khan, I. A., M. Moretto, X. Q. Wei, M. Williams, J. D. Schwartzman, and F. Y. Liew. 2002. Treatment with soluble interleukin-15Ralpha exacerbates intracellular parasitic infection by blocking the development of memory CD8+ T cell response. *J Exp Med* 195:1463-1470.

88. Lieberman, L. A., E. N. Villegas, and C. A. Hunter. 2004. Interleukin-15-deficient mice develop protective immunity to Toxoplasma gondii. *Infect Immun* 72:6729-6732.

89. Yi, J. S., M. Du, and A. J. Zajac. 2009. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324:1572-1576.

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

91. Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol* 157:798-805.

92. Jankovic, D., M. C. Kullberg, C. G. Feng, R. S. Goldszmid, C. M. Collazo, M. Wilson, T. A. Wynn, M. Kamanaka, R. A. Flavell, and A. Sher. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* 204:273-283.

93. 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.

94. Wilson, D. C., S. Matthews, and G. S. Yap. 2008. IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii Infection. *J Immunol* 180:5935-5945.

95. Deckert-Schluter, M., C. Buck, D. Weiner, N. Kaefer, A. Rang, H. Hof, O. D. Wiestler, and D. Schluter. 1997. Interleukin-10 downregulates the intracerebral immune response in chronic Toxoplasma encephalitis. *J Neuroimmunol* 76:167-176.

96. Cui, W., and S. M. Kaech. 2010. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunol Rev* 236:151-166.

97. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* 27:393-405.

98. 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.

99. 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.

100. Bullock, T. N., T. A. Colella, and V. H. Engelhard. 2000. The density of peptides displayed by dendritic cells affects immune responses to human tyrosinase and gp100 in HLA-A2 transgenic mice. *J Immunol* 164:2354-2361.

101. Jordan, K. A., and C. A. Hunter. 2010. Regulation of CD8+ T cell responses to infection with parasitic protozoa. *Exp Parasitol* 126:318-325.

102. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

103. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291:2413-2417.

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

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

106. Vaccari, M., C. J. Trindade, D. Venzon, M. Zanetti, and G. Franchini. 2005. Vaccine-induced CD8+ central memory T cells in protection from simian AIDS. *J Immunol* 175:3502-3507.

107. Seaman, M. S., F. W. Peyerl, S. S. Jackson, M. A. Lifton, D. A. Gorgone, J. E. Schmitz, and N. L. Letvin. 2004. Subsets of memory cytotoxic T lymphocytes elicited by vaccination influence the efficiency of secondary expansion in vivo. *J Virol* 78:206-215.

108. Schade, B., and H. G. Fischer. 2001. Toxoplasma gondii induction of interleukin-12 is associated with acute virulence in mice and depends on the host genotype. *Vet Parasitol* 100:63-74.

109. Sharma, S. D., J. R. Catterall, and J. S. Remington. 1986. Parasiticidal activity of macrophages against Toxoplasma. *Methods Enzymol* 132:626-637.

110. Remington, J. S., and T. C. Merigan. 1968. Interferon: protection of cells infected with an intracellular protozoan (Toxoplasma gondii). *Science* 161:804-806.

111. Krahenbuhl, J. L., and J. S. Remington. 1971. In vitro induction of nonspecific resistance in macrophages by specifically sensitized lymphocytes. *Infect Immun* 4:337-343.

112. Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med* 158:670-689.

113. Pfefferkorn, E. R. 1984. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc Natl Acad Sci U S A* 81:908-912.

114. Murray, H. W., G. L. Spitalny, and C. F. Nathan. 1985. Activation of mouse peritoneal macrophages in vitro and in vivo by interferon-gamma. *J Immunol* 134:1619-1622.

115. Pfefferkorn, E. R., S. Rebhun, and M. Eckel. 1986. Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts. *J Interferon Res* 6:267-279.

116. Anderson, S. E., Jr., and J. S. Remington. 1974. Effect of normal and activated human macrophages on Toxoplasma gondii. *J Exp Med* 139:1154-1174.

117. Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl. 1990. Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J Immunol* 144:2725-2729.

118. Butcher, B. A., R. I. Greene, S. C. Henry, K. L. Annecharico, J. B. Weinberg, E. Y. Denkers, A. Sher, and G. A. Taylor. 2005. p47 GTPases regulate Toxoplasma gondii survival in activated macrophages. *Infect Immun* 73:3278-3286.

119. Scharton-Kersten, T. M., T. A. Wynn, E. Y. Denkers, S. Bala, E. Grunvald, S. Hieny, R. T. Gazzinelli, and A. Sher. 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol* 157:4045-4054.

120. Gazzinelli, R., Y. Xu, S. Hieny, A. Cheever, and A. Sher. 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. *J Immunol* 149:175-180.

121. Suzuki, Y., F. K. Conley, and J. S. Remington. 1989. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. *J Immunol* 143:2045-2050.

122. Badovinac, V. P., and J. T. Harty. 2001. Detection and analysis of antigen-specific CD8+ T cells. *Immunol Res* 24:325-332.

123. Sibley, L. D., L. B. Adams, Y. Fukutomi, and J. L. Krahenbuhl. 1991. Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. *J Immunol* 147:2340-2345.

124. Johnson, L. L. 1992. A protective role for endogenous tumor necrosis factor in Toxoplasma gondii infection. *Infect Immun* 60:1979-1983.

125. Rehr, M., J. Cahenzli, A. Haas, D. A. Price, E. Gostick, M. Huber, U. Karrer, and A. Oxenius. 2008. Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy. *J Virol* 82:3391-3404.

126. Sutherland, J. S., J. M. Young, K. L. Peterson, B. Sanneh, H. C. Whittle, S. L. Rowland-Jones, R. A. Adegbola, A. Jaye, and M. O. Ota. 2010. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *J Immunol* 184:6537-6544.

127. Montoya, J. G., K. E. Lowe, C. Clayberger, D. Moody, D. Do, J. S. Remington, S. Talib, and C. S. Subauste. 1996. Human CD4+ and CD8+ T lymphocytes are both cytotoxic to Toxoplasma gondii-infected cells. *Infect Immun* 64:176-181.

128. Purner, M. B., R. L. Berens, P. B. Nash, A. van Linden, E. Ross, C. Kruse, E. C. Krug, and T. J. Curiel. 1996. CD4-mediated and CD8-mediated cytotoxic and proliferative immune responses to Toxoplasma gondii in seropositive humans. *Infect Immun* 64:4330-4338.

129. Denkers, E. Y., G. Yap, T. Scharton-Kersten, H. Charest, B. A. Butcher, P. Caspar, S. Heiny, and A. Sher. 1997. Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. *J Immunol* 159:1903-1908.

130. Wang, X., H. Kang, T. Kikuchi, and Y. Suzuki. 2004. Gamma interferon production, but not perforin-mediated cytolytic activity, of T cells is required for prevention of toxoplasmic encephalitis in BALB/c mice genetically resistant to the disease. *Infect Immun* 72:4432-4438.

131. Suzuki, Y., X. Wang, B. S. Jortner, L. Payne, Y. Ni, S. A. Michie, B. Xu, T. Kudo, and S. Perkins. 2010. Removal of Toxoplasma gondii cysts from the brain by perforin-mediated activity of CD8+ T cells. *Am J Pathol* 176:1607-1613.

132. Schaeffer, M., S. J. Han, T. Chtanova, G. G. van Dooren, P. Herzmark, Y. Chen, B. Roysam, B. Striepen, and E. A. Robey. 2009. Dynamic imaging of T cell-parasite interactions in the brains of mice chronically infected with Toxoplasma gondii. *J Immunol* 182:6379-6393.

133. Jost, C., I. Reiter-Owona, and O. Liesenfeld. 2007. The timing of sulfadiazine therapy impacts the reactivation of latent Toxoplasma infection in IRF-8-/- mice. *Parasitol Res* 101:1603-1609.

134. Scharton-Kersten, T., C. Contursi, A. Masumi, A. Sher, and K. Ozato. 1997. Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. *J Exp Med* 186:1523-1534.

135. Jordan, K. A., E. H. Wilson, E. D. Tait, B. A. Fox, D. S. Roos, D. J. Bzik, F. Dzierszinski, and C. A. Hunter. 2009. Kinetics and phenotype of vaccine-induced CD8+ T-cell responses to Toxoplasma gondii. *Infect Immun* 77:3894-3901.

136. 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.

137. 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.

138. 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.

139. Ichii, H., A. Sakamoto, Y. Kuroda, and T. Tokuhisa. 2004. Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. *J Immunol* 173:883-891.

140. Yoshida, K., A. Sakamoto, K. Yamashita, E. Arguni, S. Horigome, M. Arima, M. Hatano, N. Seki, T. Ichikawa, and T. Tokuhisa. 2006. Bcl6 controls granzyme B expression in effector CD8+ T cells. *Eur J Immunol* 36:3146-3156.

141. Kirak, O., E. M. Frickel, G. M. Grotenbreg, H. Suh, R. Jaenisch, and H. L. Ploegh. 2010. Transnuclear mice with predefined T cell receptor specificities against Toxoplasma gondii obtained via SCNT. *Science* 328:243-248.

142. Dzierszinski, F., M. Pepper, J. S. Stumhofer, D. F. LaRosa, E. H. Wilson, L. A. Turka, S. K. Halonen, C. A. Hunter, and D. S. Roos. 2007. Presentation of Toxoplasma gondii antigens via the endogenous major histocompatibility complex class I pathway in nonprofessional and professional antigen-presenting cells. *Infect Immun* 75:5200-5209.

143. Bhadra, R., J. P. Gigley, and I. A. Khan. 2011. The CD8 T-cell road to immunotherapy of toxoplasmosis. *Immunotherapy* 3:789-801.

144. Singh, U., J. L. Brewer, and J. C. Boothroyd. 2002. Genetic analysis of tachyzoite to bradyzoite differentiation mutants in Toxoplasma gondii reveals a hierarchy of gene induction. *Mol Microbiol* 44:721-733.

145. Almeida, J. R., D. Sauce, D. A. Price, L. Papagno, S. Y. Shin, A. Moris, M. Larsen, G. Pancino, D. C. Douek, B. Autran, A. Saez-Cirion, and V. Appay. 2009. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood* 113:6351-6360.

146. Rodrigue-Gervais, I. G., H. Rigsby, L. Jouan, D. Sauve, R. P. Sekaly, B. Willems, and D. Lamarre. 2010. Dendritic cell inhibition is connected to exhaustion of CD8+ T cell polyfunctionality during chronic hepatitis C virus infection. *J Immunol* 184:3134-3144.

147. Precopio, M. L., M. R. Betts, J. Parrino, D. A. Price, E. Gostick, D. R. Ambrozak, T. E. Asher, D. C. Douek, A. Harari, G. Pantaleo, R. Bailer, B. S. Graham, M. Roederer, and R. A. Koup. 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 204:1405-1416.

148. Sui, Y., Q. Zhu, S. Gagnon, A. Dzutsev, M. Terabe, M. Vaccari, D. Venzon, D. Klinman, W. Strober, B. Kelsall, G. Franchini, I. M. Belyakov, and J. A. Berzofsky. 2010. Innate and adaptive immune correlates of vaccine and adjuvant-induced control of mucosal transmission of SIV in macaques. *Proc Natl Acad Sci U S A* 107:9843-9848.

149. Luft, B. J., and J. S. Remington. 1992. Toxoplasmic encephalitis in AIDS. *Clin Infect Dis* 15:211-222.

150. Grant, I. H., J. W. Gold, M. Rosenblum, D. Niedzwiecki, and D. Armstrong. 1990. Toxoplasma gondii serology in HIV-infected patients: the development of central nervous system toxoplasmosis in AIDS. *Aids* 4:519-521.

151. Zangerle, R., F. Allerberger, P. Pohl, P. Fritsch, and M. P. Dierich. 1991. High risk of developing toxoplasmic encephalitis in AIDS patients seropositive to Toxoplasma gondii. *Med Microbiol Immunol* 180:59-66.

152. Scharton-Kersten, T. M., G. Yap, J. Magram, and A. Sher. 1997. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. *J Exp Med* 185:1261-1273.

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

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

155. Tsushima, F., H. Iwai, N. Otsuki, M. Abe, S. Hirose, T. Yamazaki, H. Akiba, H. Yagita, Y. Takahashi, K. Omura, K. Okumura, and M. Azuma. 2003. Preferential contribution of B7-H1 to programmed death-1-mediated regulation of hapten-specific allergic inflammatory responses. *Eur J Immunol* 33:2773-2782.

156. Dias, P., F. Giannoni, L. N. Lee, D. Han, S. Yoon, H. Yagita, M. Azuma, and S. R. Sarawar. 2010. CD4 T-cell help programs a change in CD8 T-cell function enabling effective long-term control of murine gammaherpesvirus 68: role of PD-1-PD-L1 interactions. *J Virol* 84:8241-8249.

157. Matsumoto, K., H. Inoue, T. Nakano, M. Tsuda, Y. Yoshiura, S. Fukuyama, F. Tsushima, T. Hoshino, H. Aizawa, H. Akiba, D. Pardoll, N. Hara, H. Yagita, M. Azuma, and Y. Nakanishi. 2004. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* 172:2530-2541.

158. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

159. Kirisits, M. J., E. Mui, and R. McLeod. 2000. Measurement of the efficacy of vaccines and antimicrobial therapy against infection with Toxoplasma gondii. *Int J Parasitol* 30:149-155.

160. Weiss, L. M., D. Laplace, P. M. Takvorian, H. B. Tanowitz, A. Cali, and M. Wittner. 1995. A cell culture system for study of the development of Toxoplasma gondii bradyzoites. *J Eukaryot Microbiol* 42:150-157.

161. Torre, D., S. Casari, F. Speranza, A. Donisi, G. Gregis, A. Poggio, S. Ranieri, A. Orani, G. Angarano, F. Chiodo, G. Fiori, and G. Carosi. 1998. Randomized trial of trimethoprim-sulfamethoxazole versus pyrimethamine-sulfadiazine for therapy of toxoplasmic encephalitis in patients with AIDS. Italian Collaborative Study Group. *Antimicrob Agents Chemother* 42:1346-1349.

162. Alexander, J., T. M. Scharton-Kersten, G. Yap, C. W. Roberts, F. Y. Liew, and A. Sher. 1997. Mechanisms of innate resistance to Toxoplasma gondii infection. *Philos Trans R Soc Lond B Biol Sci* 352:1355-1359.

163. Gallimore, A., A. Glithero, A. Godkin, A. C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner, 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* 187:1383-1393.

164. Su, C., D. Evans, R. H. Cole, J. C. Kissinger, J. W. Ajioka, and L. D. Sibley. 2003. Recent expansion of Toxoplasma through enhanced oral transmission. *Science* 299:414-416.

165. Dubey, J. P., D. S. Lindsay, and C. A. Speer. 1998. Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11:267-299.

166. Ferguson, D. J., J. Huskinson-Mark, F. G. Araujo, and J. S. Remington. 1994. A morphological study of chronic cerebral toxoplasmosis in mice: comparison of four different strains of Toxoplasma gondii. *Parasitol Res* 80:493-501.

167. Fleige, T., K. Fischer, D. J. Ferguson, U. Gross, and W. Bohne. 2007. Carbohydrate metabolism in the Toxoplasma gondii apicoplast: localization of three glycolytic isoenzymes, the single pyruvate dehydrogenase complex, and a plastid phosphate translocator. *Eukaryot Cell* 6:984-996.

168. Dzierszinski, F., M. Mortuaire, N. Dendouga, O. Popescu, and S. Tomavo. 2001. Differential expression of two plant-like enolases with distinct enzymatic and antigenic properties during stage conversion of the protozoan parasite Toxoplasma gondii. *J Mol Biol* 309:1017-1027.

169. Fux, B., J. Nawas, A. Khan, D. B. Gill, C. Su, and L. D. Sibley. 2007. Toxoplasma gondii strains defective in oral transmission are also defective in developmental stage differentiation. *Infect Immun* 75:2580-2590.

170. Tait, E. D., K. A. Jordan, C. D. Dupont, T. H. Harris, B. Gregg, E. H. Wilson, M. Pepper, F. Dzierszinski, D. S. Roos, and C. A. Hunter. 2010. Virulence of Toxoplasma gondii is associated with distinct dendritic cell responses and reduced numbers of activated CD8+ T cells. *J Immunol* 185:1502-1512.

171. Ferguson, D. J., and W. M. Hutchison. 1987. The host-parasite relationship of Toxoplasma gondii in the brains of chronically infected mice. *Virchows Arch A Pathol Anat Histopathol* 411:39-43.

172. Courret, N., S. Darche, P. Sonigo, G. Milon, D. Buzoni-Gatel, and I. Tardieux. 2006. CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 107:309-316.

173. Langhorne, J., F. M. Ndungu, A. M. Sponaas, and K. Marsh. 2008. Immunity to malaria: more questions than answers. *Nat Immunol* 9:725-732.

174. Bustamante, J. M., L. M. Bixby, and R. L. Tarleton. 2008. Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease. *Nat Med* 14:542-550.

175. Janumyan, Y. M., C. G. Sansam, A. Chattopadhyay, N. Cheng, E. L. Soucie, L. Z. Penn, D. Andrews, C. M. Knudson, and E. Yang. 2003. Bcl-xL/Bcl-2 coordinately regulates apoptosis, cell cycle arrest and cell cycle entry. *EMBO J* 22:5459-5470.

176. Andersen, J. L., J. L. DeHart, E. S. Zimmerman, O. Ardon, B. Kim, G. Jacquot, S. Benichou, and V. Planelles. 2006. HIV-1 Vpr-induced apoptosis is cell cycle dependent and requires Bax but not ANT. *PLoS Pathog* 2:e127.

177. Galea, I., M. Bernardes-Silva, P. A. Forse, N. van Rooijen, R. S. Liblau, and V. H. Perry. 2007. An antigen-specific pathway for CD8 T cells across the blood-brain barrier. *J Exp Med* 204:2023-2030.

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

179. Hakim, F. T., R. T. Gazzinelli, E. Denkers, S. Hieny, G. M. Shearer, and A. Sher. 1991. CD8+ T cells from mice vaccinated against Toxoplasma gondii are cytotoxic for parasite-infected or antigen-pulsed host cells. *J Immunol* 147:2310-2316.

180. Lamoril, J., J. M. Molina, A. de Gouvello, Y. J. Garin, J. C. Deybach, J. Modai, and F. Derouin. 1996. Detection by PCR of Toxoplasma gondii in blood in the diagnosis of cerebral toxoplasmosis in patients with AIDS. *J Clin Pathol* 49:89-92.

181. Glimcher, L. H., M. J. Townsend, B. M. Sullivan, and G. M. Lord. 2004. Recent developments in the transcriptional regulation of cytolytic effector cells. *Nat Rev Immunol* 4:900-911.

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

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

184. Bhadra, R., J. P. Gigley, L. M. Weiss, and I. A. Khan. 2011. Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. *Proc Natl Acad Sci U S A* 108:9196-9201.

185. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107:4781-4789.

186. Nissapatorn, V. 2009. Toxoplasmosis in HIV/AIDS: a living legacy. *Southeast Asian J Trop Med Public Health* 40:1158-1178.

187. Montoya, J. G., and O. Liesenfeld. 2004. Toxoplasmosis. *Lancet* 363:1965-1976.

188. Meythaler, M., Z. Wang, A. Martinot, S. Pryputniewicz, M. Kasheta, H. M. McClure, S. P. O'Neil, and A. Kaur. 2011. Early induction of polyfunctional simian immunodeficiency virus (SIV)-specific T lymphocytes and rapid disappearance of SIV from lymph nodes of sooty mangabeys during primary infection. *J Immunol* 186:5151-5161.

189. Makedonas, G., and M. R. Betts. 2006. Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. *Springer Semin Immunopathol* 28:209-219.

190. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. 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.

191. Harrington, L. E., M. Galvan, L. G. Baum, J. D. Altman, and R. Ahmed. 2000. Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J Exp Med* 191:1241-1246.

192. Puga, I., A. Rao, and F. Macian. 2008. Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells. *Immunity* 29:193-204.

193. Kietselaer, B. L., L. Hofstra, E. A. Dumont, C. P. Reutelingsperger, and G. A. Heidendal. 2003. The role of labeled Annexin A5 in imaging of programmed cell death. From animal to clinical imaging. *Q J Nucl Med* 47:349-361.

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

195. Hunter, C. A., Y. Suzuki, C. S. Subauste, and J. S. Remington. 1996. Cells and cytokines in resistance to Toxoplasma gondii. *Curr Top Microbiol Immunol* 219:113-125.

196. Khan, I. A., and M. Moretto. 1999. Role of gamma interferon in cellular immune response against murine Encephalitozoon cuniculi infection. *Infect Immun* 67:1887-1893.

197. 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.

198. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* 297:2060-2063.

199. Lee, B. O., L. Hartson, and T. D. Randall. 2003. CD40-deficient, influenza-specific CD8 memory T cells develop and function normally in a CD40-sufficient environment. *J Exp Med* 198:1759-1764.

200. de Totero, D., R. Meazza, S. Zupo, G. Cutrona, S. Matis, M. Colombo, E. Balleari, I. Pierri, M. Fabbi, M. Capaia, B. Azzarone, M. Gobbi, M. Ferrarini, and S. Ferrini. 2006. Interleukin-21 receptor (IL-21R) is up-regulated by CD40 triggering and

mediates proapoptotic signals in chronic lymphocytic leukemia B cells. *Blood* 107:3708-3715.

201. Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480-483.

202. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138-149.

203. Mueller, S. N., and R. Ahmed. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 106:8623-8628.

204. Shin, H., and E. J. Wherry. 2007. CD8 T cell dysfunction during chronic viral infection. *Curr Opin Immunol* 19:408-415.

205. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity* 29:650-659.

206. Zhu, J., D. M. Koelle, J. Cao, J. Vazquez, M. L. Huang, F. Hladik, A. Wald, and L. Corey. 2007. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med* 204:595-603.

207. Wherry, E. J., J. N. Blattman, and R. Ahmed. 2005. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *J Virol* 79:8960-8968.

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

209. Mordue, D. G., F. Monroy, M. La Regina, C. A. Dinarello, and L. D. Sibley. 2001. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. *J Immunol* 167:4574-4584.

210. Brooks, D. G., S. J. Ha, H. Elsaesser, A. H. Sharpe, G. J. Freeman, and M. B. Oldstone. 2008. IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. *Proc Natl Acad Sci U S A* 105:20428-20433.

211. Teixeiro, E., M. A. Daniels, S. E. Hamilton, A. G. Schrum, R. Bragado, S. C. Jameson, and E. Palmer. 2009. Different T cell receptor signals determine CD8+ memory versus effector development. *Science* 323:502-505.

212. Sheppard, K. A., L. J. Fitz, J. M. Lee, C. Benander, J. A. George, J. Wooters, Y. Qiu, J. M. Jussif, L. L. Carter, C. R. Wood, and D. Chaudhary. 2004. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett* 574:37-41.

213. Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, D. Costagliola, C. Rouzioux, H. Agut, A. G. Marcelin, D. Douek, B. Autran, and V. Appay. 2007. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 204:2473-2485.

214. Franco, D., W. Li, F. Qing, C. T. Stoyanov, T. Moran, C. M. Rice, and D. D. Ho. 2010. Evaluation of yellow fever virus 17D strain as a new vector for HIV-1 vaccine development. *Vaccine* 28:5676-5685.

215. Young, J. M., I. M. Adetifa, M. O. Ota, and J. S. Sutherland. 2010. Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. *PLoS One* 5:e11237.

216. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med* 203:2263-2269.

217. Cui, W., N. S. Joshi, A. Jiang, and S. M. Kaech. 2009. Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. *Vaccine* 27:2177-2187.

218. McLeod, R., R. G. Estes, D. G. Mack, and H. Cohen. 1984. Immune response of mice to ingested Toxoplasma gondii: a model of toxoplasma infection acquired by ingestion. *J Infect Dis* 149:234-244.

219. Ohteki, T., H. Tada, K. Ishida, T. Sato, C. Maki, T. Yamada, J. Hamuro, and S. Koyasu. 2006. Essential roles of DC-derived IL-15 as a mediator of inflammatory responses in vivo. *J Exp Med* 203:2329-2338.

220. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J Exp Med* 204:1625-1636.

221. Onami, T. M., L. E. Harrington, M. A. Williams, M. Galvan, C. P. Larsen, T. C. Pearson, N. Manjunath, L. G. Baum, B. D. Pearce, and R. Ahmed. 2002. Dynamic regulation of T cell immunity by CD43. *J Immunol* 168:6022-6031.

222. Fisicaro, P., C. Valdatta, M. Massari, E. Loggi, E. Biasini, L. Sacchelli, M. C. Cavallo, E. M. Silini, P. Andreone, G. Missale, and C. Ferrari. 2010. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. *Gastroenterology* 138:682-693, 693 e681-684.

223. Lukens, J. R., M. W. Cruise, M. G. Lassen, and Y. S. Hahn. 2008. Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model. *J Immunol* 180:4875-4884.

224. Beuneu, H., F. Lemaitre, J. Deguine, H. D. Moreau, I. Bouvier, Z. Garcia, M. L. Albert, and P. Bousso. 2010. Visualizing the functional diversification of CD8+ T cell responses in lymph nodes. *Immunity* 33:412-423.

225. Pearce, E. L., M. C. Walsh, P. J. Cejas, G. M. Harms, H. Shen, L. S. Wang, R. G. Jones, and Y. Choi. 2009. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460:103-107.