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Tissue-dependent T Cell Apoptosis and Transcriptional Regulation of Memory CD8+T Cell Differentiation During Viral Infections: A Dissertation

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TISSUE-DEPENDENT T CELL APOPTOSIS AND TRANSCRIPTIONAL REGULATION OF MEMORY CD8⁺T CELL DIFFERENTIATION DURING VIRAL INFECTIONS

A Dissertation presented

By

Varun Kapoor

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Program in Immunology and Virology
December 10, 2013

DEDICATION

This thesis is dedicated to my grandmother, who would have loved to see this day and she will be greatly missed. I also dedicate this thesis to my wonderful parents and my loving wife.

ACKNOWLEDGMENTS

The road to completing my PhD. has been challenging, but also the most exciting journey of my life so far. In this journey I have made friends and shared moments, which will be cherished all my life.

My first debt of gratitude must go to my advisor, Dr. Raymond M. Welsh. Ray has always been supportive and patiently provided the vision, encouragement and advice necessary for me to proceed through the doctoral program and complete my dissertation. Like any PhD. project, my project in the lab had its ups and downs but Ray always provided unflagging encouragement and showed faith in me. He always encouraged critical thinking and has given me great freedom to pursue independent work. Ray is not only a great scientist but a wonderful human being, and the years spend in his lab will always be dear to me.

I would like to thank Dr. Leslie J. Berg for letting me work in close collaboration with her lab. Very special thanks to Dr. HyunMu Shin, who has been a great friend and collaborator, our 'secret scientific research' (as Ray would call it) finally paid off, and I guess our obsession with Blimp-1 did work out well in the end.

Special thanks to my committee, Dr. Francis Ka-Ming Chan, Dr. Joonsoo Kang, Dr. Eva Szomolanyi-Tsuda, Dr. Richard Dutton and Dr. Brian Sheridan for their support, guidance and helpful suggestions. Their guidance has served me well and I owe them my heartfelt appreciation.

Past and present members of Welsh, Selin and Tsuda Lab also deserve my sincerest thanks, their friendship and assistance has meant more to me than I could ever express.

I want to thank my friends and family who have been a source of support all these years. I wish to thank my parents, Pradeep and Shipra Kapoor and my brother Kunal. Their love provided me inspiration and was my driving force. I owe them everything and wish I could show them just how much I love and appreciate them. My wife Rasika, whose love and encouragement allowed me to finish this journey. She has stood by me through good times and the bad and she deserves my heartfelt thanks.

"PhD projects are never completed they are abandoned" and as I close this chapter in life I feel the same. I wish I could do so much more but then it is time to move on and I believe this experience has made me a better person and hopefully a better scientist.

ABSTRACT

Activation and proliferation of antigen-specific T cells is the hallmark of an anti-viral immune response. Effector T cells generated during an immune response are heterogeneous in regards to their ability to populate the memory pool once the immune response has resolved. Initial T cell activation takes place in the lymphoid organs, after which T cells migrate into the non-lymphoid tissues. The presence of memory T cells at non-lymphoid tissue sites has been shown to be critical for protection against secondary virus challenge. Our lab has previously demonstrated that during and after the resolution of the immune response to Lymphocytic choriomeningitis virus (LCMV) CD8$^+$T cells in the non-lymphoid tissues are more resistant to apoptosis than those in the lymphoid organs. This stability of T cells in the non-lymphoid tissues may be critical in ensuring protection against a secondary virus challenge.

Mechanisms regulating tissue-dependent differences in CD8$^+$T cell apoptosis were studied in an acute LCMV infection model. Virus-specific CD8$^+$T cells from lymphoid (spleen, mesenteric lymph nodes (MLN), inguinal lymph nodes (ILN)) and non-lymphoid tissues (peritoneal exudate cells (PEC), fat-pads) were compared for expression of surface antigenic markers known to correlate with a memory phenotype. Non-lymphoid tissues were enriched in IL-7R^{hi}, KLRG-1^{lo}, CD27^{hi} and CXCR3^{hi} virus-specific CD8$^+$ T cells, and the presence of these antigenic markers correlated with increased memory potential and survival. Transcription factors in addition to cell surface antigens were assessed as

correlates of resistance to apoptosis. Virus-specific CD8$^+$T cells in the non-lymphoid tissues were enriched in cells expressing T cell factor-1 (TCF-1), which correlated with increased memory potential and survival. CD8$^+$T cells in the peritoneum of TCF-1-deficient mice had decreased survival during resolution of the immune response to LCMV, suggesting a role for TCF-1 in promoting survival in the non-lymphoid tissues.

As an additional mechanism, I investigated whether apoptosis-resistant CD8$^+$T cells migrate to non-lymphoid tissues and contribute to tissue-dependent apoptotic differences. CXCR3$^+$ CD8$^+$T cells resisted apoptosis and accumulated in the lymph nodes of mice treated with FTY720, which blocks the export of lymph node cells into the peripheral tissues. The PECs expressed increased amounts of CXCR3 ligands, CXCL9 and CXCL10, which may have recruited the non-apoptotic cells from the lymph nodes. By adoptively transferring splenic T cells into the spleen or PEC environment I showed that the peritoneal environment through a yet undefined factor promoted survival of CD8$^+$T cells. In this study I have elucidated the mechanisms by which CD8$^+$T cells preferentially survive in the non-lymphoid tissues. I found that non-lymphoid tissues were enriched in memory-phenotype CD8$^+$T cells which were intrinsically resistant to apoptosis irrespective of the tissue environment. Furthermore, apoptosis-resistant CD8$^+$T cells may preferentially migrate into the non-lymphoid tissues where the availability of tissue-specific factors may enhance memory cell survival.

Few transcription factors have been identified that regulate CD8$^+$T cell effector-memory differentiation during an immune response. In this thesis, I have also studied the mechanism by which the transcription factor Blimp-1 regulates the generation of effector and memory CD8$^+$T cells. Blimp-1 is known to repress a large number of target genes, and ChIP (chromatin immunoprecipitation) sequencing analysis done by Dr. HyunMu Shin in the lab of Dr. Leslie J. Berg identified CD25 (IL-2Rα) and CD27 as potential targets of Blimp-1. I found that Blimp-1-deficient CD8$^+$T cells had sustained expression of CD25 (IL-2Rα) and CD27 during peak and resolution of the immune response to LCMV. By performing adoptive transfers of CD25^{hi} and CD27^{hi} CD8$^+$T cells I showed that CD25 and CD27 expression on CD8$^+$T cells during resolution of the immune response correlates with enhanced survival. Silencing *Il2rα* and *Cd27* expression reduced the Blimp-1-deficient CD8$^+$T cell response, suggesting that sustained expression of CD25 and CD27 was in part responsible for the enhanced CD8$^+$T cell response seen in the Blimp-1-deficient mice. Furthermore, our collaborator Dr. HyunMu Shin showed that CD25 and CD27 are direct targets of Blimp-1, and that Blimp-1 recruits histone modifying enzymes to *Il2rα* and *Cd27* loci to suppress their expression during the peak of the anti-viral immune response. This study identifies one of the mechanisms by which Blimp-1 regulates the balance between generation of effector and memory CD8$^+$T cells.

In this thesis work I also studied the function of the transcription factor ROG (Repressor of GATA-3) in regulating *in vivo* T cell responses during both

acute and chronic LCMV infection. ROG-deficient mice had increased CD8^{+}T cell responses during an acute LCMV infection. ROG deficiency also led to the generation of memory T cells with an enhanced recall response compared to WT controls. By using LCMV-specific P14^{+} TCR transgenic ROG-deficient CD8^{+}T cells these defects were shown to be T cell intrinsic. ROG-deficient mice had enhanced CD8^{+}T cell responses and viral clearance during a persistent high dose LCMV Clone 13 infection. During chronic LCMV infection ROG-deficient mice also had increased lung pathology and mortality. The results indicate that ROG negatively regulates T cell responses and memory generation during both acute and chronic LCMV infection.

The studies highlighted in this thesis elucidate the mechanisms promoting CD8^{+}T cell survival in non-lymphoid tissues as well as transcription factor-mediated regulation of memory CD8^{+}T cell differentiation. Knowledge of this will help us better understand T cell immunity after infections and may eventually help develop better vaccines.

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ABBREVIATIONS

7AAD   (7-aminoactinomycin D)

APC   (antigen presenting cells)

Bcl-2   (B cell lymphoma 2)

Bcl-6   (B cell lymphoma 6)

BALT   (bronchus-associated lymphoid tissue)

Blimp-1   (B lymphocyte-induced maturation protein 1)

BrdU   (5-bromo-2-deoxyuridine)

BTB   (broad-Complex, tramtrack and bric-a-brac)

ChIP   (chromatin immunoprecipitation)

CFSE   (carboxyfluorescein succinimidyl ester)

CTL   (cytotoxic T lymphocytes)

CXCR3   ((C-X-C motif)-chemokine receptor-3)

CXCL9   ((C-X-C motif)-chemokine-9)

CXCL10   ((C-X-C motif)-chemokine-10)

EAE   (experimental autoimmune encephalomyelitis)

ELISA   (enzyme-linked immunosorbent assay)

EOMES   (eomesodermin)

FADD   (Fas-associated protein with death-domain)

GP   (glycoprotein)

H&E   (hematoxylin and eosin)

HBSS   (Hanks balanced salt solution)

HDAC   (histone deacetylase)

HSV   (Herpes simplex virus)

ICS   (intracellular cytokine secretion assay)

IFN   (interferon)

IFNα   (interferon-α)

IFNβ   (interferon-β)

IFNγ   (interferon-γ)

ILN   (inguinal lymph nodes)

IL-2   (interleukin-2)

IL-7   (interleukin-7)

IL-15   (interleukin-15)

IL-2Rα   (interleukin-2 receptor α)

IL-7Rα   (interleukin-7 receptor α)

i.p   (intraperitoneal)

i.v   (intravenous)

KLRG-1   (killer cell lectin-like receptor subfamily G member 1)

LCMV   (Lymphocytic choriomeningitis virus)

LEF-1   (lymphoid-enhancer binding factor 1)

MACS   (magnetic-activated cell sorting)

MFI   (mean fluorescent intensity)

MHC   (major histocompatibility complex)

MLN   (mesenteric lymph nodes)

MPEC   (memory precursor effector cells)

NP   (nucleoprotein)

NK   (natural killer)

PEC   (peritoneal exudate cells)

PMA   (phorbol myristate acetate)

PLZF   (promyelocytic leukemia zinc finger protein)

POZ   (Pox virus and zinc finger)

Prdm-1   (PR domain Zn finger protein 1)

ROG   (repressor of GATA-3)

SLEC   (short-lived effector cells)

STAT5   (signal transducer and activator of transcription 5)

Tcm   (T central memory)

TCF-1   (T cell factor-1)

TCR   (T cell receptor)

Tem   (T effector memory)

TNF   (tumor necrosis factor)

TRADD   (tumor necrosis factor receptor type 1-associated death domain protein)

TUNEL   (terminal deoxynucleotidyl transferase dUTP nick end labeling)

VACV   (Vaccinia virus)

PREFACE

Parts of this dissertation have appeared in separate publications:

Shin H*, **Kapoor VN***, Guan T, Kaech SM, Welsh RM, Berg LJ. **(*Co-First Author)** Epigenetic modifications induced by Blimp-1 regulate CD8$^+$T cell memory progression during acute virus infection. **Immunity 39, 1–15, October 17, 2013**

Kapoor VN, Kang J, Welsh RM. Regulation of tissue-dependent differences in CD8$^+$T cell apoptosis during acute LCMV infection. (Manuscript to be submitted)

Kapoor VN*, Shin H*, Welsh RM, Berg LJ. **(*Co-First Author)** Transcription factor ROG/*zbtb32* negatively regulates CD8$^+$T cell responses during acute and chronic LCMV infections. (Manuscript under preparation)

CHAPTER I: INTRODUCTION

A. LCMV

LCMV is an old world Arenavirus with *Mus musculus* being its natural host. Studies with the LCMV infection model system in mice have contributed extensively to our knowledge of immune tolerance, MHC restriction, T cell exhaustion, T cell memory, and NK cell activation and specificity [1, 2]. Nobel Prize winning studies by Burnet and Fenner on immunological tolerance and Zinkernagel and Doherty on T cell recognition of immune infected cells were done using the LCMV infection model [1].

Originally LCMV was isolated by Charles Armstrong in 1933 while studying epidemic encephalitis in St.Louis, MO [2]. Subsequently other LCMV stains were independently isolated, and the most commonly used LCMV strains in the laboratory include Armstrong, Clone 13, Traub, and WE. In mice a low to medium inoculum of LCMV causes an acute infection which is cleared by a strong cytotoxic T lymphocyte (CTL) response. Intracranial infection in mice leads to severe acute meningoencephalitis and *in utero* infection within 24 hours of birth leads to a persistent life-long infection in mice [3]. LCMV can be transmitted to humans by direct contact or by inhalation of infected rodent excreta and can cause subclinical infection or moderate to severe meningitis. LCMV has also been known to cause birth defects or spontaneous abortion of the human fetus [4].

LCMV particles are pleomorphic in appearance and are roughly spherical with spiked projections formed by the envelope glycoproteins (5). LCMV is an ambisense enveloped RNA virus which encodes four genes from two RNAs (6). The large 31S L RNA codes for two proteins, the L protein which is the RNA dependent RNA polymerase and the small zinc-binding Z protein. The short 23S RNA encodes for nucleoprotein (NP) and glycoprotein (GP). The glycoprotein is further cleaved to form the GP1 (integral membrane protein) and GP2 (peripheral membrane protein) (6). GP are trimeric Class-I fusion proteins wherein GP-1 functions as receptor binding and GP-2 functions as fusion mediating protein (7). The glycosylated peripheral plasma membrane protein α-dystroglycan has been identified as the host cellular receptor for LCMV and some other arenavirus members (8). Entry into the host cell is mediated through large smooth walled vesicles followed by pH mediated fusion (9). Recently, it was shown that LCMV can use clathrin-independent late endosomes for entry into the host cells (10). Viral replication occurs in the cytoplasm after which LCMV buds from the plasma membrane.

LCMV-variant Clone 13 has been widely used to study T cell responses during persistent infection (11). The strain was originally identified in spleens of LCMV Armstrong infected adult carrier mice (12). Clone 13 differs from the parent Armstrong strain due to a single amino acid change in the polymerase (lysine to glutamine at amino acid 1079) and glycoprotein GP-1 (phenylalanine to leucine at 260 position) (13). The mutation in the GP-1 of Clone 13 variant leads to higher

affinity of the viral glycoprotein for α-dystroglycan expressed on macrophages and CD11c^{+} and DEC-205^{+} dendritic cells (14, 15). LCMV Clone 13 has increased growth in the lymphoid tissues and can systemically spread and replicate in both lymphoid and non-lymphoid tissues to cause persistent infection (13) as opposed to the parent Armstrong strain which replicates in lymphoid tissues and fails to cause T cell exhaustion (11). For most of my studies in this thesis I have used LCMV Armstrong, and in Chapter V some studies have been performed with LCMV Clone 13 strain.

B. Immune response to LCMV

The LCMV model system has contributed immensely to our knowledge of T cell responses to viral infection. Low to moderate inoculum of LCMV via intraperitoneal (i.p) or intravenous (i.v) route induces a strong CTL response leading to viral clearance and development of T cell memory to LCMV (Figure I-1). LCMV is a strong type I IFN (Interferon) inducer, and unlike some other viruses, LCMV does not modulate host Class I MHC expression (16), thereby leading to induction of a strong CTL response and sterilizing immunity.

Early responses to viral replication are characterized by induction of type I IFN α/β, the levels of which peak around day 2 post infection (17). Type I IFN is important for regulating early viral load, as mice deficient in type-I IFN receptor had a reduced CTL response to LCMV, and had increased viral loads leading to a persistent infection (18, 19). Type I IFNs are also important for induction of NK

(Natural killer) cell activation and proliferation during early stages of the immune response [17]. Viral load in NK depleted mice was found to be similar to control animals, suggesting that NK cells do not play a role in regulating the response to acute LCMV infection [20]. However, IFNγ production by NK cells has been shown to reduce viral burden at the site of peripheral inoculation [21]. In addition, NK cells have been shown to be important in regulating T cell responses during persistent LCMV infection [22].

Viral antigens presented in the context of Class-I MHC by antigen-presenting cells (APC) lead to activation and proliferation of CD8^{+}T cells. As shown in Figure I-1 CD8^{+}T cell responses can be divided into three phases: a) activation and proliferation of CD8^{+}T cells, b) silencing of immune response by CD8^{+}T cell apoptosis on clearance of viral antigen, and c) generation of memory CD8^{+}T cells, which provide long lasting immunity to secondary challenge. CD8^{+}T cell apoptosis and memory generation has been covered in greater details in later sections.

During early stages of LCMV infection activated T cells undergo attrition, and this attrition is dependent on type I IFN and the pro-apoptotic molecule Bim [23-25]. T cell numbers increase by day 6 post infection and reach a peak around day 8 or 9. At the peak of the immune response CD8^{+}T cell numbers increase 5-20 fold in the spleen as compared to a naïve mouse [26]. CD8^{+}T cells express several markers which allow detection of activated T cells (CD44^{hi}, CD11a^{hi},
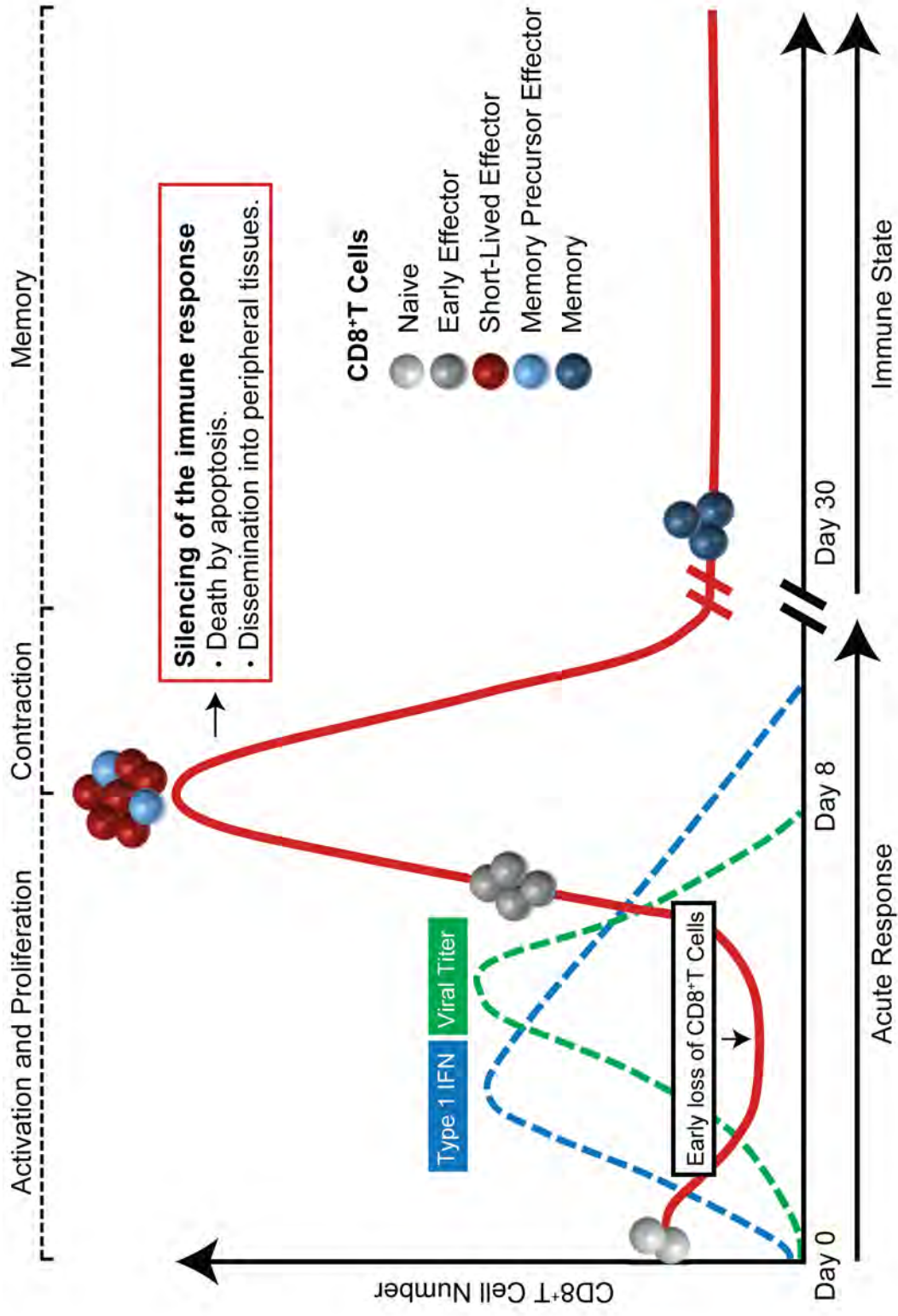
Figure I-1. Kinetics of CD8⁺T cell response during acute LCMV infection.

CD11b^{hi}, VLA-4^{hi}, CD62L^{lo}, IL-7R^{lo}, KLRG-1^{hi}), but recent studies have shown that there is considerable heterogeneity in the surface antigen expression of the activated T cell pool [27], allowing for detection of cells that might preferentially survive to become memory T cells.

The immune response to LCMV is mainly a Th1 type response with CD4^{+} and CD8^{+}T cells producing high amounts of IFNγ and IL-2. CTL kill LCMV-infected target cells by direct contact and secrete perforins and granzymes. Perforin-mediated killing seems to be the major cytotoxic mechanism, as perforin deficient mice develop a CTL response but do not clear the virus [28]. IFNγ-mediated killing of targets may also be a cytotoxic mechanism, as mice deficient in IFNγ receptor or depleted of IFNγ by antibody had increased LCMV titers [29, 30].

CD4^{+}T cells are activated and proliferate in response to LCMV infection, and approximately 10% of CD4^{+}T cells can be shown to be virus-specific at the peak of the response [26]. CD4^{+}T cells do not seem to regulate acute LCMV infection, as mice lacking CD4^{+}T cells (due to CD4 deficiency or CD4 antibody depletion) have a functional CD8^{+}T cell response and clear the virus [31-33]. However, survival and maintenance of functional CD8^{+}T cell memory requires CD4^{+}T cells, as CD8^{+}T cells generated in absence of CD4^{+}T cells are progressively lost and mount a defective secondary response [34-36]. Humoral responses also develop in the host against LCMV nucleoprotein and

glycoproteins, and IFNγ induced IgG2a isotype is the predominant response (1). CD8⁺T cell responses are normal in B cell-deficient or B cell-depleted mice, suggesting against a role of B cells in LCMV immunity (37, 38). However, as seen for CD4⁺ T cells, B cells have been shown to be important for CD8⁺T cell memory generation and long term viral control (39, 40).

C. T cell apoptosis and silencing of the immune response

Upon clearance of the viral antigen silencing of the immune response takes place to restore homeostasis in the T lymphocyte compartment. Most of the virus-specific CD8⁺T cells die by apoptosis (41), and it is estimated that approximately 90-95% of effector T cells are eliminated in the process (42).

Apoptosis is a form of programmed cell death, which is characterized by typical morphological changes that include membrane blebbing, DNA fragmentation and degradation of cellular components without the loss of membrane integrity. This form of cell death is advantageous, as apoptotic cells are rapidly cleared by macrophages and other phagocytes without inducing inflammation or an immune response. Depending on the upstream signals apoptosis can be initiated by one of the two pathways: a) extrinsic pathway or b) intrinsic pathway (42-44).

Induction of the extrinsic cell death pathway requires signals from soluble ligands and/or other cells. The signals for cell death are initiated at the cell surface by binding of death receptors belonging to Tumor Necrosis Factor (TNF)

superfamily such as TNFR and Fas to their respective ligands (42). The aggregation of death receptors at the cell surface initiates downstream signaling, leading to recruitment of death-domain containing adaptors such as FADD (Fas associated protein with death-domain) or TRADD (Tumor Necrosis Factor Receptor Type 1-associated death-domain protein) along with pro Caspase-8. Homodimerization and self-cleavage of pro-Caspase 8 releases active Caspase-8, which activates downstream caspases leading to apoptosis (45). The role of extrinsic apoptotic pathway has been evaluated in CD8$^+$T cell contraction. Activated T cells when re-stimulated through their TCR undergo cell death via Fas and TNFR1 by a mechanism known as activation-induced cell death (AICD) (45, 46). IL-2 (Interleukin-2), a T cell growth factor was shown to sensitize T cells to AICD by down-regulating apoptotic inhibitor c-FLIP (47). However, CD8$^+$T cells undergo contraction during silencing of the immune response in mice deficient in TNFR, Fas and FasL (48-50). Thus, the *in vivo* data suggests that AICD does not play a role in CD8$^+$T cell contraction as antigen is no longer present. AICD and extrinsic cell death pathway might be more relevant to a persistent infection setting or autoimmunity where the antigen will be chronically present to trigger the TCR.

The intrinsic cell death pathway is induced upon signals of cellular stress and damage. The balance between pro- and anti-apoptotic members of the Bcl-2 family of proteins are known to regulate the intrinsic pathway of apoptosis (51).The anti-apoptotic members of the Bcl-2 family include: Bcl-2, Bcl-xl, Mcl-1,

A1 which are known to inhibit the function of pro-apoptotic members. Some of the pro-apoptotic members include Bax, Bak, Bim, Bad and Bid. The members of the Bcl-2 family share at least one Bcl-2 homology domain (BH). Under conditions of stress anti-apoptotic members of the Bcl-2 family are antagonized to initiate a cascade of events leading to mitochondrial depolarization, release of cytochrome c and activation of Caspase-9, which then activates downstream effector caspases to induce apoptosis (51). During silencing of the immune response the antigen is no longer present and growth or survival factors become limited. Therefore it is thought that CD8$^+$T cell contraction occurs due to cytokine deprivation (52) leading to induction of the intrinsic apoptotic pathways. Bcl-2 is expressed in naïve T cells, down-regulated in effector T cells and surviving memory T cells express high levels of Bcl-2 (53). Bcl-2 transgene-expressing T cells are protected from IL-2 deprivation induced apoptosis, though CD8$^+$T cell contraction was not blocked in Bcl-2 overexpressing mice (50). Higher levels of the anti-apoptotic Bcl-2 in effector CD8$^+$T cells have been shown to be important in counteracting the effects of pro-apoptotic Bim, thereby allowing for survival of cells during contraction of the immune response (54). Further supporting the role of intrinsic cell death pathway in silencing of the immune response, Bim deficient T cells were resistant to cytokine withdrawal induced apoptosis, and Bim was necessary for T cell death during resolution of the immune response to Herpes simplex virus (HSV) infection (55, 56). Mice deficient in both Bim and Fas had a lymph node-specific block in contraction of the LCMV-specific immune response

(57). Overall, this suggests that the cytokine withdrawal induced intrinsic cell death pathway plays a major role in T cell death during silencing of the immune response, but, under certain infection conditions and at certain tissue sites both intrinsic and extrinsic pathways may act concurrently to regulate silencing of the immune response.

Caspases are cystinyl-aspartate-requiring proteinases that are synthesized as inactive zymogens which release the active form upon proteolytic cleavage (58). Caspases can be divided into initiator caspases (Caspase-2,-8,-9,-10), which interact with upstream adaptor molecules, or executioner caspases (Caspase-3,-6,-7), which cleave various cellular substrates to induce apoptosis (59). The extrinsic and intrinsic cell death pathways converge to activate downstream executioner caspases. Levels of activated Caspase-3 increase in effector T cells, directly correlating with their sensitivity to apoptotic stimuli (60), and T cells from Caspase-3-deficient mice have reduced susceptibility to activation-induced cell death (AICD) driven by CD3 crosslinking and Fas ligands (61). Therefore, assays to detect active-Caspases have been reliably used to identify apoptotic cells.

Large numbers of assays are available to identify apoptotic cells. During the early stages of apoptosis phosphatidylserine residues externalize to outer leaflet of the plasma membrane, marking the cells for phagocytic uptake. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein and has high affinity

for phosphatidylserine. Fluorescent-conjugate-labeled Annexin V can be used to identify pre-apoptotic cells via flocytometry or fluorescent microscopy [62]. Since Annexin V can bind to phosphatidylserine expressed on the surface of necrotic cells, this assay is usually performed along with nucleic acid stains such as 7AAD (7-aminoactinomycin D) to identify pre-apoptotic cells which have not lost their membrane integrity. Though Annexin V has been shown to bind to activated and proliferating cells [63, 64], Annexin V staining is still a reliable assay to identify pre-apoptotic cells [64]. Moreover, our lab has shown that sorted and adoptively transferred Annexin V$^+$ T cells do not develop into functional memory [65], confirming the reliability of this assay. End stage apoptotic cells undergo DNA fragmentation, and this can be visualized using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. Terminal transferase is used to add fluorescent-labeled UTP to the 3' end of DNA fragments which can be visualized by flocytometry or fluorescent microscopy [66].

In this thesis I have used Annexin V, TUNEL and active Caspase-3 staining assays to identify apoptotic cells. Wherever possible multiple assays have been used to reliably confirm the presence of apoptotic cells.

D. Generation of CD8$^+$T cell memory

Upon clearance of the viral antigen most of the virus-specific CD8$^+$T cells die by apoptosis, leaving behind a small (5-10% of the peak response) but stable population of memory CD8$^+$T cells to provide protection to secondary challenge.

The initial TCR engagement triggers the up-regulation of co-stimulatory molecules and cytokine receptors, which are critical for the clonal expansion, survival and memory generation of the responding CD8$^+$ T cells (67). Heterogeneous populations of effector CD8$^+$T cells are generated during an immune response, and various models have been proposed to explain CD8$^+$T cell differentiation into effector and memory populations (68). Terminally differentiated effector CD8$^+$T cells, which are destined to die, can be distinguished from memory precursor CD8$^+$T cells based on expression of surface markers and transcription factors (Figure I-2) (68, 69), which characterize their activation state and highlight their conversion into memory.

IL-2, IL-7 and IL-15 belong to the common γ chain family of cytokines and are required at various stages for an optimal T cell immune response. IL-7 and IL-15 are known to mediate survival and self-renewal of memory CD8$^+$T cell populations (70, 71). CD8$^+$T cells expressing IL-7Rα preferentially give rise to memory CD8$^+$T cells (72), but IL-7Rα expression is necessary but not sufficient for CD8$^+$T cell memory formation (73). Surface expression of IL-7Rα along with KLRG-1 (inhibitory receptor found on T and NK cells) has been used to identify cells which will preferentially make it into memory (Memory Precursor effector cells: IL-7R^{hi} KLRG-1^{lo}) or die during contraction of the immune response (Short-Lived effector cells: IL-7R^{lo} KLRG-1^{hi}) (69, 74). IL-2 is a T cell growth factor and plays an important role in regulating CD8$^+$ T cell responses during the different stages of viral infection (75). *In vivo* administration of IL-2 during expansion phase

Signal 1: Antigen
Signal 2: Co-stimulation
Signal 3: Cytokines
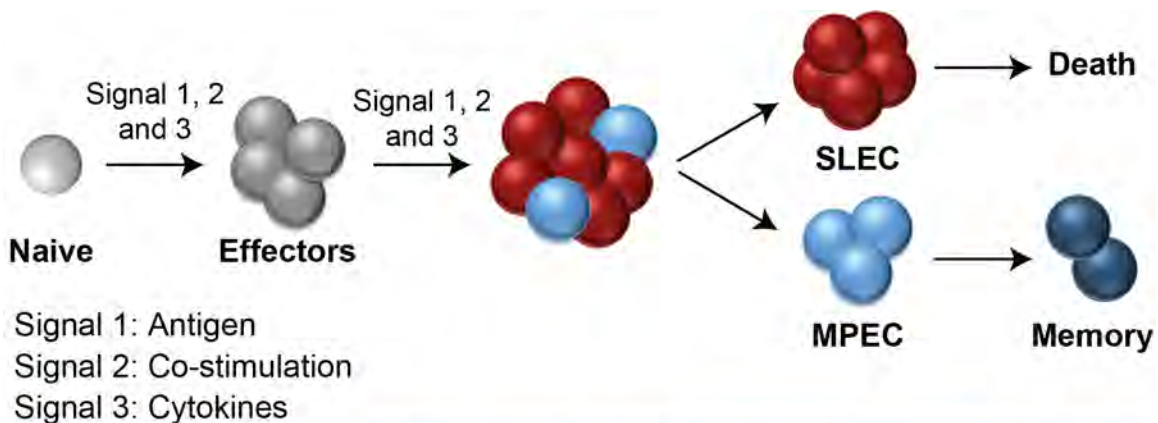
Antigenic Markers/ Transcription Factors	Short-Lived Effector Cells (SLEC)	Memory Precursor Effector Cells (MPEC)
IL-7Rα	Low	High
KLRG-1	High	Low
CD27	Low	High
CXCR3	Low	High
IL-2 (cytokine)	Low	High
TCF-1	Low	High
Blimp-1	High	Low
Bcl-6	Low	High
T-bet	High	Low
EOMES	Low	High
ID3	Low	High
ID2	High	Low
FoxO1	Low	High
FoxO3	High	Low
STAT3	Low	High
STAT4	High	Low

Figure I-2. Surface antigenic markers and transcription factors regulating CD8+T cell effector-memory generation.

of the viral response is detrimental to the survival of CD8^{+}T cells; however, IL-2 therapy during the contraction and memory stages of the response promotes CD8^{+}T cell survival (76). Additional studies have indicated that both primary and secondary CD8^{+}T cell responses are impaired in the absence of IL-2 receptor signaling (77, 78). Moreover, IL-2-producing CD8^{+}T cells are more likely to become memory cells than those that do not make IL-2 (79). CD25, a subunit of the IL-2 receptor is up-regulated by IL-2 in conjunction with TCR stimulation (75), and at early stages of the response to LCMV infection, CD25 expression promotes the development of terminally-differentiated effector CD8^{+}T cells (80). We have recently shown that following the peak of the CD8^{+}T cell response to LCMV, higher expression of CD25 correlates with enhanced survival and/or persistence of virus-specific CD8^{+}T cells (81).

Apart from cytokine receptors, various co-stimulatory and chemokine receptors have been shown to be important in generation of memory CD8^{+}T cells. CD27 is a co-stimulatory receptor of the TNFR family and has also been used as a marker to identify memory precursor CD8^{+}T cells (27). Expression of CD27 on virus-specific CD8^{+}T cells promotes survival, induces IL-7R expression, and protects against Fas-dependent apoptosis (82-84). CD27-CD70 interactions have been shown to induce autocrine IL-2 production in CD8^{+}T cells, thereby promoting their survival (85). Activated T cells may express CXCR3, a chemokine receptor required for T cell chemotaxis to the site of antigen (86, 87). Moreover,

expression of IL-7R, CD27 and CXCR3 has been used to identify memory CD8^{+}T cell populations with an efficient recall response (88).

Transcriptional regulation of effector and memory CD8^{+}T cell differentiation is beginning to be elucidated. Transcriptional profiling of effector and memory CD8^{+} T cells in both acute and chronic virus infection models has recently provided insight into the gene expression programs characterizing distinct cell subsets (89).

Blimp-1 (B lymphocyte induced maturation protein-1, encoded by *Prdm1*) is a transcription factor important for regulating differentiation of various cell types (90). Blimp-1 is expressed at high levels in terminally differentiated CD8^{+}T cells and can be induced by IL-2, IL-12 and IL-21 (80, 91-93). Blimp-1 suppresses memory generation and Blimp-1-deficient CD8^{+}T cells are better at producing IL-2 and develop into CD62L^{hi} IL-7Rα^{hi} memory precursor effector CD8^{+}T cells (94, 95). In an Influenza infection model Blimp-1 was shown to be important for recruitment of effector CD8^{+}T cells to the lung (95). Blimp-1 is also expressed in exhausted CD8^{+}T cells, which express inhibitory receptors during chronic LCMV infection (96). One recent study shows that Blimp-1 negatively regulates expression of the DNA-binding inhibitor Id3, thereby contributing to the development of short-lived effector T cells (97). Bcl-6 is a transcription factor expressed in memory T cells and is an antagonist of Blimp-1 activity (94, 98). Bcl-6 has been shown to be important for generation and maintenance of memory

CD8$^+$T cells (99, 100). ChIP-sequencing analysis done by Dr. HyunMu Shin also identified Bcl-6 as a potential direct target of Blimp-1 in activated CD8$^+$T cells (81).

T cell factor-1 (TCF-1, encoded by *Tcf7*) and lymphoid enhancer-binding factor-1 (LEF-1, encoded by *Lef1*) are transcription factors acting downstream in the Wnt signaling pathway (101). TCF-1 and LEF-1 have redundant roles in promoting thymocyte maturation (102). Apart from its role in thymocyte differentiation (103), TCF-1 was shown to be important for generation and maintenance of memory CD8$^+$T cells (104, 105), and TCF-1 deficient CD8$^+$T cells had impaired expansion after a secondary antigen challenge (104-106). Memory CD8$^+$T cells were progressively lost due to TCF-1 deficiency, which was associated with decreased expression of the transcription factor EOMES and the loss of IL-15 driven proliferation (105). Loss of TCF-1 and LEF-1 in CD8$^+$T cells was shown to completely abrogate development of memory precursor effector cells (107).

T-bet and EOMES are T-box transcription factors which can also regulate effector and memory CD8$^+$T cell generation. T-bet and EOMES co-operate to induce CD122 (IL-2Rβ) expression and IL-15 responsiveness in CD8$^+$T cells (108). However, T-bet and EOMES have antagonistic roles in generation and maintenance of memory CD8$^+$T cells. T-bet promotes the generation of terminally

differentiated effector cells (108, 109), whereas EOMES expression promotes generation of memory precursor effector CD8⁺T cells (110).

The forkhead box (Fox) family of transcription factors have been show to play a role regulating T cell homeostasis and tolerance (111). FoxO1 has been shown to be important for generation of competent memory T cells. Primary CD8⁺T cell responses to Listeria and LCMV were normal in mice lacking FoxO1 in CD8⁺T cells (112, 113). However, loss of FoxO1 decreased the generation of MPEC, and memory T cells were lost over time primarily due to decreased TCF-1 and CCR7 expression (112, 113). FoxO3a also belongs to the Fox family of transcription factors and was shown to negatively impact CD8⁺T cell memory formation by promoting contraction of CD8⁺T cells during resolution of immune response to Listeria infection (114).

Transcription factors of the JAK (Janus kinases) - STAT (Signal transducers and activators of transcription) family of transcription factors transmit signals from cell surface receptors to in response to a large number of growth factors and cytokines (115). Once activated in response to extracellular signals STAT proteins dimerize and translocate to nucleus to trans-activate a number of target genes. STAT proteins are known to play a key role in CTL differentiation in response to extracellular cytokine signals (68). STAT4 is induced downstream of pro-inflammatory cytokines IL-12 and type I IFN signaling (98, 116). At the peak of the immune response to acute LCMV infection STAT4 deficient mice had

increased frequency and number of MPEC suggesting that STAT4 negatively regulates memory development (117). STAT5 can be induced downstream of γ chain family of cytokines such as IL-2, IL-7 and IL-15 (115). STAT5 was shown to promote effector and memory T cell survival during acute LCMV infection (118) (119). Furthermore, STAT5 was shown to be critical for IL-7 and IL-15 driven upregulation of Bcl-2 thereby promoting survival of effector CD8+T cells (118, 119). STAT3 can be induced downstream of IL-10 and IL-21 and STAT-3 deficient CD8+T cells were defective in generating a memory cell pool (98). In the same study STAT-3 was shown to be important for maintenance of EOMES and Bcl-6 expression, which are transcription factors associated with memory development. Overall these studies show that signals induced by cytokines can induce cues to the cells to regulate the generation of effector and memory CD8+T cells.

Recent studies have identified that strength of TCR signals can inscript a transcriptional program in effector CD8+T cells governing their fate. In this regard IRF4 (Interferon regulatory factor 4) has been shown to play a critical role in orchestrating expansion and effector differentiation of CD8+T cells in response to TCR signals (120-122). IRF4 was shown to be induced in a dose dependent manner depending on the strength of TCR signaling and antigen specific IRF4 deficient CD8+T cells failed to expand and differentiate during Influenza and LCMV immune response (120, 121). Furthermore, IRF4 was shown to directly bind to *Prdm1* and *FoxO1* which are genes known to be involved in effector T cell differentiation and metabolic programming respectively (120, 121). Several studies

have also identified an inter-relationship between pathways involved in cellular metabolism and T cell differentiation. mTOR (mammalian target of rapamycin) kinases integrate nutrient sensing pathways and coordinate cell metabolism [123]. Inhibition of the mTOR pathway by rapamycin during acute LCMV infection increased the magnitude of LCMV specific CD8^{+}T cell response and led to generation of increased proportion of MPEC [124]. mTOR activity was shown inhibit the transcription factor FoxO1 which led to induction of T-bet and suppression of EOMES thereby promoting terminal effector differentiation [125, 126]. Overall these studies show that TCR signals and enzymes involved in sensing the metabolism of T cells can also instruct a transcriptional program to regulate their differentiation.

Though we still do not completely understand how memory CD8^{+}T cells are generated the studies mentioned above have shown that strength and duration of TCR signals, exposure to inflammatory cytokines and co-stimulatory molecules instruct a transcriptional program within the T cells governing their fate conversion to memory. With the advent of technologies for high throughput screening of transcriptional profiles and the availability of vast array of gene expression data it has now become possible to define transcriptional networks which regulate T cell differentiation during various stages of the immune response. A recent study identified key transcriptional signatures and networks which can be used to predict memory potential of effector CD8^{+}T cells [127]. In this study ten core gene clusters and their predicted transcriptional regulators

were identified based on their gene expression profiles in OVA-specific CD8$^+$T cells during various stages of immune response to Listeria infection. Each gene cluster was enriched in genes with a specific biological function during the immune response, and by profiling effector and memory T cell populations it was shown that expression of these core gene clusters can be used to predict effector or memory potential of CD8$^+$T cells. Consistent with previously published results Blimp-1, STAT4, ID2, T-bet were identified as regulators of terminal effector differentiation and TCF-1 was identified as a critical regulator of memory differentiation. Furthermore, these studies also helped identify new potential regulators of T cell function during an immune response. Overall, these studies highlight the fact that transcription factors can have overlapping functions and form a network to imprint a developmental program in T cells regulating their fate and memory differentiation.

E. Immunity at non-lymphoid tissues

T cells are initially activated in the spleen and lymph nodes by APCs, after which they migrate to non-lymphoid tissues to eliminate foreign antigens [128]. In response to both viral and bacterial infections antigen-specific CD8$^+$T cells have been shown to migrate to non-lymphoid tissues [129], and these T cells are uniquely geared to produce effector cytokines [129-133]. Activated T cells express chemokine receptors, which under the influence of chemokine ligands promote T cell migration to sites of antigen. CXCR3 is a chemokine receptor expressed on activated Th1 CD4$^+$T cells and on effector CD8$^+$T cells [86]. CXCR3 promotes

migration of activated CD8^{+}T cells into non-lymphoid tissue infection sites under the influence of its chemokine ligands CXCL9 and CXCL10 (134, 135). Distinct subsets of CD8^{+}T cells have been thought to circulate in lymphoid and non-lymphoid tissues and can be distinguished based on their surface expression of CD62L and CCR7 (136). CD62L^{hi}CCR7^{hi} central memory T cells (Tcm), thought to be cytolytically inactive, lack immediate effector functions and circulate through lymphoid organs, whereas CD62L^{lo}CCR7^{lo} effector memory T cells (Tem) can immediately respond to secondary challenge and circulates through non-lymphoid tissues. However, recent studies have shown that both Tcm and Tem mediate an efficient recall response and that CD62L is not the best predictor of a memory T cell pool (88). Consistent with this study, I also found that CD62L and CCR7 were uniformly down-regulated on virus-specific CD8^{+}T cells from both lymphoid and non-lymphoid tissues and could not be used as a predictor of memory precursors during resolution of the immune response.

Presence of memory T cells at non-lymphoid tissues is critical for protection against secondary challenge. Tissue-retentive memory CD4^{+} and CD8^{+}T cells have been shown to be important in protection against HSV and respiratory virus infections in mice (131, 137, 138). Resident memory CD8^{+}T cells in the non-lymphoid tissues were shown to be important for induction of a rapid local recall response and recruitment of circulating memory CD8^{+}T cells to the site of infection (133). Our lab has shown that during and after silencing of the immune response, LCMV-specific CD8^{+}T cells in the non-lymphoid tissues are

more resistant to apoptosis than those in the lymphoid organs (139). Virus-specific CD8$^+$T cells in the non-lymphoid tissues expressed lower levels of Fas and FasL and were resistant to activation-induced cell death (AICD). This differential in apoptosis could account for persistence of long-lived virus-specific memory CD8$^+$T cells at high frequencies in the non-lymphoid tissues, serving as frontline defense against secondary virus challenge.

F. P14 and SMARTA TCR transgenic mice

LCMV induces a strong CTL response which comprises of T cells capable of recognizing distinct viral epitopes. The T cell receptor (TCR) recognizes the viral peptides presented in the context of Class I (for CD8$^+$T cells) or Class II (for CD4$^+$T cells) MHC. The TCR comprises of disulfide linked α and β glycoproteins generated by somatic rearrangement of germline-encoded variable and constant gene segments. Due to the polymorphic nature of α and β chain rearrangement, different TCR on the surface of CD8$^+$T cells can be utilized to generate an epitope specific response against the virus. Therefore, the presence of T cells with different TCR re-arrangements recognizing the same epitope along with the generation of a T cell response to distinct viral epitopes makes quantitating and analyzing the total antigen-specific T cell response difficult.

The identification of CD8$^+$T and CD4$^+$T cell epitopes for LCMV in the last 20 years has allowed us to study the generation and maintenance of virus-specific T cell responses (140-144). Amongst the LCMV epitopes identified

immunodominant NP396 and GP33 and subdominant GP276 and NP205 have been widely used to study LCMV specific CD8$^+$T cell responses. The utilization of at least two techniques: a) Class I MHC tetramers containing viral peptides to identify antigen-specific cells and b) intracellular cytokine secretion assays (ICS) to measure IFNγ secretion at a single cell level in response to viral peptides have shown that at the peak of the immune response to LCMV, 80-95% of CD8$^+$T cells are virus-specific (145, 146)(147).

TCR transgenic mice which express single TCR α and β chains generated from a single antigen-specific T cell clone have further helped study various aspects of the immune response. P14 TCR transgenic mice express Vα2 and Vβ8.1 TCR chains under the control of the H$_2$K^b promoter and IgG enhancer (148-150). CD8$^+$T cells in the P14 TCR transgenic mice are specific for the immunodominant GP$_{33-41}$ epitope. Therefore, adoptive transfer of P14 CD8$^+$T cells into a congenic host allows us to track a single-TCR expressing LCMV-specific CD8$^+$T cell response. SMARTA TCR transgenic mice expressing a MHC Class II restricted TCR to study LCMV-specific CD4$^+$T cell responses have also been generated (151). The CD4$^+$T cells in these mice express Vα2 and Vβ8.1 TCR chain specific for the LCMV GP$_{61-80}$ epitope. Therefore, adoptive transfer of SMARTA CD4$^+$T cells into a congenic host allows us to track a single-TCR expressing LCMV-specific CD4$^+$T cell response. For studies presented in this thesis, we have done adoptive transfer studies with P14 CD8$^+$T and SMARTA CD4$^+$T cells to track LCMV-specific CD8$^+$ and CD4$^+$T cell responses.

G. Transcription factor ROG/*Zbtb32*

ROG is a transcription factor belonging to the POZ (Pox virus and zinc finger) family of proteins. Members of the POZ family have a BTB (broad complex, tramtrack, and bric-a-brac) domain at the N terminus for protein-protein interaction and a Zn finger domain at the C terminus for DNA binding (152). The majority of the POZ family members are transcriptional repressors. In yeast two-hybrid screens ROG was identified as a GATA-3 interacting protein (transcription factor regulating development, differentiation and activation of Th2 cells). ROG was shown to repress GATA-3 transactivation and hence the name repressor of GATA-3 (ROG) was coined (152).

ROG is expressed in T and B cells upon activation and in *in vitro* assays was shown to inhibit both Th1 and Th2 cytokine production (152). In type 2 CD8$^+$T cells (Tc2) ROG inhibited IL-4 gene activation by recruiting histone deacetylase (HDAC) 1 and 2 to induce repressive histone modifications (153). ROG-deficient T cells could differentiate into Th1 and Th2 cells, but had enhanced proliferation and cytokine production in response to *in vitro* anti-CD3 stimulation, suggesting that ROG negatively regulated T cell activation (154-156). Th1 immune responses of ROG-deficient and wild-type control mice were similar in an EAE (experimental autoimmune encephalomyelitis) induction model, suggesting that ROG-deficient mice had no defect in mounting a Th1 immune response (156). A recent study showed that in B cells, ROG interacts with the transcription factor Blimp-1 and

promotes plasma cell differentiation by suppressing Class II MHC expression (157).

Therefore, current literature supports a model where ROG is an early induced gene upon T cell activation and may negatively regulate T cell activation. However, our understanding of the function of ROG in regulating *in vivo* T cell responses during both acute and chronic viral infections is currently lacking.

H. Thesis objectives

Generation of memory T cells is the hallmark of a successful immune response. Long-lived memory CD8^{+}T cells populate both lymphoid organs and non-lymphoid tissues to provide protection to secondary challenge by the same pathogen. Our lab has previously shown that during and after resolution of the immune response to LCMV, virus-specific CD8^{+}T cells in the non-lymphoid tissues (fat-pad and peritoneum cavity) are more resistant to apoptosis than those in the lymphoid organs (spleen, inguinal lymph nodes and mesenteric lymph nodes), allowing for a higher frequency of memory T cells to persist in the non-lymphoid tissues. However, the mechanisms regulating tissue-dependent differences in CD8^{+}T cell apoptosis have not yet been elucidated. I questioned whether tissue-dependent apoptotic differences in LCMV-specific T cells were due to enrichment of non-lymphoid tissues with CD8^{+}T cells of a phenotype intrinsically resistant to apoptosis, due to preferential migration of non-apoptotic CD8^{+}T cells into peripheral sites, and/or due to tissue environment factors that

protect against cell death. I found that multiple mechanisms account for tissue-dependent differences in CD8$^+$T cell apoptosis seen during resolution of the immune response. Non-lymphoid tissues were enriched in memory phenotype CD8$^+$T cells which were intrinsically resistant to apoptosis irrespective of the tissue environment. Furthermore, I found that apoptosis-resistant CD8$^+$T cells may preferentially migrate into the non-lymphoid tissues where the availability of tissue-specific factors may enhance memory cell survival.

Transcription factors can regulate the balance between effector-memory CD8$^+$T cell generation. Blimp-1 is a transcriptional factor, and drives the differentiation of CD8$^+$T cells towards the short-lived effector fate, and the absence of Blimp-1 leads to the development of memory precursor CD8$^+$ T cells that are better at producing IL-2. Although Blimp-1 is known to regulate a number of target genes mediating plasma cell differentiation [90], only a few targets of Blimp-1 in CD8$^+$ T cells have been identified. The objective of this project was to identify the mechanism by which Blimp-1 regulates CD8$^+$T cell differentiation. Genome-wide ChIP-sequencing analysis done by Dr. HyunMu Shin identified CD25 and CD27 genes as direct targets of Blimp-1. Furthermore, we could show that suppression of CD25 and CD27 expression by Blimp-1 is one of the mechanisms by which Blimp-1 dictates the fate of effector CD8$^+$ T cells.

ROG is a transcription factor, and several *in vitro* studies have suggested that ROG may regulate the activation of CD4$^+$ and CD8$^+$ T cells. I investigated

the role of ROG in the development of anti-viral CD8$^+$ T cell responses during acute and chronic LCMV infection. By using mice deficient in ROG I showed that ROG plays a unique non-redundant role in negatively regulating CD8$^+$T cell responses during acute and chronic LCMV infection, and that it might be an important regulator of effector and memory CD8$^+$T cell generation.

Overall the studies highlighted in this thesis elucidate the mechanisms which may regulate tissue-dependent differences in CD8$^+$T cell apoptosis during acute LCMV infection. These studies will also help us gain insight into transcriptional regulation of CD8$^+$T cell effector-memory differentiation during viral infections.

CHAPTER II: MATERIALS AND METHODS

A. Mice

C57BL/6J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). P14 transgenic mice were bred onto CD90.1 (Thy1.1) and CD45.1 (Ly5.1) C57BL/6 backgrounds to distinguish the transgenic cells from wild-type (WT) cells in the C57BL/6 (CD90.2$^+$ (Thy1.2) CD45.2$^+$ (Ly5.2)) mice. $Tcf7^{-/-}$ mice on the C57BL/6 background have been described previously [158], and were bred at the UMMS. $Tcf7^{+/+}$ littermate mice were used as WT controls. The LCMV GP61-specific CD4 transgenic SMARTA CD45.1$^+$ mice [151] were bred at UMMS. CD4-Cre mice were a gift from Joonsoo Kang at UMMS. $Prdm1^{fl/fl}$ mice have been described previously [159]. This line was crossed to CD4-cre$^+$ transgenic mice, thereby deleting Blimp-1 in all αβ T cells (referred to as $Prdm1^{-/-}$). In Chapter IV, unless otherwise indicated, C57BL/6 CD4-Cre$^+$ transgenic mice were used as WT controls. For some experiments, P14$^+$ $Prdm1^{fl/fl}$ mice were crossed to Granzyme B-Cre$^+$ transgenic mice for the deletion of Blimp-1 in activated CD8$^+$T cells, as previously described [94]. $Zbtb32^{-/-}$ mice have been described previously [156], and were crossed to P14 TCR transgenic CD90.1 or CD45.1 mice to study the role of ROG in intrinsic CD8$^+$T cell function. Mice were bred and housed in specific pathogen free conditions at the UMMS in accordance with the guidelines of the Institutional Animal Care and Use Committee of UMMS (IACUC).

B. Preparation of leukocytes

Mice were sacrificed by cervical dislocation. PECs were collected by lavaging with 10 ml cold RPMI 1640 medium (Gibco). Spleen, lymph nodes and fat-pads were ground between glass microscope slides to prepare single cell suspensions. To remove contaminating erythrocytes, leukocyte preparations were treated with 0.84% ammonium chloride (NH_4Cl).

C. Virus

LCMV, strain Armstrong, was propagated in BHK cells as previously described [160]. Mice were inoculated intraperitoneally (i.p) with 0.1 ml containing 5×10^4 PFU (plaque forming units) of the Armstrong strain of LCMV in PBS. The Clone 13 variant of LCMV was propagated in baby hamster kidney BHK21 cells [12] and titrated by plaque assay on Vero cells. Mice were infected intravenously (i.v) with 2×10^6 (high dose) PFU of LCMV, strain Clone 13. In some experiments, mice were inoculated i.p with 1×10^6 PFU of Vaccinia virus (VACV), strain Western Reserve.

D. Antibodies, flocytometry and intracellular cytokine staining

Leukocytes were stained for CD8α (53-6.7; BD Pharmigen), CD8β (YTS156.7.7; Biolegend), CD4 (RM4-5; BD Pharmigen), CD44 (IM7; eBiosciences), Thy1.1/CD90.1 (HIS51; eBiosciences), Ly5.1/CD45.1 (A20; Biolegend), KLRG-1 (2F1; eBiosciences), CD127 (A7R34; eBiosciences), CD27 (LG.310; BD Pharmingen), CXCR3 (CXCR3-173; Biolegend), CXCL9 (MIG-2F5.5; eBiosciences), TCF-1

(C63D9; Cell Signaling), EOMES (Dan11mag; eBiosciences), Bcl-2 (3F11; BD Pharmingen), CD122 (TM.BETA-1; BD Pharmigen), CD70 (FR70; Biolegend), CD25 (PC61; BD Pharmigen), Granzyme B (NGZB; eBiosciences), CD62L (MEL-14; BD Pharmigen), CD155 (TX56; Biolegend). For TCF-1 intracellular staining AF647 conjugated goat-anti rabbit (Invitrogen) secondary antibody was used. To stain for intracellular antigens FOXP3/Transcription factor staining buffer kit (eBiosciences) was used. For intracellular cytokine secretion assays leukocytes were incubated for 5 hours ex-vivo with 1µM LCMV-specific $NP_{396-404}$, GP_{33-41} or GP_{61-80} peptide or 1µM VACV-specific B8R or K3L peptide in the presence of Golgi Plug (BD Biosciences). Cells were then permeabilized using the cytofix/cytoperm kit (BD Biosciences) followed by intracellular staining for IFNγ (XMG1.2; eBiosciences), IL-2 (JES65H4; BD Pharmigen) and TNF (MP6-XT22; Biolegend). Samples were analyzed on an LSRII flow cytometer (Becton Dickinson), and data were further analyzed using FlowJo (Tree Star).

E. H2D^b MHC tetramer staining

For identification of virus-specific CD8$^+$T cells by Class I MHC tetramer staining $2x10^6$ leukocytes were incubated with PE- or APC-conjugated H-2D^b-restricted $NP_{396-404}$ or GP_{33-41} tetramers for 1 hr at 4C.

F. Annexin V, TUNEL and Caspase-3 assay

Leukocytes were stained for Annexin V (eBiosciences) and samples were analyzed live according to the manufacturers' protocol. 7AAD (BD Pharmingen)

was used to gate out dead and late-apoptotic populations. For TUNEL and Caspase-3 staining, leukocytes were incubated at 37C directly ex-vivo for 5 hours in culture media. TUNEL assay (APO-DIRECT KIT; BD Pharmigen) and active Caspase-3 (C92-605; BD Pharmigen) staining was carried out according to the manufacturers' protocol. For active Caspase-3 and TCF-1 co-staining, after rabbit TCF-1 primary and goat anti-rabbit secondary antibody staining samples were incubated with unlabeled rabbit IgG (Southern Biotech) to saturate the goat anti-rabbit secondary antibody. Cells were then incubated with rabbit active Caspase-3 antibody. For inhibiting Caspase dependent apoptosis leukocytes were cultured for 5 hours ex-vivo in the presence of 100µM Z-VAD-FMK (Enzo Life Sciences).

G. pSTAT5 staining

Freshly isolated leukocytes were plated in 96 well plates and stimulated with IL-7 cytokine (Pepro Tech) as shown in Figure III-30. Cells were fixed on ice with BD cytofix (BD Biosciences) for 15 min and stained with surface antibodies after which cells were permeabilized with BD Phosflow perm buffer III (BD Biosciences) for 30 mins on ice. Cells were then stained intracellularly for pSTAT5 (pY964; BD Biosciences).

H. Adoptive transfer studies, T cell isolation and cell labeling

Unless otherwise noted virus-specific CD8^{+}T cell responses were tracked by transferring 2 x10^{5} P14 splenocytes (CD90.1 or CD45.1) into a congenic

C57BL/6 host (CD90.2$^+$ CD45.2$^+$). Splenocytes from day 7 LCMV-infected mice were labeled with CFSE (2µM) or DDAO (0.5µM), washed with HBSS and adoptively transferred into an infection matched C57BL/6 host as shown in Figure III-28. The schematic for adoptive transfer of CD25$^{hi/lo}$ and CD27$^{hi/lo}$ CD8$^+$T cells is shown in Figure IV-13 and IV-20, respectively. Recall responses of $Zbtb32^{-/-}$ or WT P14 CD8$^+$T cells at day 30 post-LCMV infection were compared as shown in the schematic in Figure V-18. Wherever noted, total CD8$^+$T cells were isolated with the CD8$^+$T Cell Isolation Kit II (Miltenyi Biotec), and LCMV-specific P14$^+$CD8$^+$T cells were further sorted on a FACS Aria cell sorter.

I. BrdU staining

WT and $Prdm1^{-/-}$ mice were infected with LCMV, and then injected via i.p with 100ul of 10mg/ml BrdU (Bromodeoxyuridine) solution (BD Pharmingen) at day 7 or 8 post-infection. Twelve hours later, splenocytes were harvested and GP$_{33-41}$ tetramer-positive CD8$^+$T cells were analyzed for BrdU incorporation according to the manufacturers' instructions.

J. Apyrase and FTY720 treatment

Day 7 LCMV infected mice were i.p injected with 5U Apyrase (New England Biolabs) or succinate buffer control and were sacrificed after 6 hours. FTY720 (Cayman Chemical Company) at 1 mg/kg was given i.v (day 5 and 6) and i.p (day7) post-LCMV infection as shown in Figure III-21.

K. *In vitro* T cell stimulation and proliferation assay.

CD8$^+$T cells were isolated from WT or *Prdm1$^{-/-}$* mice at day 7 after LCMV infection. CD8$^+$T cells were labeled with CFSE (2uM), washed, and were cultured for 2 days with or without the indicated cytokines. IL-1β, IL-6, IL-12, IL-18, IL-21, and IL-23 were purchased from R&D. IL-2 and IL-4 were purchased from BD Biosciences. IFNγ, IL-7, and IL-15 were purchased from PeproTech. IFNβ was purchased from PBL InterferonSource. Cytokine concentrations were as follows: IFNβ (200 U), IFNγ (200 U), IL-1β (50 ng/ml), IL-2(40 ng/ml), IL-4 (40 ng/ml), IL-6 (40 ng/ml), IL-7 (20 ng/ml), IL-12 (20 ng/ml),IL-15 (20 ng/ml), IL-18 (20 ng/ml), IL-21 (20 ng/ml), and IL-23 (40 ng/ml).

L. Plaque assay for LCMV

Spleens from LCMV Armstrong infected WT or *Prdm1$^{-/-}$* or *Zbtb32$^{-/-}$* mice were ground and titrated by plaque assay on Vero cells, as previously described [160]. The same protocol was used to analyze viral titers from spleen and liver of day 10 LCMV Clone 13 infected WT or *Zbtb32$^{-/-}$* mice.

M. Histology

Lung and liver tissues from high dose LCMV Clone 13 infected mice were fixed in 10% paraformaldehyde and stained with hematoxylin and eosin (H&E).

N. RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen), converted to cDNA (Quantitect Reverse Transcription kit, Qiagen), and analyzed by real-time

quantitative PCR amplification on a Bio-Rad iCycler by using Quanti-Fast RT-PCR SYBR green kit (Qiagen), according to the manufacturers' protocol. Primers for *Cxcl9* and *Cxcl10* were purchased from Qiagen. *Zbtb32* and *Actb* primers were purchased from Real Time Primers, LLC.

O. Microarray analysis

Day 6 LCMV infected P14 TCR transgenic WT or *Zbtb32*$^{-/-}$ CD8$^+$T cells were sorted on FACS Aria cell sorter and total RNA was isolated using an RNeasy mini kit (Qiagen). Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix) was used, and data were analysed on Affymetrix Transcriptome Analysis Console (TAC) software.

P. Statistical analysis

In chapter III data significance (p values) was calculated using an unpaired Students t test. All error bars in chapter III of the manuscript represent Standard Deviation from the mean (StDev). In Chapters IV and V the statistical difference between samples was analyzed with an unpaired Students t test. All error bars in Chapter IV and V represent the Standard Error of the Mean (SEM).

CHAPTER III: MECHANISMS REGULATING TISSUE-DEPENDENT DIFFERENCES IN CD8^{+}T CELL APOPTOSIS DURING ACUTE LCMV INFECTION

The majority of intracellular pathogen infections are initiated at non-lymphoid tissue sites. T cells get primed and activated in the lymphoid organs after which they migrate to non-lymphoid tissues and produce effector cytokines to eliminate the pathogen (128, 129, 131, 132). The presence of memory T cells in non-lymphoid tissues has been shown to be critical for protective immunity in various viral infection models (133, 137, 138, 161). Our lab has previously shown that virus-specific CD8^{+}T cells in the non-lymphoid tissues were more resistant to apoptosis as compared to the lymphoid organs during the resolution and memory phase of the immune response to an acute LCMV infection (139). That study provided a mechanism by which memory T cells would be protected and retained at a higher frequency in the non-lymphoid tissues to provide protection to secondary challenges. However, the mechanisms which regulate tissue-dependent differences in CD8^{+}T cell apoptosis have not been investigated so far.

I hypothesized that the increased resistance to apoptosis seen in virus-specific CD8^{+}T cells in the non-lymphoid tissues could be due to any of several factors: 1) enrichment of non-lymphoid tissues with a phenotype of cells which are intrinsically resistant to apoptosis, 2) preferential migration of apoptosis-resistant CD8^{+}T cells to non-lymphoid tissues during an immune response, 3) the presence of tissue-specific survival factors which promoted stability of CD8^{+}T

cells in the non-lymphoid tissues, 4) efficient removal of pre-apoptotic T cells from non-lymphoid tissue sites contributing to the phenotype. In this chapter I provide evidence that increased resistance to apoptosis seen in virus-specific CD8^{+}T cells in the non-lymphoid tissues was due to a combination of several factors. Non-lymphoid tissues were enriched in memory-phenotype CD8^{+}T cells, which were intrinsically resistant to apoptosis irrespective of the tissue environment. Furthermore, I show that apoptosis-resistant CD8^{+}T cells preferentially migrate into the non-lymphoid tissues where the availability of tissue-specific factors may enhance memory cell survival.

A. CD8^{+}T cells in the non-lymphoid tissues are resistant to apoptosis

Our lab has previously documented that LCMV-specific CD8^{+}T cells in the non-lymphoid tissues reacted less with the pre-apoptotic marker Annexin V (139) and that adoptively transferred Annexin V-reactive cells failed to develop into memory cells (65). To track virus-specific CD8^{+}T cell responses, splenocytes of P14 transgenic mice (harboring CD8^{+}T cells specific for the LCMV GP$_{33-41}$ epitope) were adoptively transferred into a congenic C57BL/6 host followed by infection with LCMV, strain Armstrong. At day 9 post-LCMV infection, lymphoid (spleen, inguinal lymph nodes, mesenteric lymph nodes) and non-lymphoid (fat-pad, PEC) tissues were harvested. Wherever possible I have tried to compare the phenotype in multiple organs, but for most of the studies PEC were used as a representative of non-lymphoid tissue and spleen was used as a representative of lymphoid organs. As shown in Figure III-1 and consistent with the previous

results, I found that LCMV-specific CD8^{+}T cells in the non-lymphoid tissues reacted less with the pre-apoptotic marker Annexin V. The plots in Figure III-1 were gated on 7AAD negative "viable" lymphocytes to distinguish pre-apoptotic cells from late-apoptotic populations. The differences in Annexin V reactivity of virus-specific CD8^{+}T cells from lymphoid and non-lymphoid tissues are further highlighted by differences in mean fluorescent intensity (MFI) of Annexin V within the antigen-specific T cell population (Figure III-1). However, because Annexin V can also bind at some level to activated and proliferating T cells (63, 64), I reassessed bona-fide end stage apoptotic cells using the TUNEL assay to detect fragmented DNA, which is a classic hallmark of apoptosis. Detection of end stage apoptotic cells directly *ex-vivo* is difficult, as most of them are efficiently cleared in vivo by phagocytes (162). I therefore cultured cells directly *ex-vivo* for 5 hours to detect TUNEL positive end stage apoptotic cells. Cells from five mice were pooled for each experiment to perform multiple assays simultaneously.   In accordance with our previous observations, I found that at day 9 post-LCMV infection, virus-specific CD8^{+}T cells in the non-lymphoid tissues including the peritoneal cavity and fat-pad were more resistant to apoptosis and less reactive with TUNEL than those in the lymphoid organs, including spleen, inguinal lymph nodes and mesenteric lymph nodes (Figure III-2). In the previous studies tissue-dependent apoptotic differences persisted at least up to 4 months post-LCMV infection, and the phenotype was shown to be long lasting (139). In accordance with the previous observations, I found that at day 35 post-LCMV infection
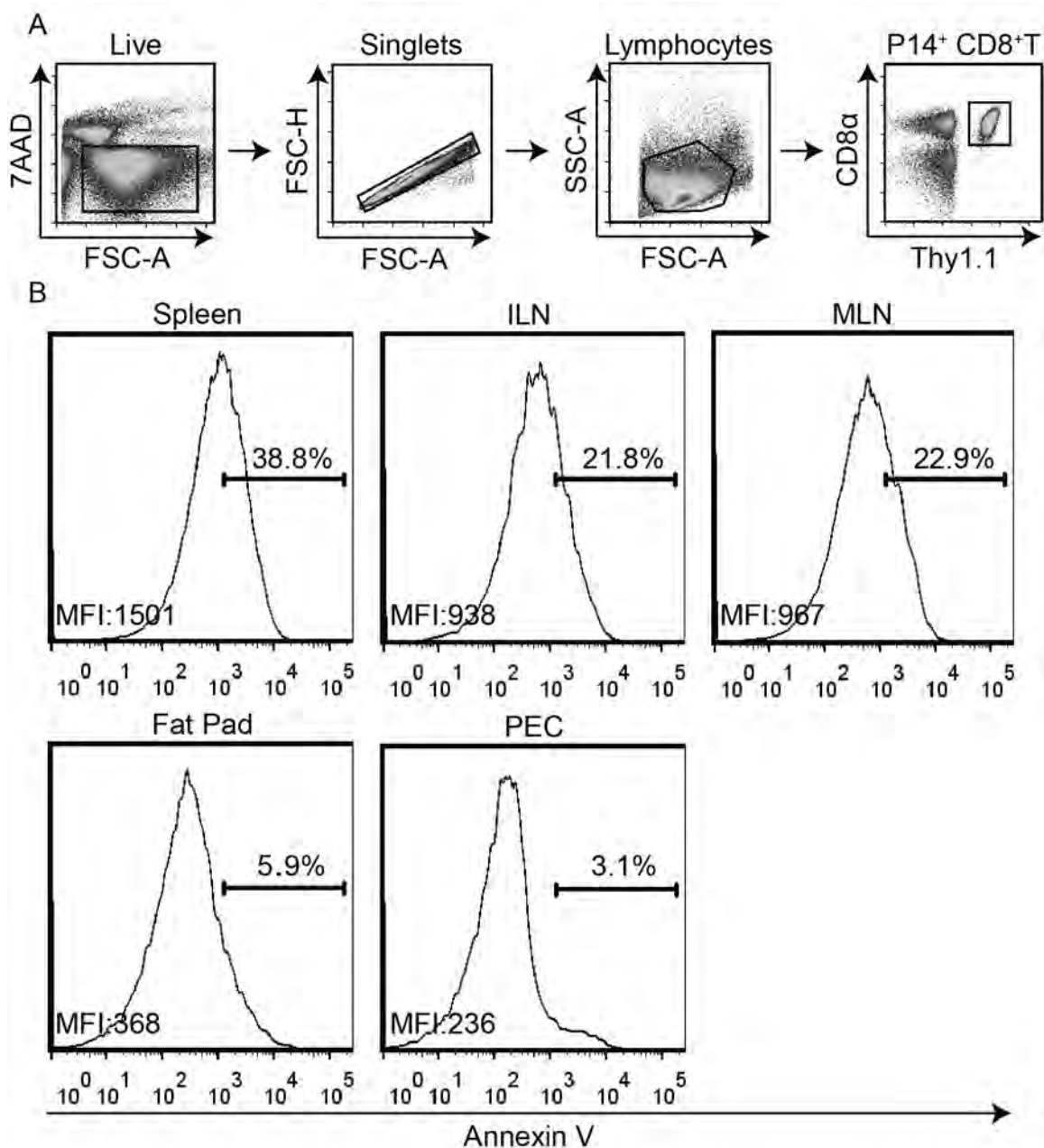
FIGURE III-1. Lower frequency of Annexin V-reactive virus-specific CD8+T cells in the non-lymphoid tissues. A) Representative plots of general gating strategy used in this thesis. B) P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Annexin V reactivity of P14+CD8+T cells in lymphoid (Spleen, ILN, MLN) and non-lymphoid (PEC, Fat Pad) tissues at day 9 post-LCMV infection. Leukocytes from five mice were pooled for the experiment, which was repeated at least three times. MFI for Annexin V reactivity of P14+CD8+T cells in each organ is listed below each histogram.
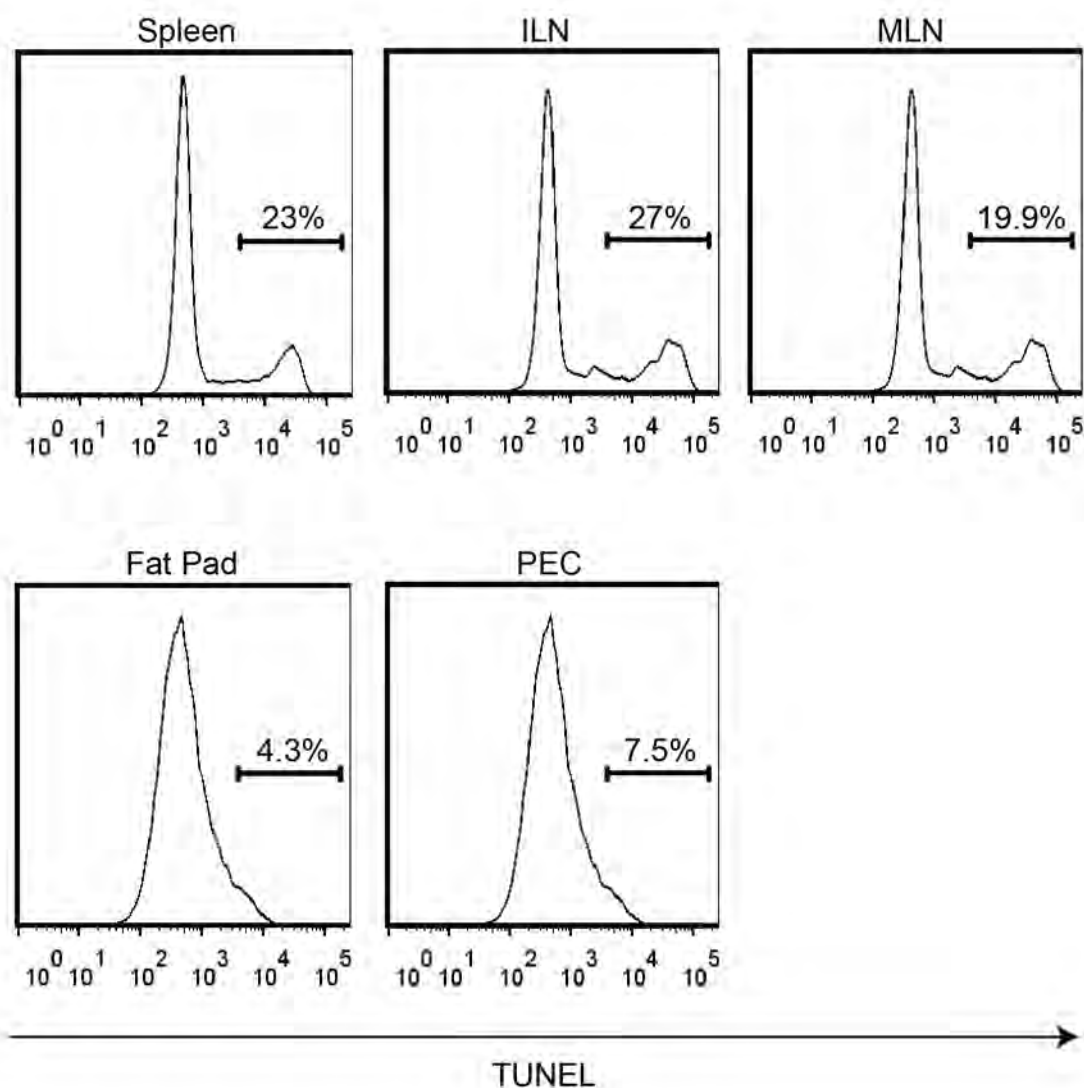
FIGURE III-2. Lower frequency of TUNEL-reactive virus-specific CD8⁺T cells in the non-lymphoid tissues. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods.Lymphocytes from lymphoid (Spleen, ILN, MLN) and non-lymphoid (PEC, Fat Pad) tissues were isolated at day 9 post LCMV infection and cultured ex-vivo for 5 hours post isolation. TUNEL reactivity of P14⁺CD8⁺T cells is represented in the histograms. Leukocytes from five mice were pooled for the experiment, and the experiment was repeated at least three times.

(memory phase), virus-specific CD8⁺T cells in the peritoneal cavity (non-lymphoid tissue) remained less apoptotic than those in the spleen (lymphoid organ) as detected by Annexin V assay (Figure III-3).

Caspases belong to a family of cysteine proteases which play an essential role in apoptosis. Apoptotic T cells have been shown to have increased levels of activated Caspase-3 (60), and T cells from Caspase-3-deficient mice have reduced susceptibility to AICD driven by CD3 crosslinking and Fas ligands (61). Therefore, I tested whether increased apoptosis of virus-specific CD8⁺T cells in lymphoid organs correlated with increased Caspase-3 activation. Virus-specific CD8⁺T cells from day 9 LCMV-infected PEC or spleen were cultured for 5 hours directly *ex-vivo* and stained for active-Caspase 3. Consistent with the increased apoptosis, virus-specific CD8⁺T cells in spleen also had higher levels of active Caspase-3, in comparison to the PEC (Figure III-4). To further confirm that the CD8⁺T cell apoptosis was caspase dependent, day 9 splenocytes from LCMV-infected mice were incubated for 5 hours in the presence of the pan-caspase inhibitor ZVAD-FMK, which irreversibly inhibits activity of caspase family proteases and blocks apoptosis. The virus-specific CD8⁺T cells from the spleen were less TUNEL reactive when incubated in the presence of ZVAD-FMK (Figure III-5) as compared to control-treated cells, suggesting that ZVAD-FMK was blocking apoptosis and that the CD8⁺T cell apoptosis required caspases.

Day 35 LCMV

Spleen

35.7%

MFI:1132

10^0 10^1 10^2 10^3 10^4 10^5

PEC

2.9%

MFI:186

10^0 10^1 10^2 10^3 10^4 10^5
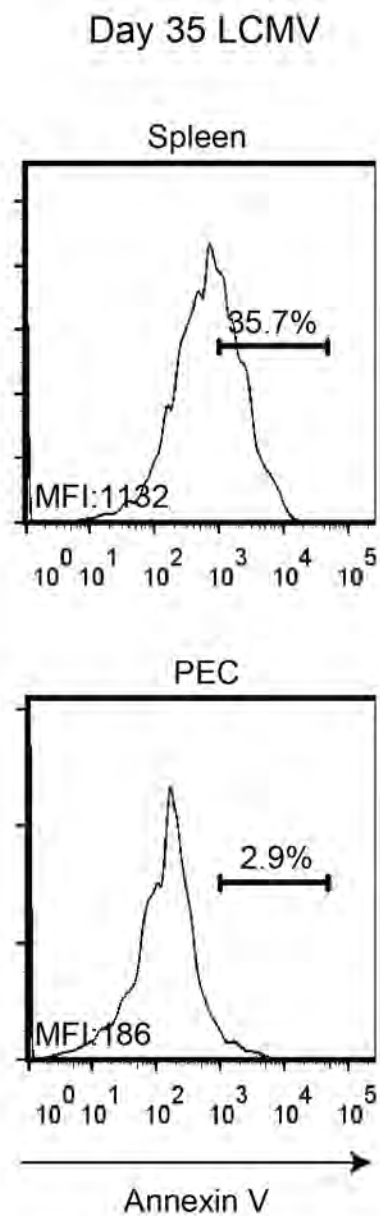
Annexin V

FIGURE III-3. Lower frequency of Annexin V-reactive virus-specific CD8+T cells in the PEC at day 35 post LCMV infection. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Annexin V reactivity of P14+CD8+T cells in the spleen and PEC at day 35 post-LCMV infection. Leukocytes from five mice were pooled for the experiment, and the experiment was done two times. MFI for Annexin V reactivity of P14+CD8+T cells in each organ is listed below each histogram.
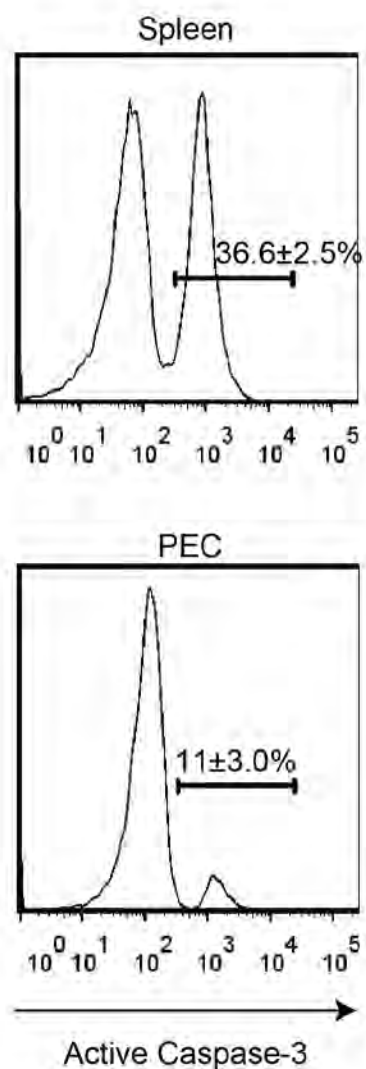
FIGURE III-4. Lower frequency of active Caspase-3 reactive virus-specific CD8+T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Lymphocytes from spleen and PEC were isolated at day 9 post-LCMV infection and cultured *ex-vivo* for 5 hours post isolation. Percentage ± StDev of P14+CD8+T cells reactive with active Caspase-3 depicted in histograms. Experiment was repeated at least three times with 3 mice in each experiment.

Cellular apoptosis results from the interplay between pro and anti-apoptotic factors, and higher levels of the anti-apoptotic Bcl-2 in effector CD8^{+}T cells have been shown to be important in counteracting the effects of pro-apoptotic Bim, thereby allowing for survival of cells during contraction of the immune response (54). Activated virus-specific CD8^{+}T cells that become memory cells also express higher levels of Bcl-2 (53). I therefore compared Bcl-2 expression in virus-specific CD8^{+}T cells from PEC and the spleen. Virus-specific CD8^{+}T cells in the PEC expressed modestly higher levels of Bcl-2 as compared to those from the spleen at day 9 post-LCMV infection as shown by the overlay in Figure III-6. The MFI of Bcl-2 expression in antigen-specific CD8^{+}T cells from PEC was also higher than in the spleen as shown by the histogram in Figure III-6. Therefore by several criteria I could show that virus-specific CD8^{+}T cells in the peripheral non-lymphoid tissues are more resistant to apoptosis than those in the lymphoid organs.

B. Non-lymphoid tissues are enriched for apoptosis-resistant memory phenotype CD8^{+}T cells

I next sought to determine why CD8^{+}T cells in the peripheral tissues were more resistant to apoptosis. I hypothesized that non-lymphoid tissues might be enriched in an antigenic phenotype of cells which were intrinsically resistant to apoptosis. CD8^{+}T cells expressing high amounts of IL-7R have been shown to express more Bcl-2 and to be relatively resistant to apoptosis (65, 72). Surface expression of IL-7Rα along with KLRG-1 (inhibitory receptor found on T and NK

SPLEEN

Control

23.4±1.8%

10^1 10^2 10^3 10^4 10^5

Z-VAD

12.2±1.7%

10^1 10^2 10^3 10^4 10^5

TUNEL
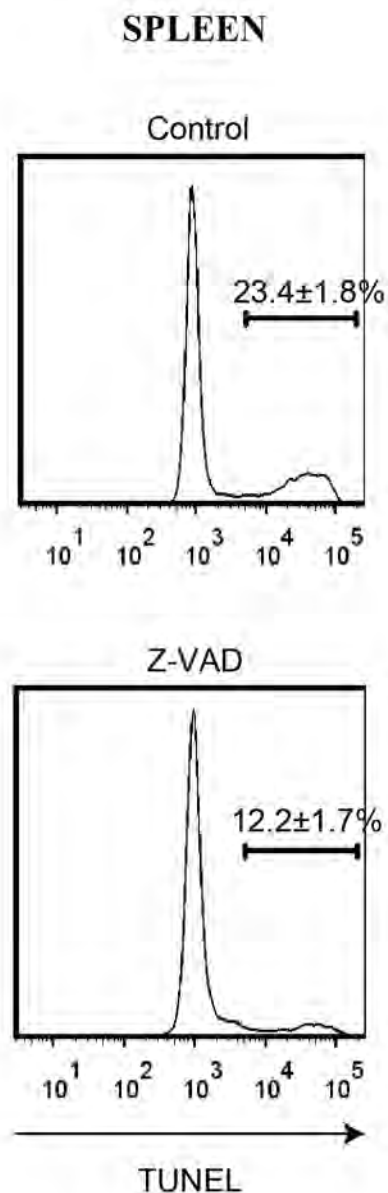
FIGURE III-5. T cell apoptosis during resolution of immune response is Caspase dependent. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Splenocytes isolated at day 9 post infection were cultured 5 hours *ex-vivo* in presence or absence of Z-VAD FMK. Percentage ± StDev of P14$^+$CD8$^+$T cells reactive with TUNEL depicted in histograms. Experiment was done twice with 3 mice in each experiment.
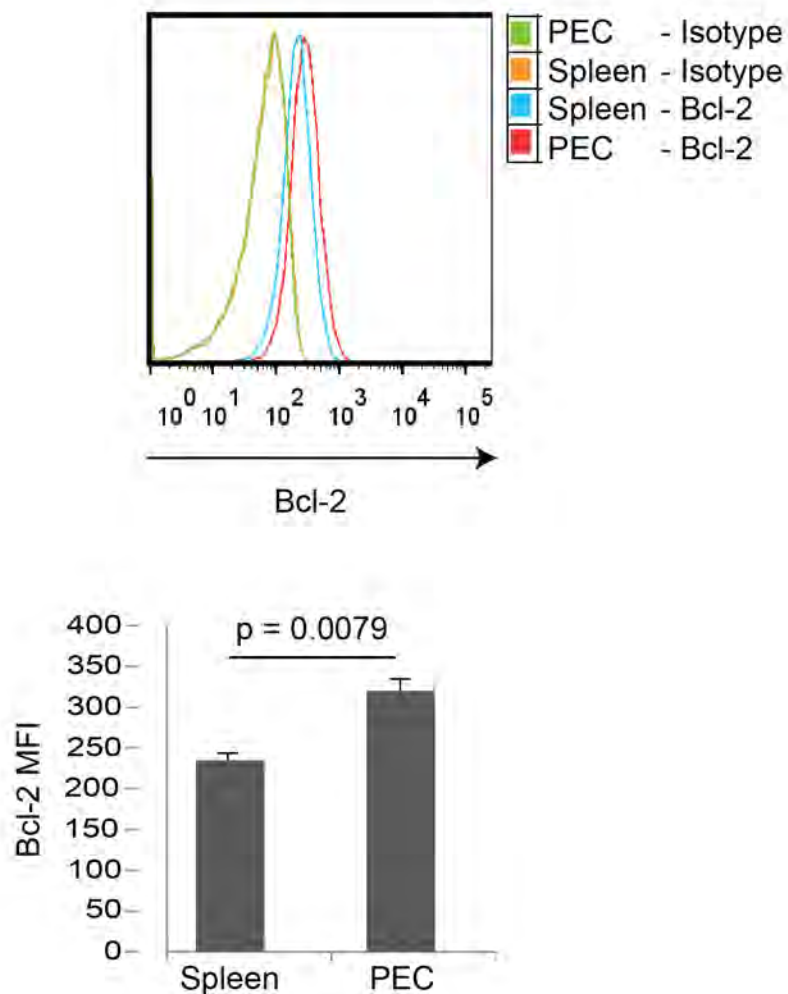
FIGURE III-6. Higher Bcl-2 expression in virus-specific CD8⁺T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Overlay of Bcl-2 expression (top) in P14⁺CD8⁺T cells in the PEC (red), spleen (blue) or isotype control (green, orange) at day 9 post-LCMV infection. MFI of Bcl-2 expression (bottom) in P14⁺CD8⁺T cells in PEC and spleen shown in the histogram. Experiment was done twice with 3 mice in each experiment.

cells) has been used to identify cells which will preferentially make it into memory (Memory Precursor effector cells: IL-7R^{hi} KLRG-1^{lo}) or die during contraction of the immune response (Short-Lived effector cells: IL-7R^{lo} KLRG-1^{hi}) [69, 74]. At day 8 post-LCMV infection virus-specific CD8$^+$T cells from lymphoid organs (spleen, ILN, MLN) and non-lymphoid tissues (PEC, fat-pad) were stained directly *ex-vivo* for IL-7Rα and KLRG-1. I found that a higher proportion of virus-specific CD8$^+$T cells with a MPEC phenotype (IL-7R^{hi} KLRG-1^{lo}) were present in the non-lymphoid tissues as compared to lymphoid organs at day 8 post-LCMV infection (Figure III-7). Tissue-dependent apoptotic differences persist long term after the resolution of the immune response and I questioned whether PEC were enriched in memory phenotype cells when compared to spleen during memory phase of the immune response. I found a higher frequency of memory phenotype virus-specific CD8$^+$T cells persisted in the PEC at day 40 post-LCMV infection compared to the spleen (Figure III-8).

Apart from IL-7Rα and KLRG-1, other phenotypic markers have been used to identify cells with a memory phenotype. CD27 is a co-stimulatory receptor of the TNF-R family, and stimulation of CD27 on CD8$^+$T cells in vitro has been shown to promote survival [84, 163]. *In vivo* blockade of CD27 during resolution of the immune response promotes apoptosis of virus-specific CD8$^+$T cells [83]. Therefore, I compared CD27 expression on virus-specific CD8$^+$T cells from PEC and spleen at day 8 post-LCMV infection. A higher proportion of virus-specific CD8$^+$T cells in the PEC expressed CD27 as compared to those in the
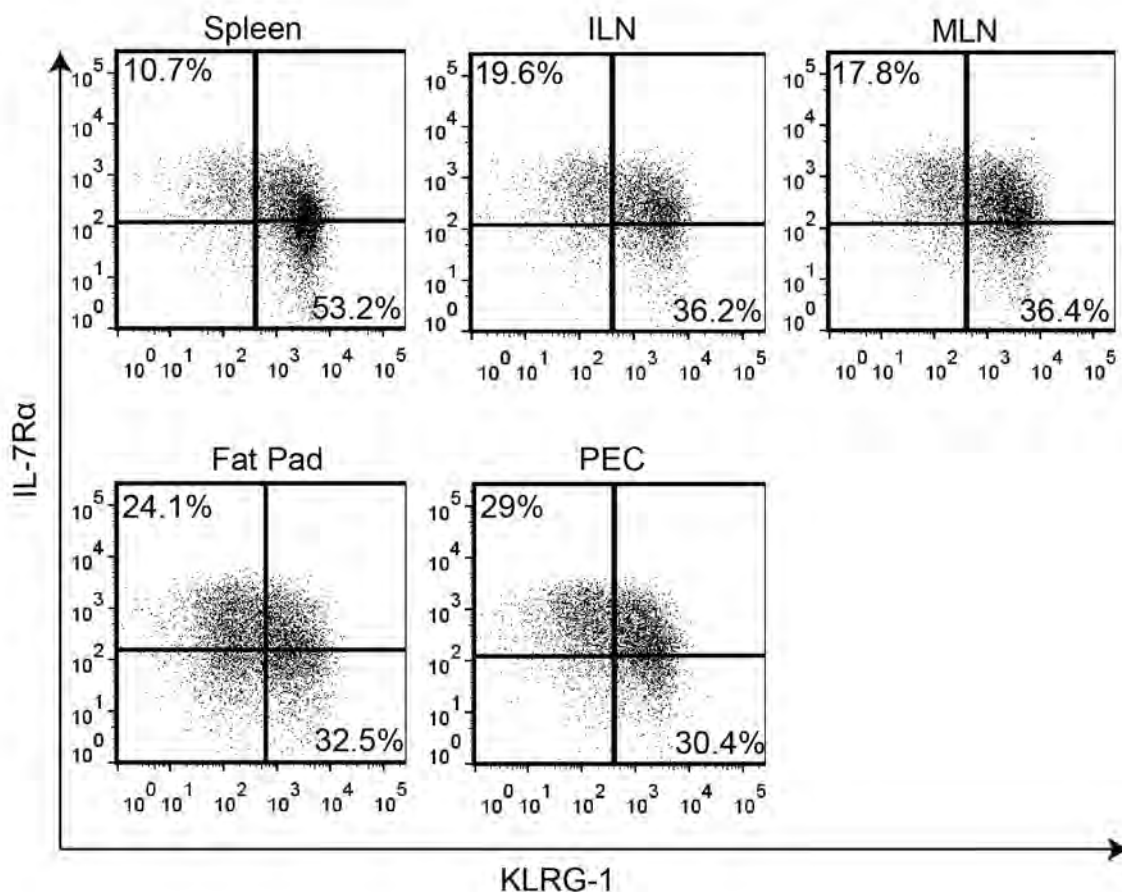
FIGURE III-7. Higher proportion of memory precursor effector cells in the non-lymphoid tissues. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. P14+CD8+T cells in lymphoid (spleen, ILN, MLN) and non-lymphoid (PEC, fat pad) tissues at day 8 post-LCMV infection were stained with antibodies for IL-7R and KLRG-1. Leukocytes from five mice were pooled for the experiment, and the experiment was repeated at least three times. Percentage of memory precursor effector cells (top left) or short-lived effector cells (bottom right) are depicted.
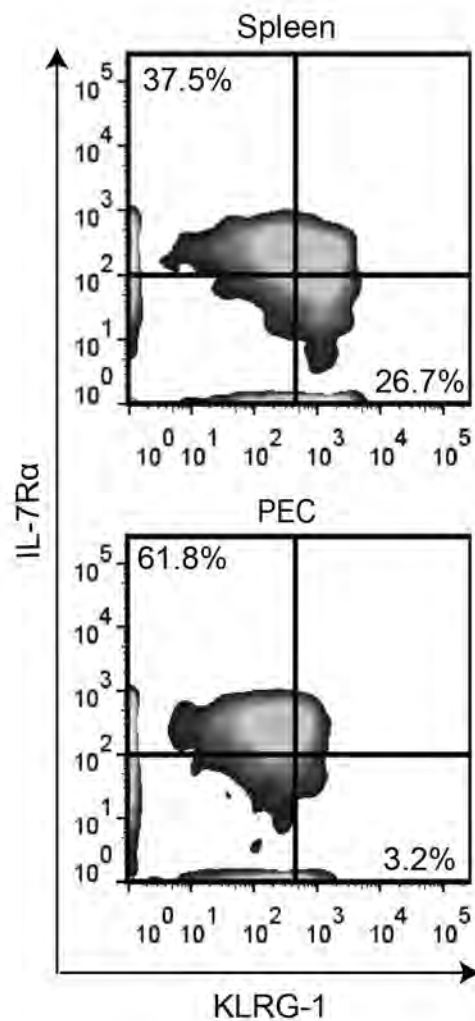
FIGURE III-8. Higher proportion of memory precursor effector cells in the PEC at day 40 post-LCMV infection. P14 splenocytes were adoptively trans-ferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. P14⁺CD8⁺T cells in spleen and PEC at day 40 post-LCMV infection were stained for IL-7R and KLRG-1. Leukocytes from five mice were pooled for the experiment, and the experiment was done twice. Percentage of memory precursor effector cells (top left) or short-lived effector cells (bottom right) are depicted.

spleen, as shown in Figure III-9. Although CD27 is known to be expressed in memory phenotype CD8$^+$T cells, correlation of high CD27 expression with decreased apoptosis during resolution of the immune response has not been tested. To confirm that CD27 expression on CD8$^+$T cells correlates with survival, I compared apoptotic profiles of CD27^{hi} and CD27^{lo} CD8$^+$ T cells. Virus-specific CD8$^+$T cells from PEC or the spleen were cultured for 5 hours directly *ex-vivo* at day 8 post-LCMV infection and stained for CD27, TUNEL and active Caspase-3. As hypothesized, I found that cells expressing higher levels of CD27 from both the PEC and spleen reacted less with apoptotic markers TUNEL and active Caspase-3 at day 8 post-LCMV infection (Figure III-9). Therefore, CD27-expressing virus-specific CD8$^+$ T cells were less apoptotic, and were present at a higher proportion in the PEC. CXCR3 is another marker preferentially expressed on memory phenotype CD8$^+$T cells (69). Therefore, I compared CXCR3 expression on virus-specific CD8$^+$T cells from PEC and spleen at day 8 post-LCMV infection. A higher proportion of virus-specific CD8$^+$T cells in the PEC expressed CXCR3 as compared to those in the spleen, as shown in Figure III-10. Although CXCR3 is known to be expressed in memory phenotype CD8$^+$T cells, correlation of high CXCR3 expression with decreased apoptosis during resolution of the immune response has not been tested. To confirm that CXCR3 expression on CD8$^+$T cells correlates with survival, I compared apoptotic profiles of CXCR3^{hi} and CXCR3^{lo} CD8$^+$ T cells. As hypothesized, I found that cells expressing higher levels of CXCR3 from both the PEC and spleen reacted less with apoptotic
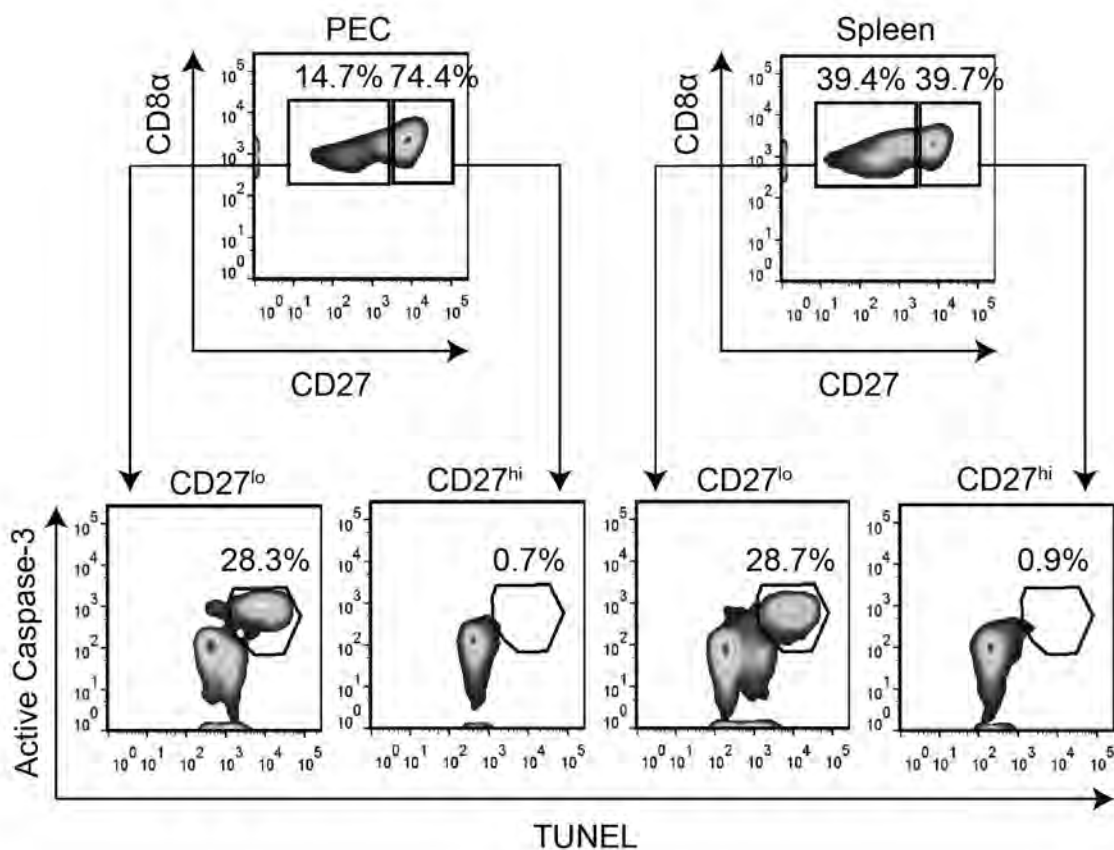
FIGURE III-9. Higher proportion of apoptosis-resistant CD27-expressing CD8+T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percentage of CD27 (top) expressing P14+CD8+T in PEC or spleen at day 8 post-LCMV infection. At day 8 post-LCMV infection, lymphocytes from PEC and spleen were cultured for 5 hours directly *ex-vivo* followed by TUNEL and active Caspase-3 staining. TUNEL and Caspase-3 reactivity of CD27-hi or -lo (bottom) P14+CD8+T cells in PEC or spleen are represented. Graphs are representative of 3 experiments with at least 3 mice in each experiment.
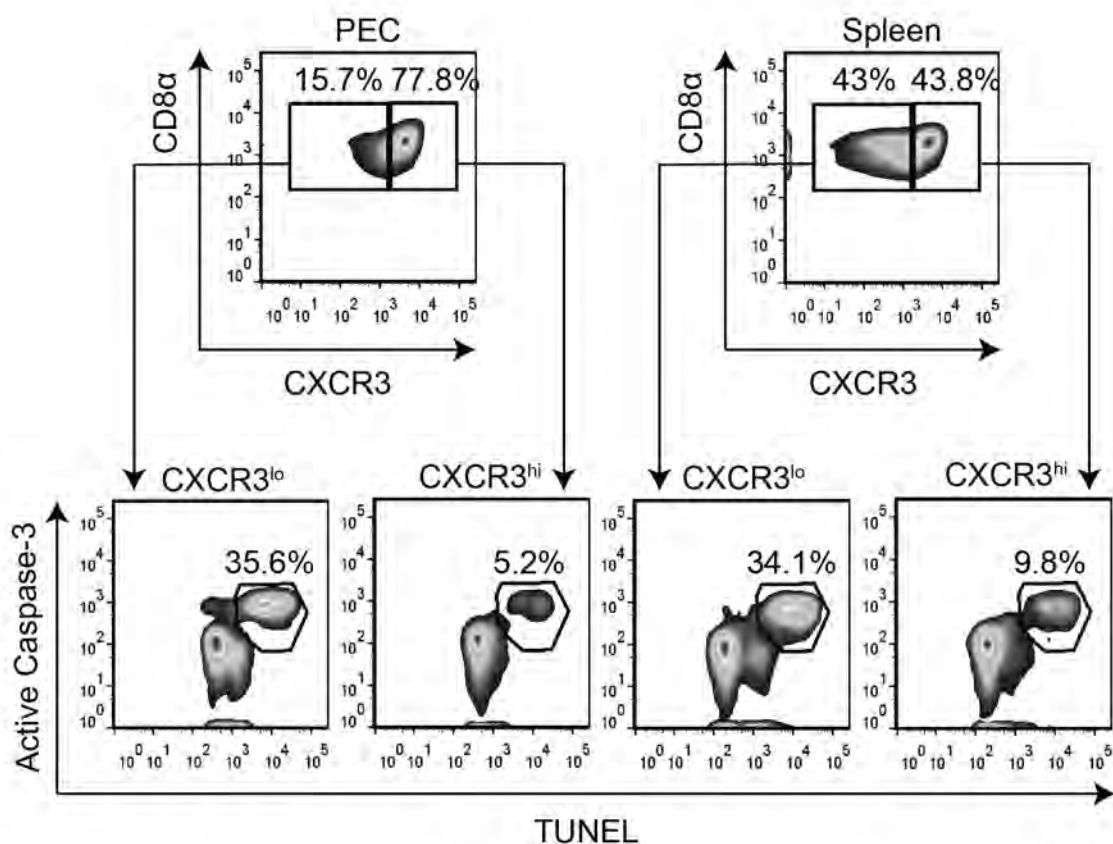
FIGURE III-10. Higher proportion of apoptosis-resistant CXCR3-expressing CD8+T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percentage of CXCR3 (top) expressing P14+CD8+T in PEC or spleen at day 8 post-LCMV infection. At day 8 post LCMV infection, lymphocytes from PEC and spleen were cultured for 5 hours directly *ex-vivo* followed by TUNEL and active Caspase-3 staining. TUNEL and Caspase-3 reactivity of CXCR3-hi or -low (bottom) P14+CD8+T cells in PEC or spleen are represented. Graphs are representative of 3 experiments with at least 3 mice in each experiment.

markers TUNEL and active Caspase-3 at day 8 post-LCMV infection (Figure III-10). Thus, CXCR3-expressing virus-specific CD8$^+$ T cells were less apoptotic, and were present at a higher proportion in the PEC. Therefore, by several criteria, I could show memory T cells resistant to apoptosis were present at a higher frequency in PEC as compared to the spleen. Moreover, during resolution of the immune response CD27 and CXCR3 expression on CD8$^+$T cells correlated with decreased apoptosis. Interestingly, CD27- and CXCR3-expressing CD8$^+$T cells reacted less with apoptotic markers TUNEL and active Caspase-3 irrespective of the tissue environment, suggesting that CD27- and CXCR3-expressing cells were intrinsically resistant to apoptosis.

The ability of virus-specific CD8$^+$T cells to produce multiple cytokines, such as IFNγ, TNF and IL-2, simultaneously during viral infection is a primary indicator of their functional capacity, and IL-2-producing CD8$^+$T cells are more likely to become memory cells than those that do not make IL-2 (79). I examined the cytokine repertoire of virus-specific CD8$^+$T cells isolated from PEC and spleen. Day 8 LCMV-infected splenocytes or PEC were cultured directly *ex-vivo* for 5 hours in the presence of LCMV-specific GP33 or NP396 peptide epitopes. As shown in Figure III-11, virus-specific CD8$^+$T cells from PEC were enriched in IFNγ, TNF and IL-2 triple producers as compared to the spleen (% indicated on top right represent triple cytokine producers), indicating that T cells in the PEC were functionally superior than in the spleen during resolution of the immune response. I have shown that during resolution of the immune response CD27 and

Gated: CD8α IFNγ[+]
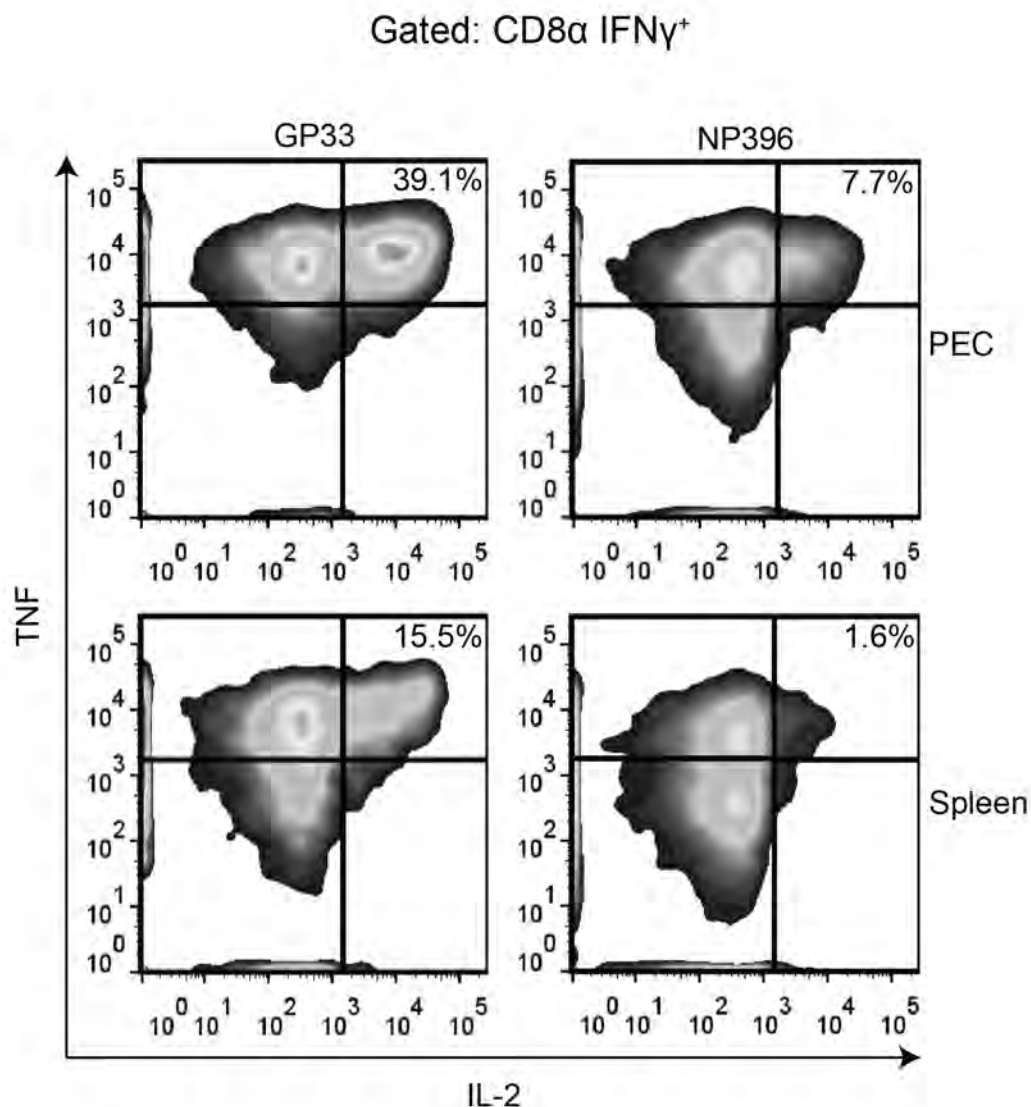
FIGURE III-11. Higher frequency of triple-cytokine producing virus-specific CD8+T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Day 9 LCMV-infected lymphocytes from PEC and spleen were cultured for 5 hours in the presence of LCMV-specific GP33 or NP396 peptides. Plots are gated on virus-specific CD8+ IFNγ+ T cells. Numbers in top right represent the percentage of TNF+ IL-2+ virus-specific CD8+T cells. Graphs are representative of 3 experiments with at least 3 mice in each experiment.

CXCR3 expression on CD8$^+$ T cells correlated with decreased apoptosis. However, as a further correlate of CD27 and CXCR3 expression with functional memory CD8$^+$T cell pool I compared IL-2 production in CD27- and CXCR3-expressing virus-specific CD8$^+$T cells. As shown in Figure III-12 CD27^{hi} and CXCR3^{hi} virus-specific CD8$^+$T cells were enriched in IFNγ, TNF and IL-2 triple cytokine producers compared to CD27^{lo} and CXCR3^{lo} populations both in the spleen and PEC, suggesting that expression of these markers represented CD8$^+$T cells that could become functional memory cells. Interestingly, a higher frequency of CD27^{hi} and CXCR3^{hi} CD8$^+$T cells in the PEC produced multiple cytokines when compared to the CD27^{hi} and CXCR3^{hi} CD8$^+$T cells in the spleen respectively (Figure III-12), suggesting that PEC tissue environment was promoting generation of functional memory CD8$^+$T cells. Overall, these results indicated that the non-lymphoid tissues are enriched in memory T cells that are more functional and resistant to apoptosis.

C. Virus-specific CD8$^+$T cells in the non-lymphoid tissues express transcription factors correlating with a memory phenotype

Few transcription factors that can regulate CD8$^+$T cell differentiation into effector and memory cells during an immune response have been identified. Therefore, in addition to examining cell surface antigens as correlates of resistance to apoptosis, I sought for transcription factors that might regulate the processes. TCF-1 is a transcription factor which promotes generation and maintenance of functional memory CD8$^+$T cells [104-106]. Since the non-lymphoid
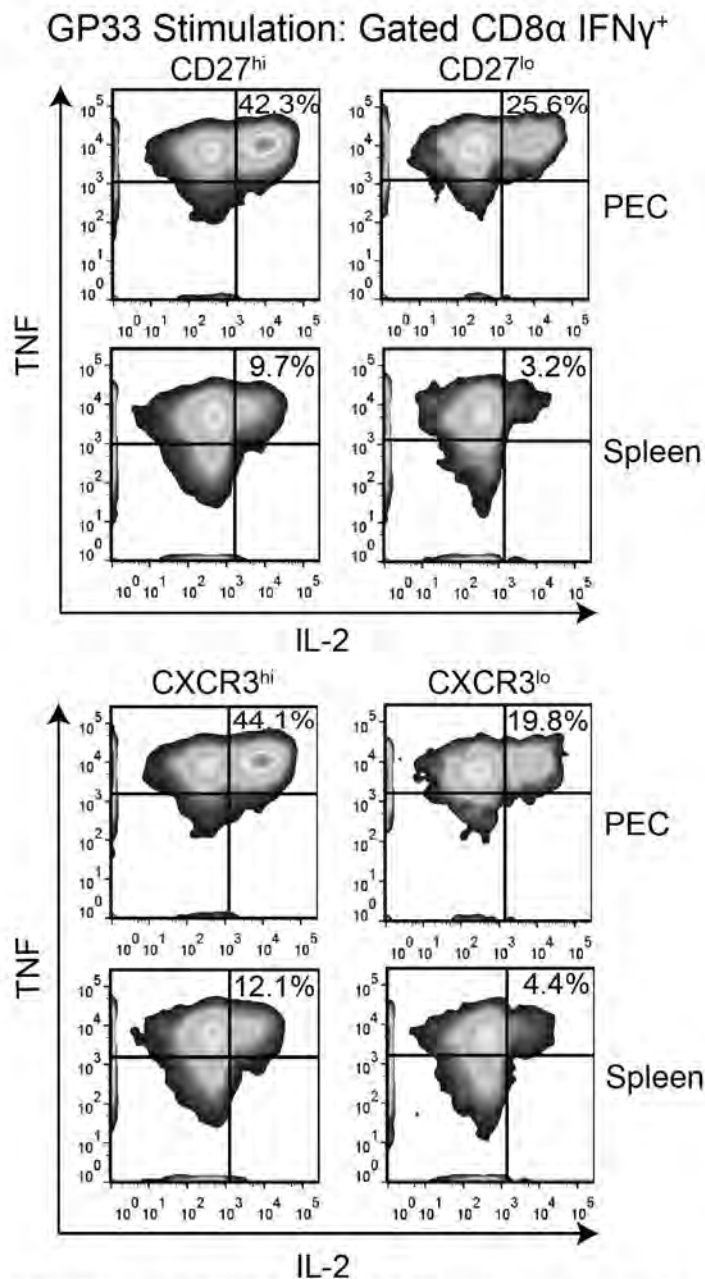
FIGURE III-12. CD27hi- and CXCR3hi- CD8+T cells are enriched in triple-cyto-kine producing cells. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods-Day 9 LCMV-infected lymphocytes from PEC and spleen were cultured for 5 hours in the presence of LCMV-specific GP33 or NP396 peptides. Plots are gated on virus-specific CD8α IFNγ+ T cells which are (top) CD27-hi or -lo and (bottom) CXCR3-hi or -lo. Numbers in top right represent percentage of TNF+ IL-2+ virus-specific CD8+T cells. Graphs are representative of 3 experiments with at least 3 mice in each experiment.

tissue environment was enriched in memory phenotype CD8$^+$T cells, I determined TCF-1 expression in the T cells from PEC as a representative of non-lymphoid tissue and spleen as a representative of lymphoid organ. Virus-specific P14$^+$CD8$^+$T cells or activated CD44^{hi} CD8$^+$T cells were intracellularly stained for TCF-1 at day 9 post-LCMV infection. I found that a higher proportion of virus-specific and activated CD44^{hi} CD8$^+$T cells expressed TCF-1 in the PEC as compared to the spleen (Figure III-13). The expression level of TCF-1 was also higher in virus-specific and activated CD44^{hi} CD8$^+$T cells in the PEC when compared to spleen as shown by the MFI (Figure III-13). Consistent with the increased survival of CD8$^+$T cells in non-lymphoid tissues during memory phase of the immune response, I found that a higher proportion of virus-specific CD8$^+$T cells in the PEC expressed TCF-1 at day 30 post-LCMV infection when compared to the spleen (Figure III-14). Although TCF-1 deficiency has been shown to limit the generation of CD44^{hi} CD62L^{hi} (central memory) CD8$^+$T cells [105], a correlation of high TCF-1 expression with memory phenotype CD8$^+$T cells and decreased apoptosis during resolution of the immune response has not been tested. At day 9 post-LCMV infection splenocytes and PEC were cultured directly *ex-vivo* for 5 hours and expression of memory phenotype markers and active Caspase-3 was compared in TCF-1^{hi} and ^{lo} P14 CD8$^+$T cells. I found that in both the tissues tested (PEC and spleen) virus-specific CD8$^+$T cells expressing high amounts of TCF-1 were enriched in MPECs (IL-7R^{hi} KLRG-1^{lo}), with most expressing CD27 and CXCR3. TCF-1^{hi} cells were also less apoptotic, as

Gated: P14⁺CD8⁺T

Gated: CD44^hi CD8⁺T

FIGURE III-13. Higher proportion of TCF-1-expressing virus-specific and activated CD8⁺T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percentage ± StDev of TCF-1-expressing P14⁺CD8⁺T cells (top) or CD44^hi CD8⁺T cells (bottom) at day 9 post-LCMV infection in the PEC and spleen. MFI for TCF-1 expression on total P14⁺CD8⁺T cells (top) or CD44^hi CD8⁺T cells (bottom) is depicted in each histogram. Data are representative of three independent experiments.

Gated: P14⁺CD8⁺T Cells : D30 LCMV
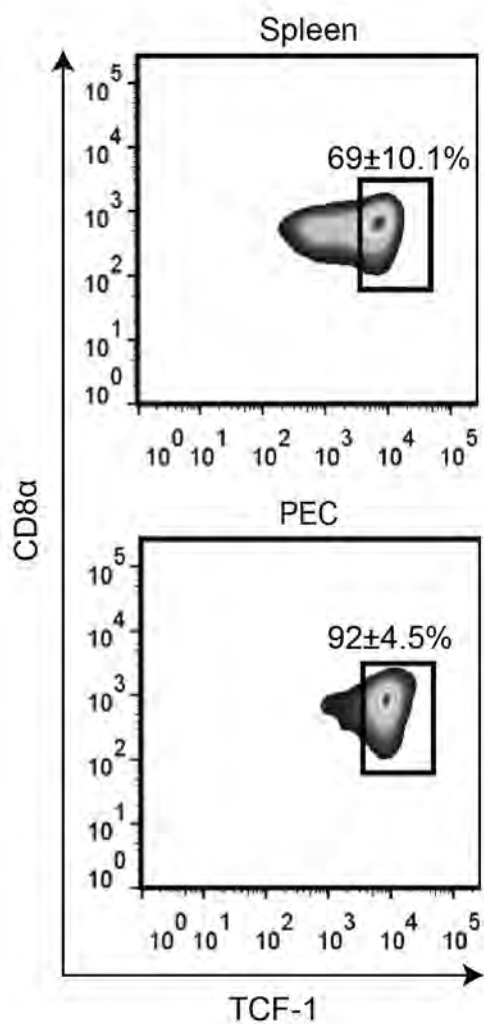
FIGURE III-14. Higher proportion of TCF-1-expressing virus-specific CD8⁺T cells in the PEC at day 30 post-LCMV infection. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percentage ± StDev of TCF-1 expressing P14⁺CD8⁺T cells at day 30 post-LCMV infection in the PEC and spleen. Data are representative of two independent experiments.

revealed by active Caspase-3 staining (Figure III-15 A and B). These results demonstrate that the PEC environment was enriched in TCF-1^{hi} CD8$^+$T cells, and that this correlated with increased memory and increased survival. LEF-1 is a transcription factor acting downstream in the Wnt signaling pathway (101), and TCF-1 and LEF-1 have redundant roles in promoting thymocyte maturation (102). Loss of TCF-1 and LEF-1 in CD8$^+$T cells was shown to completely abrogate development of memory precursor effector cells (164). Since LEF-1 and TCF-1 are known to co-operate in generation of memory CD8$^+$T cells, I wanted to compare LEF-1 expression in virus-specific CD8$^+$T cells in the PEC and spleen. I found that at day 10 post-LCMV infection, PECs had a higher proportion of LEF-1 expressing virus-specific CD8$^+$T cells as compared to the spleen (Overlay in Figure III-16). The expression level of LEF-1 was also higher in virus-specific CD8$^+$T cells in the PEC when compared to spleen, as shown by the MFI in Figure III-16. TCF-1 has been shown to be necessary and sufficient for expression of EOMES, a transcription factor associated with memory CD8$^+$T cells (105). EOMES has been shown to be important for induction of CD122 (IL-2Rβ) expression and IL-15 responsiveness in CD8$^+$T cells (108), thereby promoting survival of memory T cells. I compared EOMES expression in virus-specific CD8$^+$T cells in the PEC and spleen. Consistent with a higher TCF-1 expression, a higher proportion of virus-specific CD8$^+$ T cells in the PEC expressed EOMES as compared to the spleen at day 9 post-LCMV infection (Figure III-17). The expression level of EOMES was also higher in virus-specific

SPLEEN
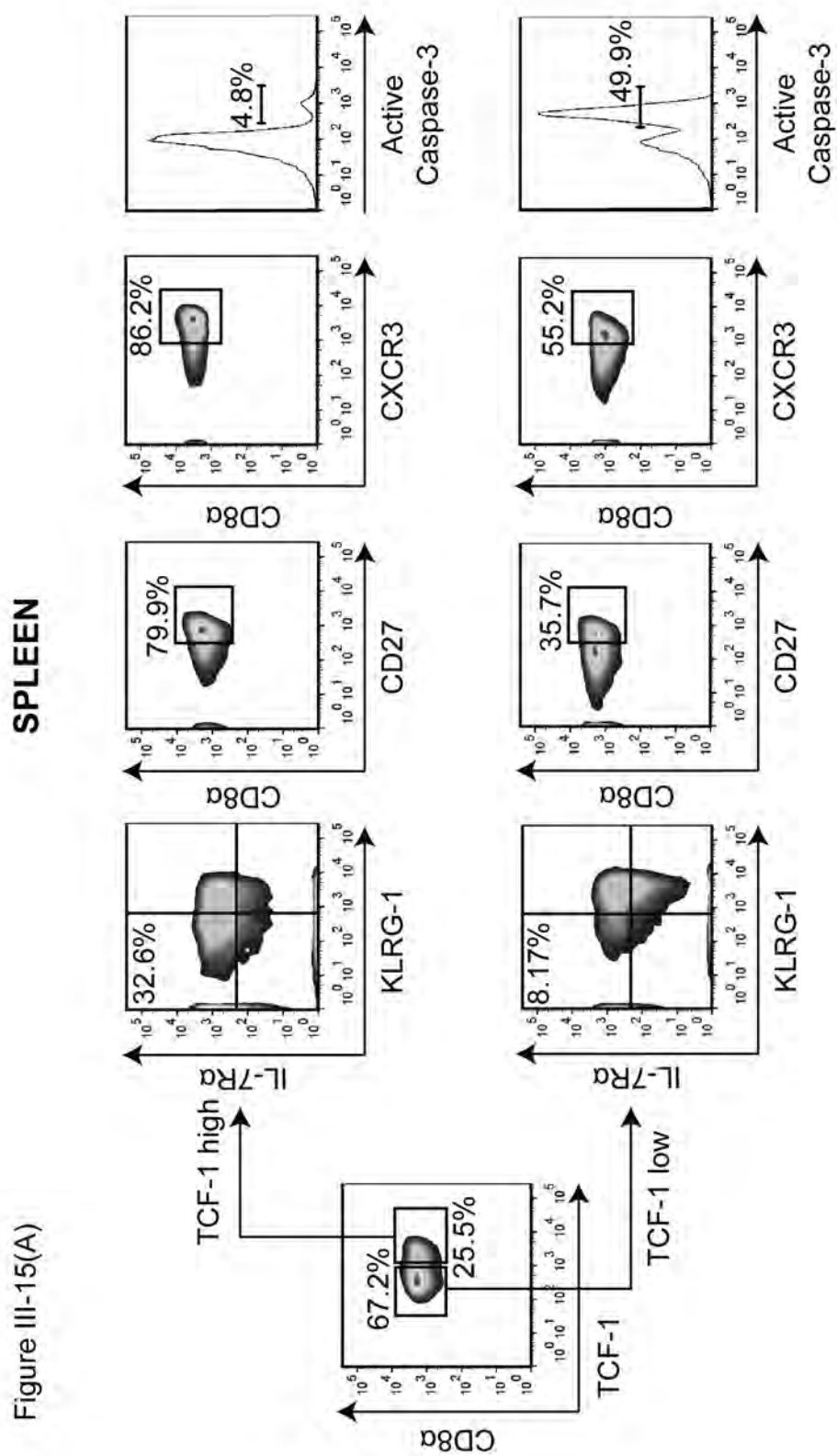
Figure III-15(A)
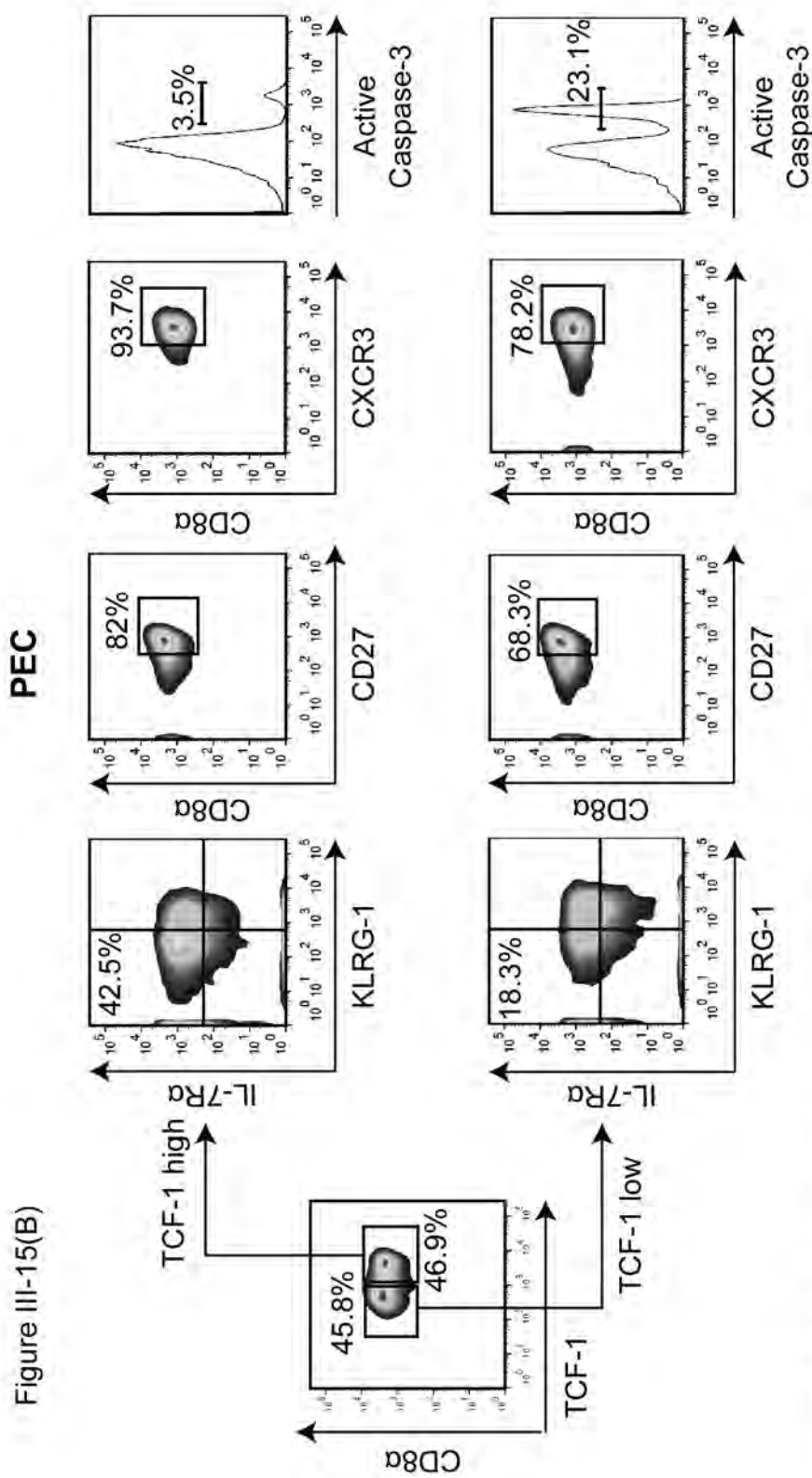
Figure III-15(B)

FIGURE III-15 (A,B). TCF-1 expression on virus-specific CD8+T cells correlates with increased memory phenotype and decreased apoptosis. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. TCF-1 staining of P14+CD8+T cells from spleen (A) and PEC (B) at day 9 post infection. IL-7R, KLRG-1, CD27, CXCR3 and active Caspase-3 staining in TCF-1hi and TCF-1lo subsets are depicted. Plots are representative of three experiments with three mice in each experiment.
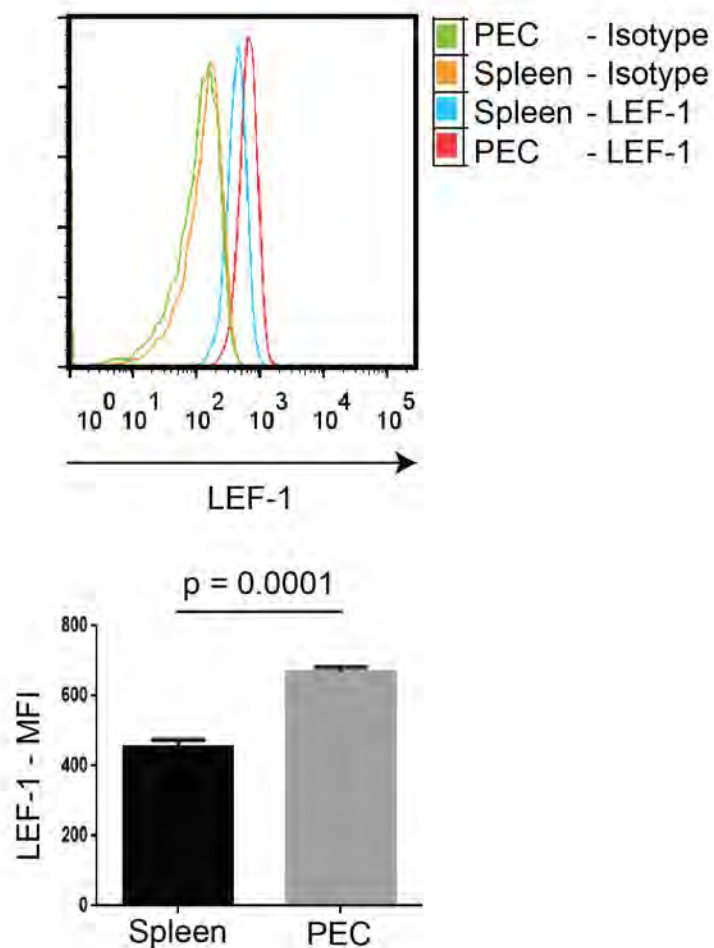
FIGURE III-16. Higher LEF-1 expression in virus-specific CD8$^+$T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Overlay of LEF-1 expression (top) in P14$^+$CD8$^+$T cells in the PEC (red), spleen (blue) or isotype control (green, orange) at day 9 post-LCMV infection. MFI of LEF-1 expression (bottom) in total P14$^+$CD8$^+$T cells in PEC and spleen shown in the histogram. Experiment was done twice with 3 mice in each experiment.

CD8$^+$T cells in the PEC when compared to spleen as shown by the MFI in Figure III-17. Therefore, non-lymphoid tissues are enriched in virus-specific CD8$^+$T cells expressing transcription factors associated with memory generation which may contribute to survival of CD8$^+$T cells in the non-lymphoid tissue environment.

D. Transcription factor TCF-1 promotes survival of CD8$^+$T cells in the non-lymphoid tissues during resolution of the immune response

Since a higher proportion of virus-specific CD8$^+$T cells in the PEC expressed TCF-1, I next sought to identify whether TCF-1 was promoting survival of CD8$^+$T cells in the non-lymphoid tissues. To address this question I infected TCF-1-deficient or littermate control mice with LCMV and compared the phenotype and apoptotic profiles of activated CD44^{hi} CD8$^+$T cells at day 9 post infection. During an immune response to LCMV approximately 70% of the activated CD44^{hi} CD8$^+$T cells are virus-specific [145], and since we did not have P14 TCR transgenic TCF-1-deficient mice to track LCMV-specific responses I decided to focus on CD44^{hi} CD8$^+$T cell responses. In accordance with previous reports [104, 105], I found that TCF-1-deficient mice at day 9 post-infection had a decreased proportion and absolute number of activated CD44^{hi} CD8$^+$T cells in both PEC and spleen (Figure III-18). I then compared expression of IL-7Rα and KLRG-1 on CD44^{hi} CD8$^+$T cells in PEC and spleen of WT and TCF-1-deficient mice. As shown in Figure III-19, the proportion of MPEC in the CD44^{hi} CD8$^+$T cell pool was reduced more dramatically in the PEC than in the spleen of the TCF-1-deficient mice at day 9 post infection.   To   test   whether   maintenance   of   TCF-1
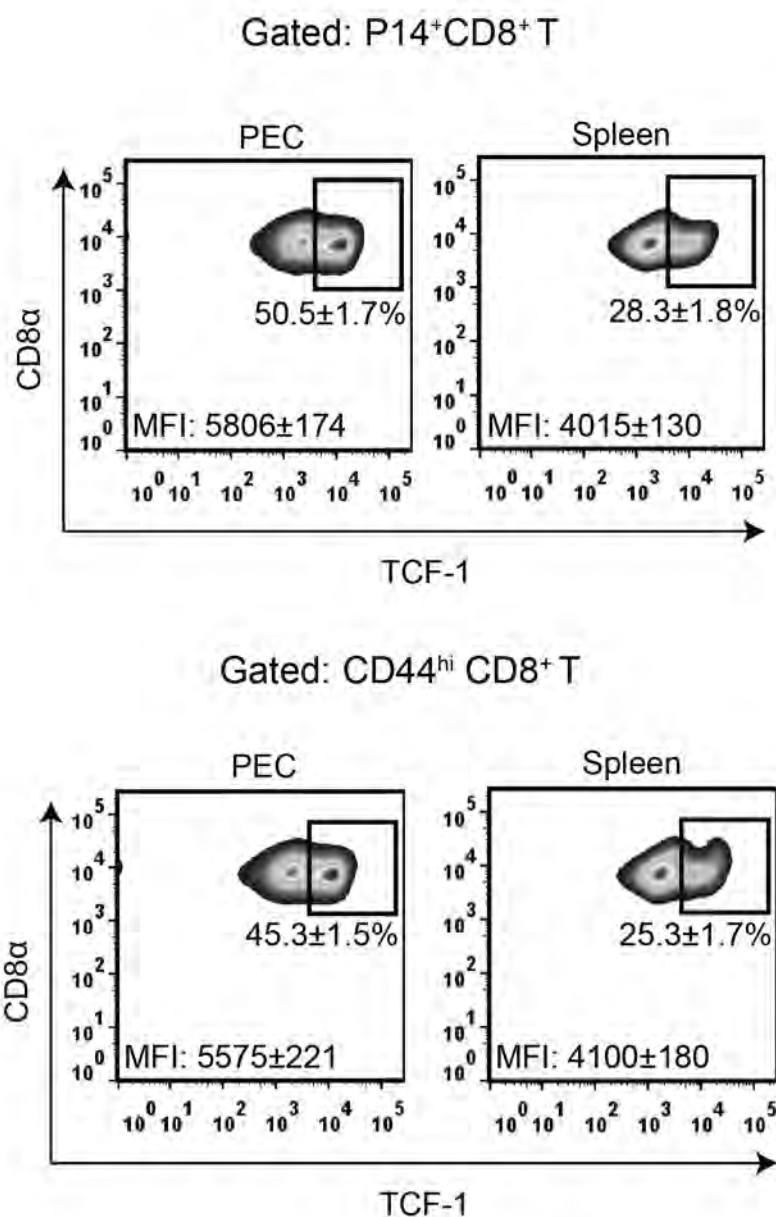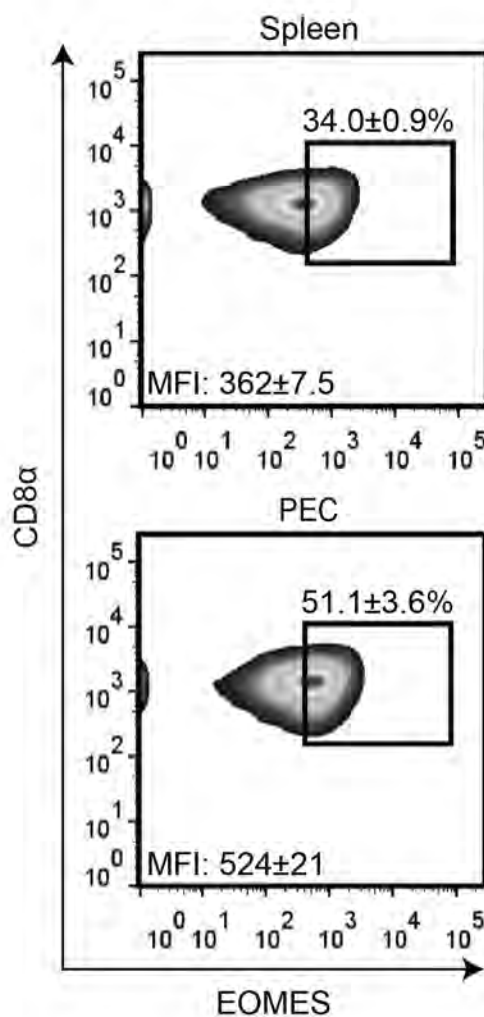
FIGURE III-17. Higher proportion of EOMES-expressing virus-specific CD8+T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percentage ± StDev of EOMES-expressing P14+CD8+T cells at day 9 post-LCMV infection in PEC and spleen. MFI of EOMES expression in P14+CD8+T cells in PEC and spleen shown in the histogram.
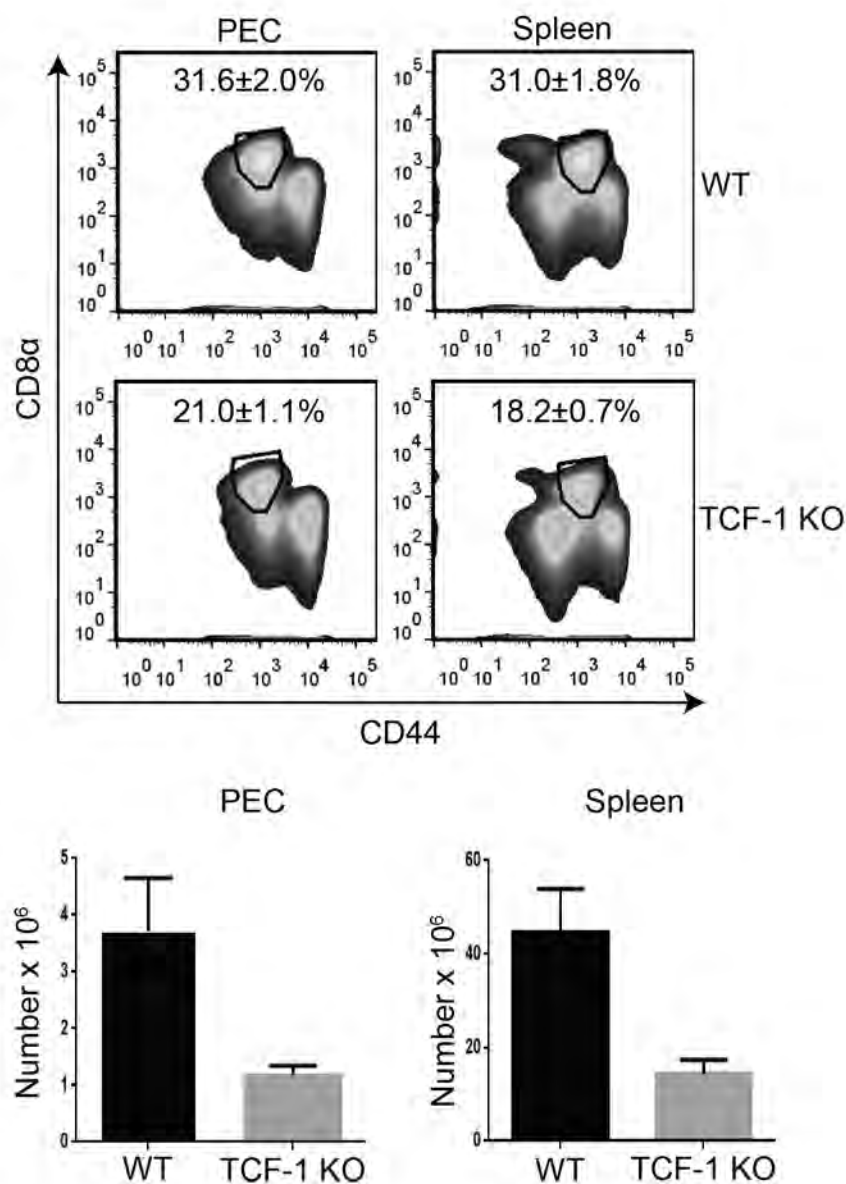
FIGURE III-18. Decreased T cell responses in the TCF-1-deficient mice during acute LCMV infection. Percentage ± StDev (top) and absolute number (bottom) of CD44^{hi} CD8$^+$T cells in PEC and spleen of WT or TCF-1 KO mice at day 9 post-LCMV infection. Graphs are representative of four experiments with at least 3 mice in each experiment.
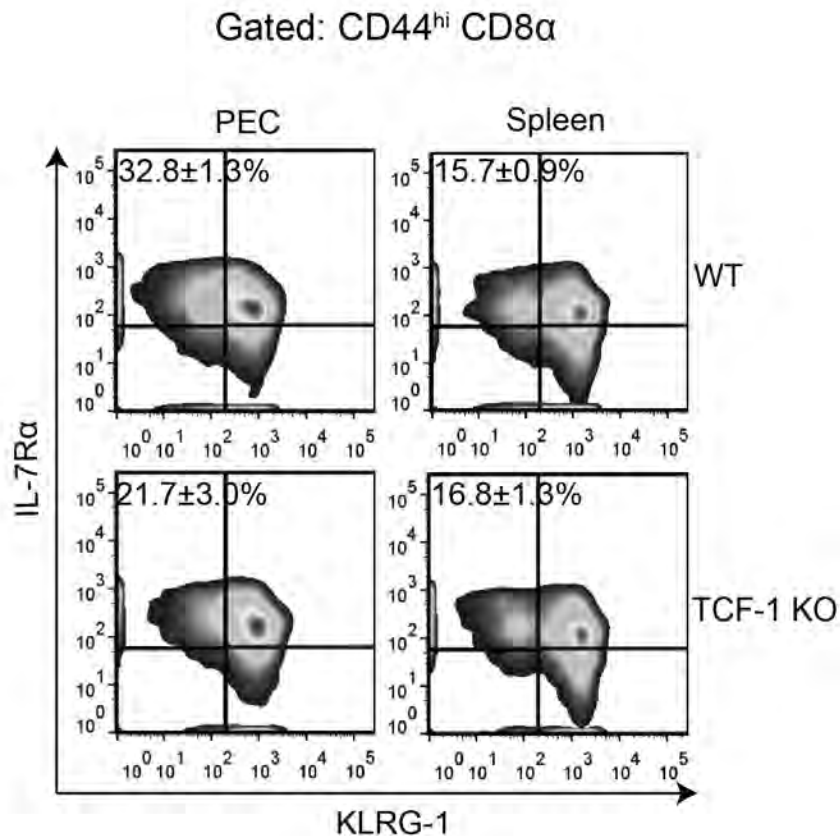
FIGURE III-19. Decreased proportion of memory precursor effector cells in the PEC of TCF-1-deficient mice. Percentage of IL-7R and KLRG-1 expressing CD44^{hi} CD8$^+$T cells in PEC and spleen of WT or TCF-1 KO mice at day 9 post-LCMV infection. Numbers represent percentage ± StDev of IL-7R^{hi} KLRG-1^{lo} CD8$^+$T cells. Graphs are representative of four experiments with at least 3 mice in each experiment.

expression promotes survival of CD8^{+}T cells in the non-lymphoid tissues, I isolated WT or TCF-1-deficient cells from spleen and PEC of day 9 LCMV-infected mice and cultured them *ex-vivo* for 5 hours, followed by TUNEL staining. As shown in Figure III-20 activated CD44^{hi} CD8^{+}T cells from PEC of TCF-1-deficient mice were more apoptotic when compared to WT littermates, with about twice the number of TUNEL^{+} cells. Supporting a previous observation (105), we found no statistically significant difference in apoptosis of CD44^{hi} CD8^{+} T cells from spleen between the TCF-1 KO and WT mice (Figure III-20). As compared to wild type littermate controls TCF-1-deficient mice had defects in generating a normal immune response, and overall numbers and proportions of CD44^{hi} CD8^{+}T cells in TCF-1-deficient mice were reduced in all the organs tested. However, these experiments were intrinsically controlled, and I compared activated CD44^{hi} CD8^{+}T cells from PEC and spleen of TCF-1-deficient mice. These results indicate that TCF-1 was more critical for the memory generation and survival of CD8^{+}T cells in the PEC during resolution of the immune response. Importantly, I saw that CD44^{hi} CD8^{+}T cells in the PEC of TCF-1 KO mice were still less apoptotic than those in the spleen, which suggested that additional mechanisms might regulate tissue-dependent differences in CD8^{+}T cell apoptosis.

E. Accumulation of non-apoptotic CXCR3^{+} CD8^{+} T cells in the inguinal lymph nodes on FTY720 treatment

I next questioned whether tissue-dependent differences in CD8^{+}T cell apoptosis were associated with preferential migration of non-apoptotic CD8^{+}T
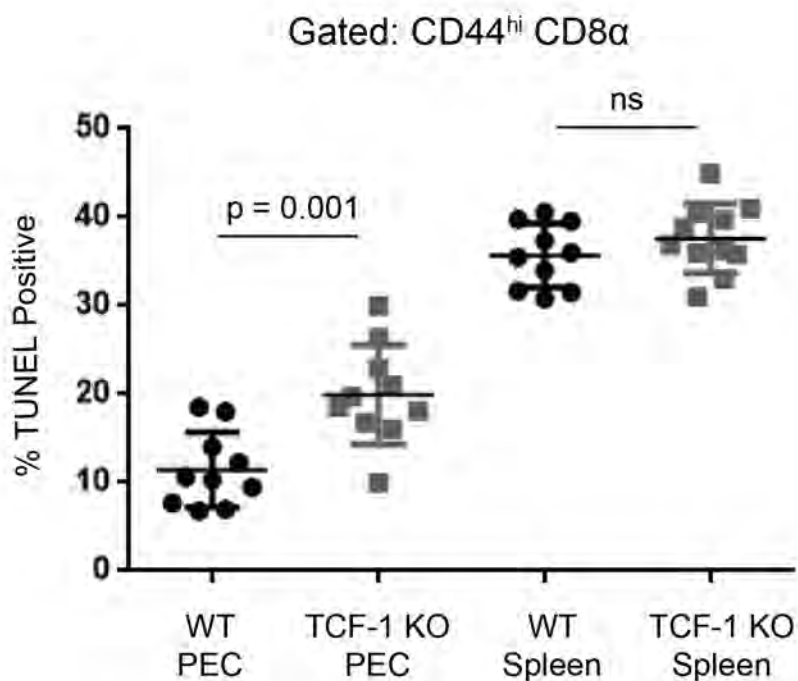
FIGURE III-20. Decreased T cell survival in the PEC of TCF-1-deficient mice during resolution of the immune response. Lymphocytes from spleen and PEC were isolated at day 9 post-LCMV infection from WT and TCF-1 KO mice and cultured *ex-vivo* for 5 hours post isolation. TUNEL reactivity of CD44^{hi} CD8^{+}T cells are represented in the histograms. Graph is a compilation of four experiments. ns (non-significant).

cells to the non-lymphoid tissues. To address this question I used FTY720, an immunosuppressive drug which can modulate trafficking of lymphocytes and prevent emigration of T cells from the lymph nodes into the periphery (165-167). Transgenic P14$^+$CD8$^+$T cells were adoptively transferred into a congenic host, which was then infected with LCMV. Mice were treated with FTY720 at 1 mg/kg on days 5, 6 and 7 post-LCMV infection according to the treatment regimen shown in Figure III-21. FTY720 treatment was more efficacious in sequestering the T cells in the lymph nodes than in the spleen; therefore, I decided to focus on lymph nodes for further analysis. There was an increase in absolute number and proportion of virus-specific CD8$^+$T cells in the inguinal lymph nodes of treated mice (Figure III-22), and a decrease in absolute number and proportion of virus-specific CD8$^+$T cells in the PEC (Figure III-22). Thus, FTY720 treatment was effective in preventing migration of T cells out of the lymph nodes. I reasoned that if non-apoptotic CD8$^+$T cells preferentially leave the lymph nodes, blocking their egress should lead to accumulation of non-apoptotic CD8$^+$T cells in those lymph nodes. Lymphocytes were isolated from PEC, ILN and MLN of D8 LCMV infected control- or FTY720-treated mice and cultured *ex-vivo* for 5 hours followed by TUNEL staining. As shown in Figure III-23, a lower percent of virus-specific CD8$^+$T cells in the inguinal and mesenteric lymph nodes were apoptotic in the FTY720-treated group as compared to the control group. There was only a modest increase in apoptosis of virus-specific CD8$^+$T cells in the PEC in the FTY720-treated mice than the control mice (Figure III-23). Thus, these data are
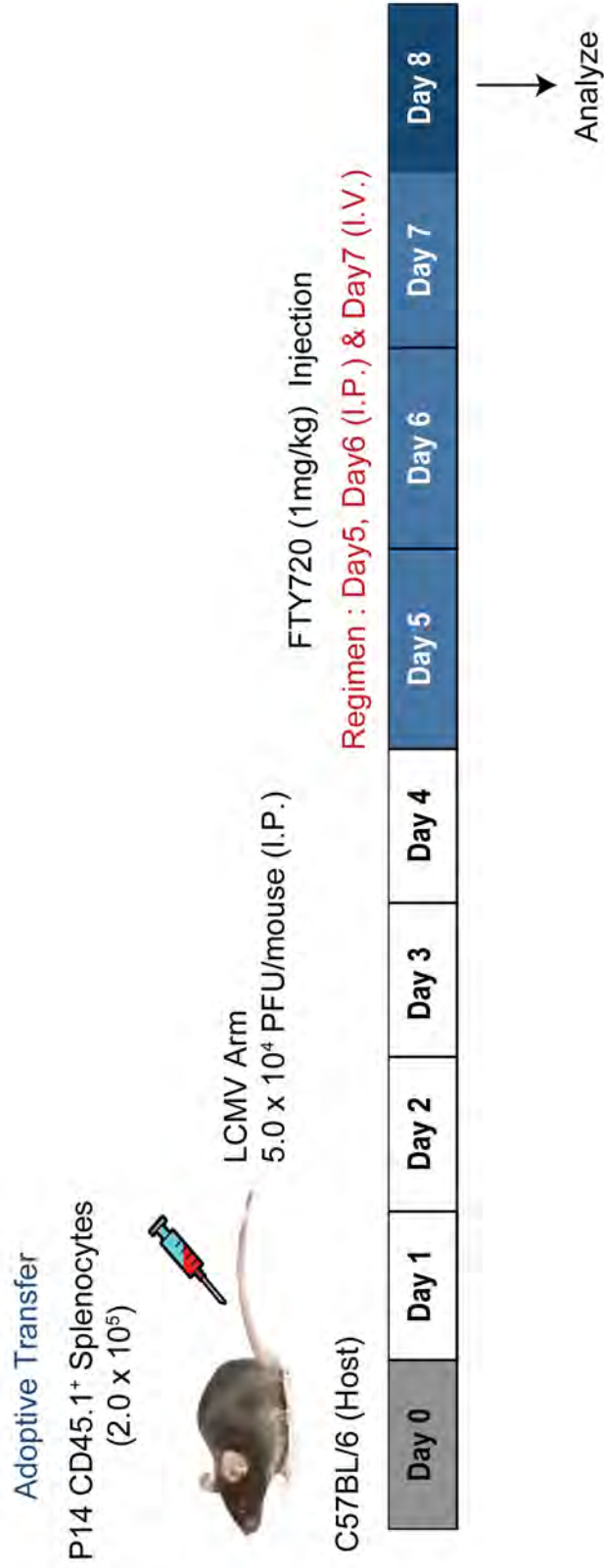
FIGURE III-21. Schematic of FTY720 treatment regimen.

ILN

PEC
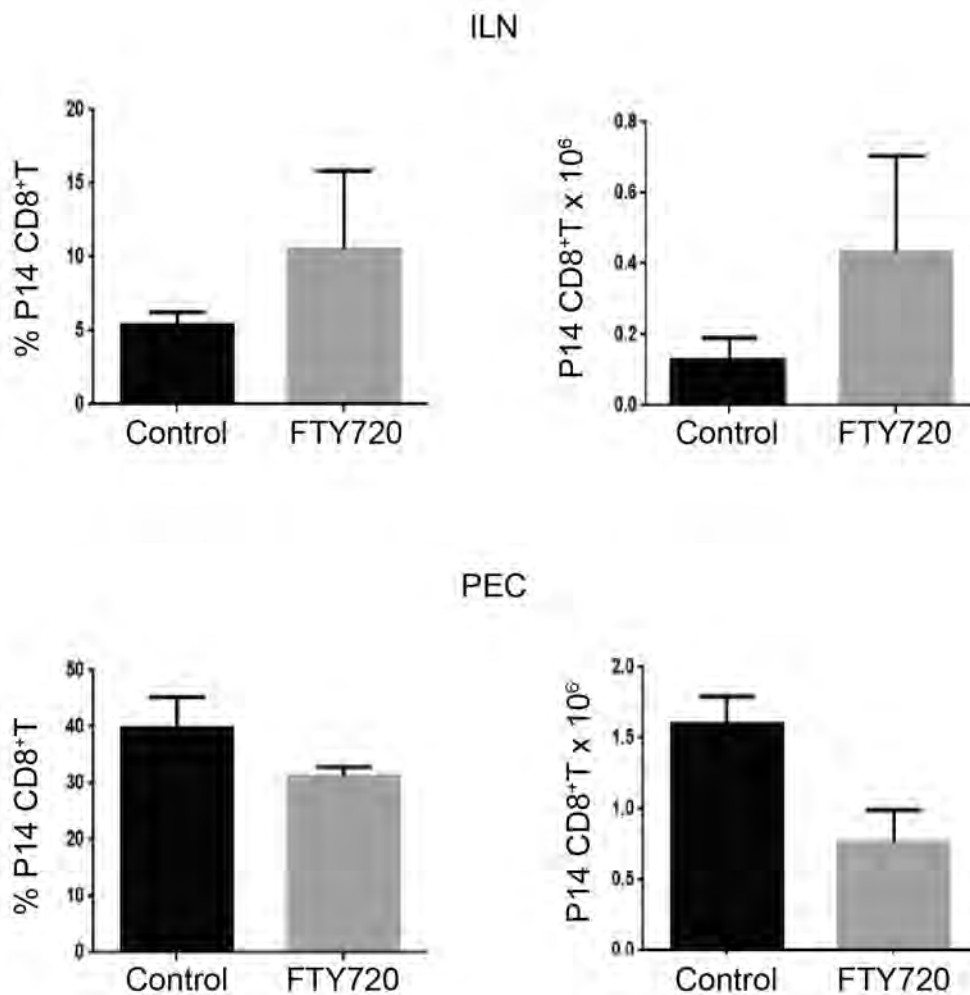
FIGURE III-22. FTY720 treatment promotes accumulation of virus-specific CD8⁺T cells in the inguinal lymph nodes (ILN). P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection. FTY720 treatment regimen is described in Figure III-21. Absolute numbers and percentage of P14⁺CD8⁺T cells in the ILN (top) and PEC (bottom) of FTY720 or control treated mice at day 8 post-LCMV infection. Data are representative of 3 experiments with 3 mice in each experimental group.
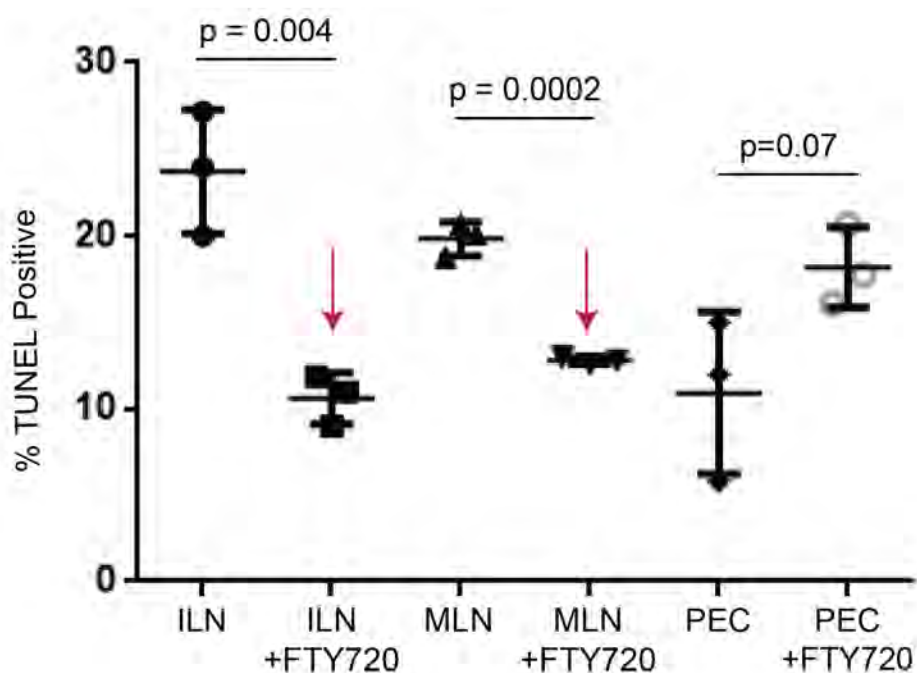
FIGURE III-23. FTY720 treatment promotes accumulation of apoptosis-resistant virus-specific CD8⁺T cells in the lymph nodes. P14 splenocytes were adoptively transferred into a C57BL/6 host ollowed by LCMV infection. FTY720 treatment regimen is described in Figure III-21. Total lymphocytes were isolated and cultured directly *ex-vivo* for 5 hours. Percentage of TUNEL⁺ P14⁺CD8⁺T cells in FTY720 and control treated mice in the ILN, MLN and PEC at day 8 post-LCMV infection. Data are representative of 3 experiments with 3 mice in each experimental group.

consistent with the hypothesis that non-apoptotic CD8$^+$T cells preferentially migrate into non-lymphoid tissues, and when migration is stopped, these apoptosis-resistant T cells accumulate in the lymph nodes.

T cells get activated in the lymph nodes and migrate to non-lymphoid tissues to eliminate the antigen, a process mediated by chemokines and their receptors. I questioned whether certain chemokine receptors might be promoting preferential migration of non-apoptotic CD8$^+$T cells to non-lymphoid tissue environment. CXCR3 is a chemokine receptor shown to be important for migration of antigen-specific CD8$^+$T cells to tissue effector sites (86)(134), and it has been previously reported that the peritoneal cavity is dominated by CXCR3$^+$ Th1 CD4$^+$ T cells (168). I have shown that a higher proportion of virus-specific CD8$^+$T cells expressed CXCR3 in the PEC and that these were less apoptotic (Figure III-10). If CXCR3 was important for migration of non-apoptotic CD8$^+$T cells out of the lymph nodes I should see an accumulation of CXCR3-expressing cells on FTY720 treatment. Figure III-24 shows that there was an increased proportion of CXCR3$^+$ CD8$^+$T cells accumulating in the inguinal lymph nodes on FTY720 treatment, suggesting that CXCR3$^+$ CD8$^+$T cells were normally preferentially migrating out of the lymph nodes into the non-lymphoid tissues. The next aim was to identify whether the PEC environment was expressing CXCR3 ligands which would attract non-apoptotic CD8$^+$T cells. CXCR3$^+$T cells migrate under the influence of the IFNγ-inducible chemokine ligands CXCL9 and CXCL10 (86, 87). I found that at day 8 post-LCMV infection there was a higher

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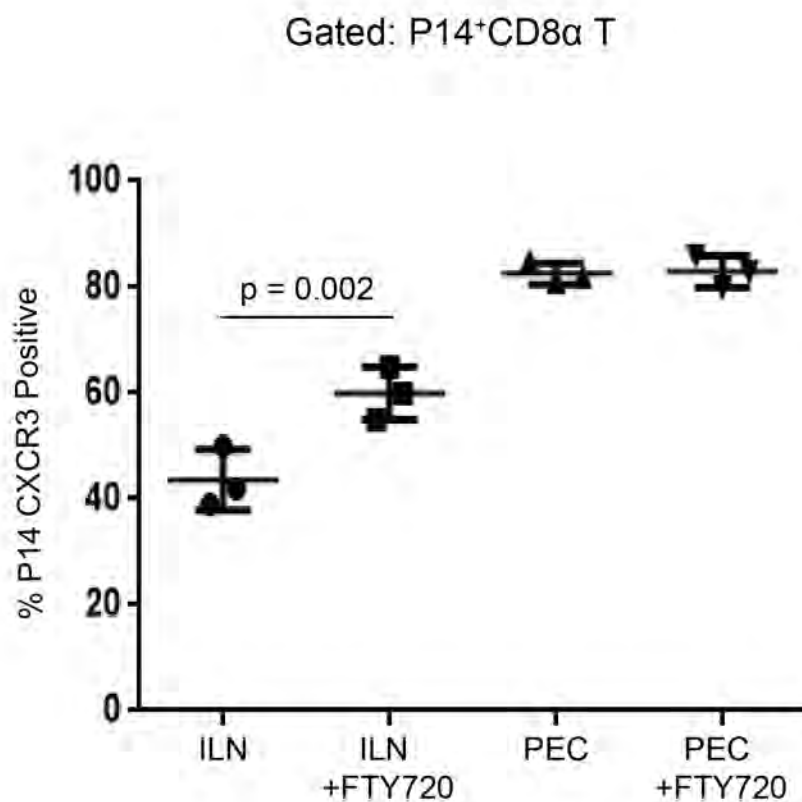
FIGURE III-24. FTY720 treatment promotes accumulation of CXCR3⁺ virus-specific CD8⁺T cells in the inguinal lymph nodes (ILN). P14 splenocytes were adoptively transferred into a C57BL/6 host ollowed by LCMV infection. FTY720 treatment regimen is described in Figure III-21. Percentage of CXCR3⁺ P14⁺CD8⁺T cells in FTY720 and control treated mice in the ILN and PEC at day 8 post-LCMV infection. Data are representative of 3 experiments with 3 mice in each experimental group.

proportion of total lymphocytes expressing CXCL9 in the PEC environment compared to the spleen (Figure III-25). Due to unavailability of a working CXCL10 antibody for FACS analysis I decided to compare the RNA message for CXCL9 and CXCL10 between spleen and PEC leukocytes. At day 7 post-LCMV infection, RNA was isolated from total PEC and spleen leukocytes and subjected to Q-PCR analysis with *Cxcl9* and *Cxcl10* primers. Figure III-26 shows that mRNA for CXCL9 and CXCL10 was higher 4.7 and 2.6 fold respectively, in the total PEC leukocytes when compared to total splenocytes at D7 post-LCMV infection. Thus, the PEC environment had a higher expression of CXCR3 ligands that could promote preferential migration of CXCR3$^+$CD8$^+$T cells. Overall, these results are consistent with the hypothesis that non-apoptotic CXCR3$^+$ CD8$^+$T cells preferentially migrate out of secondary lymphoid organs into the non-lymphoid tissues under the influence of chemokines CXCL9 and CXCL10. This phenomenon may provide an additional mechanism by which healthy non-apoptotic memory CD8$^+$T cells populate non-lymphoid tissues to provide protection to secondary challenge.

F. *In Vivo* PEC environment promotes survival of CD8$^+$T cells

So far, I have shown that virus-specific CD8$^+$T cells in the non-lymphoid tissues are intrinsically more resistant to apoptosis, and that this could in part be due to preferential migration of non-apoptotic memory phenotype CD8$^+$T cells from lymph nodes into the non-lymphoid tissues. I next addressed whether the PEC environment is particularly enriched with factors that can promote survival
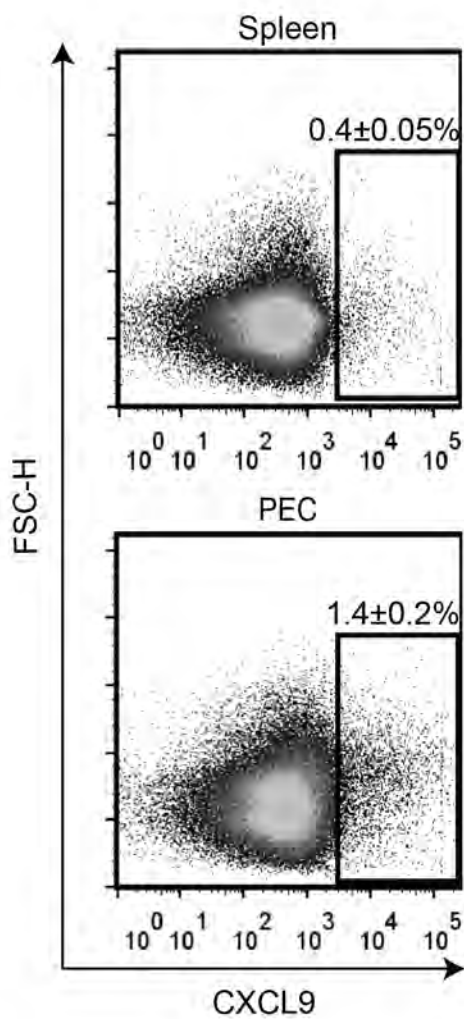
FIGURE III-25. Higher proportion of CXCL9-expressing leukocytes in the PEC. Percent ± StDev of total leukocytes from spleen and PEC expressing CXCL9 at day 8 post-LCMV infection. Data are representative of 2 experiments with 3 mice in each experimental group. Plots are gated on FSC-A vs SSC-A total lymphocytes.
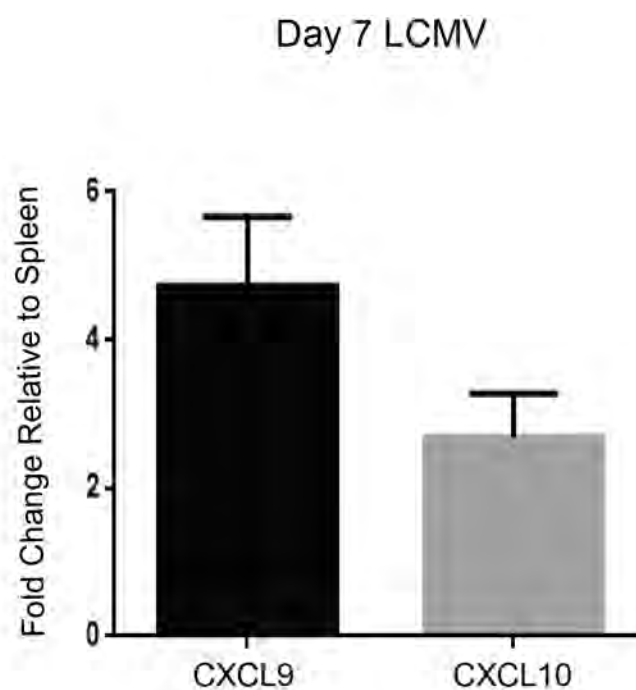
FIGURE III-26. Higher expression of CXCL9 and CXCL10 mRNA in total PEC leukocytes. Fold change in CXCL9 and CXCL10 mRNA in the total PEC leukocytes with respect to spleen leukocytes at day 7 post-LCMV infection. Experiment was performed two times with three mice in each experiment.

and maintenance of memory CD8$^+$T cells. To test whether the in *vivo* PEC environment promoted survival of CD8$^+$T cells, LCMV-specific P14$^+$ CD8$^+$T cells were adoptively transferred into a congenic host followed by LCMV infection. At day 7 post infection, I isolated splenocytes which were labeled with tracking dyes CFSE or DDAO and transferred back into an infection-matched host via the i.v or i.p route respectively, as shown in Figure III-27. Splenocytes and PEC were isolated from the infection-matched host mice 36 hours post transfer. I used tracking dyes to distinguish between cells which were transferred into the same host via the i.v or i.p route. Thus, day 7 infected splenocytes (which are highly apoptotic) were labeled and introduced into the spleen or the PEC environment of the same infection-matched host to address whether the tissue environment contributed to CD8$^+$T cell survival. As shown in Figure III-28, donor P14$^+$CD8$^+$T splenocytes which were introduced directly into the PEC environment via the i.p route were less apoptotic as compared to the P14 CD8$^+$T cells which were present in the spleen and introduced via the i.v route. A small number of donor P14$^+$CD8$^+$T splenocytes given intravenously were found in the PEC and were also less apoptotic when compared to the donor CD8$^+$T cells which populated the spleen (data not shown). As shown in Figure III-28, I found that transferred P14 CD8$^+$T cells which were present in the PEC environment had higher expression of the memory marker CD27 when compared with P14 CD8$^+$T cells present in the spleen. Therefore CD8$^+$T cells from the same source when introduced into the PEC were less apoptotic and more memory like when compared to those
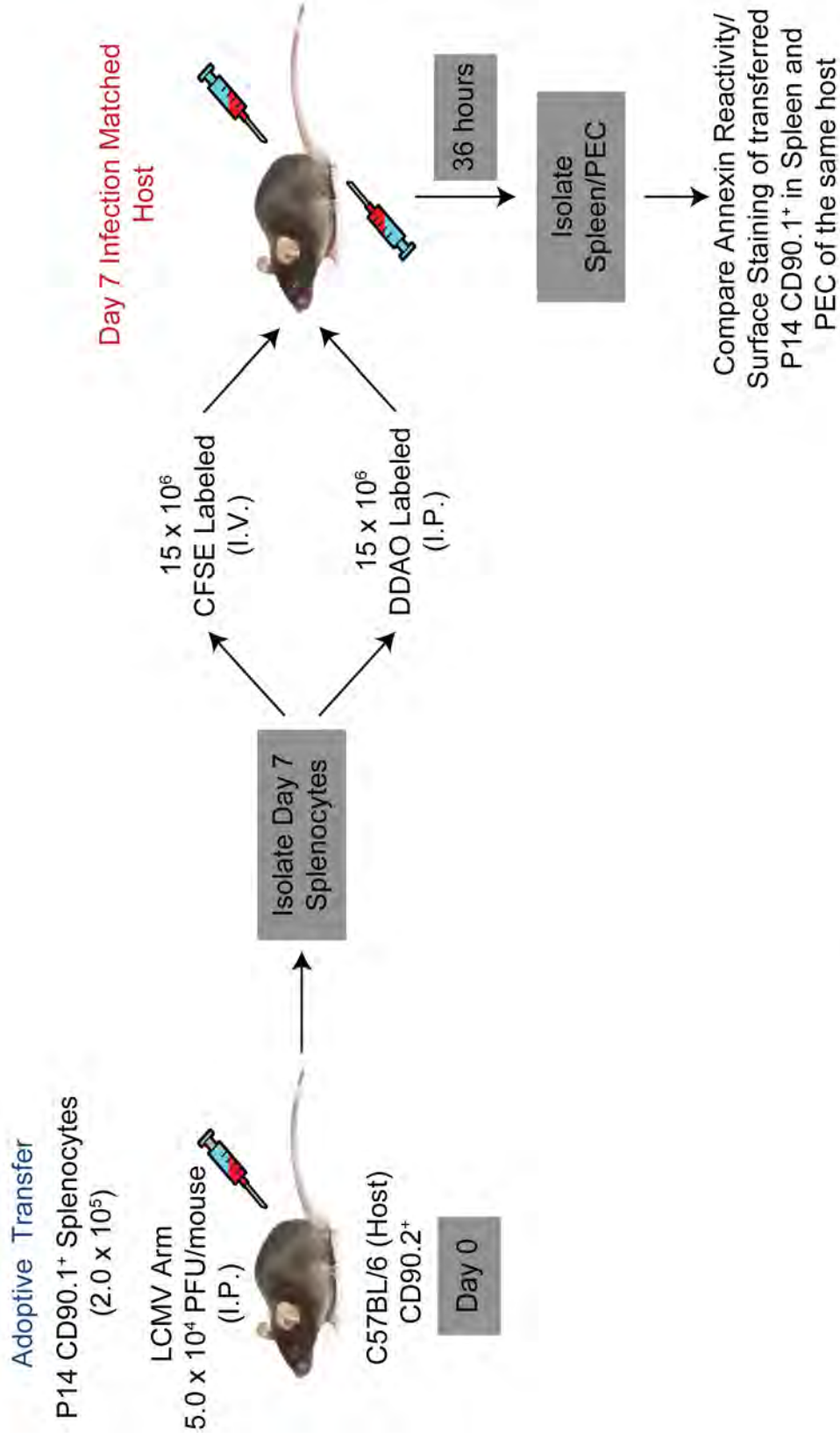
FIGURE III-27. Schematic of experimental setup to test effect of *in-vivo* tissue environment in promoting survival of CD8⁺T cells.
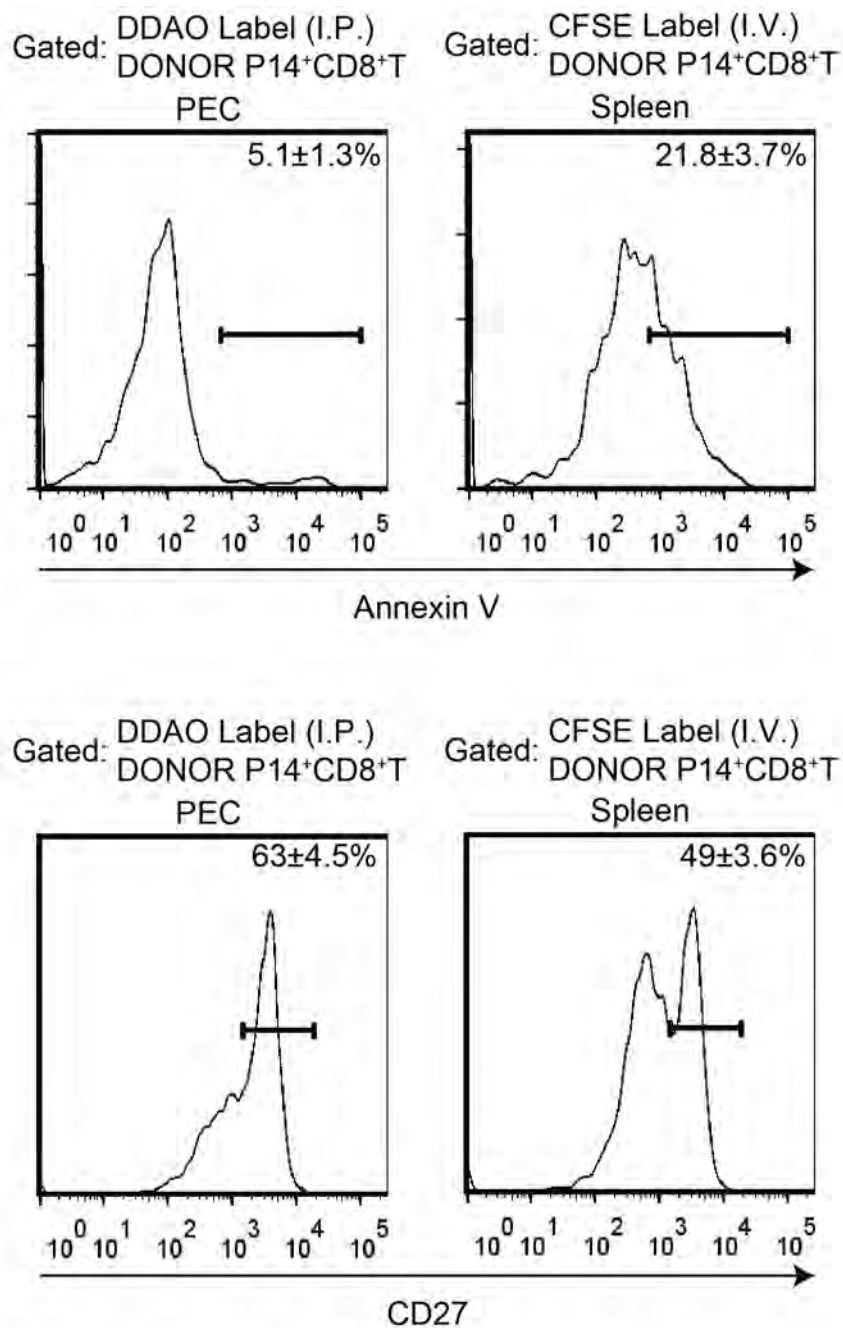
FIGURE III-28. *In vivo* PEC environment promotes survival and memory generation of T cells. Annexin V reactivity (top) and CD27 expression (bottom) on P14+CD8+T cells which were labeled and adoptively transferred from a day 7 LCMV-infected donor into an infection-matched host as shown in schematic in Figure III-27. Data are representative of at least three experiments with 3 mice in each experiment. Percentages are represented with ± StDev.

which were introduced into the spleen. Thus, the in vivo PEC environment was not only promoting survival of CD8$^+$T cells but selectively retained cells expressing memory markers.

I reasoned that another possibility for the observation of fewer apoptotic cells in the PEC environment could be due to enhanced phagocytic clearance of pre-apoptotic T cells. If, for example, there were more apoptotic cell-scavenging phagocytes in the PEC than in the spleen, cells in early stages of apoptosis might be quickly scavenged and removed from the system. To test this possibility I adoptively transferred P14 CD8$^+$T cells into a congenic host which was subsequently infected with LCMV. Nucleotides released by apoptotic cells can be recognized by phagocytic cells to promote apoptotic cell clearance (169). Apyrase, an enzyme which hydrolyses nucleoside tri phosphates, can be used to prevent phagocytic clearance of apoptotic cells (169). Thus, by using Apyrase to block phagocytic uptake of apoptotic cells I questioned whether enhanced phagocytic clearance in the PEC tissue environment was contributing to tissue-dependent apoptotic differences. At day 8 post infection mice were treated with Apyrase or succinate buffer control for 6 hours, following which splenocytes and PEC were isolated. Virus-specific CD8$^+$T cells in the PEC and spleen of Apyrase-treated mice were, as expected, more apoptotic when compared to the control group (Figure III-29), thus providing evidence that Apyrase inhibited phagocytic clearance. However, virus-specific, apoptosis-resistant CD8$^+$T cells were still enriched in the PEC as compared to spleen in the Apyrase-treated group (Figure
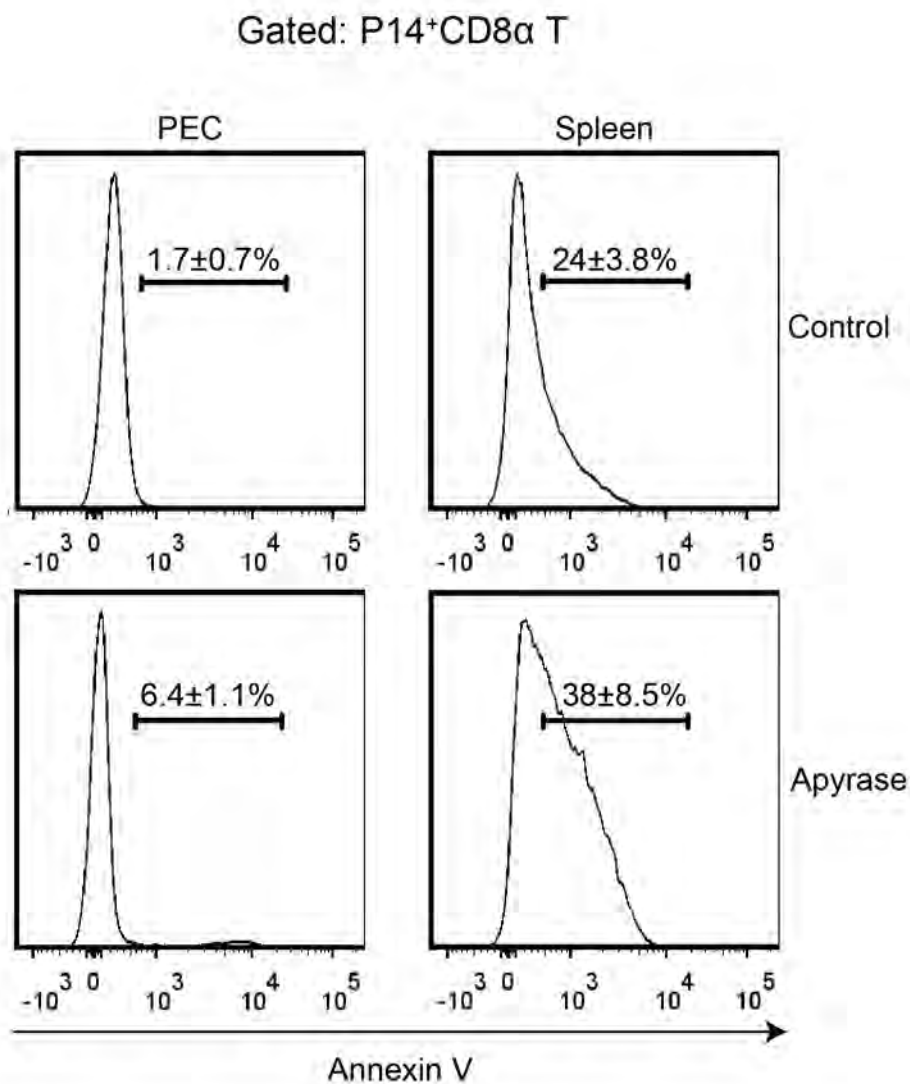
FIGURE III-29. Enhanced phagocytic clearance does not contribute to tissue-dependent apoptotic differences. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. The FACS plots depict Annexin V reactivity of P14$^+$CD8$^+$T cells in PEC and spleen from day 8 LCMV-infected mice which were treated *in-vivo* with Apyrase or buffer control for 6 hours. Data are representative of two experiments with 3 mice in each experiment. Percentages are represented with ± StDev.

III-29). This suggests that enhanced phagocytic clearance was not a major contributor to tissue-dependent differences in CD8^{+}T cell apoptosis.

G. Potential factors promoting CD8^{+}T cell survival in the *in vivo* PEC environment

Overall my results indicate that enhanced survival of CD8^{+}T cells in the non-lymphoid tissues was a consequence of enrichment of this niche by cells which had an apoptosis-resistant antigenic and transcription factor phenotype. Furthermore, I show that apoptosis-resistant CD8^{+}T cells may preferentially migrate into the non-lymphoid tissue where they will be stably maintained due to a yet undefined protective factor, that I have not been able to clearly resolve. Some preliminary experiments suggest that there may be some cytokines, co-stimulatory factors and cell types which might be actively promoting CD8^{+}T cell stability in the non-lymphoid tissues.

IL-7 is a cytokine belonging to common γ chain family of cytokines and is known to mediate survival and self-renewal of memory CD8^{+}T cell populations [70, 71]. IL-7 therapy during contraction of the immune response enhances the generation of memory CD8^{+}T cells [170]. IL-7 signaling induces STAT5 phosphorylation, which is known to be associated with survival of effector and memory CD8^{+} T cells [118, 119]. I questioned whether virus-specific CD8^{+}T cells in the PEC had higher STAT5 phosphorylation when compared to the spleen counterparts in response to IL-7. Day 8 LCMV-infected MACS-sorted CD8^{+}T

cells were stimulated directly *ex-vivo* with IL-7 for multiple time points and then stained intracellularly to identify STAT5 phosphorylation. As shown in Figure III-30, I found that a higher proportion of virus-specific CD8$^+$T cells in the PEC exhibited STAT5 phosphorylation in response to IL-7 directly ex-vivo as compared to the spleen. The expression level of pSTAT5 was also higher in virus-specific CD8$^+$T cells in the PEC when compared to spleen, as shown by the MFI in Figure III-30. STAT5 signaling activates Akt, which can inhibit apoptosis and promote cell survival (171, 172). Thus IL-7 mediated induction of STAT5 phosphorylation may promote survival of CD8$^+$T cells in the PEC environment.

CD27 is a co-stimulatory receptor, and stimulation through CD27 requires the presence of its co-stimulatory ligand CD70, which is expressed on dendritic cells, B cells, and activated CD4 and CD8 T cells. A study by another group showed that blocking CD70-CD27 co-stimulation during acute LCMV infection decreased the CD8$^+$T cell response and the IL-7Rα expression on virus-specific CD8$^+$T cells (173). Therefore, I stained total PEC and spleen leukocytes for CD70 at day 9 post-LCMV infection. As shown by the overlay and MFI in figure III-31, I found that at day 9 post-LCMV infection total PEC leukocytes had a higher expression of CD70 as compared to spleen leukocytes. Thus, sustained CD27 stimulation due to continued presence of CD70-expressing cells in the PEC environment might promote survival of virus-specific CD8$^+$T cells.
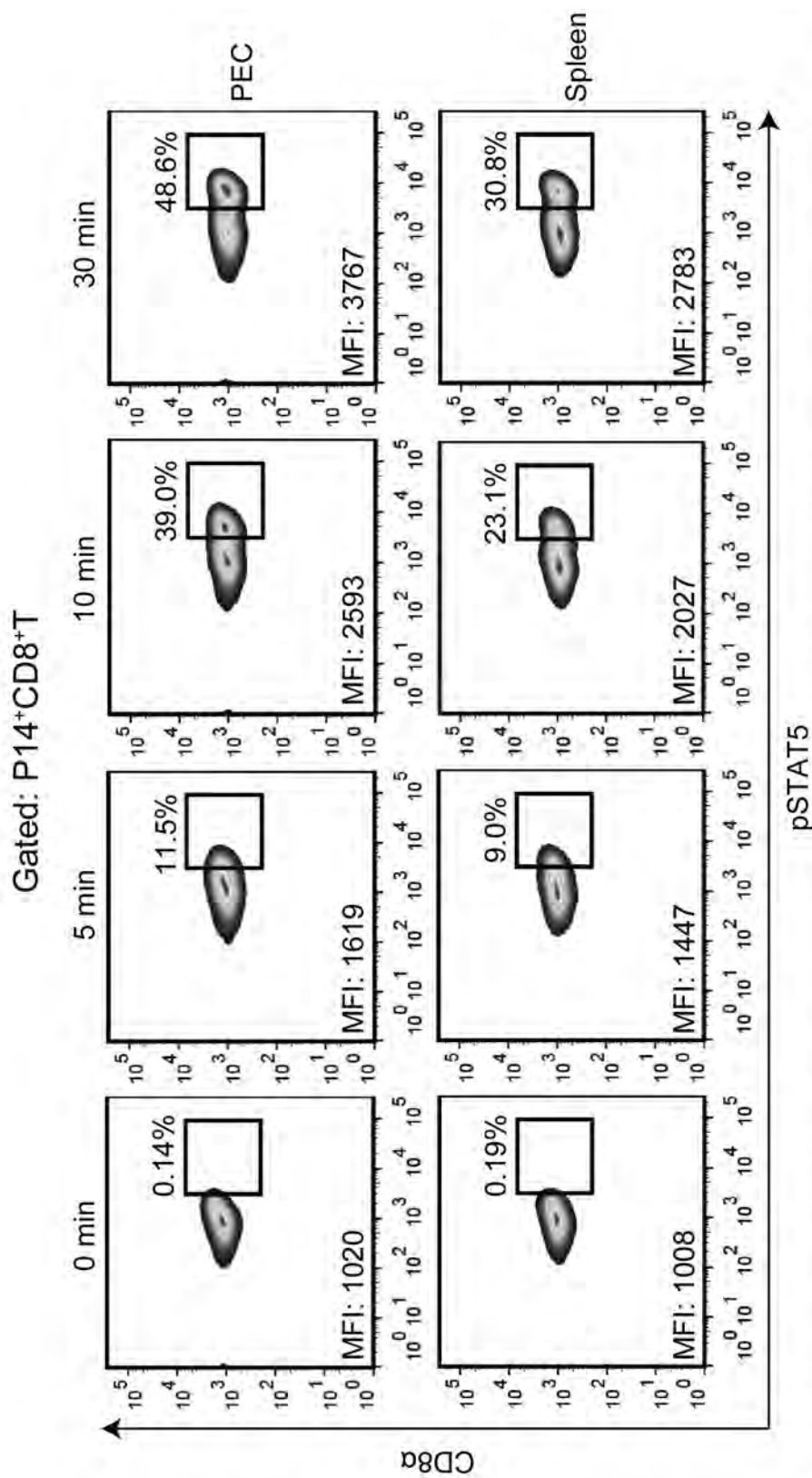
FIGURE III-30. Increased IL-7-induced STAT5 phosphorylation in virus-specific CD8+T cells from PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods.Lymphocytes isolated from PEC and spleen were cultured directly *ex-vivo* in presence of IL-7 (2.5 ng/ml) for the indicated time points. Graphs depict percentage of pSTAT5 expressing P14+CD8+T cells. Data are representative of two independent experiments. MFI of pSTAT5 expression on P14+CD8+T cells is depicted in the plots.
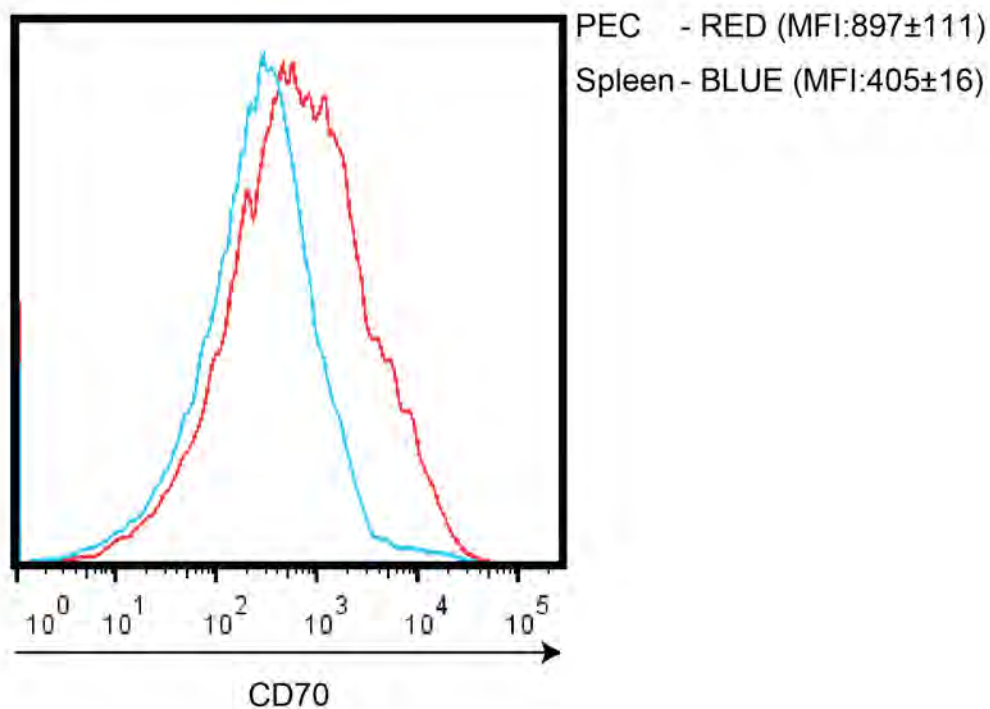
FIGURE III-31. Higher CD70 expression in the PEC leukocyte environment.
Graph depicts overlay of CD70 expression in PEC (red) and spleen (blue) leuko-
cytes at day 9 post-LCMV infection. MFI of CD70 expression is depicted in the
plots. Data are representative of three independent experiments with 3 mice in
each experiment. Plots were gated on FSC-A vs SSC-A leukocytes.

Survival and proliferation of CD8^{+}T cells can also be influenced by other cell types present in the tissue environment. CD4^{+}T cell help has been shown to be important for survival, maintenance and generation of functional memory CD8^{+}T cells (34-36)(174), and the absence of CD4^{+}T cells decreased the proportion and survival of CD8^{+}T cells at tissue effector sites (175, 176). I questioned whether CD4^{+}T cells in the non-lymphoid tissues were also resistant to apoptosis during resolution of the immune response. At day 8 post-LCMV infection, lymphoid (spleen, inguinal lymph nodes, mesenteric lymph nodes) and non-lymphoid (fat-pad, PEC) tissues were harvested and stained directly *ex-vivo* for Annexin V. CD44^{hi} CD4^{+}T cells in the non-lymphoid tissues reacted much less with the apoptotic marker Annexin V compared to the lymphoid organs (Figure III-32). Day 8 LCMV-infected CD44^{hi} CD4^{+}T cells from non-lymphoid tissues were also less TUNEL reactive compared to lymphoid organs (data not shown), further confirming stability of CD4^{+}T cells in the non-lymphoid tissues. Therefore, similar to CD8^{+}T cells, CD4^{+}T cells were also less apoptotic in the non-lymphoid tissues.

I explored the possibility of CD4^{+}T cells promoting CD8^{+}T cell survival in the PEC by performing some additional studies. To track virus-specific CD4^{+}T cell responses I adoptively transferred transgenic LCMV GP61 epitope-specific CD4^{+}T cells (SMARTA) into a C57BL/6 congenic host, which was subsequently infected with LCMV. As shown in Figure III-33, at day 9 and day 15 post-LCMV infection a higher proportion of activated virus-specific CD4^{+}T cells were present in the PEC relative to the spleen. Moreover, when stimulated with cognate

Gated: CD44^{hi} CD4⁺T
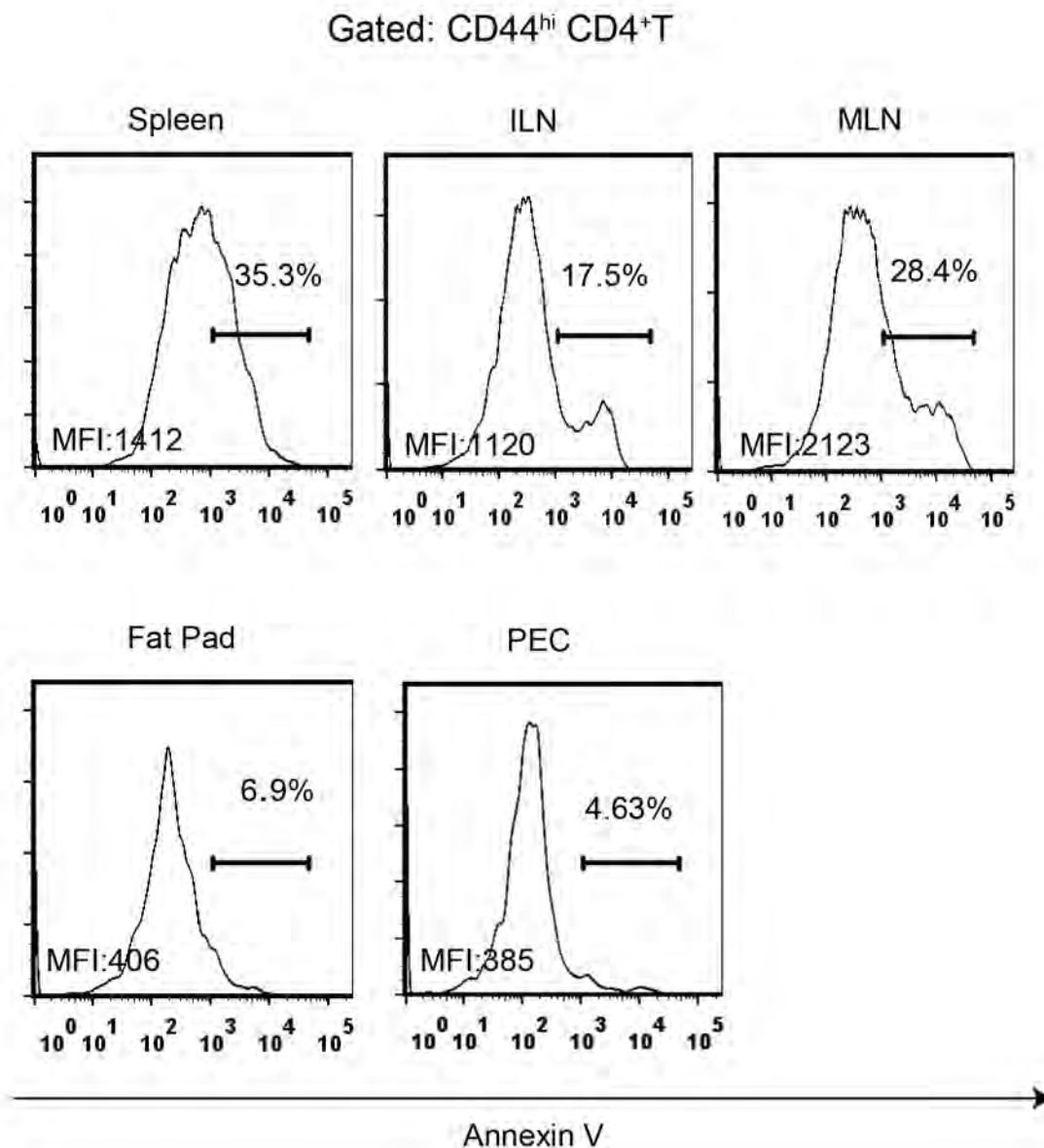
FIGURE III-32. Lower frequency of Annexin V-reactive CD4+ CD44hi T cells in the non-lymphoid tissues. Annexin V reactivity of CD44hi CD4+T cells in lymphoid (spleen, ILN, MLN) and non-lymphoid (PEC, fat pad) tissues at day 8 post-LCMV infection. Leukocytes from five mice were pooled for the experiment, which was repeated at least two times. MFI for Annexin V reactivity on CD44hi CD4+T cells is listed in each histogram.
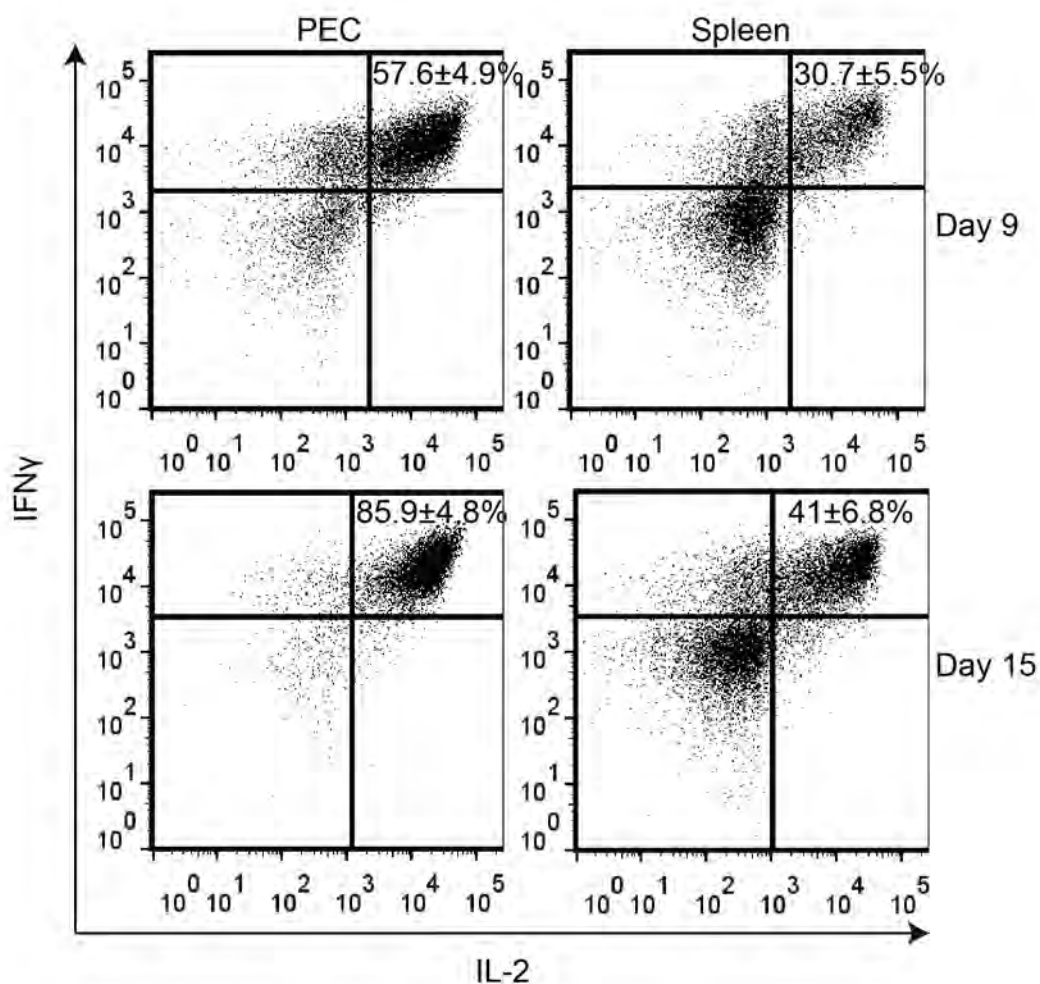
FIGURE III-33. Higher proportion of IL-2-producing virus-specific CD4+T cells in the PEC. 2×10^5 transgenic SMARTA splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection. Percentage ± StDev of IL-2+ IFNγ+ double cytokine producers in the spleen and PEC at day 9 and day 15 post-LCMV infection responding to 5 hours *ex-vivo* GP61 stimulation. Plots are gated on CD4 TCR transgenic SMARTA T cells. Data are representative of two independent experiments with at least three mice in each experiment.

peptide virus-specific CD4$^+$T cells in the PEC made much more IL-2 than those in the spleen (Figure III-33). IL-2 is a T cell growth factor and, while it can promote AICD of T cells under some circumstances, IL-2 therapy during contraction of the immune response has been shown to increase CD8$^+$T cell proliferation and survival [76]. Earlier studies in mice lacking the IL-2 gene showed no role of IL-2 in regulating *in vivo* T cell responses to LCMV or VACV [177]. However, these studies were confounded by presence of autoimmune and inflammatory conditions due to defective Treg function [178]. Subsequent studies in which IL-2R deficient T cells were present in a wild type environment showed that both primary and secondary CD8$^+$T cell responses are affected in absence of IL-2 signaling during LCMV infections [77, 78]. Therefore, higher proportions of virus-specific CD4$^+$T and CD8$^+$T cells in the PEC are capable of making IL-2, and this could be another factor in the enhanced survival of CD8$^+$T cells seen in the PEC environment. CD4$^+$T cell help to CD8$^+$T cells can be direct by providing cytokines and co-stimulation or indirect by activating APC. I found that CD8$^+$T cell apoptosis in the PEC trended higher at day 8 post-LCMV infection in mice depleted of CD4$^+$T cells at day 6 post infection (18.1±5.7% TUNEL$^+$ in control mice vs 25.2±3.8% TUNEL$^+$ in CD4 depleted mice) but no change was observed in the spleen (34.3±1.2% TUNEL$^+$ in control mice vs 35.1±2.4% TUNEL$^+$ in CD4 depleted mice). Thus, the increased proportion of IL-2-producing CD4$^+$T cells in the PEC environment could account for some of the increased resistance to apoptosis of virus-specific CD8$^+$T cells in the PEC. IL-2 signals are transduced

by high-affinity IL-2R, which is comprised of CD25 (IL-2Rα), CD122 (IL2Rβ) and CD132 (common γ chain). CD25 expression is uniformly down-regulated in virus-specific CD8⁺T cells during resolution of the immune response (81). However, the intermediate affinity IL-2R comprising of CD122 and CD132 is sufficient for responsiveness to IL-2 and IL-15 (179). I compared CD122 expression on virus-specific CD8⁺T cells during the resolution and memory phase of the immune response. As shown in Figure III-34, I found a modest increase in the proportion of virus-specific CD8⁺T cells expressing CD122 in the PEC at day 9 post-LCMV infection compared to spleen, and this difference was enhanced at day 30 post-LCMV infection. Expression of CD122 on a per-cell basis (as shown by MFI) was also increased in virus-specific CD8⁺T cells in the PEC compared to the spleen (Figure III-34). This might allow for IL-2- and IL-15-mediated CD8⁺T cell survival in the PEC environment. Overall these results indicate that decreased CD4⁺T cell apoptosis and their enhanced ability to produce IL-2 in the non-lymphoid tissues might be another factor promoting stability of CD8⁺T cells in the peripheral tissue environment.

H. Discussion to Chapter III

T cells persist at high frequencies in non-lymphoid tissues after resolution of the immune response, and these T cells are uniquely geared to produce effector cytokines (128, 129, 131, 132). The presence of memory T cells at non-lymphoid tissue sites is critical for protection against secondary virus challenge (133, 137, 138, 161). Our lab has previously shown that during and after silencing of
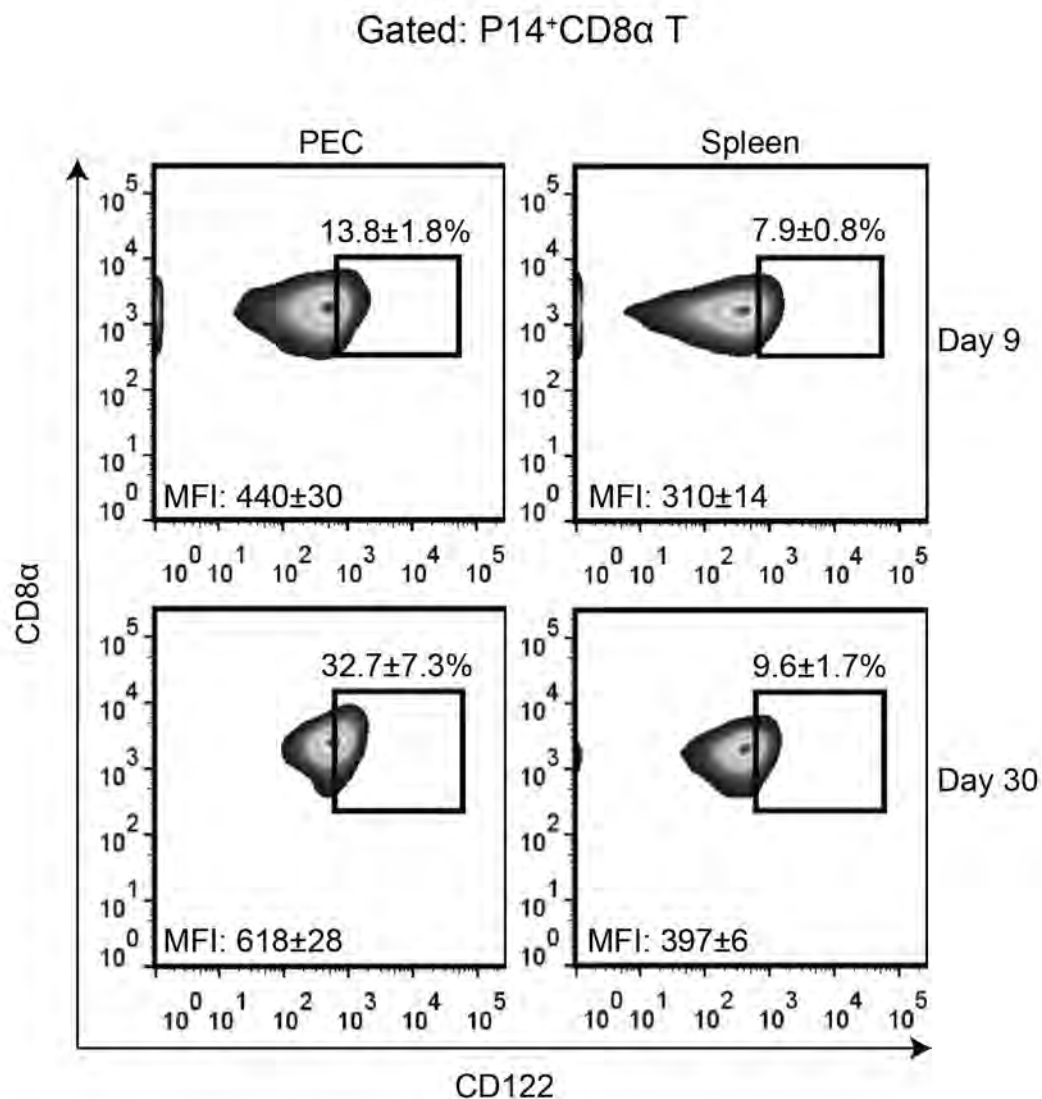
FIGURE III-34. Higher proportion CD122-expressing virus-specific CD8⁺T cells in the PEC. P14 splenocytes were adoptively transferred into a C57BL/6 host followed by LCMV infection as described in materials and methods. Percent ± StDev of CD122 expressing P14⁺CD8⁺T cells in PEC and spleen at day 9 and day 30 post-LCMV infection. MFI of CD122 expression on P14⁺CD8⁺T cells is depicted in the plots. Data are representative of two independent experiments with at least three mice in each experiment.

the immune response, LCMV-specific CD8$^+$T cells in non-lymphoid tissues are more resistant to apoptosis than those in the lymphoid organs (139). This differential in apoptosis could account for persistence of long-lived virus-specific memory CD8$^+$T at high frequencies in the non-lymphoid tissues, serving as frontline defense against secondary virus challenge.

The studies performed in this chapter reveal the mechanisms underlying tissue-dependent differences in CD8$^+$T cell apoptosis. I have shown here that higher proportions of virus-specific CD8$^+$T cells in the non-lymphoid tissues expressed surface markers that correlated with an apoptosis-resistant phenotype. CD8$^+$T cells which are IL-7R^{hi} and KLRG-1^{lo} are thought to preferentially become memory cells (27, 72, 74), and these were found in higher proportions in non-lymphoid tissues (Figure III-7 and 8). IL-7 is important for survival and homeostatic proliferation of memory CD8$^+$T cells (170). IL-7 signaling induces STAT5 phosphorylation, which is known to be associated with survival of effector and memory CD8$^+$ T cells (118, 119). I found that a higher proportion of virus-specific CD8$^+$T cells in the PEC exhibited STAT5 phosphorylation in response to IL-7 directly *ex-vivo* as compared to the spleen counterparts (Figure III-30). Though I have not directly tested IL-7 cytokine levels in PEC, IL-7 is constitutively produced by many cell types, and it is possible that stromal cells in the PEC environment might produce high amounts of IL-7, thereby promoting optimal survival of virus-specific CD8$^+$T cells. I found that a higher proportion of virus-specific CD8$^+$T cells in the PEC expressed CD27, which were less

apoptotic (Figure III-9). It has also been shown that CD27 sustains survival of virus-specific CD8$^+$T cells in the non-lymphoid tissues by inducing autocrine IL-2 production (85). Stimulation through CD27 requires the presence of its co-stimulatory ligand CD70, and I found that at day 9 post-LCMV infection total PEC leukocytes had a higher expression of CD70 as compared to spleen leukocytes (Figure III-31). Thus, sustained CD27 stimulation due to continued presence of CD70-expressing cells in the PEC environment might promote survival of virus-specific CD8$^+$T cells. The collagen binding α1β1 integrin VLA-1 has been shown to be important in maintenance and survival of influenza A virus-specific CD8$^+$T cells in lungs (180). However, I did not find VLA-1 to be highly expressed on LCMV-specific CD8$^+$T cells in the PEC or spleen, suggesting that different mechanisms might regulate tissue-dependent survival in different tissues and during different viral infections.

I also explored the possibility of CD4$^+$T cells promoting CD8$^+$T cell survival in the PEC. Similar to CD8$^+$T cells, CD4$^+$T cells in the non-lymphoid tissues resisted apoptosis during resolution of the immune response to LCMV (Figure III-32). Furthermore, I found that a higher proportion of virus-specific CD8$^+$T cell and CD4$^+$T cells in the PEC are capable of making IL-2 (Figure III-11 and 33), and this could be another factor in the enhanced survival of CD8$^+$T cells seen in the PEC environment. However, I was unable to show the presence of IL-2 in the PEC environment directly *ex-vivo* by ELISA assays at day 7 post-LCMV infection. The inability to detect IL-2 could have been due to extremely low

levels of free IL-2 present in the tissue environment during resolution of the immune response. However, I also found that depleting CD4+T cells during resolution of the immune response modestly increased CD8+T cell apoptosis in the PEC but no change was observed in the spleen. Thus, increased resistance to apoptosis of virus-specific CD8+T cells in the PEC may be accounted by the increased proportion of IL-2-producing CD4+T cells in the PEC environment. Higher proportion of virus-specific CD8+T cells in the PEC expressed CD122 (IL2Rβ) (Figure III-34) which might allow for IL-2- and IL-15- mediated CD8+T cell survival in the PEC environment if trace levels of these cytokines were made.

Initially I had performed experiments with mice immunized with serum-containing LCMV stocks. I found that in *in vitro* assays CD4+T cells from the PEC but not the spleen responded strongly to serum (Figure III-35), thereby inducing IL-2 production that contributed to CD8+T cell survival in the PEC but not the spleen. Serum-free LCMV stocks were thus used for the experiments in the current study. Since CD4+T cells in the PEC had a dramatic response to serum antigen, we can speculate that a similar response to limited amounts of viral antigen in the PEC might promote CD4+T cell dependent CD8+T cell survival in the PEC. Influenza virus-derived antigens are known to be presented for a prolonged period in the lung after apparent resolution of infection (181), and whether similar persistence of LCMV antigen contributes to T cell survival is not clear. I also found that tissue-dependent differences in CD8+T cell apoptosis persisted even if the peritoneal cavity was not the initial site for antigen delivery.
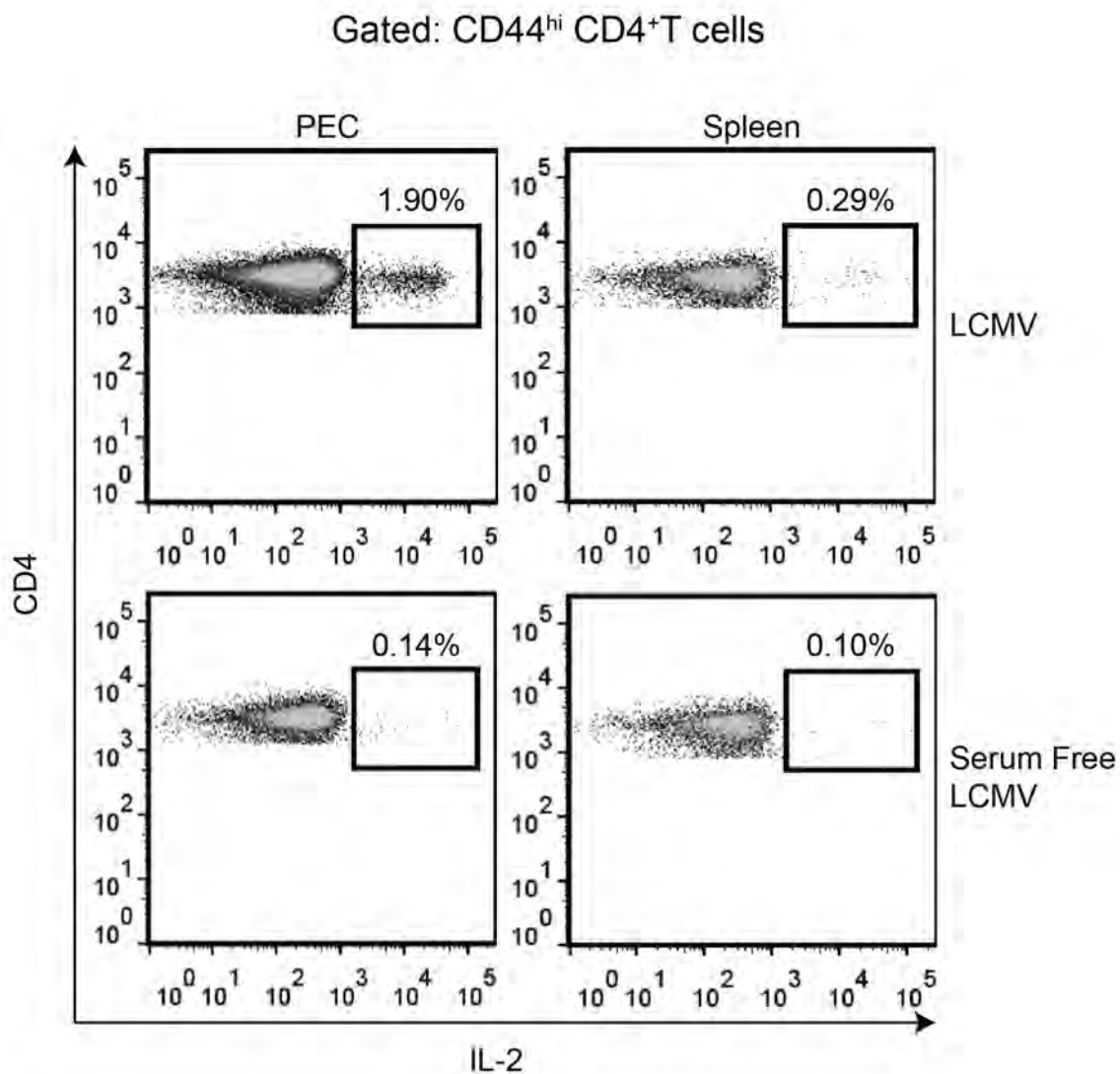
FIGURE III-35. Spontaneous serum-induced IL-2 production by CD4$^+$T cells in the PEC. Lymphocytes were isolated from spleen and PEC of day 8 LCMV-infected mice and cultured directly ex-vivo for 5 hours. Graphs depict day 8 LCMV-infected IL-2 producing CD4$^+$T cells in the PEC or spleen. The mice were infected with serum-diluted or serum-free LCMV Armstrong. Data are representative of two independent experiments with at least three mice in each experiment.

In mice challenged with LCMV via i.v route the influx of activated CD8$^+$T cells in the PEC was decreased, but, CD8$^+$T cells in the PEC were still more resistant to apoptosis as compared to the spleen (10.1% Annexin V$^+$ CD44^{hi} CD8$^+$T in the PEC vs 43.4% Annexin V$^+$ CD44^{hi} CD8$^+$T in the spleen).

Transcription factors can regulate CD8$^+$T cell effector-memory fate decisions. I found that a higher proportion of CD8$^+$T cells in the PEC express high amounts of TCF-1, LEF-1 and EOMES (Figure III-15,16,17), which are transcription factors known to correlate with T cell memory potential. By using the *Tcf7$^{-/-}$* mice I showed that TCF-1 promoted survival of CD8$^+$T cells in the non-lymphoid tissues during resolution of the immune response. Moreover, by day 30 post-LCMV infection nearly 100% of virus-specific CD8$^+$T cells in the PEC were TCF-1 positive (Figure III-14) as compared to 61% IL-7R^{hi} KLRG-1^{lo} (MPEC) (Figure III-8), suggesting that increased survival in non-lymphoid tissues was not simply due to presence of a higher proportion of MPECs. Expression of TCF-1 can be modulated by Wnt ligands and by TCR and cytokine signals (105, 182, 183). It has been suggested that Wnt proteins might induce TCF-1-Eomes-IL2Rβ axis to induce cytokine responsiveness in memory CD8$^+$T cells (105). However, I did not observe significant differences in the expression of the canonical WNT signaling target *Axin2* (data not shown) in T cells from the PEC relative to spleen, raising the possibility that TCF-1 has a Wnt-independent function in promoting memory CD8$^+$T cell survival in the non-lymphoid tissues. How do virus-specific CD8$^+$T cells maintain higher TCF-1 expression in the PEC? The PEC

environment is enriched in macrophages and dendritic cells, which *in vitro* are known to produce Wnt5a, which induces the non-canonical Wnt pathway (184). Alternatively, it is possible that TCF-1-expressing cells might preferentially populate the peritoneal cavity. Future studies will need to define the underlying mechanism for the biased recruitment and/or maintenance of TCF-1^{hi} cells in non-lymphoid tissues.

T cells migrate into tissue effector sites post-activation to eliminate foreign antigens, and I provide evidence that non-apoptotic CXCR3$^+$CD8$^+$T cells accumulated in the lymph nodes if their egress was blocked by FTY720 treatment. CXCR3$^+$CD8$^+$T cells are known to migrate in response to IFNγ-inducible chemokines CXCL9 and CXCL10. CXCL9 and CXCL10 are produced by activated macrophages and DC's, and I found that the PEC environment had higher expression of these chemokines. Thus, during resolution of the immune response non-apoptotic memory phenotype CD8$^+$T cells likely migrate preferentially into non-lymphoid tissues. This trafficking pattern, in conjunction with tissue-intrinsic differences in promoting cell survival, provides an attractive mechanism for promoting long-lived immunity at non-lymphoid tissues.

I provide evidence that multiple mechanisms account for tissue-dependent differences in CD8$^+$T cell apoptosis seen during resolution of the immune response (Figure III-36). These mechanisms would work in conjunction to promote survival and overall maintenance of CD8$^+$T cell populations in the non-

lymphoid tissues. The presence of functional long-lived memory CD8$^+$T cells at non-lymphoid tissue effector sites would ensure a productive response on encounter with the antigen providing protection against secondary virus challenge.
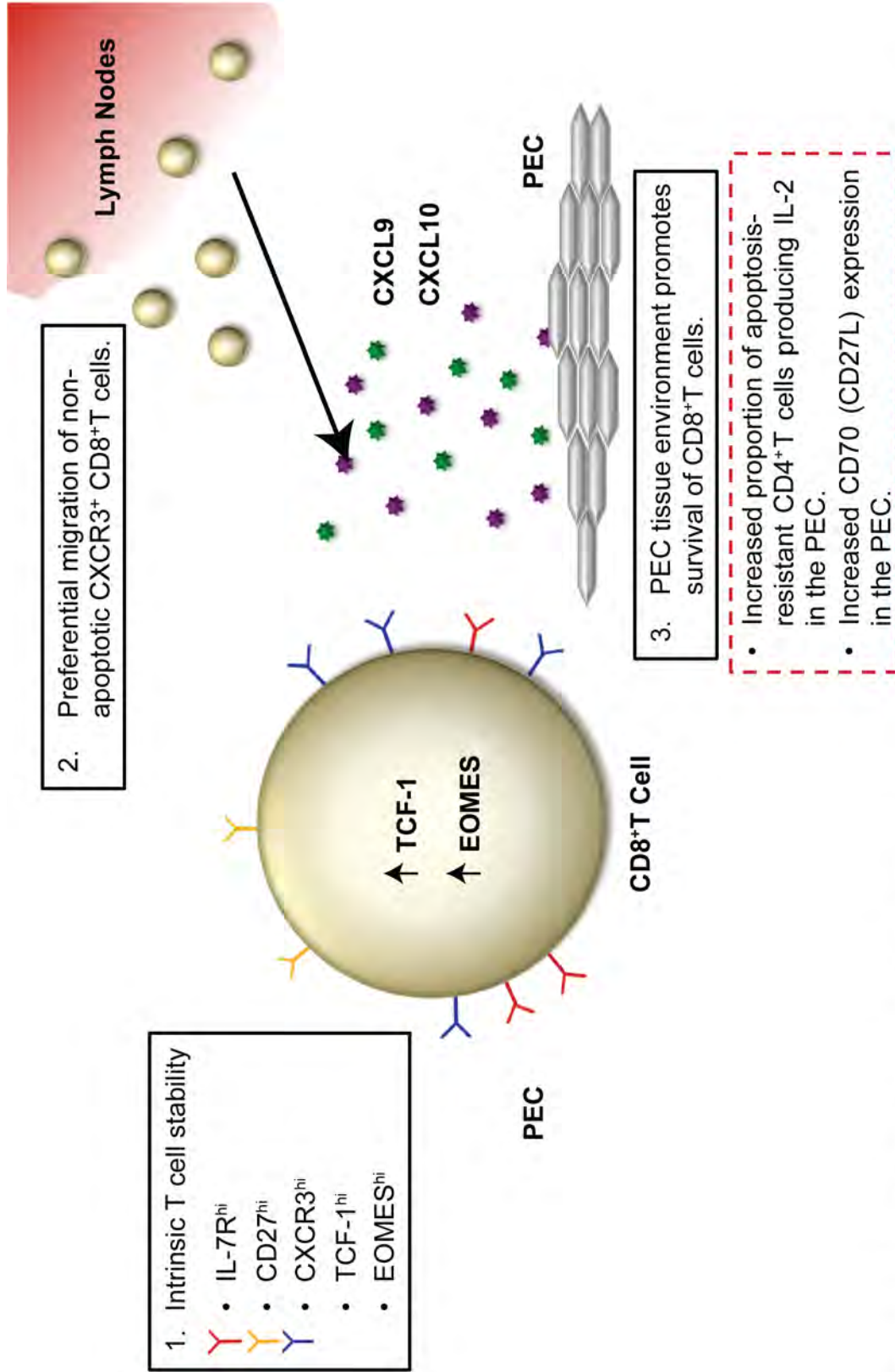
Figure III-36. Mechanisms regulating tissue-dependent differences in CD8+T cell apoptosis during acute LCMV infection.

CHAPTER IV: BLIMP-1 SUPPRESSES CD25 AND CD27 EXPRESSION TO NEGATIVELY REGULATE CD8$^+$T CELL MEMORY DIFFERENTIATION

In response to a virus infection, CD8$^+$T cells proliferate and differentiate into effector cells that eradicate the pathogen. The population of effector CD8$^+$T cells generated during an immune response is heterogeneous; the majority of effector cells die, whereas a small population survive and become memory cells. Transcriptional profiling of effector and memory CD8$^+$T cells in both acute and chronic virus infection has recently provided insight into the gene expression programs characterizing distinct cell subsets (89). Nonetheless, the precise mechanisms by which these transcriptional programs are established and maintained during CD8$^+$T cell differentiation remain largely unknown.

The transcription factor Blimp-1 (encoded by *Prdm1* gene) drives CD8$^+$T cell differentiation towards short-lived effector cells at the expense of memory precursors (94, 95). Although Blimp-1 is known to regulate a number of target genes mediating plasma cell differentiation (90), only a few targets of Blimp-1 in CD8$^+$T cells have been identified. One recent study has shown that Blimp-1 directly regulates expression of the DNA-binding inhibitor Id3 and thereby contributes to the development of short-lived effector T cells (97). However, more global information on the targets of Blimp-1 in CD8$^+$T cells is currently lacking.

In this study I wanted to identify the mechanisms by which Blimp-1 regulates differentiation of effector-memory CD8$^+$T cells. Preliminary genome-

wide ChIP-sequencing studies done by my collaborator Dr. HyunMu Shin in the lab of Dr. Leslie J. Berg identified novel Blimp-1 target genes. During contraction of the CD8$^+$T cell response, when viral antigens are no longer present, cytokine stimulation and/or co-stimulatory signals become critical for T cell survival and memory development (52, 67). Therefore, to address the functional importance of specific Blimp-1 target genes in CD8$^+$ T cells, I chose to focus on cytokine and co-stimulatory receptors, particularly CD25 (IL-2Rα) and CD27, as these are known to be critical in regulating T cell function and differentiation. In this study I show that CD25 and CD27 expression is dysregulated in *Prdm1*$^{-/-}$ T cells responding to LCMV infection, that T cells expressing CD25 and CD27 preferentially survive during acute viral infection, and that silencing of *IL-2Rα* and/or *Cd27* in Blimp-1-deficient CD8$^+$T cells decreases the magnitude of the virus specific CD8$^+$T cell response. Furthermore, by using genome-wide ChIP-sequencing analysis our collaborating group identified *IL-2Rα* (CD25) and *CD27* genes as direct targets of Blimp-1. At the peak of the anti-viral response, but not earlier, Blimp-1 recruited the histone modifying enzymes G9a (methyl transferase) and HDAC2 (Histone deacetylase 2) to the *Il2rα* and *Cd27* loci, thereby repressing expression of these genes. Therefore, we found that Blimp-1 suppresses CD25 and CD27 expression by inducing histone modifications to negatively regulate CD8$^+$T cell differentiation. These studies were done in collaboration with Dr. HyunMu Shin and Dr. Leslie J. Berg, and are a part of the manuscript titled "Epigenetic Modifications Induced by Blimp-1 Regulate CD8$^+$T

Cell Memory Progression during Acute Virus Infection" (Immunity 39, 1-15, October 17, 2013).

A. Increased CD8^{+}T cell response and memory development in Blimp-1-deficient mice

To investigate the role of Blimp-1 in regulating CD8^{+}T cell responses during a virus infection, we used mice carrying a conditional *Prdm1* allele in which exon 5 is flanked by loxP sites (159). This line was crossed to *Cd4-cre*[+] transgenic mice, thereby deleting *Prdm1* in all αβ T cells (referred to as *Prdm1*^{-/-}), and differed from those previously used in studies of Blimp-1 function in B and T lymphocytes (185, 186). We used *Cd4-cre*[+] littermate mice as wild type controls (referred to as WT) for our studies. Since the conditional knockout (CKO) line generated by us was different from previously used lines we had to verify that these Blimp-1 CKO mice had a phenotype similar to what has been previously reported. As previously reported (185, 187), I did not detect any changes in the proportion of lymphocytes in various lymphoid organs, although naïve *Prdm1*^{-/-} mice had a higher proportion of CD44^{hi} CD4^{+} and CD8^{+}T cells when compared to WT controls (data not shown). I also tested CD8^{+}T cell response in the *Prdm1*^{-/-} mice during an acute LCMV infection. Consistent with previous studies (94, 96), I found that there was a higher absolute number of CD8^{+}T cells in *Prdm1*^{-/-} mice at day 7 post-LCMV infection (Figure IV-1). I also found a marked increase in the proportion of activated CD44^{hi} CD8^{+}T and LCMV-specific NP396^{+} and GP33^{+} CD8^{+}T cells in *Prdm1*^{-/-} at day 7 after LCMV Armstrong infection (Figure IV-1)

Day 7 LCMV (P.I.)
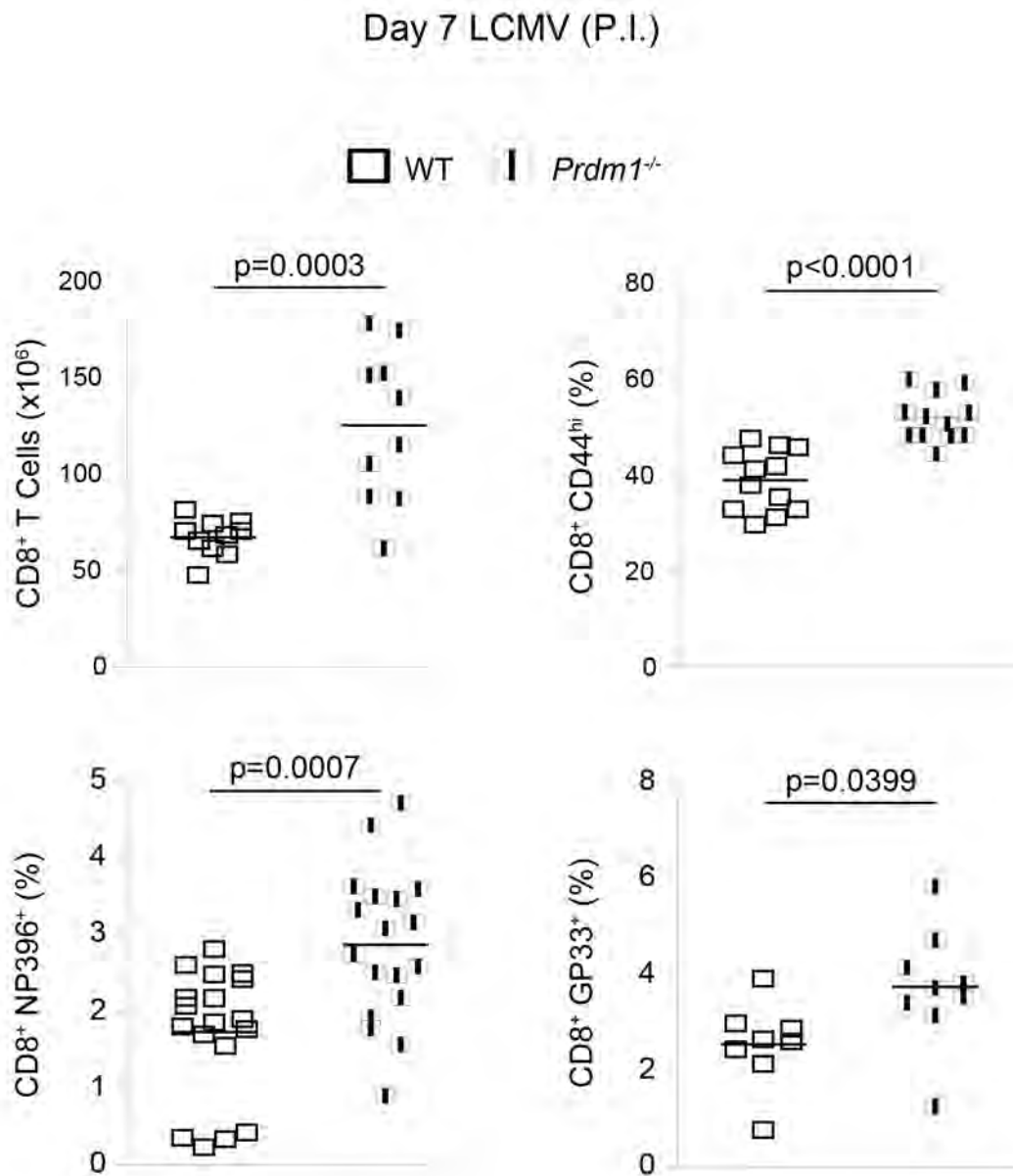
FIGURE IV-1. Higher proportion and absolute number of T cells in the Blimp-1 conditional knockout mice during acute LCMV infection. The total number of CD8+T cells and frequencies of CD8+CD44hi, GP33+CD8+T, and NP396+CD8+T cells in the spleen at day 7 after LCMV infection are shown for WT and *Prdm1-/-* mice. NP396 and GP33 LCMV-specific CD8+T cells were identified by Class-I MHC tetramer staining. Data are compilation of three experiments.

and these differences were also seen at day 14 post infection (data not shown). Blimp-1 deficiency leads to generation of higher proportion of MPEC CD8$^+$T cells (KLRG-1^{lo} IL-7Rα^{hi}; (94)), and as shown in figure IV-2, I also found a higher proportion of LCMV-specific NP396$^+$CD8$^+$T cells with a MPEC phenotype in the *Prdm1*$^{-/-}$ mice at day 7 and day 14 post infection. Therefore, the phenotype seen in *Prdm1*$^{-/-}$ mice during acute LCMV infection was similar to previous reports. The transcription factor EOMES promotes persistence of memory CD8$^+$T cells (110). Since, a higher proportion of Blimp-1-deficient T cells had a memory phenotype I compared EOMES expression in virus-specific T cells from *Prdm1*$^{-/-}$ and WT mice. As shown in Figure IV-3, I found that a higher proportion of LCMV-specific NP396$^+$ CD8$^+$T cells from *Prdm1*$^{-/-}$ mice expressed EOMES compared to WT controls at days 7 and 14 post infection. The expression level of EOMES was also higher in virus-specific CD8$^+$T cells in the *Prdm1*$^{-/-}$ mice when compared to WT controls, as shown by the MFI in Figure IV-3. Increased magnitude of the CD8$^+$T cell response in *Prdm1*$^{-/-}$ mice during an acute LCMV infection could have been due to defective viral clearance. Therefore, I enumerated viral-titers in *Prdm1*$^{-/-}$ by plaque assays at day 4, 7 and 8 post-LCMV infection. As shown in Figure IV-4, viral clearance in the spleen of *Prdm1*$^{-/-}$ mice was similar to WT controls, indicating that the increased magnitude of the CD8$^+$T cell response to LCMV in *Prdm1*$^{-/-}$ mice was not due to impaired viral clearance.
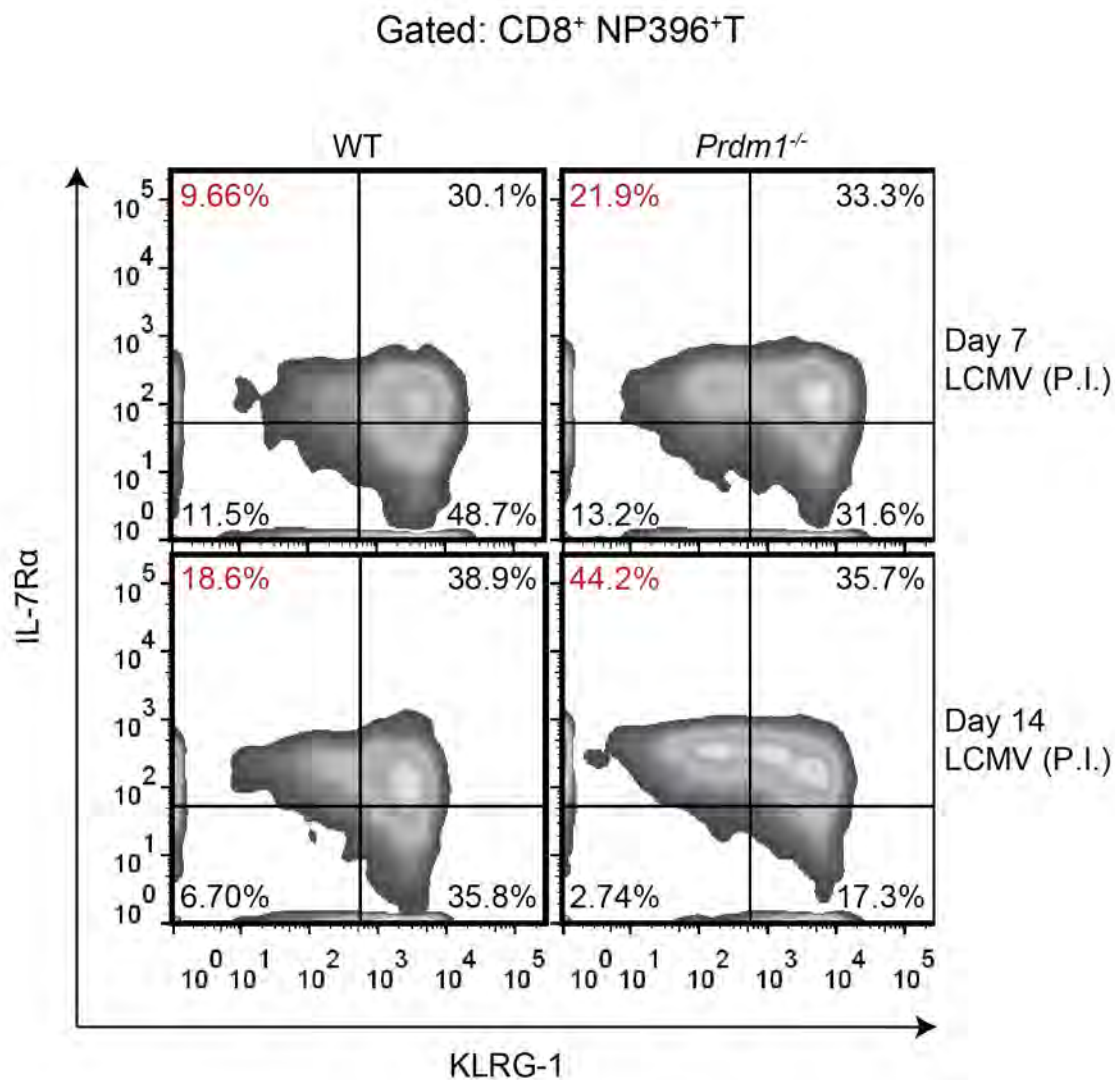
FIGURE IV-2. Higher proportion of memory precursor effector T cells in the Blimp-1 conditional knockout mice. NP396 tetramer-binding CD8+T cells in splenocytes from WT and *Prdm1-/-* mice were analyzed for IL-7R and KLRG-1 expression at days 7 and 14 after LCMV infection. The numbers indicate the percentage of NP396 tetramer-binding CD8+T cells in each subset. The numbers in the top left quadrant (marked in red) are indicative of memory precursor effector cells. Data are representative of three independent experiments.
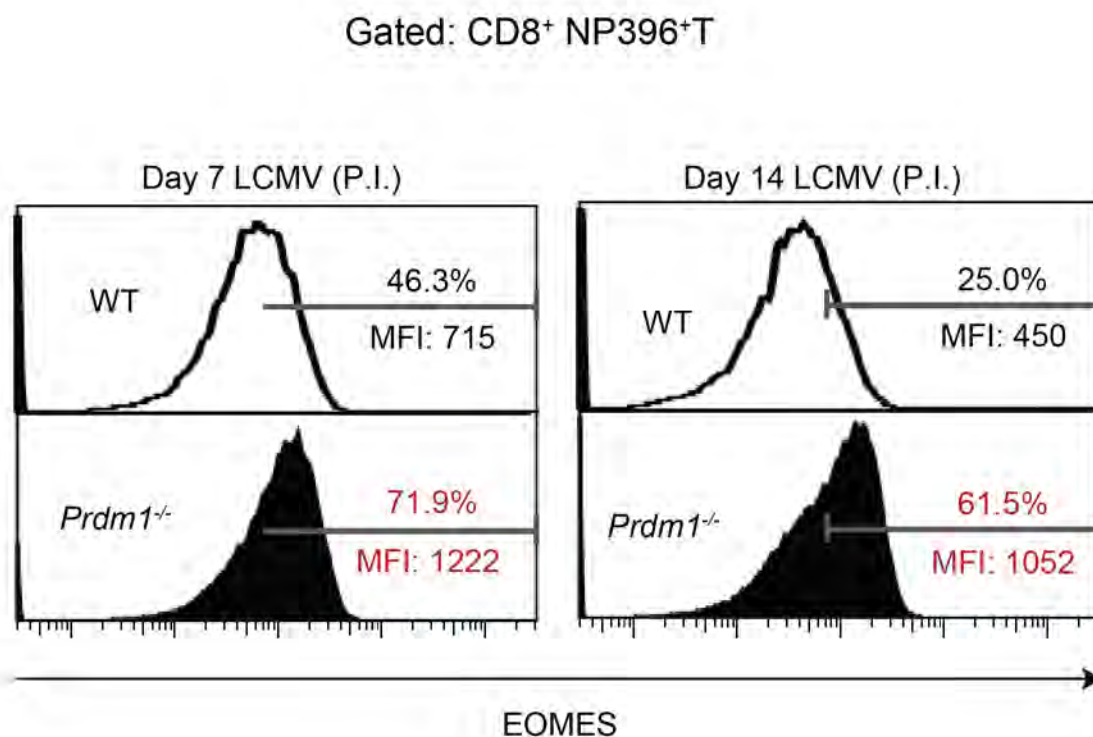
FIGURE IV-3. Higher proportion of EOMES-expressing virus-specific CD8+T cells in the Blimp-1 conditional knockout mice. Percentages of EOMES+ NP396 tetramer-positive CD8+T cells from spleen at days 7 and 14 post-infection were determined by intracellular transcription factor staining; the MFI of EOMES staining on total NP396+CD8+T cells is indicated. Data are representative of three independent experiments.
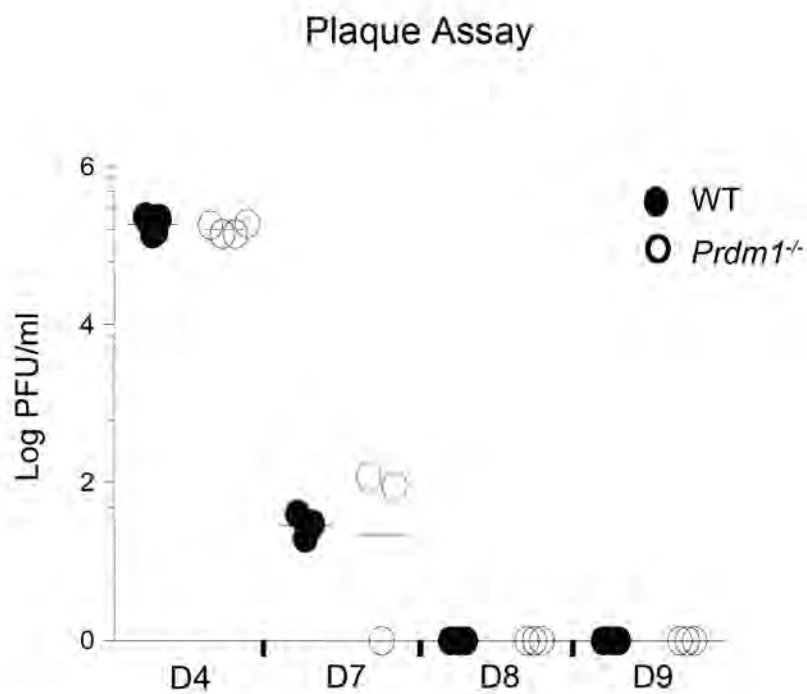
FIGURE IV-4. Viral clearance in the Blimp-1 conditional knockout mice.
LCMV titers in spleen from infected WT and *Prdm1-/-* mice were determined by
plaque assay at the indicated days post infection. Data are representative of three
independent experiments.

B. Increased proliferation and decreased CD8+T cell apoptosis in the Blimp-1-deficient mice during resolution of the immune response

Blimp-1 deficiency in CD8+T cells leads to an increased magnitude of the CD8+T cell response. This could have been due to increased proliferation or decreased apoptosis of T cells due to Blimp-1 deficiency. A previous study has shown that Blimp-1-expressing virus-specific CD8+T cells had decreased proliferative capabilities *in vitro* (94).To test whether Blimp-1 deficiency leads to increased T cell proliferation *in vivo*, day 7 LCMV infected $Prdm1^{-/-}$ and WT control mice were injected i.p with BrdU and sacked after 11 hours. I found that a higher proportion of LCMV-specific GP33+CD8+T cells from $Prdm1^{-/-}$ mice incorporated BrdU compared to WT controls (Figure IV-5). Therefore, enhanced proliferation of T cells contributes to the increased magnitude of the CD8+T cell response seen in Blimp-1-deficient mice. Alternatively, decreased T cell apoptosis can also contribute to increased CD8+T cell responses. To test whether Blimp-1-deficient CD8+T cells were less apoptotic during resolution of the immune response, splenocytes from day 9 LCMV-infected $Prdm1^{-/-}$ and WT control mice were cultured directly *ex-vivo* for 5 hours. As shown by decreased TUNEL reactivity, CD44^hi CD8+T cells from Blimp-1-deficient mice were less apoptotic as compared to WT controls (Figure IV-6). Decreased apoptosis can result from the presence of pro-survival factors such as Bcl-2, which has been shown to be important in counteracting the effects of pro-apoptotic Bim, thereby allowing for survival of cells during contraction of the immune response (54). In

Gated: GP33+CD8+T
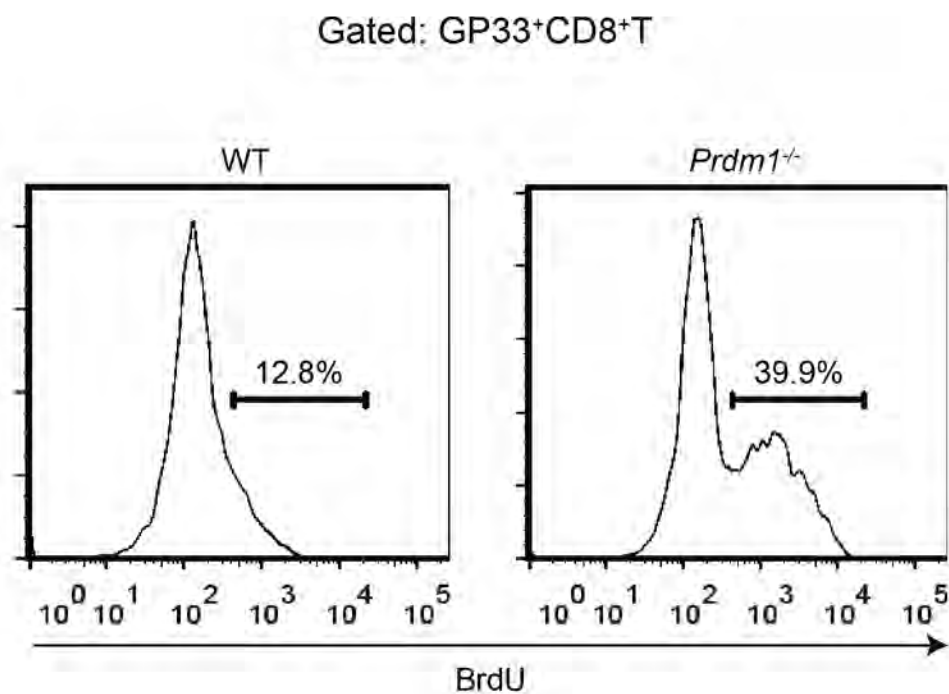
FIGURE IV-5. Increased T cell proliferation due to Blimp-1 deficiency. Day 7 LCMV-infected WT or *Prdm1*-/- mice were given BrdU (i.p) and sacked after 11 hours. Graphs represent BrdU incorporation by LCMV-specific GP33+CD8+T splenocytes at day 7 post-LCMV infection. Data are representative of three independent experiments.

Day 9 LCMV (P.I.)

Gated: CD8⁺ CD44^hiT
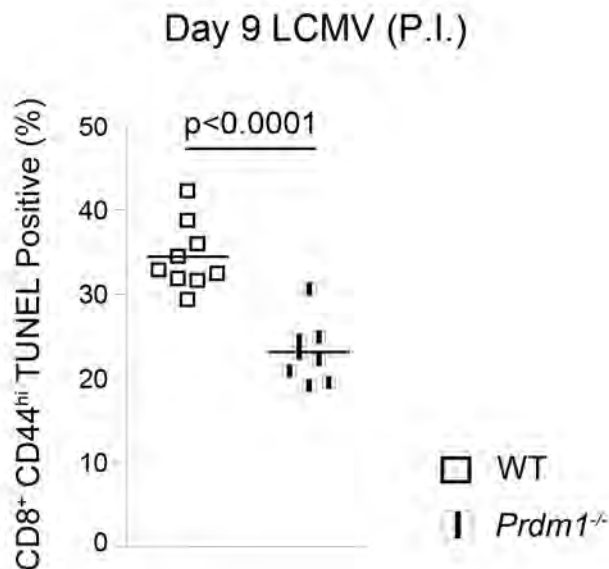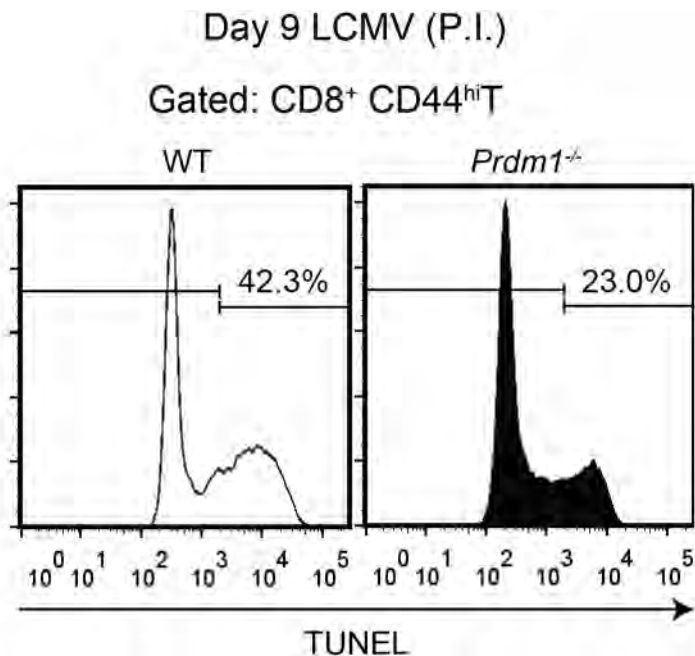
FIGURE IV-6. Decreased T cell apoptosis in the Blimp-1 conditional knock-out mice. Splenocytes from day 9 LCMV-infected WT or *Prdm1*⁻/⁻ mice were cultured directly *ex-vivo* for 5 hours. Histograms (top) show representative results of TUNEL staining in WT and *Prdm1*⁻/⁻ CD44^hi CD8⁺T cells at day 9 after LCMV infection. The histogram (bottom) shows a compilation of data from three independent experiments.

accord with the decreased apoptosis, a higher proportion of LCMV-specific CD8$^+$T cells in the *Prdm1$^{-/-}$* mice expressed Bcl-2 at day 7 post infection when compared to WT controls (Figure IV-7). Therefore, these results show that the increased magnitude of the CD8$^+$T cell response seen in the Blimp-1-deficient mice was due to a combined effect of increased proliferation and decreased T cell apoptosis.

C. Blimp-1 suppresses cytokine responsiveness of CD8$^+$T cells at the peak of the anti-viral response

Since CD8$^+$T cells from the *Prdm1$^{-/-}$* mice had increased proliferation and survival, I questioned whether differential cytokine responsiveness in WT versus *Prdm1$^{-/-}$* T cells contributes to altered T cell survival and/or proliferation. To address this question I isolated CD8$^+$T cells from WT and *Prdm1$^{-/-}$* mice infected with LCMV Armstrong at day 7 (peak expansion) post infection. Sorted CD8$^+$T cells were then labeled with CFSE, and cultured *in vitro* for 2 days with a panel of cytokines (Figure IV-8). As shown in Figure IV-8, *Prdm1$^{-/-}$* CD8$^+$T cells had enhanced proliferation in response to the cytokines IL-2, IL-12, and IL-15 at day 7 post infection when compared to WT controls. This suggested that Blimp-1 suppresses cytokine responsiveness of CD8$^+$T cells at the peak of the anti-viral response, and that the ability of Blimp-1-deficient T cells to respond to certain cytokines during the peak and resolution of the immune response might contribute to their enhanced survival and/or proliferation.

Gated: CD8⁺ NP396⁺T
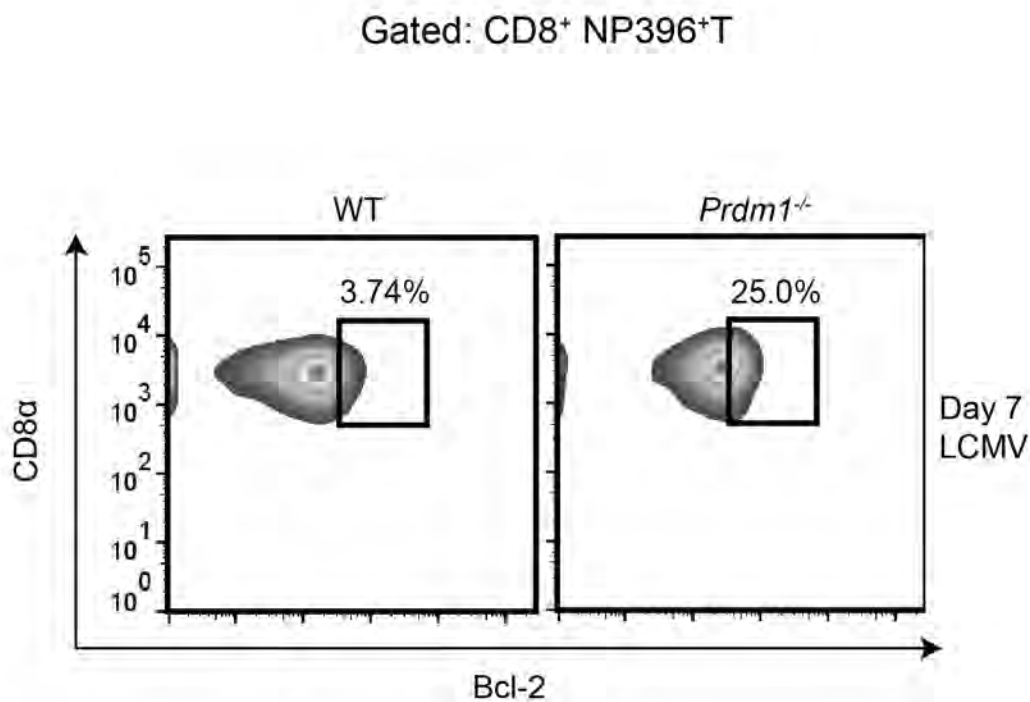
FIGURE IV-7. Higher proportion Bcl-2 expressing T cells in the Blimp-1 conditional knockout mice. NP396 tetramer-binding splenic CD8⁺T cells in WT and *Prdm1⁻/⁻* mice were analyzed for intracellular Bcl-2 expression at day 7 after LCMV infection. The numbers indicate the percentage of Bcl-2⁺ T cells in each sample. Data are representative of two independent experiments.

Day 7 LCMV
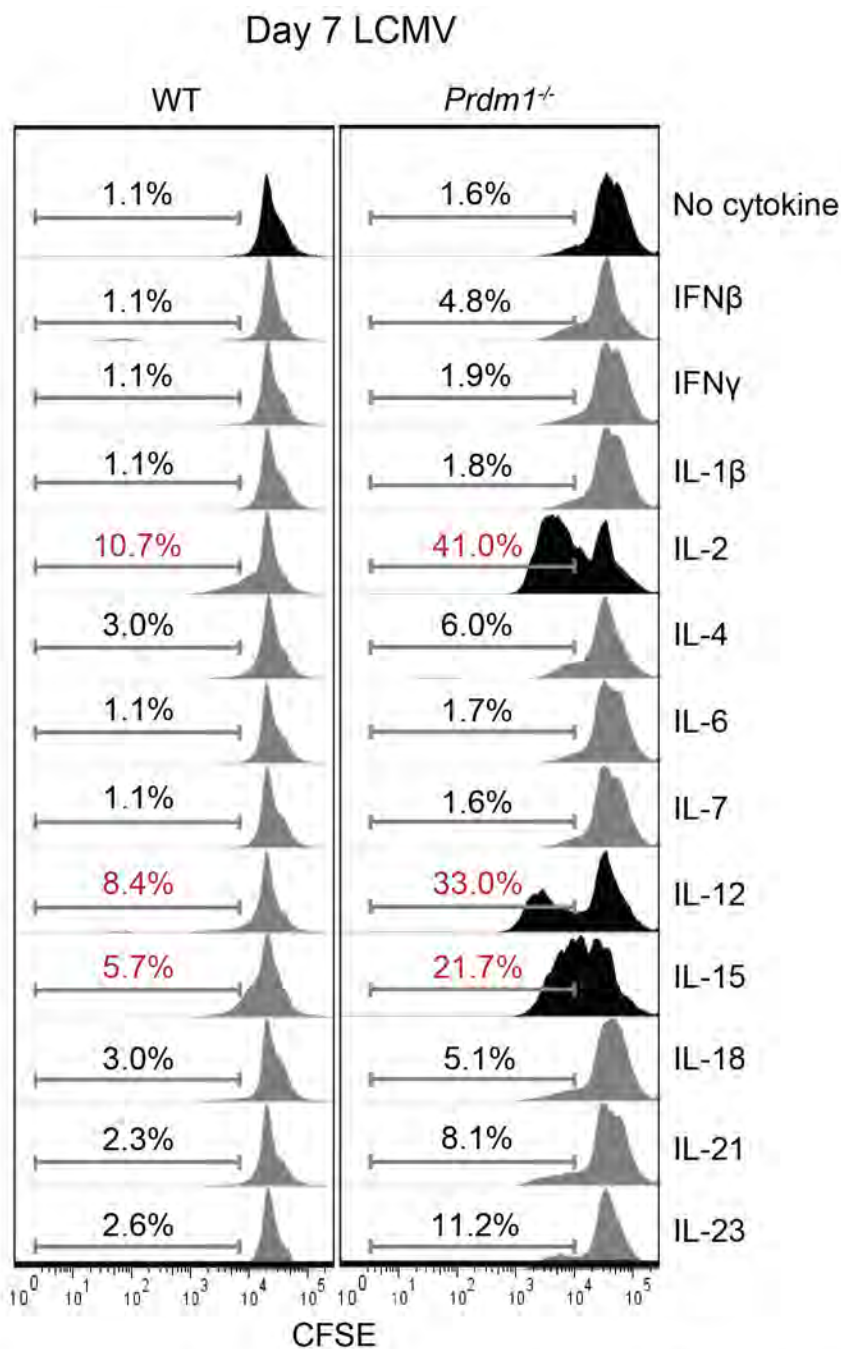
FIGURE IV-8. Sustained cytokine responsiveness of Blimp-1-deficient CD8⁺T cells after LCMV infection. Splenic CD8⁺T cells were isolated from WT or *Prdm1⁻ᐟ⁻* mice at day 7 after LCMV infection. Cells were labeled with CFSE and cultured for 2 days with or without the indicated cytokines. Numbers indicate the percentage of divided cells. Histograms are representative of three independent experiments. Plots are gated on live CD8⁺T cells.

D. Blimp-1 regulates CD25 expression in virus-specific CD8$^+$T cells during anti-viral immune response

Because the cytokine responsiveness of T cells during viral infections can be regulated by the modulation of cytokine receptor expression (74, 80), I questioned whether Blimp-1 might function to suppress cytokine receptor expression during the anti-viral immune response. I chose to focus on CD25, which is the high affinity IL-2Rα for the following reasons: a) Blimp-1-deficient T cells were more responsive to IL-2 at the peak of the immune response when compared to WT controls (Figure IV-8), b) IL-2 is a cytokine which is known to promote survival and proliferation of T cells, c) genome wide ChIP-sequencing analysis performed by our collaborating group identified CD25 as a potential target of Blimp-1. Therefore, to test whether CD25 expression was dysregulated in CD8$^+$T cells lacking Blimp-1, I infected *Prdm1$^{-/-}$* and WT mice with LCMV and analyzed virus-specific CD8$^+$T cells for CD25 expression at days 7-9 post infection. Consistent with the increased responsiveness to IL-2, approximately twice as many LCMV-specific GP33$^+$ and NP396$^+$ CD8$^+$T cells from *Prdm1$^{-/-}$* mice expressed CD25 when compared to WT controls (Figure IV-9). As shown in Figure IV-9, compared to WT controls the *Prdm1$^{-/-}$* T cells continued to have proportionally higher CD25 expression until day 9 post infection, although the overall proportion of CD25$^+$CD8$^+$T cells diminished dramatically after day 7. Since the conditional Blimp-1-deficient mice used in this study lacked Blimp-1 in both CD4$^+$ and CD8$^+$T cells, I wanted to confirm that dysregulated CD25
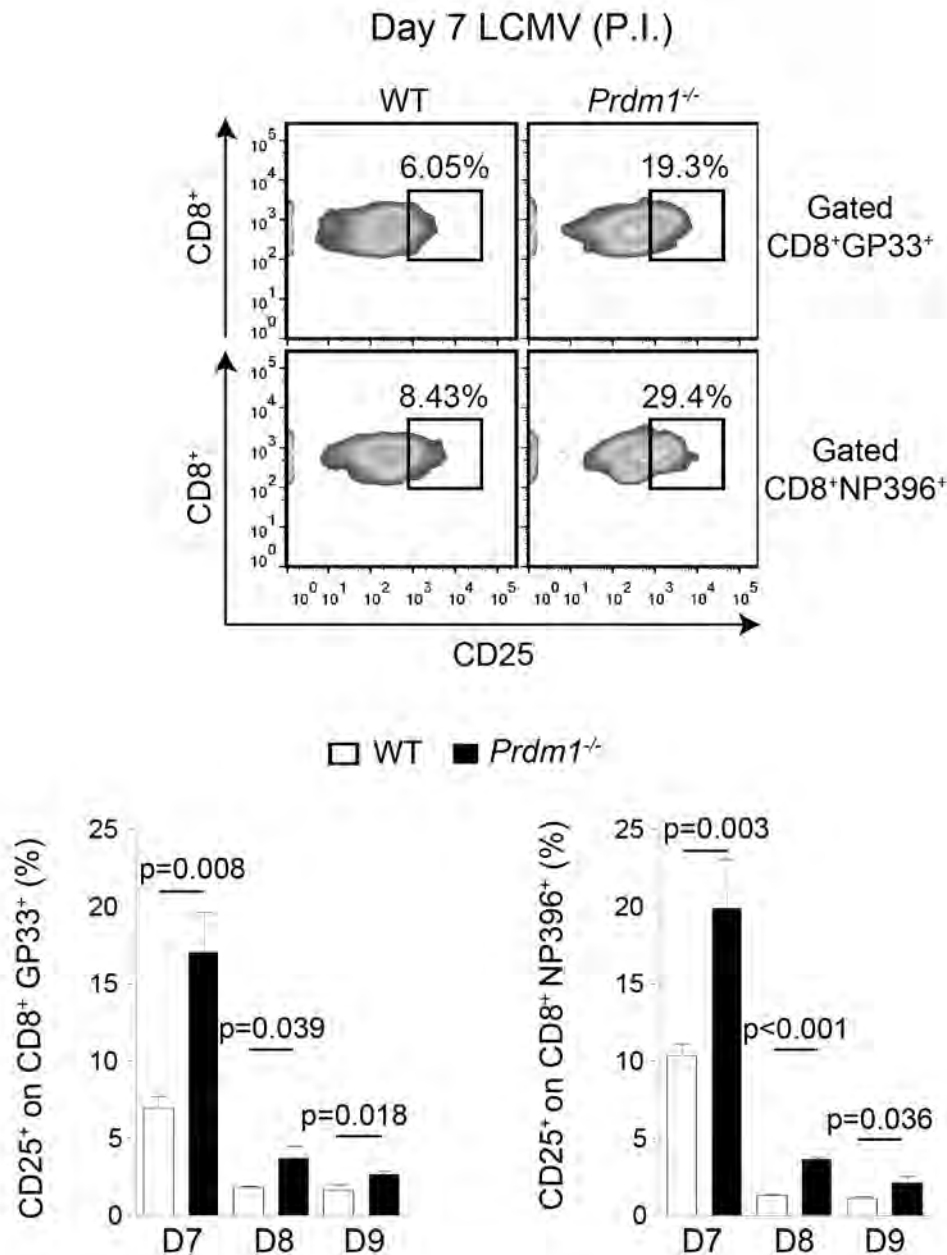
FIGURE IV-9. Increased CD25 expression on Blimp-1-deficient CD8⁺T cells during anti-viral immune response. Splenic CD8⁺T cells from WT and *Prdm1⁻/⁻* mice were isolated at days 7–9 after LCMV infection and stained with CD25 antibody plus LCMV-specific GP33 or NP396 tetramers. CD25 expression on tetramer-positive CD8⁺T cells at day 7 is shown (top) along with a compilation of CD25 expression kinetics from three independent experiments (bottom). All error bars represent the SEM.

expression due to Blimp-1 deficiency was CD8^{+}T cell intrinsic and not due to the presence of Blimp-1-deficient CD4^{+}T cells in the environment. To address this question I adoptively transferred P14 TCR transgenic WT or *Prdm1*[flox/flox] *x GzmB-cre*^{+} CD8^{+}T cells (in which Cre expression was under the control of the human Granzyme B promoter (94), therefore activated CD8^{+}T cells will become Blimp-1 deficient) into a WT host which were then infected with LCMV. I found that at day 9 post infection a higher proportion of virus-specific P14 *Prdm1*[flox/flox] *x GzmB-cre*^{+} CD8^{+}T cells expressed CD25 compared to WT controls (Figure IV-10), supporting the idea that this function of Blimp-1 was intrinsic to CD8^{+}T cells. To further confirm the inverse correlation between Blimp-1 and CD25 expression, I infected Blimp-1 GFP mice in which GFP expression is under the control of *Prdm1* promoter (187), thereby allowing us to track Blimp-1-expressing cells. As shown in Figure IV-11, a higher proportion of CD25-expressing CD8^{+}T cells were present in the Blimp-1^{lo} subset as compared to Blimp-1^{hi} subset at days 7 and 9 post infection. Therefore, CD25 expression inversely correlated with Blimp-1 expression. These data together indicate that Blimp-1 represses CD25 expression at late stages during the anti-viral CD8^{+}T cell response.

Recent studies have demonstrated that CD25 expression during the early expansion phase of the antiviral CD8^{+}T cell response is critical in regulating cell fate in that CD25^{hi} cells become short-lived effector cells (80, 91). To confirm these findings I compared expression of memory markers IL-7R and CD62L on CD25^{hi}- and CD25^{lo}-CD44^{hi} CD8^{+}T cells at day 5 post-LCMV infection.

Day 9 LCMV (P.I.)

Gated: P14⁺CD8⁺ T
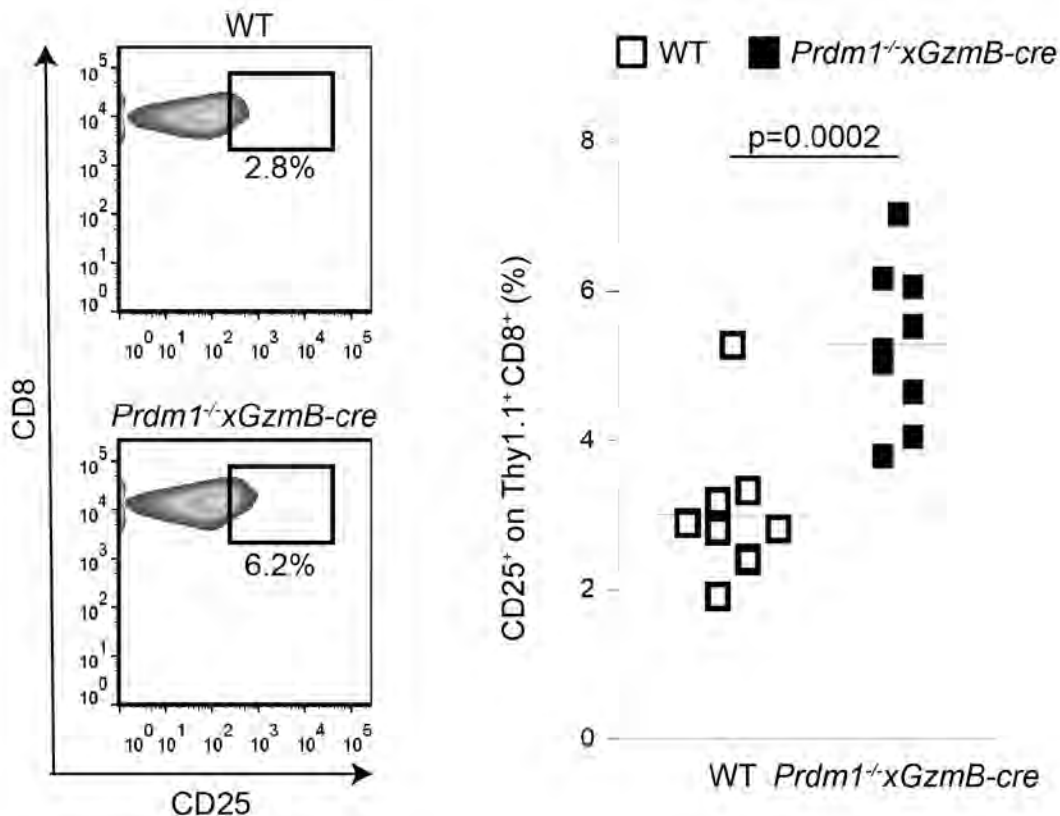
FIGURE IV-10. Increased CD25 expression is due to an intrinsic defect of Blimp-1 in CD8⁺T cells. 2 x10^5 P14 TCR transgenic CD90.1⁺ *Prdm1^{flox/flox}xGzmB-cre⁺* (*Prdm1^{-/-}*) or CD90.1⁺ *Prdm1^{flox/flox}xGzmB-cre⁻* (WT) littermate splenocytes were adoptively transferred into CD90.2⁺ congenic WT mice, and infected with LCMV. At day 9 post infection, CD90.1⁺CD8⁺T cells were analyzed for CD25 expression (left panels). On the right, the percentages of CD25⁺CD90.1⁺CD8⁺T cells from two independent experiments each with four to five mice are shown.
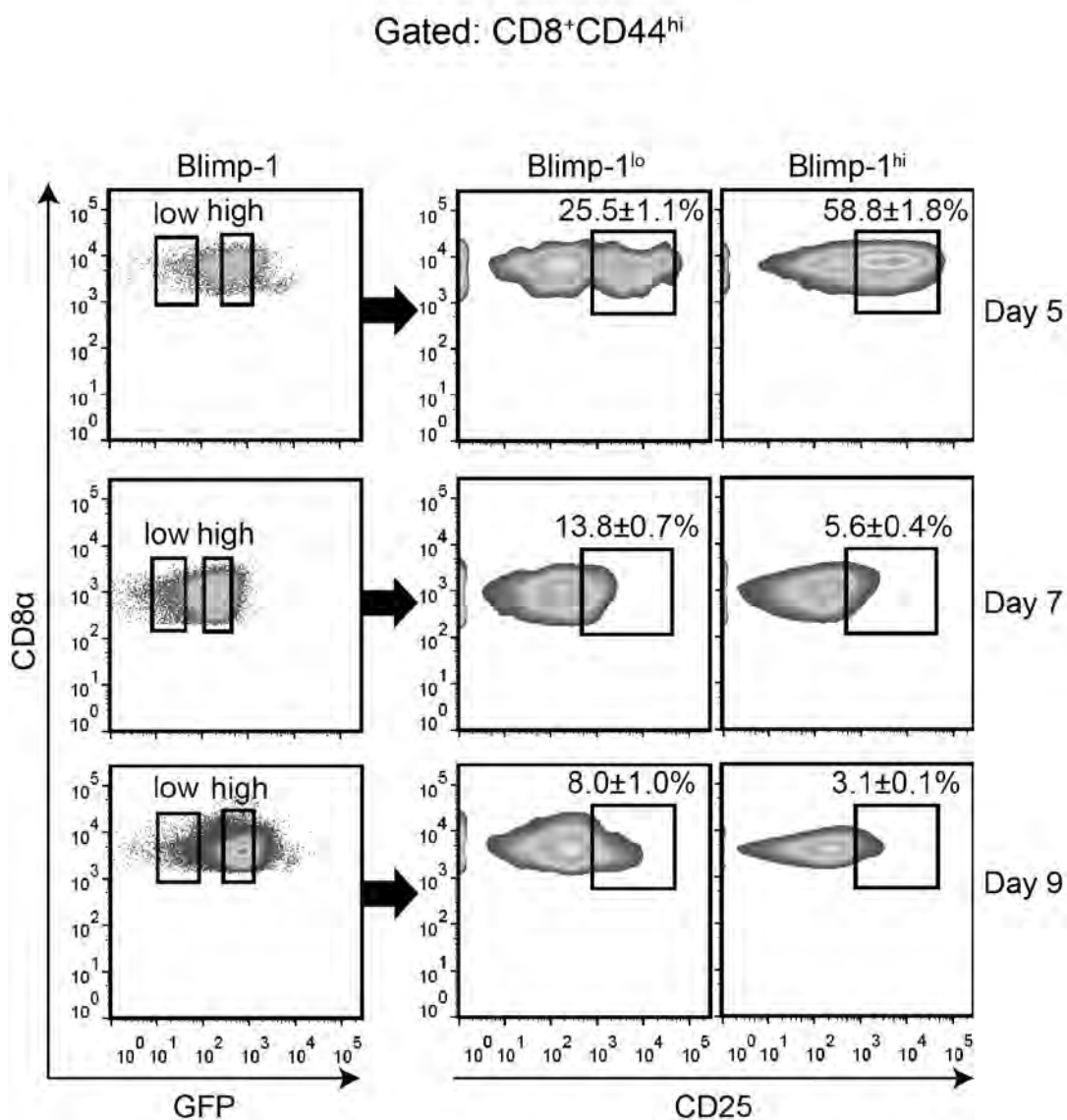
FIGURE IV-11. Inverse correlation between Blimp-1 and CD25 expression during the peak and resolution of the immune response to LCMV. Blimp-1-GFP reporter mice were infected with LCMV, and splenic CD44^{hi}CD8^{+}T cells were analyzed at days 5, 7, and 9 post infection for GFP expression (left panels). On the right, Blimp-1^{lo} and Blimp-1^{hi} populations were analyzed for CD25 expression. The percentages ± SEM of CD25^{+} cells are indicated on each plot. Data are representative of two independent experiments.

Consistent with previous findings, I also observed that high CD25 expression at day 5 post-infection correlated with decreased IL-7Rα and CD62L expression (Figure IV-12); further, at this stage of the response, cells expressing higher Blimp-1 also expressed more CD25 (Figure IV-11) [80]. These data indicate that strong IL-2R signaling during the early stage of the antiviral CD8$^+$T cell response promotes the development of short-lived effector cells. However, I considered whether the role of IL-2R signaling might be different at the later stage of the response, at which time CD8$^+$T cells undergo attrition. In support of this possibility, I found that at day 9 post infection, CD25 expression on CD8$^+$ T cells positively correlated with IL-7Rα and CD62L expression (Figure IV-12), indicating that, compared to the CD25^{lo} population, the CD25^{hi} population at this stage was enriched with memory precursor cells.

Although during resolution of the immune response CD25^{hi} CD8$^+$T cells were enriched with memory precursor cells, I wanted to directly test whether differences in CD25 expression on virus-specific CD8$^+$T cells are associated with differences in T cell survival and/or persistence. To address this question splenocytes from P14 TCR transgenic mice were adoptively transferred into a congenic host which was subsequently infected with LCMV. CD25^{hi} and CD25^{lo} P14 TCR transgenic CD8$^+$T cells were then sorted from mice at day 7 after LCMV infection, as shown in the schematic in Figure IV-13. Sorted T cells were adoptively transferred into infection-matched congenic hosts, and the number of P14$^+$ T cells was assessed at day 15 post infection (8 days post transfer). As
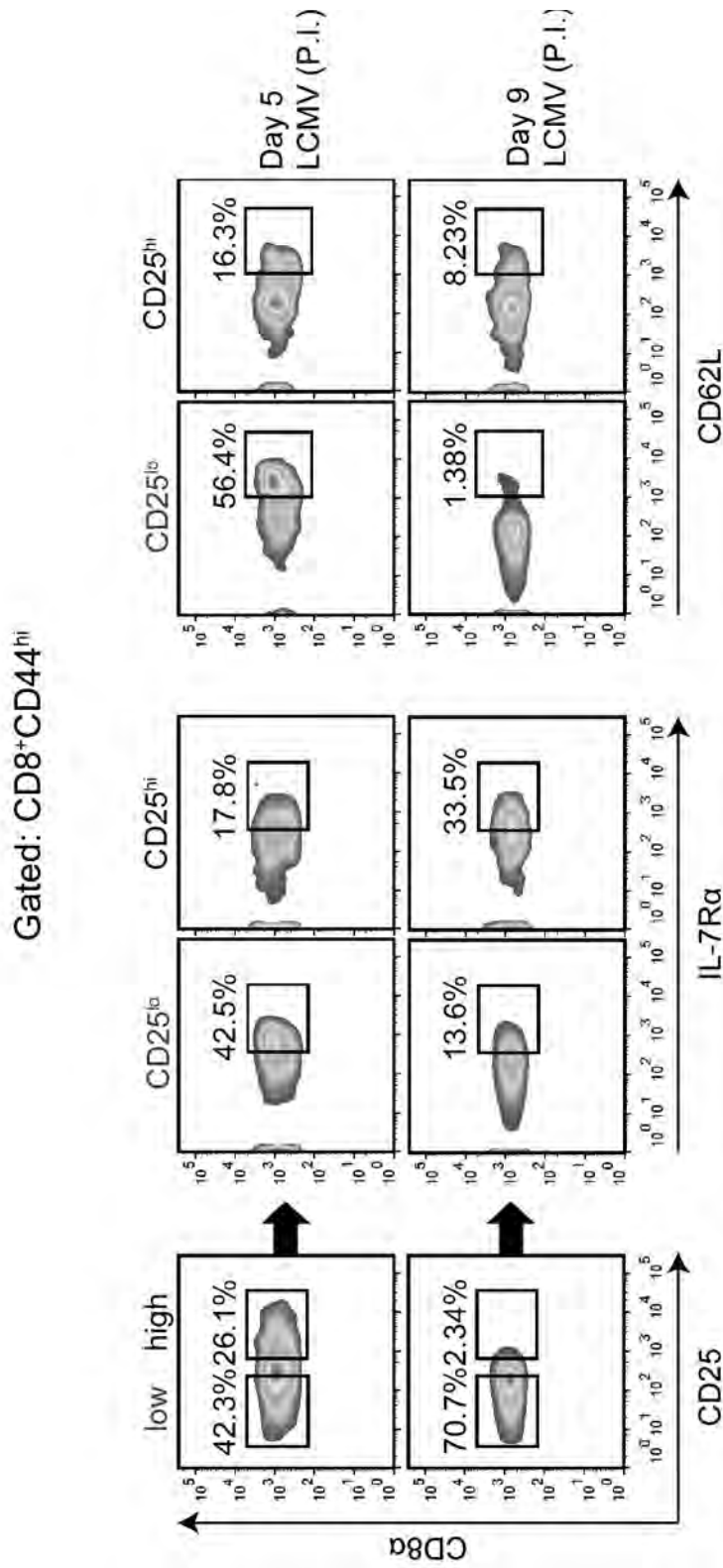
FIGURE IV-12. CD25 expression on CD8+T cells directly correlates with a memory precursor phenotype during resolution of the immune response. WT mice were infected with LCMV, and splenic CD44hiCD8+T cells were analyzed on days 5 and 9 post infection for CD25 expression (left panels). On the right, CD25lo and CD25hi populations were analyzed for IL-7Rα and CD62L expression. The percentages of positive cells are indicated on each plot. Data are representative of four independent experiments.
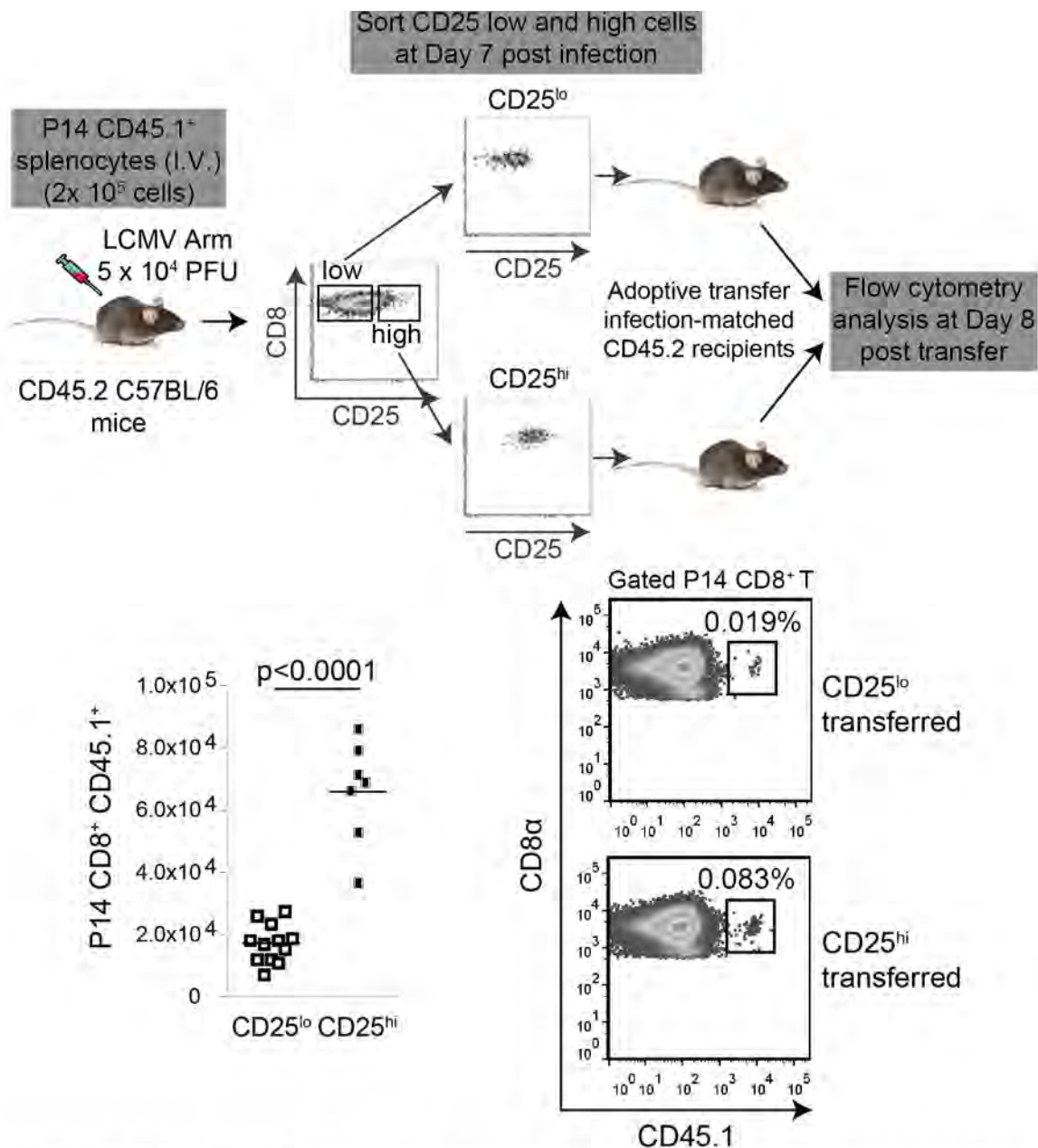
FIGURE IV-13. CD25 expression during resolution of the immune response correlates with enhanced CD8+T cell survival. P14 TCR transgenic CD45.1+ splenocytes were adoptively transferred into CD45.2+ congenic WT mice, and infected with LCMV. At day 7 post infection, 1×10^6 CD25^{lo} P14+CD8+ or CD25^{hi} P14+CD8+ T cells were isolated by cell sorting and adoptively transferred into a infection-matched congenic recipient mice. Eight days later, absolute number and proportion of transferred P14+ cells was assessed. Data shown include a compilation of two independent experiments and representative flow cytometry analysis.

shown in Figure IV-13, I found that a higher proportion and absolute number of virus-specific CD25^{hi} CD8$^+$ T cells than of CD25^{lo} transferred cells survived in the infection-matched host. These results indicate that, at day 7 post-infection, CD25 expression on virus-specific CD8$^+$T cells correlated with their enhanced survival and/or persistence. To determine whether enhanced proliferation contributed to this effect, I injected LCMV-infected WT and *Prdm1$^{-/-}$* mice with BrdU at day 7 post-infection and analyzed LCMV-specific GP33$^+$ CD8$^+$T cells 12 hour later for BrdU incorporation, which is indicative of proliferation. A higher proportion of CD25^{hi} T cells than of CD25^{lo} cells exhibited BrdU incorporation in the WT and *Prdm1$^{-/-}$* mice (Figure IV-14). Therefore, a higher proportion of CD25 expressing cells are present in the Blimp-1-deficient mice, which I show here are more proliferative during resolution of the immune response. Though overall as shown in Figure IV-5, Blimp-1-deficient CD8$^+$T cells were more proliferative when compared to WT controls irrespective of their CD25 expression. The ability of virus-specific CD8$^+$T cells to produce multiple cytokines, such as IFNγ, TNF and IL-2, simultaneously during viral infection is a primary indicator of their functional capacity. I questioned whether CD25^{hi} cells represented a functional memory pool compared to CD25^{lo} populations. As shown in Figure IV-15, I found that in response to *ex-vivo* LCMV peptide stimulation at day 8 post infection, a higher proportion of CD25^{hi} CD8$^+$T cells than of CD25^{lo} CD8$^+$T cells produced IFNγ and IL-2, correlating high expression of CD25 with a functional memory T cell response. Overall these data indicate that Blimp-1 represses CD25 expression at
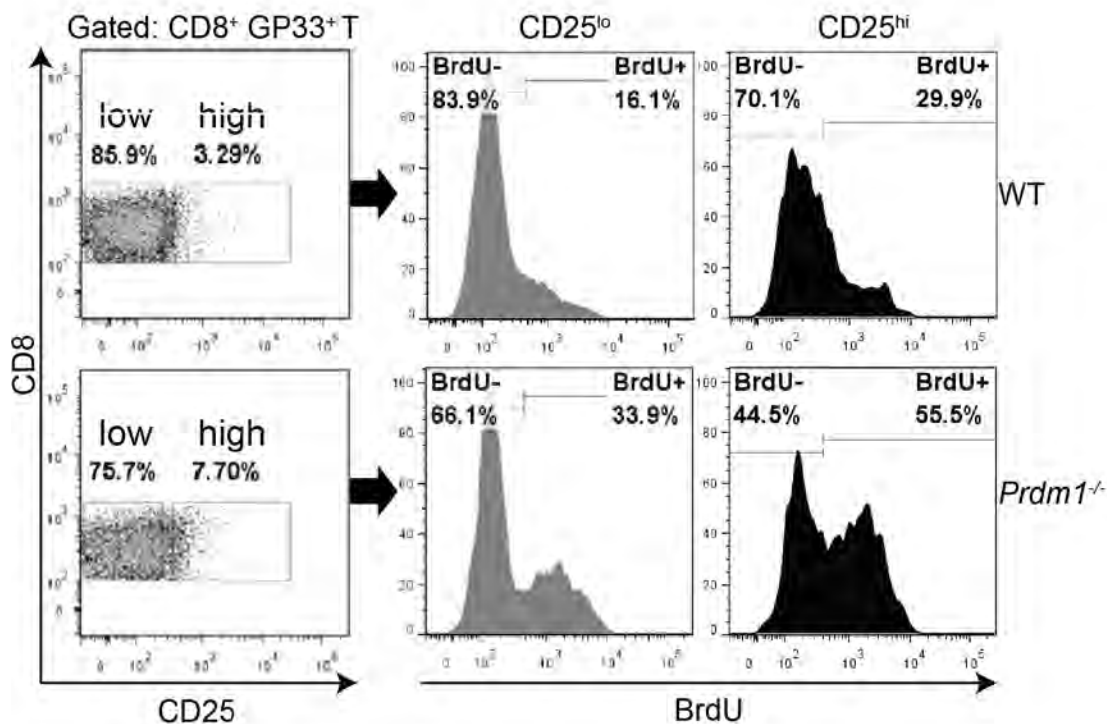
FIGURE IV-14. Enhanced proliferation of CD25^{hi} CD8$^+$T cells. WT and *Prdm1$^{-/-}$* mice were infected with LCMV and then injected with BrdU for 12 hours at day 7 post infection. Splenic GP33-tetramer-positive CD8$^+$T cells were analyzed for BrdU incorporation. Dot-plots show CD25 expression on virus-specific CD8$^+$ T cells (left); histograms show BrdU staining on CD25^{lo} versus CD25^{hi} subsets (right). Numbers indicate the percentage of cells in each subpopulation. Data are representative of two independent experiments with at least 3 mice in each experiment.

Gated: CD8⁺ CD44ʰⁱ
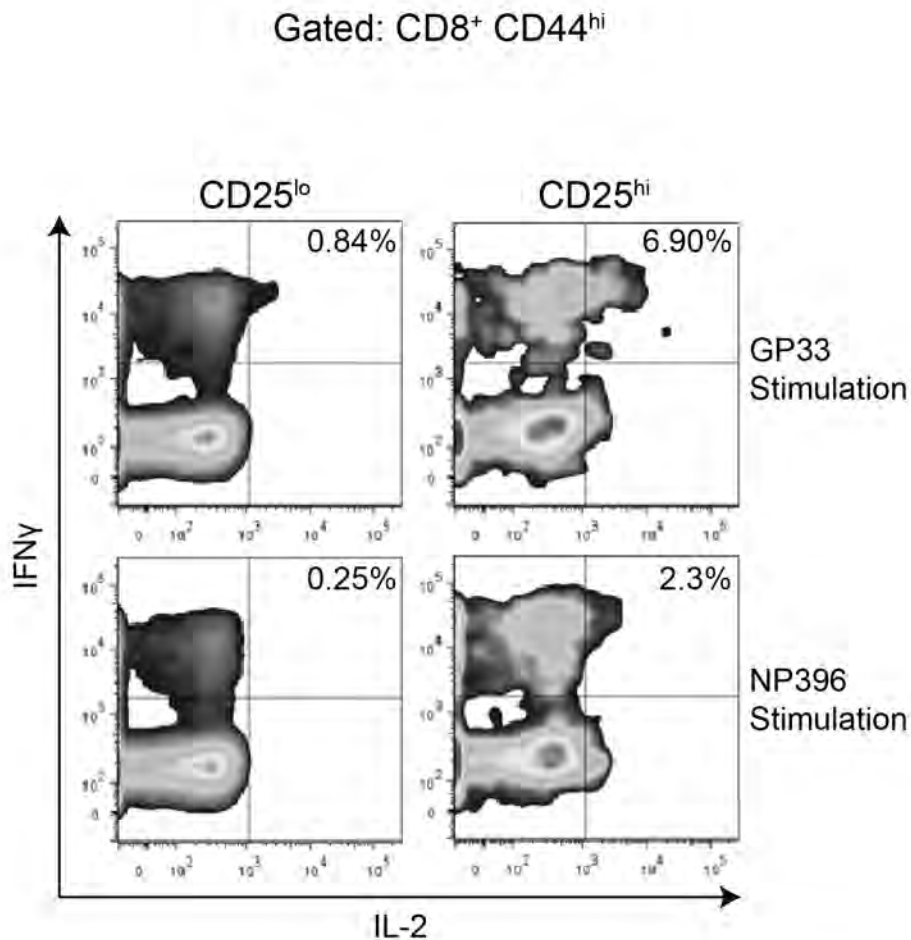
FIGURE IV-15. Enhanced IL-2 production by CD25ʰⁱ CD8⁺T cells. Splenocytes from LCMV-infected WT mice were stimulated with GP33 or NP396 peptides for 5 hours *in vitro*, followed by intracellular cytokine staining. Dot-plots show IL-2 versus IFNγ staining on CD25ˡᵒ versus CD25ʰⁱ subsets of CD44ʰⁱCD8⁺T cells at day 8 post infection. Numbers indicate the percentage of double cytokine producing cells. Plots are representative of three independent experiments with at least 3 mice in each experiment.

late stages during antiviral CD8^{+}T cell response, and following the peak of the CD8^{+}T cell response to LCMV, higher expression of CD25 correlates with enhanced survival and/or persistence of virus-specific T cells.

E. Blimp-1 regulates CD27 expression in virus-specific CD8^{+}T cells during anti-viral immune response

Apart from cytokine receptors, co-stimulatory molecules are known to be critical for the clonal expansion and survival of CD8^{+}T cells (67). CD27, a co-stimulatory molecule of the TNF receptor family promotes memory generation and survival of CD8^{+}T cells (67). Genome-wide ChIP-sequencing analysis performed by Dr. HyunMu Shin identified CD27 as a potential target of Blimp-1. Therefore, to test whether CD27 expression was dysregulated in CD8^{+}T cells lacking Blimp-1, I infected *Prdm1^{-/-}* and WT mice with LCMV and analyzed virus-specific CD8^{+}T cells for CD27 expression at days 7.5-9 post infection. As shown in Figure IV-16, at day 7.5 post-LCMV infection a higher proportion of LCMV-specific GP33^{+} and NP396^{+} CD8^{+}T cells from *Prdm1^{-/-}* mice expressed CD27 when compared to WT controls, and the differences became more apparent by days 8 and 9 post infection. Since the conditional Blimp-1-deficient mice used in the study lacked Blimp-1 in both CD4^{+} and CD8^{+}T cells, I wanted to confirm that dysregulated CD27 expression due to Blimp-1 deficiency was CD8^{+}T cell intrinsic and not due to the presence of Blimp-1-deficient CD4^{+}T cells in the environment. To address this question I adoptively transferred P14 TCR transgenic WT or *Prdm1[flox/flox] x GzmB-cre^{+}* CD8^{+}T cells into a WT host which were then infected
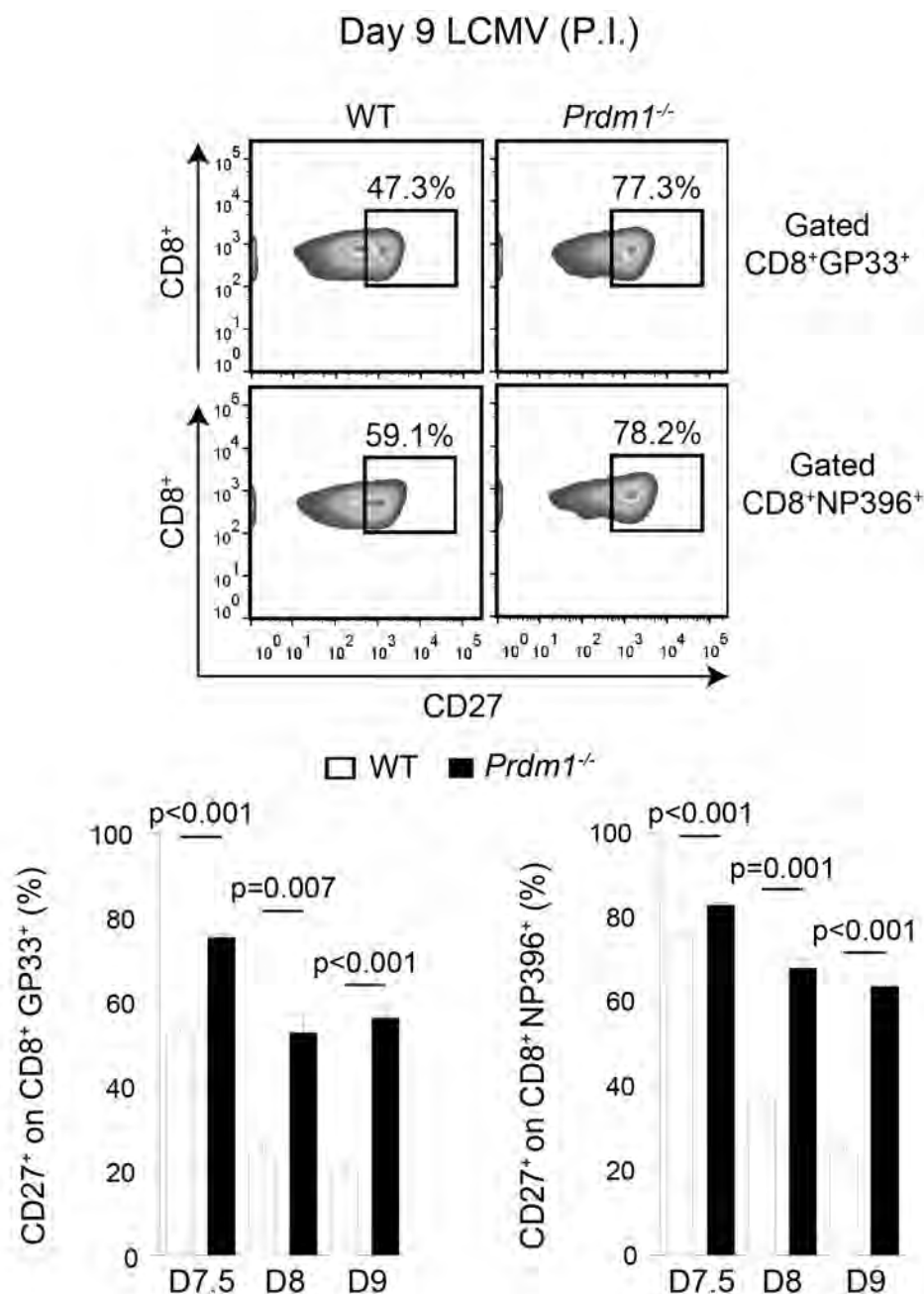
FIGURE IV-16. Increased CD27 expression on Blimp-1-deficient CD8⁺T cells during the anti-viral immune response. Splenic CD8⁺T cells from WT and *Prdm1⁻/⁻* mice were isolated at days 7.5–9 after LCMV infection and stained with CD27 antibody plus LCMV-specific GP33 or NP396 tetramers. CD27 expression on tetramer-positive CD8⁺T cells at day 9 is shown (top) along with a compilation of CD27 expression kinetics from three independent experiments (bottom). All error bars represent the SEM.

with LCMV. I found that at day 9 post-infection a higher proportion of virus-specific P14 *Prdm1*$^{flox/flox}$ *x GzmB-cre*$^+$ CD8$^+$T cells expressed CD27 compared to WT controls (Figure IV-17), supporting the idea that this function of Blimp-1 was intrinsic to CD8$^+$T cells. To further confirm the inverse correlation between Blimp-1 and CD27 expression, I infected Blimp-1 GFP mice in which GFP expression is under the control of the *Prdm1* promoter (187), thereby allowing us to track Blimp-1-expressing cells. As shown in Figure IV-18, a higher proportion of CD27-expressing CD8$^+$T cells were present in the Blimp-1^{lo} subset as compared to Blimp-1^{hi} subset at day 9 post infection. Furthermore, CD27 expression on CD8$^+$T cells was higher in the Blimp-1^{lo} subset as compared to Blimp-1^{hi} subset as represented by the MFI in Figure IV-18. Therefore, CD27 expression inversely correlated with Blimp-1 expression. These data together indicate that Blimp-1 represses CD27 expression during the peak and resolution of the anti-viral CD8$^+$T cell response.

It has been previously shown that CD27-expressing CD8$^+$T cells represent a functional memory pool (88). To confirm that CD27 expression on virus-specific CD8$^+$T cells at day 9 post infection represents a subset enriched with memory precursor cells, I examined IL-7Rα expression on LCMV-specific CD8$^+$T cells with varying CD27 expression. As shown in Figure IV-19, increasing amounts of IL-7Rα on LCMV-specific GP33$^+$CD8$^+$T cells correlated with increased CD27 expression. Although during resolution of the immune response CD27^{hi} CD8$^+$T cells were enriched with memory precursor cells, I wanted to directly test whether
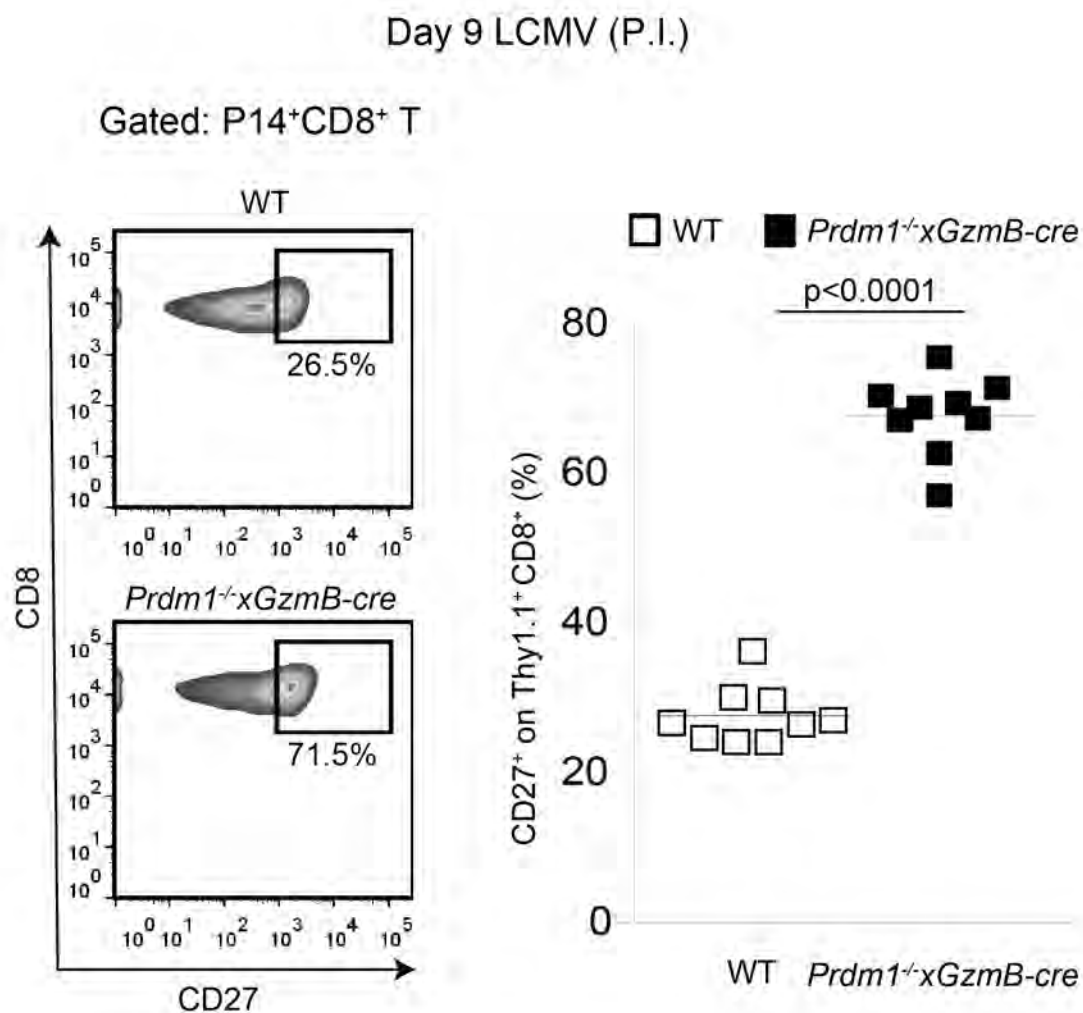
FIGURE IV-17. Increased CD27 expression is due to an intrinsic defect of Blimp-1 in CD8⁺T cells. 2×10^5 P14 TCR transgenic CD90.1⁺ *Prdm1^{flox/flox}xG-zmB-cre⁺* (*Prdm1^{-/-}*) or CD90.1⁺ *Prdm1^{flox/flox}xGzmB-cre⁻* (WT) littermate splenocytes were adoptively transferred into CD90.2⁺ congenic WT mice, and infected with LCMV. At day 9 post infection, splenic CD90.1⁺CD8⁺T cells were analyzed for CD27 expression (left panels). On the right, the percentages of CD27⁺CD90.1⁺CD8⁺T cells from two independent experiments each with four to five mice are shown.

Day 9 LCMV (P.I.)

Gated: CD8+ CD44hi

Blimp-1

Blimp-1lo

Blimp-1hi

62.2%

MFI: 2246

49.6%

MFI: 1571
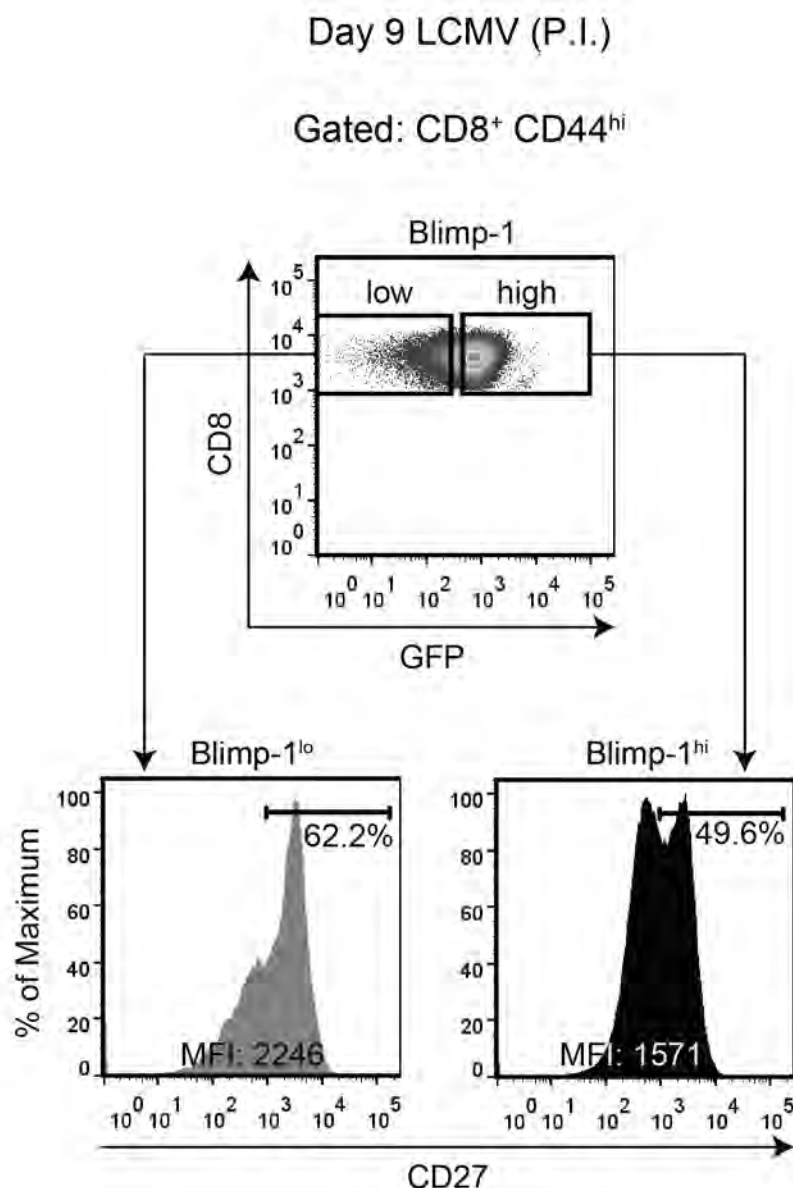
CD27

FIGURE IV-18. Inverse correlation between Blimp-1 and CD27 expression during resolution of the immune response to LCMV. Blimp-1-GFP reporter mice were infected with LCMV, and splenic CD44hiCD8+T cells were analyzed at day 9 post infection for GFP expression (top panel). On the bottom, Blimp-1lo and Blimp-1hi populations were analyzed for CD27 expression. The percentages of CD27-positive cells and the MFI of CD27 expression on Blimp-1lo and Blimp-1hi populations are indicated on each histogram. Data are representative of two independent experiments with at least 3 mice in each experiment.
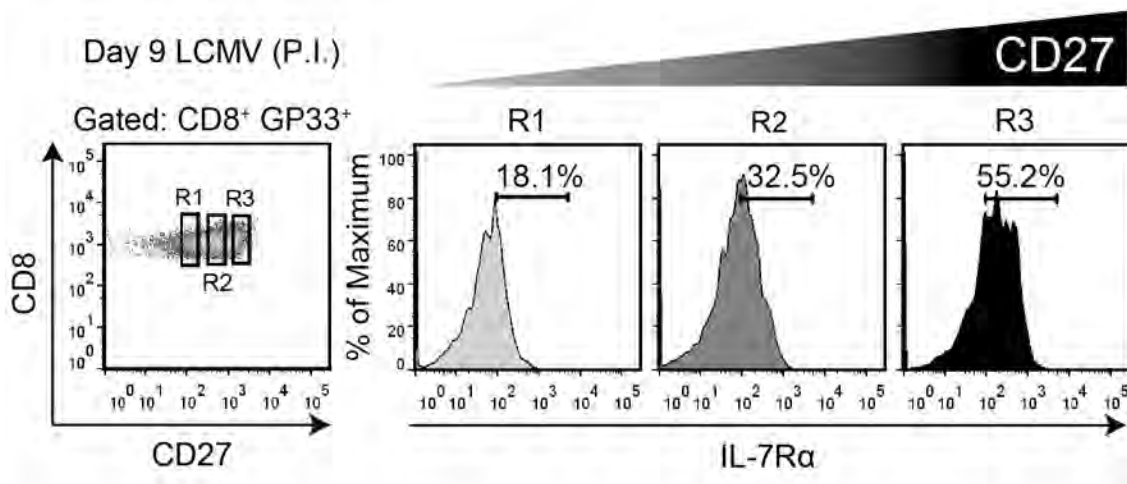
FIGURE IV-19. CD27 expression on CD8+T cells directly correlates with a memory precursor phenotype during resolution of the immune response. WT mice were infected with LCMV, and splenic GP33+CD8+T cells were analyzed on day 9 post infection for CD27 expression (left panel). On the right, CD27^{lo} (R1), CD27^{int} (R2), and CD27^{hi} (R3) populations were analyzed for IL-7Rα expression. The percentages of IL-7R-positive cells are indicated on each histogram. Data are representative of four independent experiments.

differences in CD27 expression on virus-specific CD8^{+}T cells are associated with differences in T cell survival and/or persistence. To address this question splenocytes from P14 TCR transgenic mice were adoptively transferred into a congenic host which was subsequently infected with LCMV. CD27^{hi} and CD27^{lo} P14 TCR transgenic CD8^{+}T cells were then sorted from mice at day 9 after LCMV infection, as shown in the schematic in Figure IV-20. Sorted T cells were adoptively transferred into infection-matched congenic hosts, and the number of P14^{+} T cells was assessed at day 16 post infection (7 days post transfer). As shown in Figure IV-20, I found that a higher proportion and absolute number of virus-specific CD27^{hi} CD8^{+}T cells than of CD27^{lo} transferred cells survived in the infection-matched host. These results indicate that, during resolution of the immune response, CD27 expression on virus-specific CD8^{+}T cells correlated with their enhanced survival and/or persistence.

F. Sustained CD25 and CD27 expression contributes to the increased magnitude of Blimp-1-deficient CD8^{+}T cell response

So far I've shown that CD25 and CD27 expression was enhanced in Blimp-1-deficient CD8^{+}T cells and that expression of CD25 and CD27 on CD8^{+}T cells during resolution of the immune response correlates with a memory phenotype. The next question addressed was whether persistent CD25 and CD27 expression contributed to the increased magnitude of the Blimp-1-deficient CD8^{+}T cell response. To address this question, we utilized small hairpin RNA (shRNA) silencing to diminish expression of CD25 and CD27 proteins in LCMV-
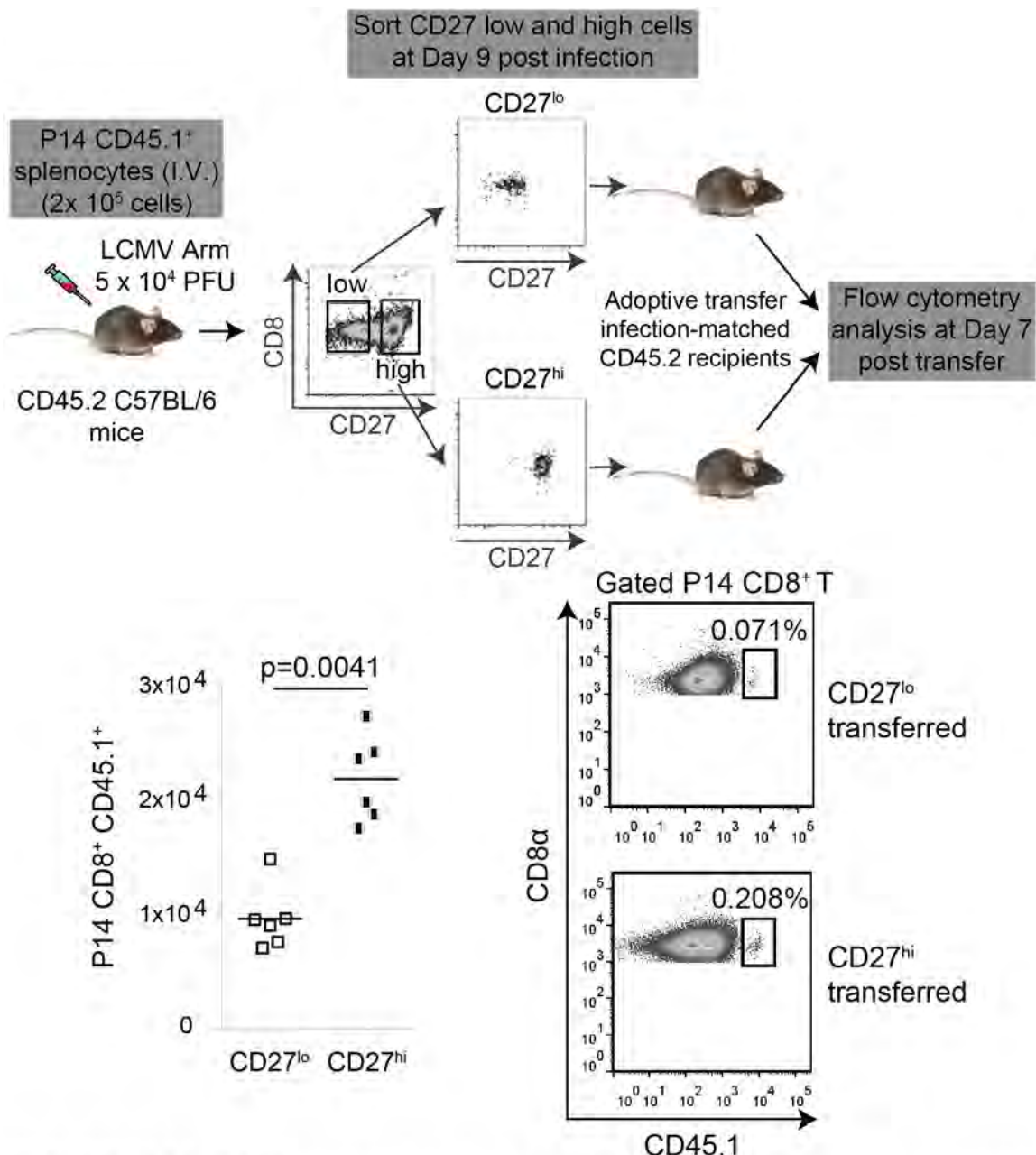
FIGURE IV-20. CD27 expression during resolution of the immune response correlates with enhanced CD8+T cell survival. P14 TCR transgenic CD45.1+ splenocytes were adoptively transferred into CD45.2+ congenic WT mice, and infected with LCMV. At day 9 post infection, 1×10^6 CD27lo P14+CD8+T or CD27hi P14+CD8+T cells were isolated by cell sorting and adoptively transferred into infection-matched congenic recipient mice. Eight days later, absolute number and proportion of transferred P14+ cells were assessed. Data shown include a compilation of two independent experiments and representative flow cytometry analysis.

specific Prdm1$^{-/-}$ T cells. The generation of shRNA RV (Retrovirus) constructs and transduction of *Prdm1*$^{-/-}$ P14^{+} T cells was performed by Dr. HyunMu Shin in the lab of Dr. Leslie J. Berg. For these experiments, P14^{+}CD90.1^{+} *Prdm1*$^{flox/flox}$ x *GzmB-cre*$^{+}$ splenocytes were adoptively transferred into a congenic WT host and were activated *in vivo* by infection of recipient mice with LCMV. At day 5 post infection, P14^{+} T cells were isolated by cell sorting, stimulated *in vitro* with CD3 and CD28 antibodies for 24 hours, infected with RV carrying shRNA to *IL-2Rα* and *CD27*, and then transferred into LCMV-infected (day 6 post-infection) recipient mice (Figure IV-21). As shown by GFP expression (RV vectors also expressed GFP, allowing us to track transduced T cells), we achieved similar transduction efficiencies for each of the RV constructs (Figure IV-21); in addition, each shRNA was able to reduce expression of the targeted gene (Figure IV-22). When analyzed at day 9 post infection, the proportion of *Prdm1*$^{-/-}$ CD8^{+}T cells transduced with shCD25 or shCD27 was lower than that of cells transduced with the scrambled shRNA control (Figures IV-22). Moreover, silencing of both *Il2rα* and *Cd27* further decreased the frequency of GFP^{+}CD8^{+}T cells (Figure IV-22), suggesting a non-redundant role of CD25 and CD27 in sustaining CD8^{+}T cell responses. Overall, these results indicate that the increased magnitude and survival of CD8^{+}T cells in *Prdm1*$^{-/-}$ mice was in part due to sustained expression of CD25 and CD27.
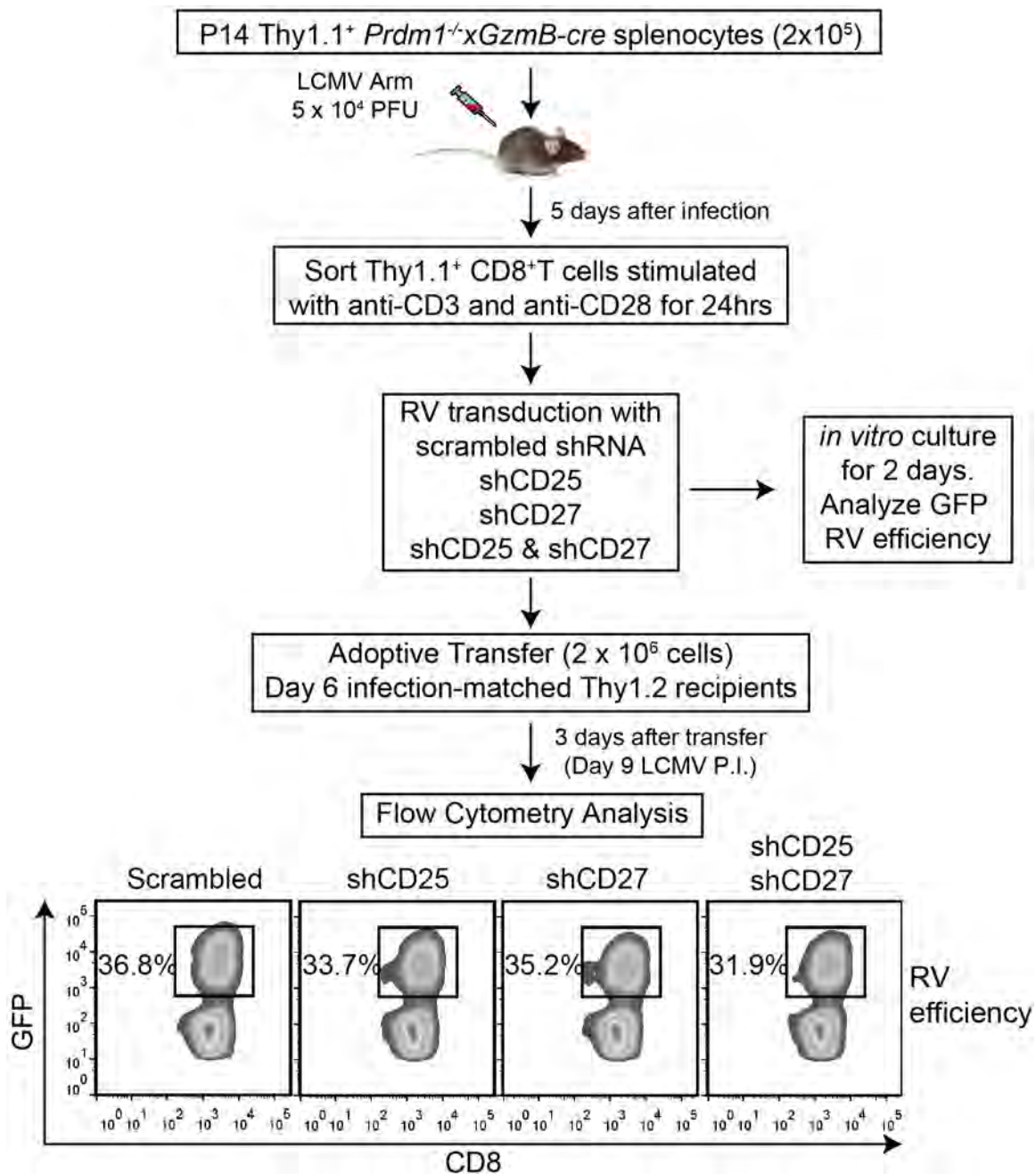
FIGURE IV-21. Schematic of protocol used for shRNA mediated silencing of CD25 and CD27 expression. P14 TCR transgenic Blimp-1-deficient T cells were transduced with retroviruses expressing shRNAs to silence *Il2ra* (shCD25), *Cd27* (shCD27), shCD25 and shCD27 or scrambled negative control as shown in the schematic (top). A subset of transduced CD90.1⁺CD8⁺T cells was cultured *in vitro* for 2 days, and the transduction efficiency was assessed by GFP fluorescence (bottom). This figure was generated by Dr. HyunMu Shin.
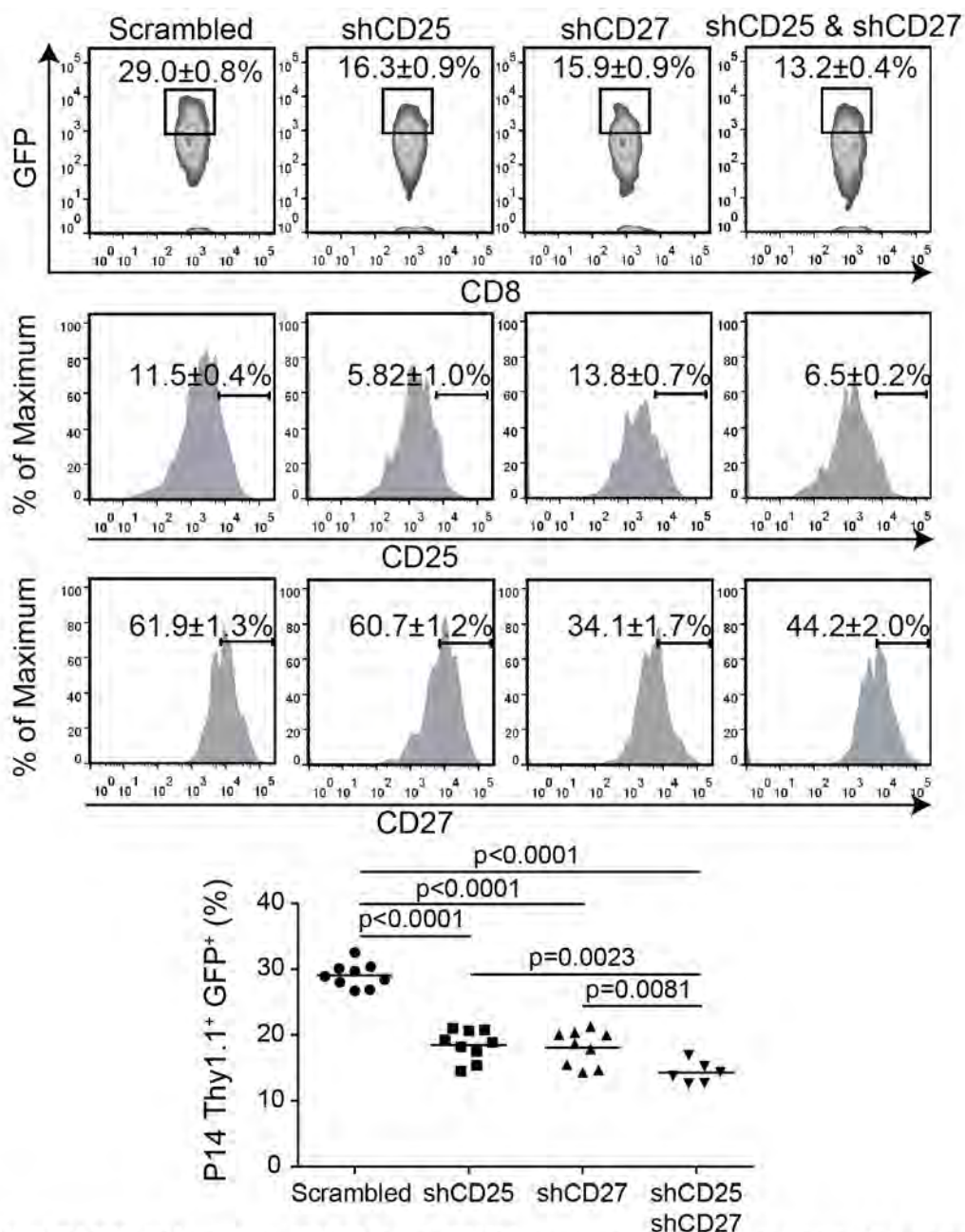
FIGURE IV-22. Silencing CD25 and CD27 expression decreases the magnitude of the Blimp-1-deficient T cell response. As shown in the schematic in Figure IV-21, three days after transfer, the percentages ± SEM of GFP+ CD90.1+CD8+T cells from spleen were determined; dot plots show a representative experiment (top), and a compilation of data from three independent experiments is shown as a scatter plot (bottom). A representative example of CD25 and CD27 expression on GFP+CD90.1+CD8+T cells, along with the percentages ± SEM from three compiled independent experiments are shown (middle). This figure was generated by Dr. Hyun Mu Shin

G. Blimp-1 directly represses *Il2rα* and *Cd27* expression by recruiting histone-modifying enzymes to induce repressive chromatin modifications

The following studies were performed by Dr. HyunMu Shin in the lab of Dr. Leslie J. Berg and are a part of the manuscript titled "Epigenetic Modifications Induced by Blimp-1 Regulate CD8^{+}T Cell Memory Progression during Acute Virus Infection" (Immunity 39, 1-15, October 17, 2013).

Transcriptional regulation by Blimp-1 has been extensively studied in B cells, leading to the identification of *Myc*, *Pax5*, *Bcl6*, *Ciita*, *Spib*, *Id3*, and *Prdm1* as direct Blimp-1 target genes (90). Substantially less is known about the direct targets of Blimp-1 in T cells, particularly CD8^{+}T cells. To identify Blimp-1 target genes in CD8^{+}T cells, Dr. HyunMu Shin performed genome-wide ChIP-seq analysis. Naïve CD8^{+}T cells from OT-I TCR transgenic Rag1^{-/-} mice were stimulated for 3 days *in vitro* in the presence of IL-2 to upregulate Blimp-1 (93). For identifying the genomic locations of Blimp-1 binding sites, chromatin was immunoprecipitated with Blimp-1 antibody and DNA was subjected to deep sequencing. Consistent with previous studies (94, 97), this analysis identified *Prdm1*,*Id3*, *Bcl6*, *Pax5*, *Myc*, and *Ciita* as having Blimp-1 binding sites in CD8^{+}T cells. In addition, the study identified *Il2rα*, *Cd27*, *Il2rb*, *Sell* (Cd62l), *Eomes*, and *Ccr6* as Blimp-1 targets. To further confirm the results of ChIP-seq analysis day 7 LCMV-infected activated CD8^{+}T cells were subjected to ChIP by Blimp-1 antibody. He found that Blimp-1 bound to the *Il2rα* and *Cd27* loci in WT CD8^{+}T cells further supporting the conclusion that Blimp-1 binding to the *Il2rα* and *Cd27*

loci represses expression of CD25 and CD27, respectively, during the anti-viral CD8^{+}T cell response.

In previously characterized systems, Blimp-1 has been shown to mediate transcriptional repression by associating with histone-modifying enzymes (188-191). Because there is differential histone modification between memory and effector CD8^{+} T cell subsets (192), we considered whether Blimp-1 might function in T cells to regulate histone lysine modification. To examine whether Blimp-1 mediates the recruitment of histone-modifying enzymes G9a (methyltransferase) or HDAC2 (histone deacetylase 2) to Blimp-1 binding sites, Dr. HyunMu Shin isolated CD8^{+}T cells from WT or *Prdm1^{-/-}* mice at day 7 after LCMV infection. ChIP assays revealed that both G9a and HDAC2 were present at the *Il2rα* and *Cd27* loci in WT CD8^{+}T cells at day 7 post-infection and we did not detect any binding of G9a and HDAC2 in *Prdm1^{-/-}* CD8^{+}T cells, indicating that Blimp-1 is required for the recruitment of G9a and HDAC2 to these genes. Furthermore, Dr. HyunMu Shin could show that Blimp-1 induced a repressive chromatin state at *Il2rα* and *Cd27* loci in CD8^{+}T cells at the peak of the immune response to LCMV. Together, these findings demonstrate that Blimp-1 suppresses CD25 and CD27 expression by inducing a repressive chromatin state at *Il2rα* and *Cd27* loci in CD8^{+}T cells at the peak of the response to LCMV infection.

H. Discussion to Chapter IV

Blimp-1-deficient CD8^{+}T cells have an enhanced capacity to differentiate into memory cells (94, 95). In this study, which was done in collaboration with Dr. HyunMu Shin and Dr. Leslie J. Berg, we have identified an important mechanism by which Blimp-1 association with histone-modifying enzymes suppresses *Il2rα* and *Cd27* expression to regulate the overall magnitude of the virus-specific memory CD8^{+}T cell response. Upon virus infection and T cell activation, co-stimulatory signals, along with the inflammatory cytokine milieu, are key components leading to the generation of CD8^{+} effector and memory precursor T cells that together promote virus clearance and provide long-term protection against reinfection. After virus clearance, CD8^{+}T cells down-regulate co-stimulatory receptor and cytokine receptor expression (67, 80). After this process, the vast majority of the virus-specific effector population undergoes apoptosis (50), most likely as a result of cytokine deprivation and the lack of co-stimulatory signals. Our data indicate that CD25 (or CD27) has an impact on the small proportion of effector cells capable of long-term survival and provides an important signal to promote the survival of cells expressing the receptor in this specific subset. We propose that Blimp-1 normally functions to down-regulate cytokine receptor expression and thereby promotes the death of effector T cells. This mechanism would function in addition to the regulation of proliferation and survival genes by Blimp-1, as proposed previously (193).

Over the past decade, numerous studies have investigated the role of IL-2 signaling in CD8$^+$T cell activation and differentiation in response to virus infections (75). These studies showed that CD25 expression and the strength of IL-2R signaling during T cell priming influences the relative generation of effector versus memory CD8$^+$T cells. Furthermore, *in vivo* administration of IL-2 during T cell contraction increases the size of the CD8$^+$ memory pool (76). These data prompted us to investigate whether IL-2 receptor expression is regulated by distinct mechanisms at different stages of the anti-viral immune response, and further, whether the function of IL-2R signaling might also vary between T cell priming and T cell contraction. CD25 expression on CD8$^+$T cells is rapidly induced during the early stage of viral infection, mediated by a combination of TCR and IL-2R signaling (75). During this early stage, cells expressing CD25 are destined to be short-lived effectors, in keeping with their high Blimp-1 expression (80, 94). Our data indicate that, at this time point, Blimp-1 expression is not able to override the positive signals promoting CD25 expression. In contrast, by day 7 post-infection, CD25 expression is nearly absent on virus-specific cells, a change that is Blimp-1 dependent. We propose that after viral clearance and the cessation of IL-2 production, CD25 expression becomes susceptible to regulation by Blimp-1. It has been shown previously that *Prdm1$^{-/-}$* mice consistently maintain higher proportions and numbers of memory CD8$^+$T cells (94). Although we have not directly looked at later time points after viral infection, the transcription factor EOMES was upregulated in *Prdm1$^{-/-}$* mice (Figure IV-3), consistent with our

ChIP-seq analysis. CD8$^+$T cells from *Prdm1$^{-/-}$* mice were also more responsive to IL-15 stimulation (Figure IV-8). Therefore, it is likely that the EOMES and IL-15 axis promotes the homeostatic proliferation and survival of the increased memory T cell pool in *Prdm1$^{-/-}$* mice.

In this study, we confirmed that CD25 and CD27 expression was significantly altered in *Prdm1$^{-/-}$* CD8$^+$T cells during the course of LCMV infection. Blimp-1 directly bound to the *Il2rα* locus, was required for recruitment of HDAC2 and G9a to this gene, and was required for the repressive chromatin modifications seen at the *Il2rα* locus in CD8$^+$T cells from day 7 LCMV-infected mice. Unlike that of *Il2rα*, the transcriptional regulation of *Cd27* is not well defined in T cells. Although present on naive T cells, TCR stimulation leads to further up-regulation of CD27 (194). The mechanisms leading to CD27 down-regulation are even more poorly characterized. Although ligation with CD70 or stimulation with phorbol myrsitate acetate (PMA) down-regulates CD27 expression (195, 196), the factors mediating this event are not known. Our work has provided evidence that Blimp-1 plays a key role in CD27 down-regulation following the peak of the anti-viral CD8$^+$T cell response. As with the *Il2rα* locus, Blimp-1 binds to *Cd27* and recruits the histone-modifying enzymes, HDAC2 and G9a, leading to repressive modifications at this locus during CD8$^+$T cell contraction.

In the study highlighted in this chapter, I show that CD25 and CD27 expression is decreased in *Prdm1$^{-/-}$* T cells responding to LCMV infection, that T

cells expressing CD25 and CD27 preferentially survive during acute viral infection, and that silencing of *Il2rα* and/or *Cd27* in Blimp-1-deficient CD8$^+$T cells decreases the magnitude of the virus-specific CD8$^+$T cell response. Our collaborating group identified *Il2rα* and *Cd27* as direct targets of Blimp-1 with the use of deep-sequencing analysis of Blimp-1-bound DNA targets in CD8$^+$T cells (ChIP-seq analysis). They could also show that at the peak of CD8$^+$T cell expansion, but not early in infection, Blimp-1 binds to regulatory regions of *Il2rα* and *Cd27* and recruits G9a or HDAC2 to promote repressive histone modifications at these loci. These data provide key insights into the mechanism by which Blimp-1 acts as an epigenetic regulator of target genes, thereby dictating the fate of CD8$^+$ effector T cells (Figure IV-23).
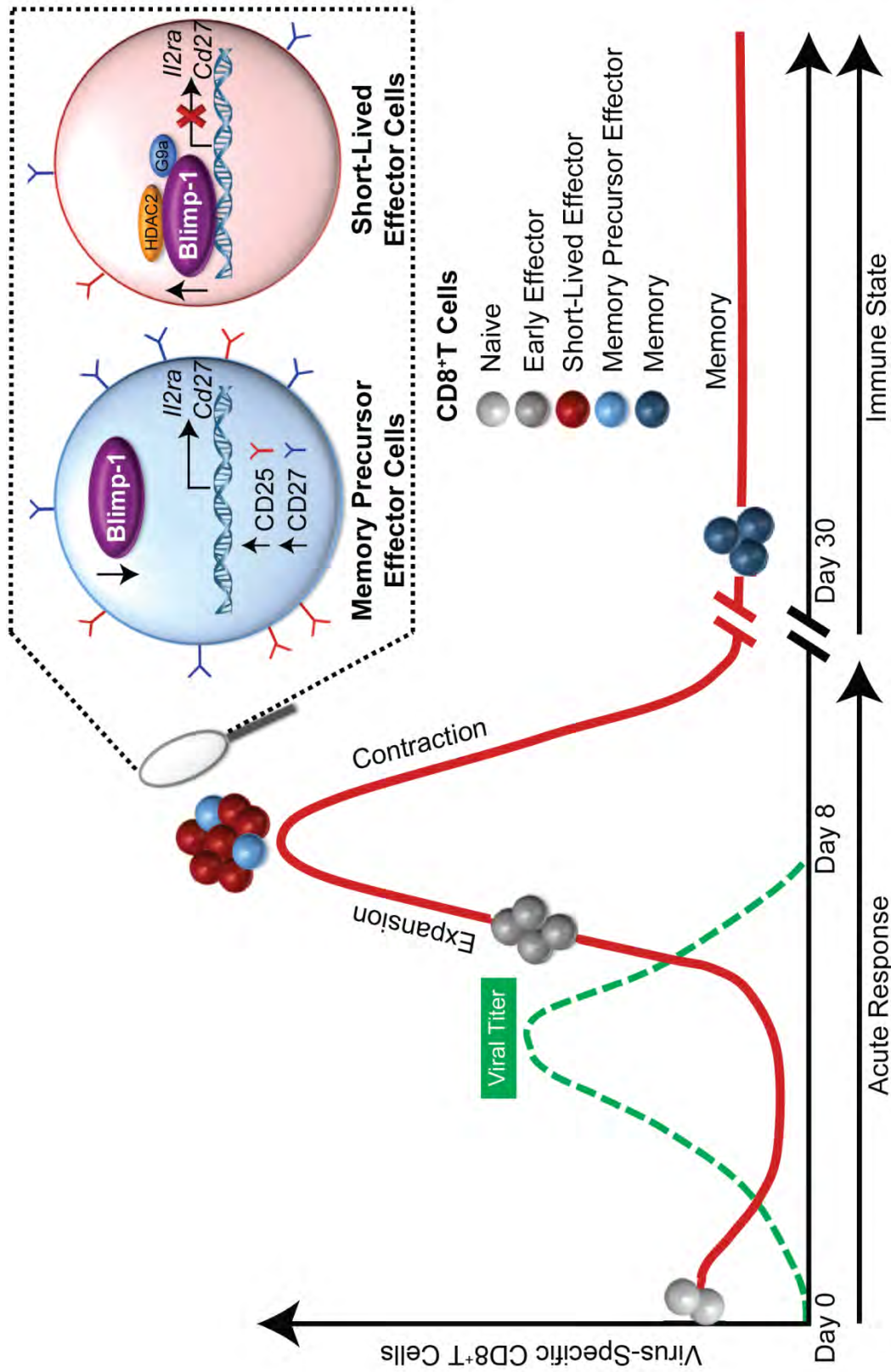
Figure IV-23. Blimp-1 suppresses CD25 and CD27 expression to negatively regulate CD8+T cell responses and memory generation.

CHAPTER V: THE TRANSCRIPTION FACTOR ROG/*Zbtb32* NEGATIVELY REGULATES THE CD8$^+$T CELL RESPONSE DURING ACUTE AND CHRONIC VIRAL INFECTION

Upon recognition of the viral antigen CD8$^+$T cells get activated and proliferate. Depending on the strength of the stimulus, cytokine milieu, and co-stimulatory factors, a transcriptional program is initiated, governing the fate of effector T cells. Transcriptional profiling of effector and memory CD8$^+$T cells in models of both acute and chronic virus infection has recently provided insight into the gene expression programs characterizing distinct cell subsets (89). ROG is a transcription factor which belongs to the POZ family of proteins (152). Most of the POZ family members are transcriptional repressors, and some of the well-known members of this family include PLZF (Promyelocytic leukemia Zn finger protein) and Bcl-6 (B cell lymphoma-6), and are known to play an important role in T cell function and differentiation.

ROG is expressed in activated T and B cells, and *in vitro* studies have suggested that ROG negatively regulates T cell activation (152, 154, 155) (156). ROG-deficient mice have been generated and were shown to have no defect in mounting a Th1 immune response in an EAE induction model (156). Our current understanding of the function of ROG in regulating *in vivo* T cell responses is extremely limited.

The studies highlighted in this chapter have been done in collaboration with Dr. HyunMu Shin in the lab of Dr. Leslie J. Berg at UMMS, and initial studies carried out in their lab suggested that ROG is an early IL-2-induced gene. Since ROG is induced upon T cell activation, I questioned whether ROG was important in regulating CD8^{+}T cell responses during acute and chronic LCMV infections. I found that upon acute infection with LCMV Armstrong, *Zbtb32*^{-/-} mice accumulated higher proportions and numbers of virus-specific CD8^{+}T cells, resulting in an increased number of memory cells. These defects were CD8^{+}T cell intrinsic. I also examined the response of *Zbtb32*^{-/-} mice to chronic infection with a high dose of LCMV Clone 13 and found that approximately two-thirds of *Zbtb32*^{-/-} mice infected with high dose LCMV Clone 13 succumbed to this infection as early as day 9 post infection and also exhibited more severe immune pathology in the lung compared to WT mice. The higher frequency of IFNγ producing virus-specific CD8^{+}T cells in *Zbtb32*^{-/-} mice also resulted in enhanced viral control following LCMV Clone 13 infection. Thus, I could show that ROG plays a unique non-redundant role in negatively regulating CD8^{+}T cell response and memory T cell generation during acute and chronic LCMV infection.

A. ROG is expressed in virus-specific CD8^{+}T cells during acute LCMV infection

Several *in vitro* studies have shown that ROG is induced in T cells upon activation. Since I wanted to study the function of ROG in regulating *in vivo* T cell responses, I questioned whether ROG was induced in virus-specific CD8^{+}T cells

upon infection. To address this question splenocytes from P14 TCR transgenic mice were adoptively transferred into a congenic C57BL/6 host, which was then subsequently infected with LCMV. Total RNA from P14 CD8$^+$T cells was then isolated at days 0, 3, 6, 8, 10 and 14 post-LCMV infection (for day 3 total CD8$^+$T cells were isolated). As shown in Figure V-1, I performed Q-PCR analysis with primers specific for *Zbtb32* and found that ROG was induced in LCMV-specific CD8$^+$T cells. As expected, ROG expression was minimal at early time points post infection but reached a peak around day 6 and then decreased during the course of the immune response. Interestingly, the pattern of ROG expression indicated that ROG is expressed in effector T cells and decreases during the memory phase of the T cell response.

B. Increased CD8$^+$T cell response and memory development in the ROG-deficient mice

Since ROG was expressed in activated virus-specific CD8$^+$T cells during an acute LCMV infection I questioned whether ROG was important in regulating *in vivo* CD8$^+$T cell responses. To address this question, I used ROG-deficient mice (referred to as *Zbtb32*$^{-/-}$) in which the coding regions (exons 2, 3, 4, 5, and a part of exon 6) of ROG were replaced with a neomycin resistance gene; these mice have been previously described (156). As reported previously, ROG-deficient mice had a normal development with no apparent defects in maturation and total number of lymphocytes in the thymus, spleen and lymph nodes when compared to littermate control WT mice (data not shown). To test *in vivo* CD8$^+$T
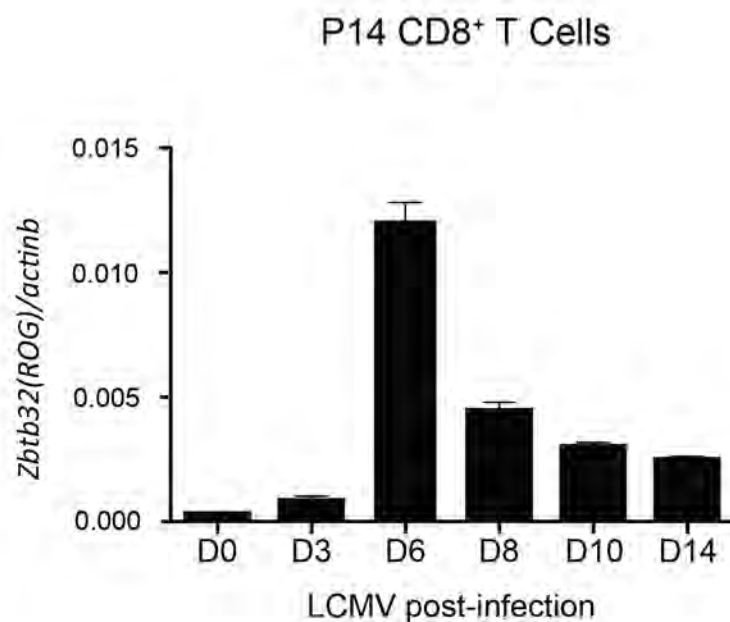
P14 CD8⁺ T Cells

FIGURE V-1. ROG is induced in virus-specific CD8⁺T cells during acute LCMV infection. Total RNA was isolated from sorted splenic P14⁺ TCR transgenic CD8⁺T cells at days 0, 6, 8, 10 and 14 post-LCMV infection (total CD8⁺T cells were sorted for day 3). The graph depicts mRNA levels of *Zbtb32* relative to *β-actin* housekeeping gene at various time points post infection. Data are representative of two independent experiments with at least three mice in each experiment. All error bars represent the SEM.

cell responses during an acute virus infection, I infected *Zbtb32*^{-/-} and WT mice with LCMV, strain Armstrong. As shown in Figure V-2, the first apparent defect I found was that at day 8 and 15 post infection ROG-deficient mice had an enlarged spleen size compared to WT controls. Using Class-I MHC tetramers for immunodominant LCMV epitopes NP396 and GP33, I enumerated the absolute number and proportion of LCMV-specific CD8+T cells at days 6, 8 (peak of response) and 45 (memory phase) post infection. As shown in Figure V-3 and 4, I found that ROG-deficient mice had a higher proportion and absolute number of LCMV-specific CD8+T cells at day 8 post infection and these differences were maintained as long as day 45 post infection. To further confirm these results LCMV-specific CD8+T cells were identified by their ability to produce IFNγ in response to *ex-vivo* stimulation with LCMV NP396 and GP33 peptides. I found that at days 8 and 45 post infection a higher proportion of IFNγ-producing LCMV-specific CD8+T cells were present in the ROG deficient mice when compared to WT controls (Figure V-5). These results indicate that ROG deficiency led to generation of an increased magnitude of a T cell response, which persisted into the memory phase, suggesting that ROG functions to negatively regulate the CD8+T cell response. The ability of virus-specific CD8+T cells to produce multiple cytokines, such as IFNγ, TNF and IL-2, simultaneously during viral infection is a primary indicator of their functional capacity, and IL-2-producing CD8+T cells are more likely to become memory cells than those that do not make IL-2 [79]. LCMV-infected splenocytes from WT or ROG-deficient mice were cultured directly *ex-*

Day 8 LCMV   Day 15 LCMV

WT   ROG KO   WT   ROG KO
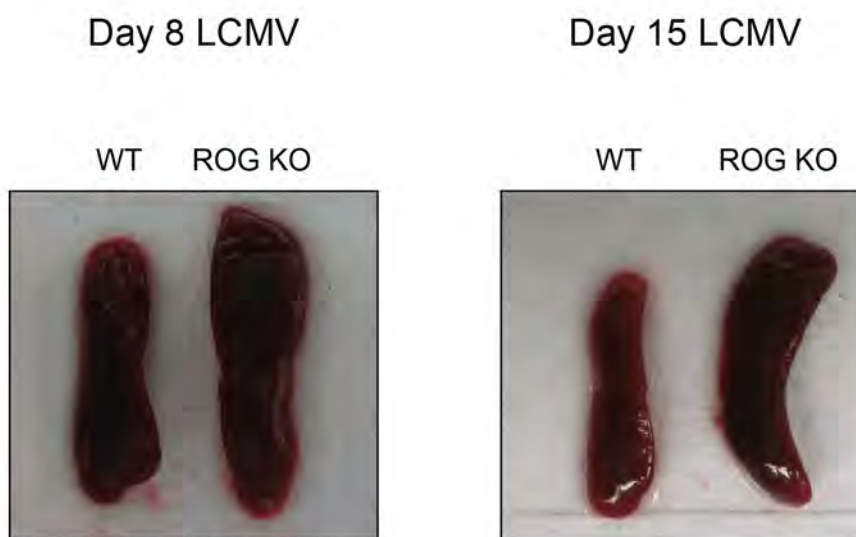
FIGURE V-2. ROG-deficient mice have an enlarged spleen compared to WT controls during acute LCMV infection. WT or ROG KO mice were infected with LCMV, strain Armstrong and spleens were isolated at day 8 and 15 post infection. Picture is a representative of at least 5 experiments with 3-5 mice in each group.
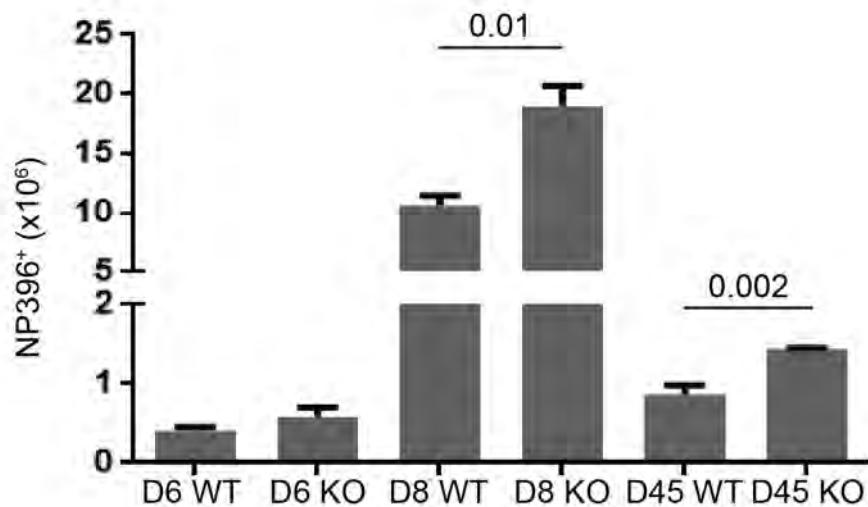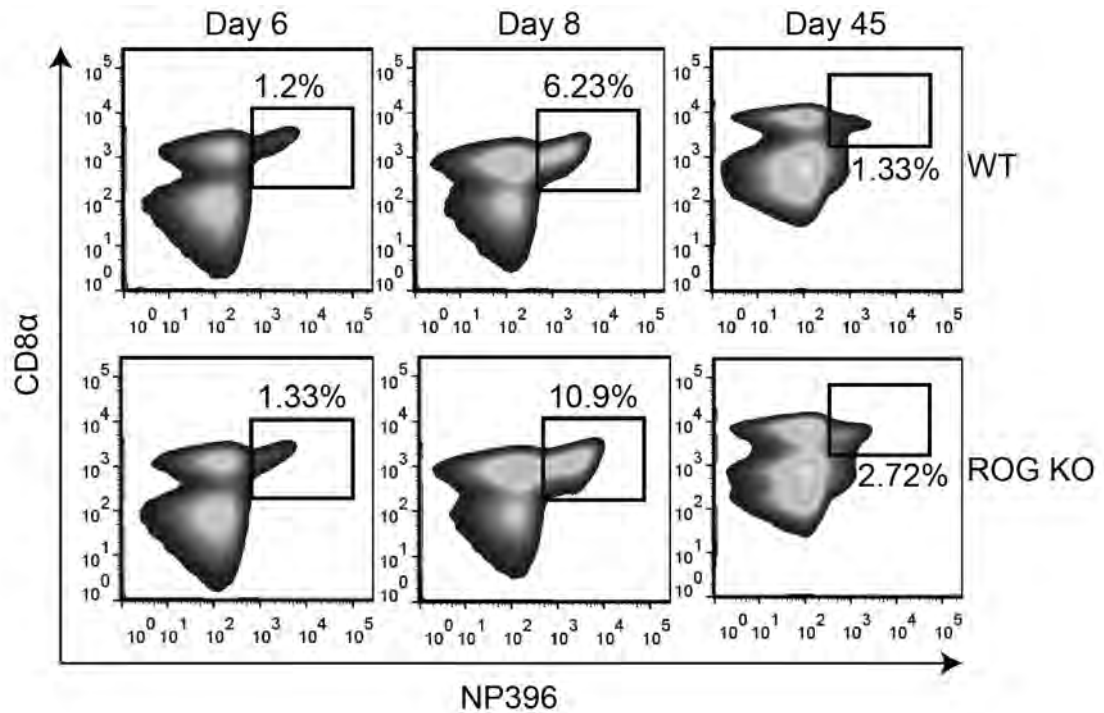
FIGURE V-3. Higher proportion and absolute number of NP396-specific T cells in the ROG-deficient mice during acute LCMV infection. The percentage (top) and total number (bottom) of LCMV-specific CD8$^+$T cells in splenocytes from WT or ROG KO mice were enumerated at days 6, 8 and 45 post-LCMV infection. NP396-specific CD8$^+$T cells were identified by Class-I MHC tetramer staining. Data are representative of three independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes.
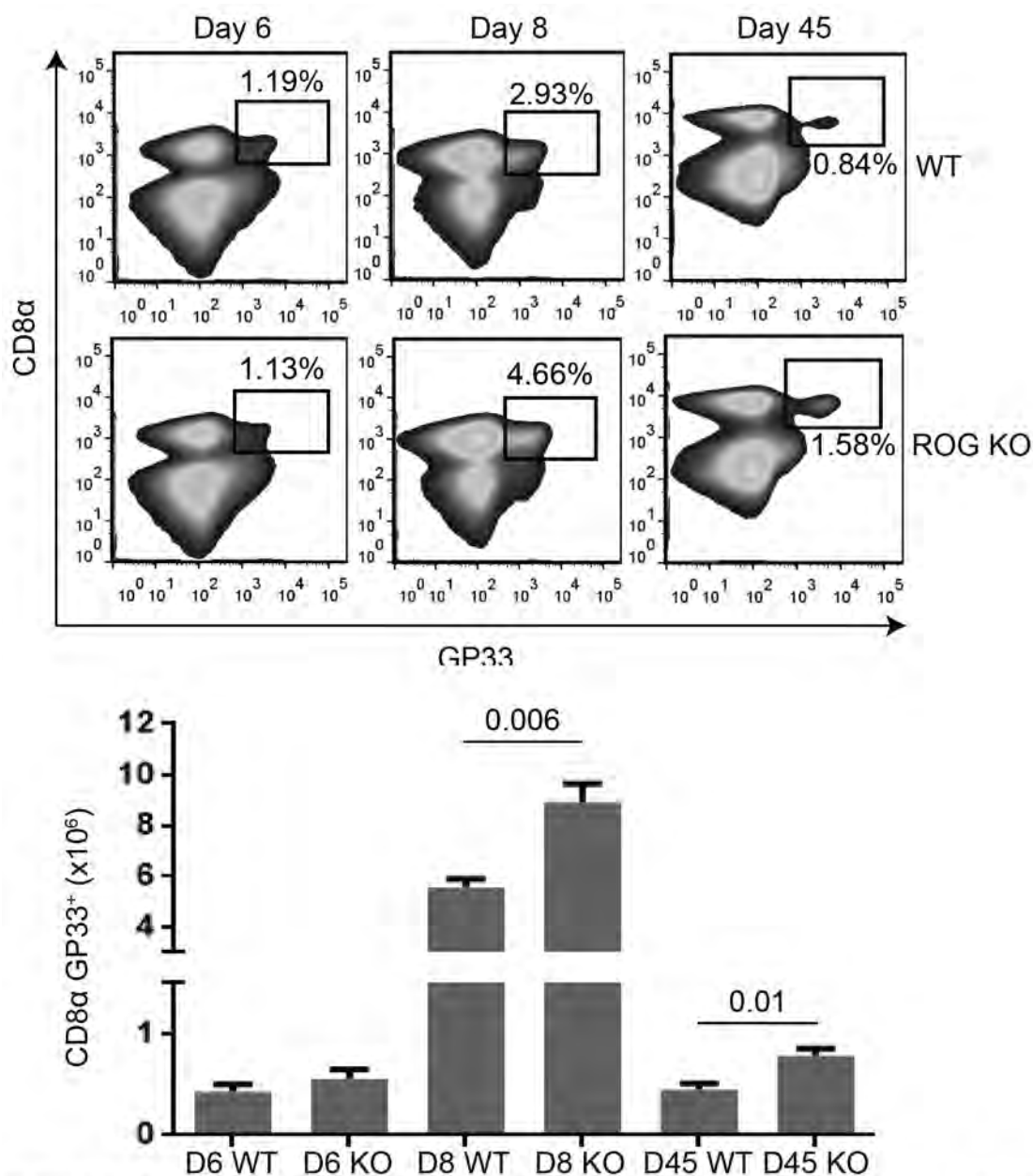
FIGURE V-4. Higher proportion and absolute number of GP33-specific T cells in the ROG-deficient mice during acute LCMV infection. The percentage (top) and total number (bottom) of LCMV-specific CD8^{+}T cells in splenocytes from WT or ROG KO mice were enumerated at days 6, 8 and 45 post-LCMV infection. GP33-specific CD8^{+}T cells were identified by Class-I MHC tetramer staining. Data are representative of three independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes
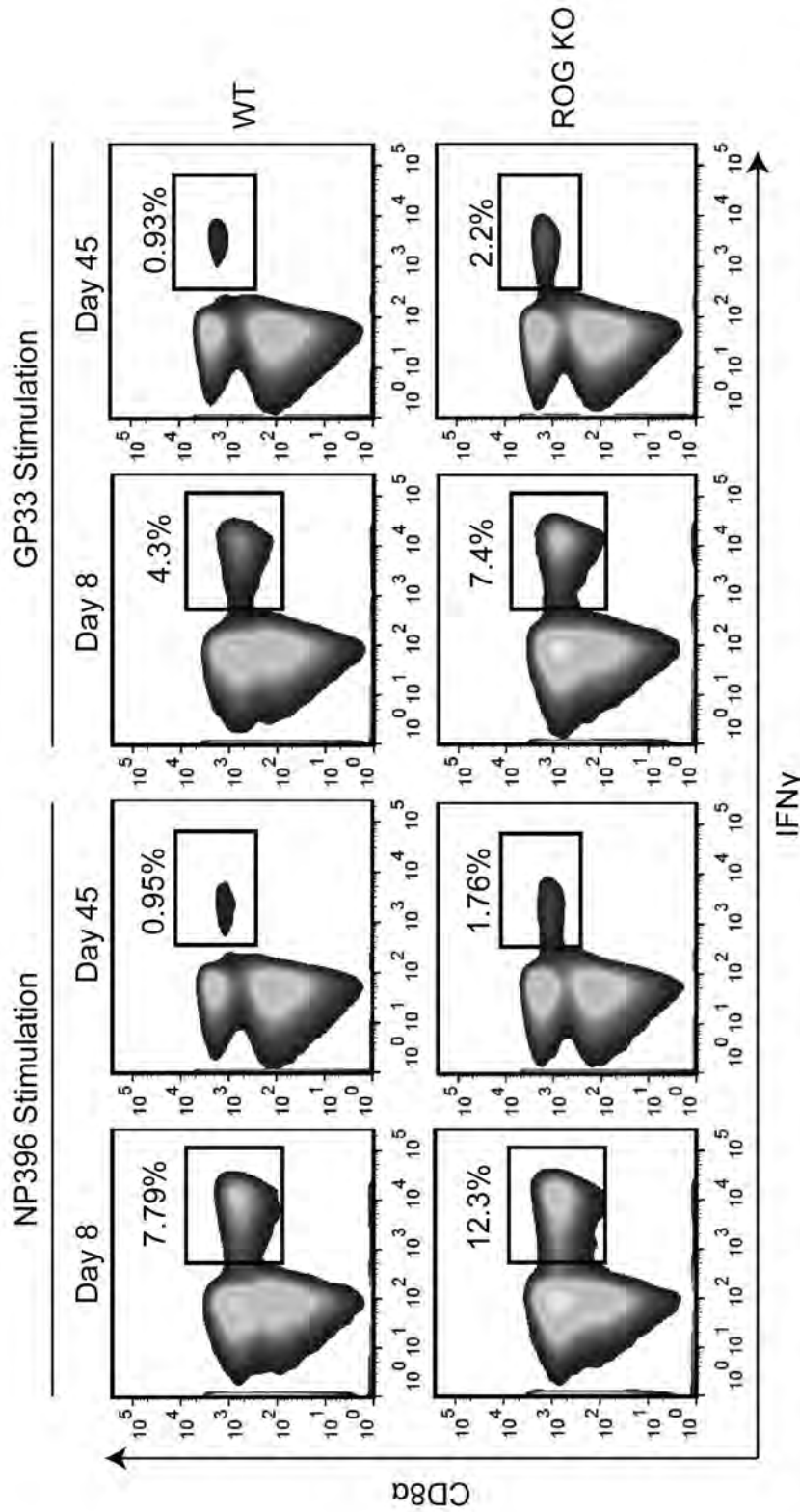
FIGURE V-5. Higher frequency of IFNγ-producing virus-specific CD8⁺T cells in the ROG-deficient mice during acute LCMV infection. Day 8 and 45 LCMV-infected splenocytes from WT and ROG deficient mice were cultured for 5 hours in the presence of LCMV-specific NP396 and GP33 peptides followed by intracellular cytokine staining. Numbers in plots represent the percentage of virus-specific CD8⁺ IFNγ⁺ T cells. Data are representative of two independent experiments with at least three mice in each experiment. FACS plots are gated on total lymphocytes.

vivo for 5 hours in the presence of LCMV-specific GP33 and NP396 peptide epitopes. As shown in Figure V-6, at days 8 and 45 post infection, virus-specific CD8$^+$T cells from *Zbtb32$^{-/-}$* mice were enriched in IFNγ, TNF and IL-2 triple producers as compared to the WT controls (% indicated on top right represent triple cytokine producers), indicating that ROG-deficient T cells were functionally superior than their WT counterparts during the peak and memory phase of the immune response. Overall these results indicate that ROG would normally function to suppress T cell responses and memory development during acute viral infection.

Increased magnitude of CD8$^+$T cell response in *Zbtb32$^{-/-}$* mice during an acute LCMV infection could have been due to defective viral clearance. Therefore, I enumerated viral-titers in *Zbtb32$^{-/-}$* by plaque assay at days 4 and 8 post-LCMV infection. As shown in Figure V-7, viral clearance in the spleen of *Zbtb32$^{-/-}$* mice was similar to WT controls, indicating that the increased magnitude of the CD8$^+$T cell response to LCMV in *Zbtb32$^{-/-}$* mice was not due to impaired viral clearance.

C. Increased CD8$^+$T cell response in the ROG-deficient mice during Vaccinia virus infection

Since I saw increased T cell responses in the ROG deficient mice during acute LCMV infection, I wanted to test whether ROG would function similarly in regulating T cell responses in an acute Vaccinia virus (VACV) infection. VACV is
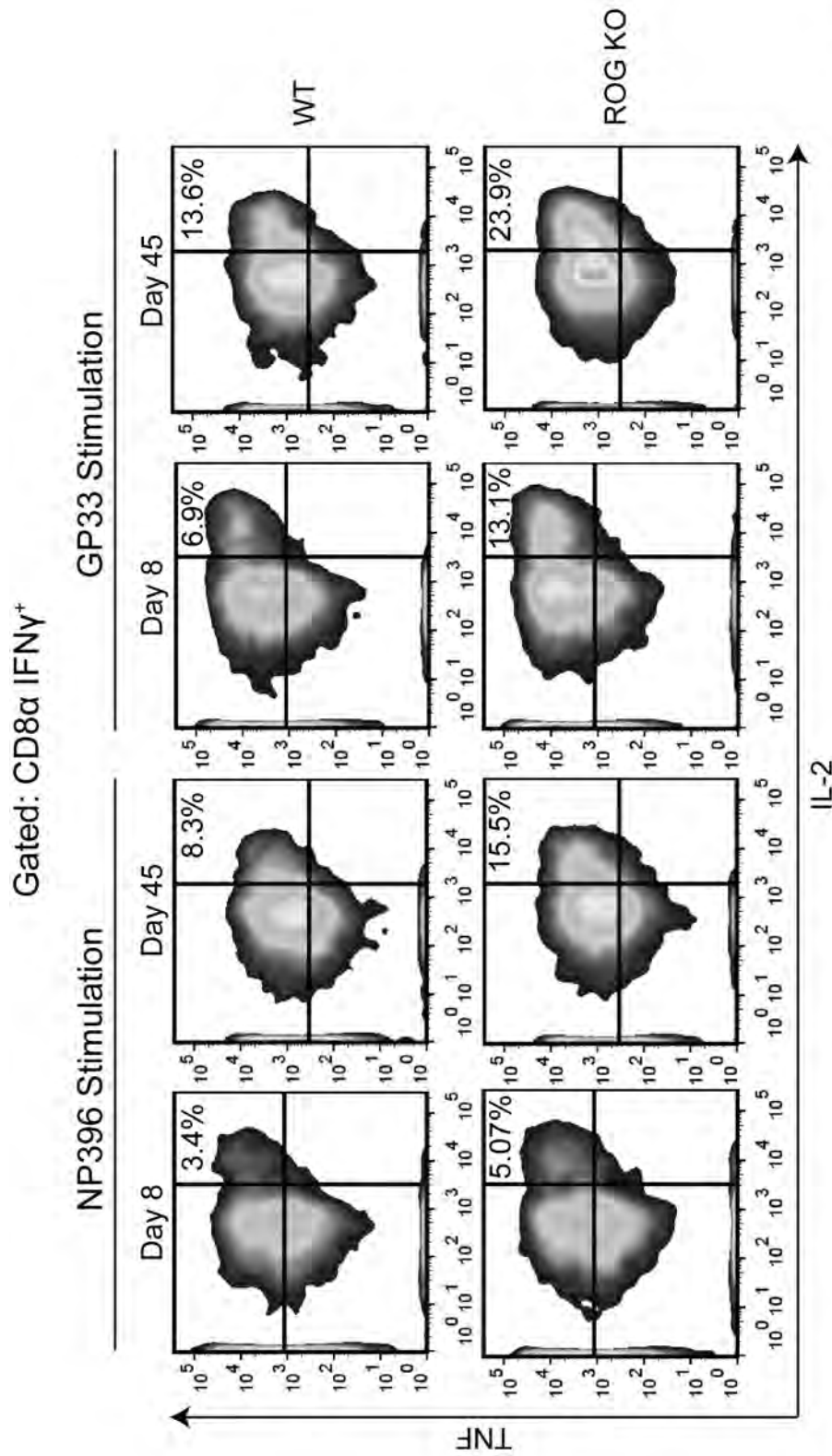
FIGURE V-6. Higher frequency of triple cytokine-producing virus-specific CD8+T cells in the ROG-deficient mice during acute LCMV infection. Day 8 and 45 LCMV-infected splenocytes from WT and ROG deficient mice were cultured for 5 hours in the presence of LCMV-specific NP396 and GP33 peptides followed by intracellular cytokine staining. Plots are gated on virus-specific CD8+ IFNγ+ T cells. Numbers in top right represent the percentage of TNF+ IL-2+ virus-specific CD8+T cells. Data are representative of two independent experiments with at least three mice in each experiment. FACS plots are gated on total lymphocytes.
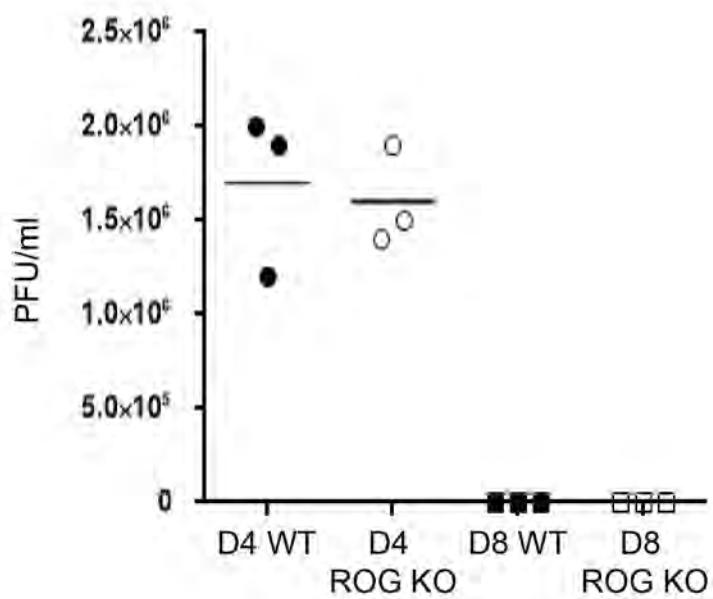
FIGURE V-7. Viral clearance in the ROG-deficient mice. LCMV titers in spleen from infected WT and *Zbtb32⁻/⁻* mice were determined by plaque assay at days 4 and 8 post infection. Data are representative of two independent experiments.

a large DNA virus of the Poxviridae family, and infection with VACV can induce a potent primary CD8$^+$T cell response and long term memory [197]. I infected WT or ROG-deficient mice with VACV, strain WR, and enumerated the proportion and absolute number of VACV-specific CD8$^+$T cells at days 7 and 14 post infection. VACV-infected splenocytes from WT or ROG-deficient mice were cultured directly *ex-vivo* for 5 hours in the presence of VACV-specific B8R and K3L peptide epitopes. As shown in Figure V-8 and 9, at days 7 and 14 post infection, a higher proportion and absolute number of IFNγ$^+$ virus-specific CD8$^+$T cells were present in the *Zbtb32$^{-/-}$* mice compared to the WT controls. Therefore, I could show that ROG functions to negatively regulate CD8$^+$T cell response in at least two different acute infection models.

D. ROG intrinsically regulates CD8$^+$T cell response and memory development

For my studies I was using ROG-deficient mice in which *Zbtb32* was ablated in both lymphoid and non-lymphoid cells. Therefore, absence of ROG in other cell types could have contributed to the increased CD8$^+$T cell responses seen in the ROG-deficient mice. To test whether ROG intrinsically regulates LCMV-specific CD8$^+$T cell responses I generated ROG-deficient P14 TCR transgenic mice. I adoptively transferred ROG-deficient and WT P14 TCR transgenic splenocytes into congenic C57BL/6 host, which were then subsequently infected with LCMV. The proportion and absolute number of *Zbtb32$^{-/-}$* and WT P14 CD8$^+$T cell responses were enumerated at days 6, 9 and
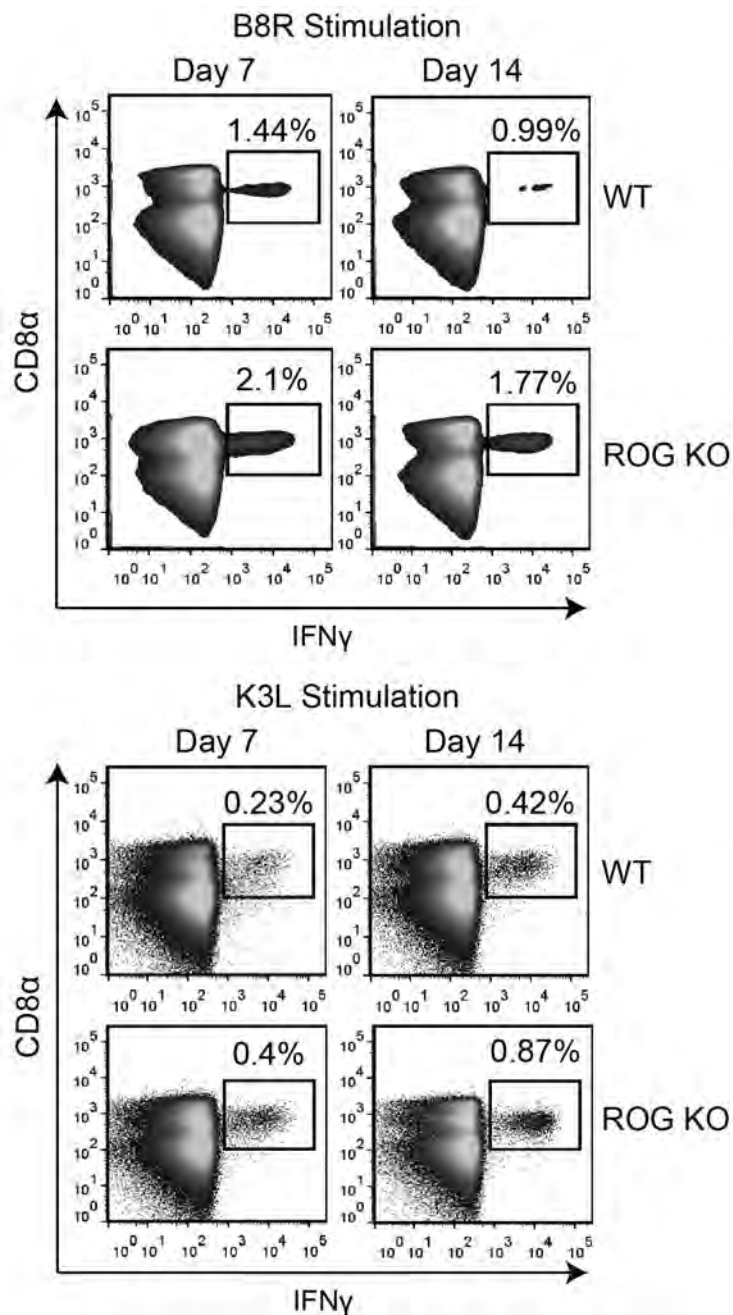
FIGURE V-8. Higher frequency of IFNγ-producing virus-specific CD8⁺T cells in the ROG-deficient mice during acute Vaccinia virus infection. Day 7 and 14 VACV-infected splenocytes from WT and ROG-deficient mice were cultured for 5 hours in the presence of VACV-specific B8R (top) and K3L (bottom) peptides followed by intracellular cytokine staining. Numbers in plots represent the percentage of virus-specific CD8⁺ IFNγ⁺ T cells. Data are representative of two independent experiments with at least three mice in each experiment. FACS plots are gated on total lymphocytes.
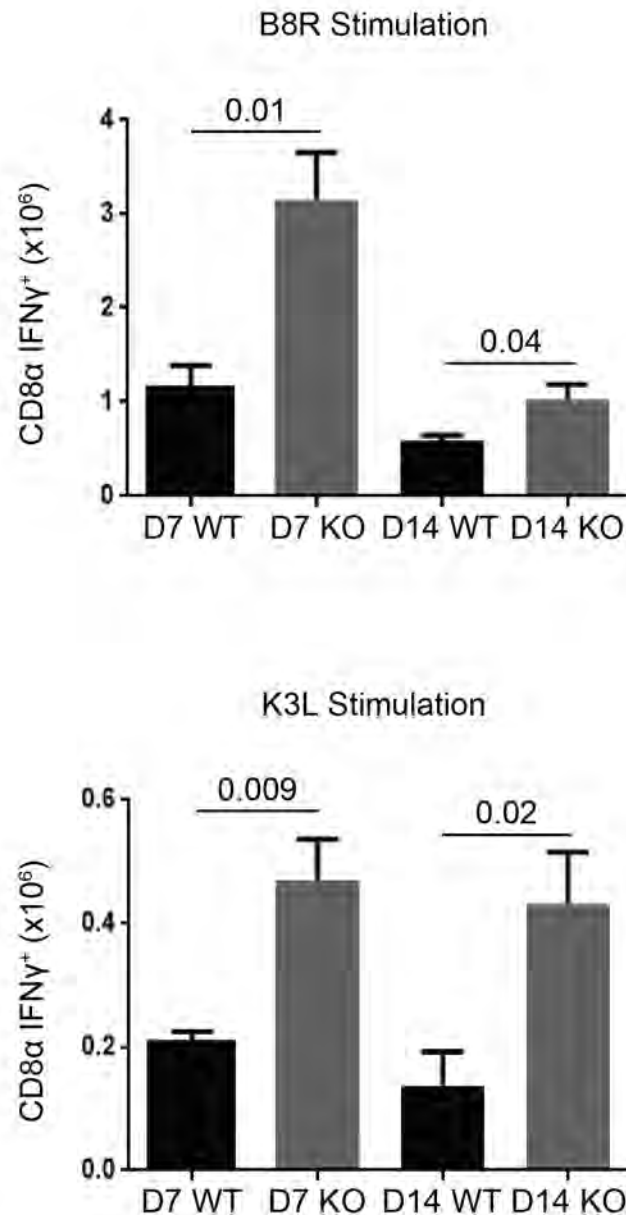
FIGURE V-9. Higher absolute numbers of IFNγ-producing virus-specific CD8+T cells in the ROG-deficient mice during acute Vaccinia virus infection. Day 7 and 14 VACV-infected splenocytes from WT and ROG-deficient mice were cultured for 5 hours in the presence of VACV-specific B8R (top) and K3L (bottom) peptides followed by intracellular cytokine staining. Histograms represent the absolute number of virus-specific CD8+ IFNγ+ T cells. Data are representative of two independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes.

15 post-LCMV infection. As shown in Figure V-10, a higher proportion and absolute number of *Zbtb32*^{-/-} P14 CD8^{+}T cells accumulated in the congenic host compared to WT controls at days 9 and 15 post infection indicating that ROG intrinsically regulated CD8^{+}T cell response during acute LCMV infection.

Recent studies have demonstrated that CD25 expression during the early expansion phase of the anti-viral CD8^{+}T cell response is critical in regulating cell fate in that CD25^{hi} cells become short-lived effector cells (80, 91). As shown in Figure V-11, a lower proportion of *Zbtb32*^{-/-} P14 CD8^{+}T cells expressed CD25 when compared to WT controls at day 6 post-LCMV infection, indicating that *Zbtb32*^{-/-} P14 CD8^{+}T might preferentially become memory T cells. Surface expression of antigenic markers such as IL-7Rα along with KLRG-1 (MPEC: IL-7R^{hi} KLRG-1^{lo}) as well as CD27 and CXCR3 have been used to identify cells which will preferentially make it into memory (88). Moreover, expression of CD62L has been used to identify central memory T cells (Tcm) which are thought to mediate long term protection to secondary challenge (198). To address whether ROG intrinsically regulates generation of memory CD8^{+}T cells, ROG-deficient and WT P14 TCR transgenic splenocytes were adoptively transferred into congenic C57BL/6 host, which were then subsequently infected with LCMV. At day 9 post-LCMV infection a higher proportion of P14[+] *Zbtb32*^{-/-} cells expressed the memory markers CD27 and CXCR3 when compared to WT control cells and no differences were observed in expression of IL-7R vs KLRG-1 and CD62L (Figure V-12). However, as shown in Figure V-13, by day 15 post-LCMV infection
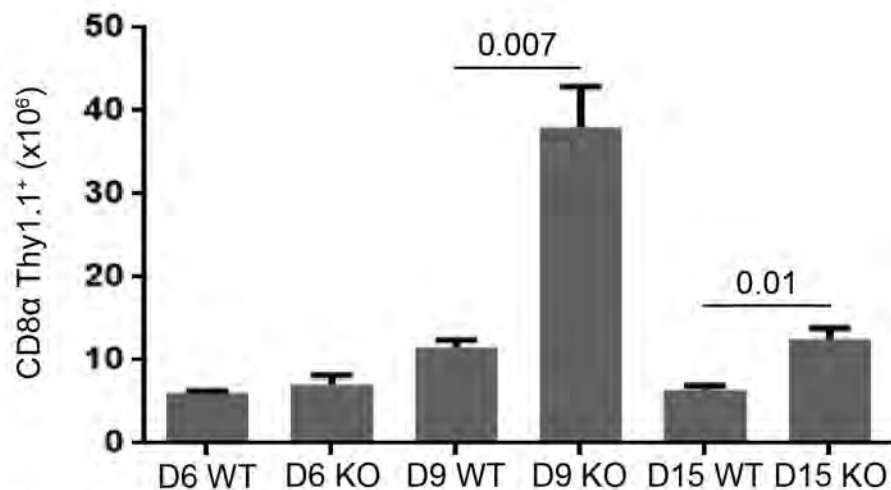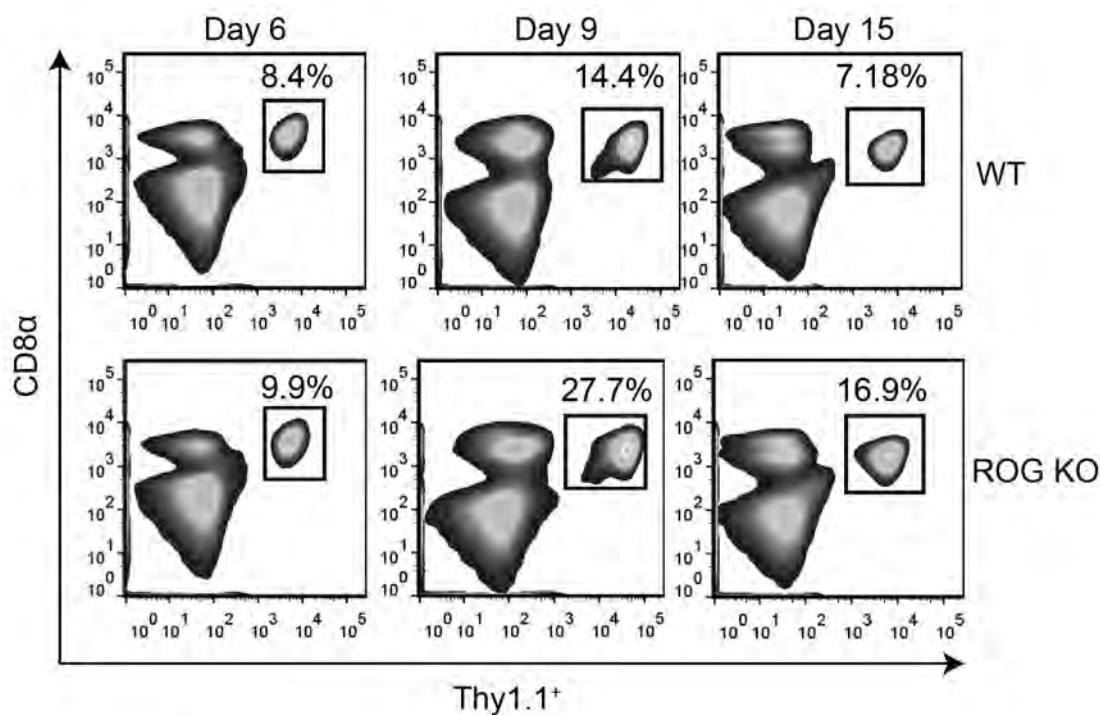
FIGURE V-10. Increased magnitude of T cell response during acute LCMV infection is due to an intrinsic defect of ROG in CD8+T cells. 2×10^5 P14 splenocytes from WT or *Zbtb32-/- mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. The percentage (top) and total number (bottom) of splenic P14 TCR transgenic LCMV-specific WT or *Zbtb32-/-* CD8+ T cells were enumerated at days 6, 9 and 15 post-LCMV infection. Data are representative of three independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes.
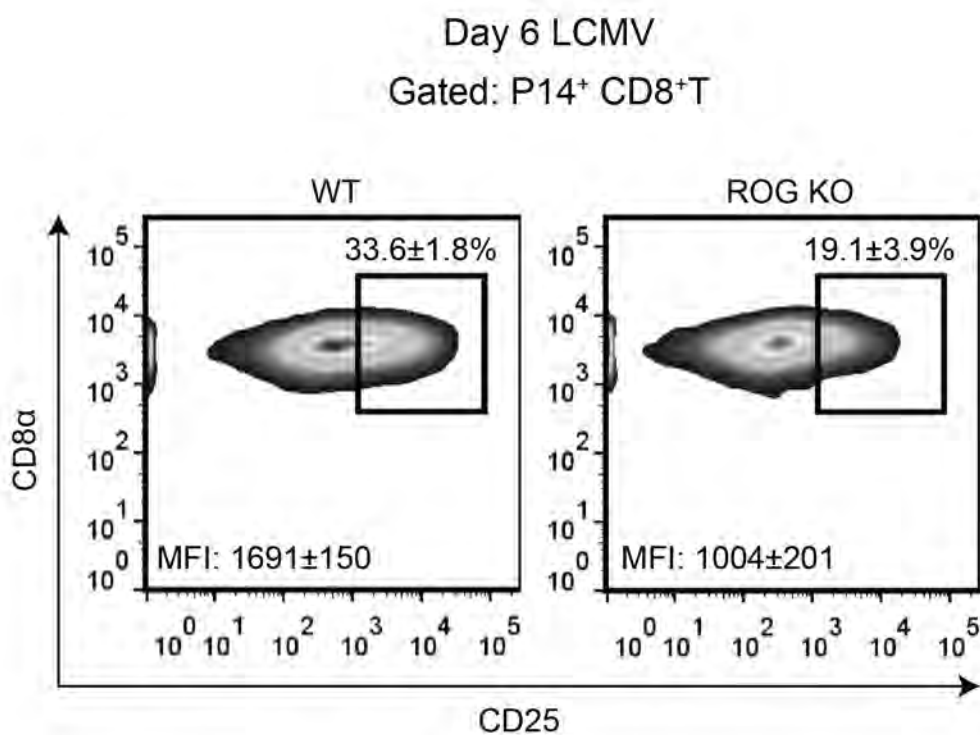
FIGURE V-11. Decreased CD25 expression on ROG-deficient CD8⁺T cells at day 6 post-LCMV infection. 2×10^5 P14 splenocytes from WT or *Zbtb32^{-/-} mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. P14 TCR transgenic LCMV-specific WT or *Zbtb32^{-/-}* CD8⁺T cells from spleen were stained for CD25 at day 6 post-LCMV infection. Percentage ± SEM of CD25-expressing cells is represented. Data are representative of three independent experiments with at least three mice in each experiment. MFI ± SEM of CD25 expression on total P14⁺CD8⁺T cells are represented in the plot.
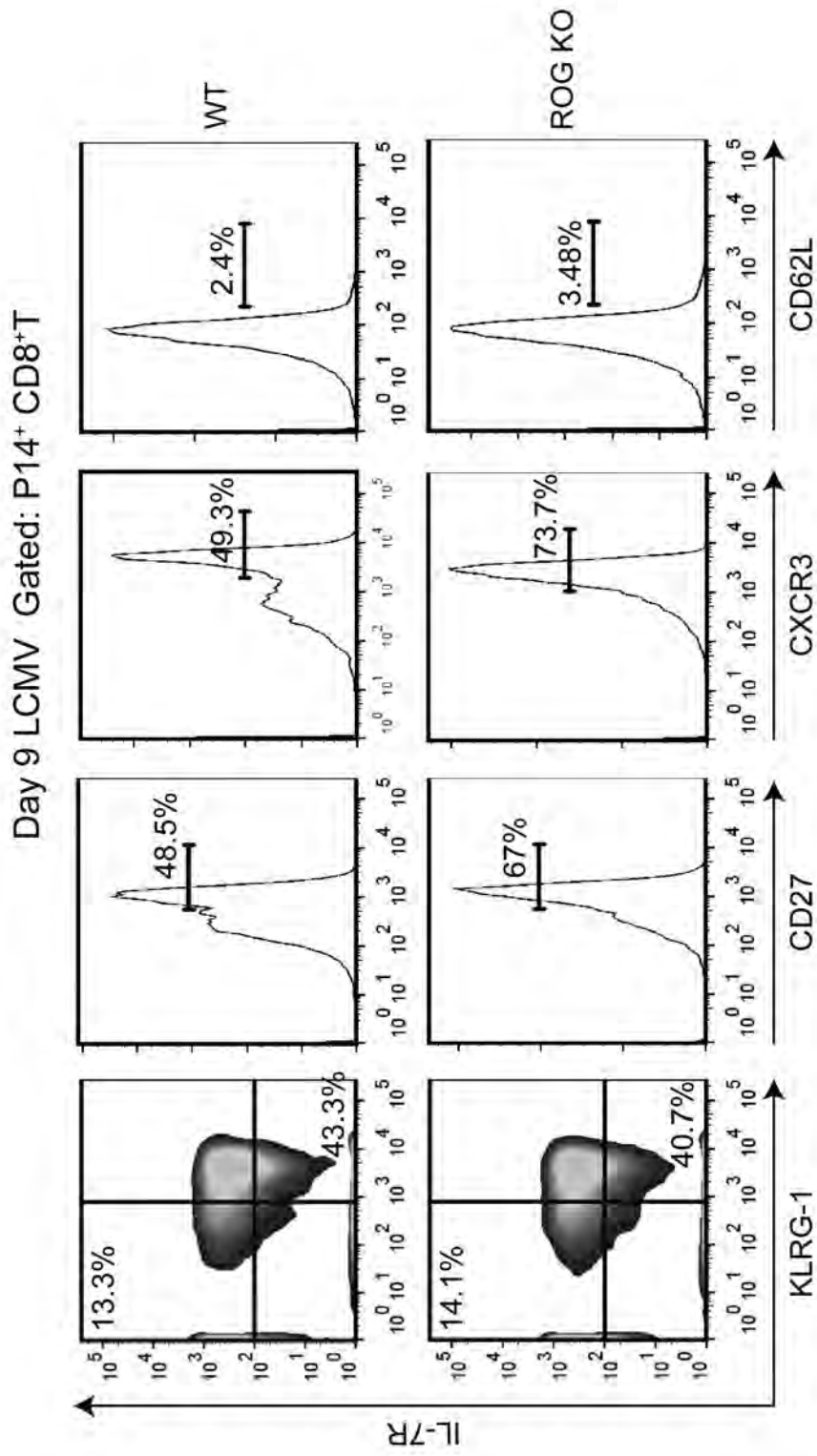
FIGURE V-12. Higher proportion of memory precursor CD8+T cells are generated due to ROG deficiency at day 9 post-LCMV infection. 2×10^5 P14 splenocytes from WT or *Zbtb32-/- mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. P14 TCR transgenic LCMV-specific WT or *Zbtb32-/-* splenic CD8+T cells were stained for memory markers (IL-7R, KLRG-1, CD27, CXCR3, CD62L) at day 9 post-LCMV infection. Data are representative of three independent experiments with at least three mice in each experiment.
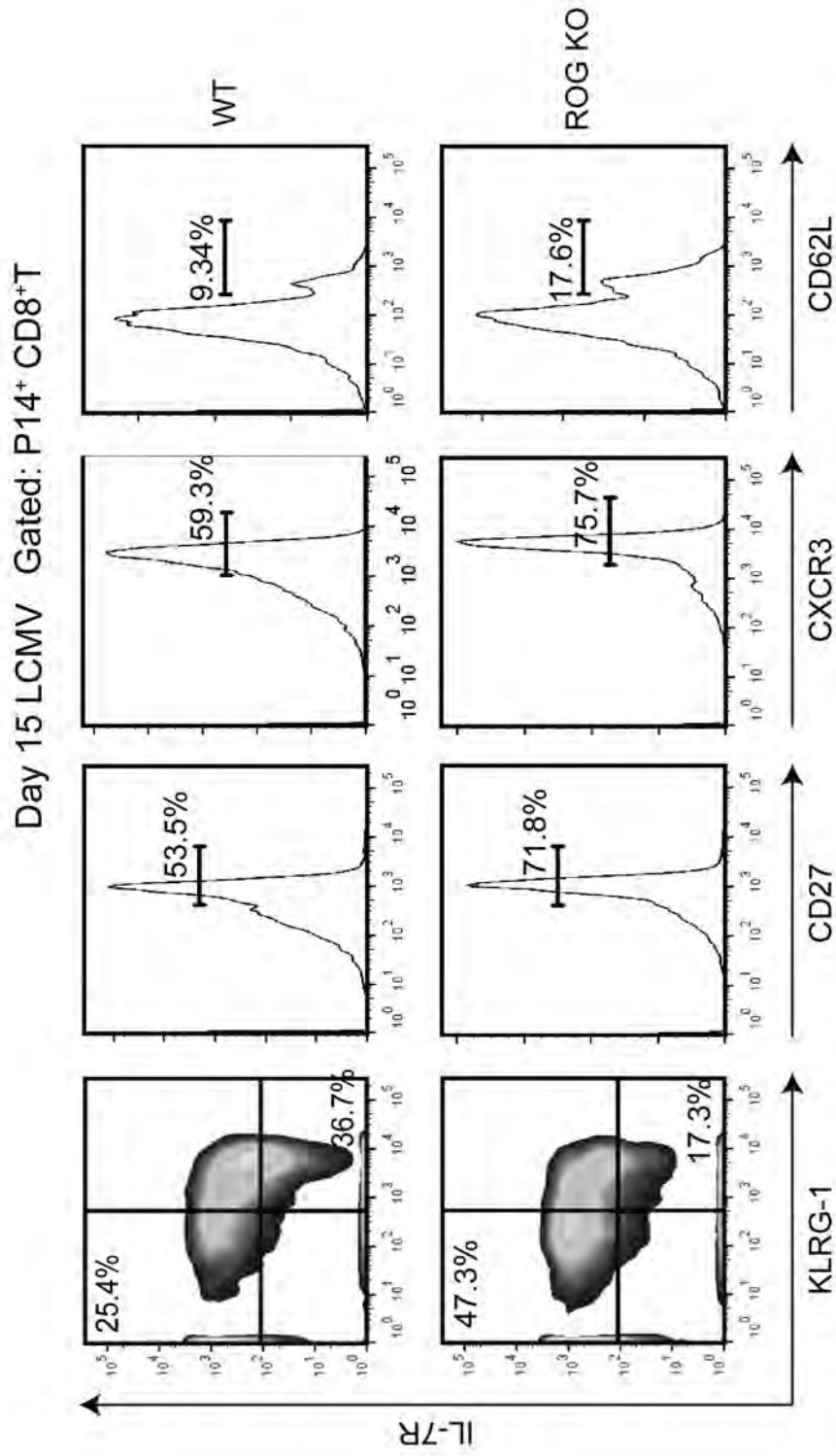
FIGURE V-13. Higer proportion of memory precursor CD8⁺T cells are generated due to ROG deficiency at day 15 post-LCMV infection. 2×10^5 P14 splenocytes from WT or *Zbtb32⁻/⁻ mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. P14 TCR transgenic LCMV-specific WT or *Zbtb32⁻/⁻* splenic CD8⁺T cells were stained for memory markers (IL-7R, KLRG-1, CD27, CXCR3, CD62L) at day 15 post-LCMV infection. Data are representative of three independent experiments with at least three mice in each experiment.

I found that P14$^+$ *Zbtb32$^{-/-}$* CD8$^+$T cells were enriched in memory precursor effector cells (IL-7R^{hi} KLRG-1^{lo}) and expressed the memory markers CD27, CXCR3 and CD62L. The ability of CD8$^+$T cells to produce multiple cytokines, such as IFNγ, TNF and IL-2 is an indicator of their functional capacity. As shown in Figure V-14, I found that a higher proportion of P14$^+$ *Zbtb32$^{-/-}$* CD8$^+$T cells produced triple cytokines when compared to WT controls at day 9 and 15 post infection (% indicated on top right represent triple cytokine producers) in response to *ex-vivo* LCMV-GP33 peptide stimulation. The transcription factor EOMES promotes persistence of memory CD8$^+$T cells [110]. Since, a higher proportion of ROG-deficient T cells had a memory phenotype I compared EOMES expression in adoptively transferred P14 TCR transgenic *Zbtb32$^{-/-}$* and WT CD8$^+$T cells. As shown in Figure V-15, I found that a higher proportion of LCMV-specific P14$^+$ CD8$^+$T cells from *Zbtb32$^{-/-}$* expressed EOMES compared to WT controls at day 9 post infection. The expression level of EOMES was also higher in *Zbtb32$^{-/-}$* P14$^+$CD8$^+$T cells when compared to WT controls, as shown by the MFI in Figure V-15. Overall, the data suggests that ROG intrinsically negatively affects the magnitude of CD8$^+$T cell response and suppresses the generation of memory precursor cells.

E. ROG deficiency leads to generation of functionally superior memory CD8$^+$T cells

So far I have shown that ROG deficiency led to generation of CD8$^+$T cells, which have a memory precursor phenotype. I questioned whether the difference

GP33 Stimulation: Gated P14⁺ CD8α IFNγ⁺
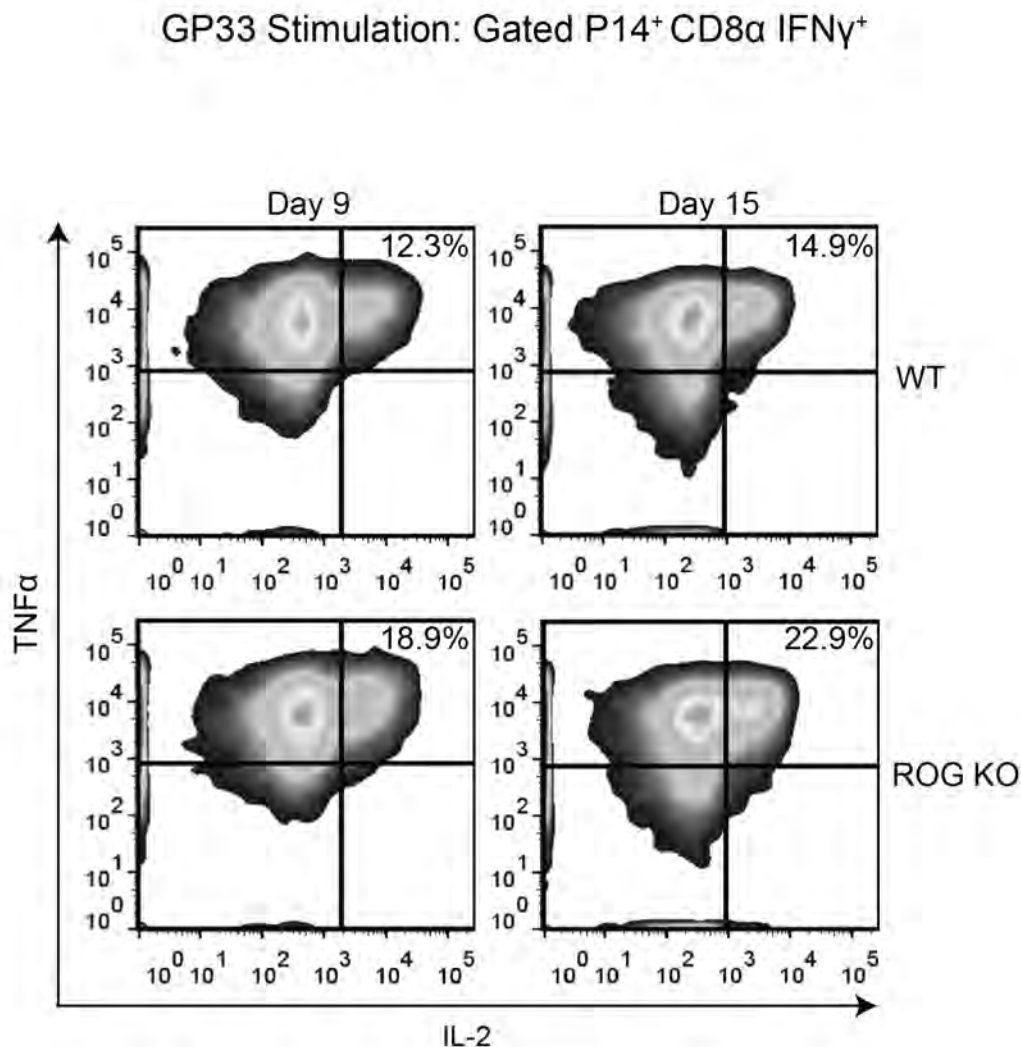
FIGURE V-14. Higher frequency of triple cytokine-producing P14 TCR transgenic ROG-deficient T cells. 2x10^5 P14 splenocytes from WT or *Zbtb32$^{-/-}$ mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. Day 9 and 15 LCMV-infected splenocytes from WT hosts which were harboring P14 TCR transgenic WT or *Zbtb32$^{-/-}$* CD8⁺T cells were cultured for 5 hours in the presence of LCMV-specific GP33 peptide. Plots are gated on virus-specific CD8⁺ IFN γ⁺ T cells. Numbers in top right represent the percentage of TNF⁺ IL-2⁺ virus-specific CD8⁺T cells. Data are representative of three independent experiments with at least three mice in each experiment.

Gated: P14⁺ CD8⁺T
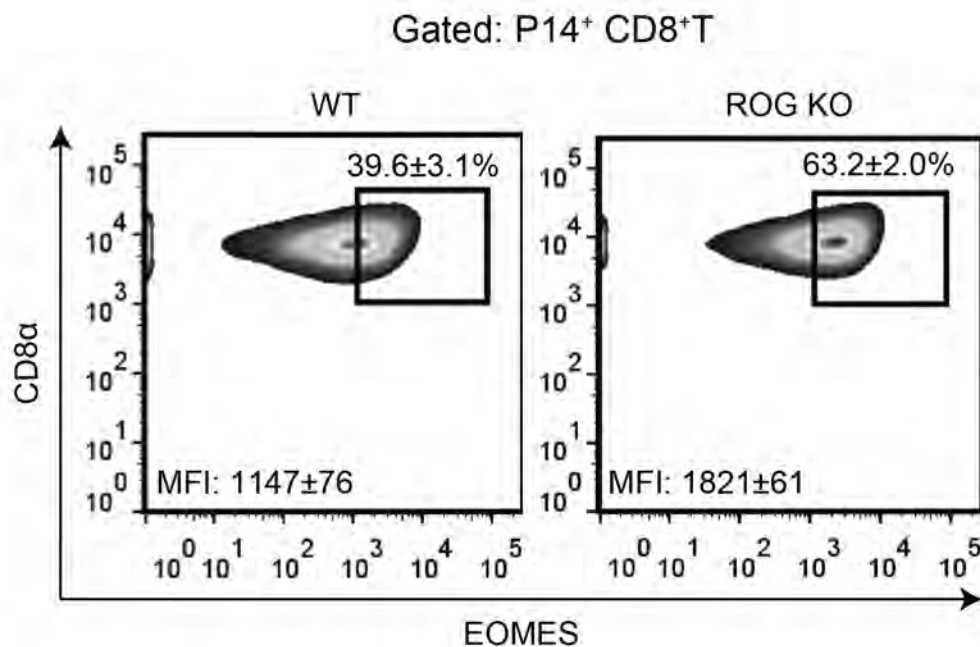
FIGURE V-15. Increased EOMES expression in P14 TCR transgenic ROG-deficient T cells. 2×10^5 P14 splenocytes from WT or *Zbtb32⁻/⁻ mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection.Percentages ± SEM of EOMES⁺ P14 TCR transgenic WT or *Zbtb32⁻/⁻* CD8⁺ T cells from spleen at day 9 post-LCMV infection was determined by intracellular transcription factor staining. The MFI ± SEM of EOMES staining on total P14⁺CD8⁺T cells is indicated. Data are representative of four independent experiments with at least three mice in each experiment.

in CD8$^+$T cell phenotype between WT and ROG-deficient T cells was restricted to early time points or whether it is maintained into the memory phase of the immune response. I adoptively transferred ROG-deficient and WT P14 TCR transgenic splenocytes into congenic C57BL/6 host, which were then subsequently infected with LCMV. At day 30 post infection (memory phase), I found approximately a three-fold higher proportion and absolute number of *Zbtb32*$^{-/-}$ P14$^+$CD8$^+$T in the congenic host when compared to WT controls (Figure V-16). Furthermore, as shown in Figure V-17 a higher proportion of P14$^+$ *Zbtb32*$^{-/-}$ CD8$^+$T cells represented functional memory and produced triple cytokines IFNγ, TNF and IL-2 (% indicated on top right represent triple cytokine producers) when compared to WT controls in response to *ex-vivo* LCMV-GP33 peptide stimulation.

ROG-deficient memory CD8$^+$T cells were enriched in IL-2 producers, which can be used as predictor of recall efficacy of a T cell. Therefore, I questioned whether ROG deficiency led to generation of bona fide superior memory T cells which had a better recall efficacy compared to WT controls. As shown in the schematic in Figure V-18, *Zbtb32*$^{-/-}$ or WT P14 CD8$^+$T cells were sorted at day 30 post-LCMV infection and adoptively transferred into naïve hosts, which were then infected with LCMV. At day 5 post-infection I enumerated the proportion and absolute number of *Zbtb32*$^{-/-}$ or WT P14 CD8$^+$T which were responding to a secondary LCMV challenge. As shown in Figure V-18, a higher proportion and absolute number of *Zbtb32*$^{-/-}$ P14 CD8$^+$T cells were present in the
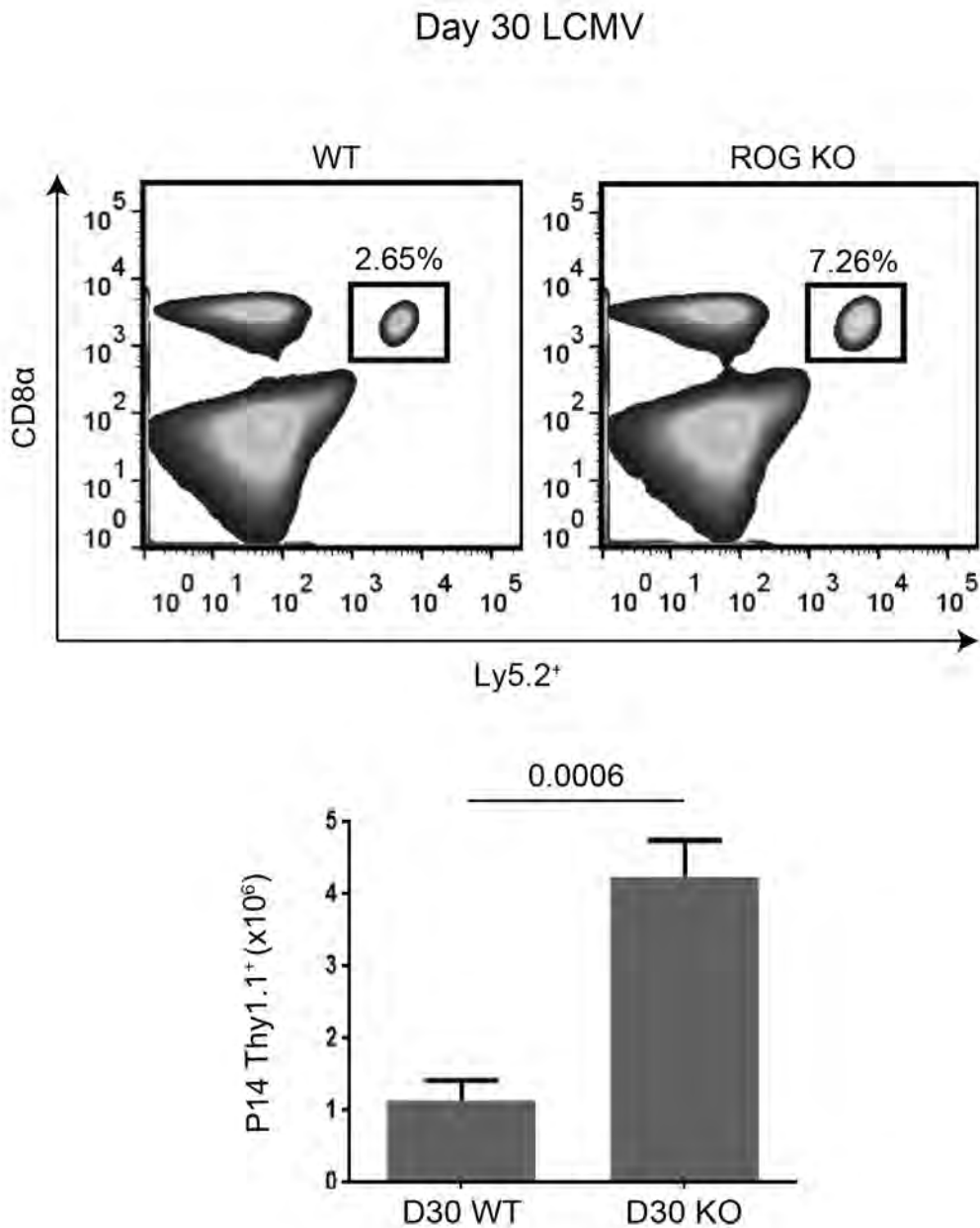
FIGURE V-16. Higher proportion and absolute number of memory CD8+T cells are generated due to ROG deficiency. $2x10^5$ P14 splenocytes from WT or *Zbtb32-/- mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. The percentage (top) and total number (bottom) of splenic P14 TCR transgenic LCMV-specific WT or *Zbtb32-/-* CD8+T cells were enumerated at day 30 post-LCMV infection. Data are representative of three independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes.

GP33 Stimulation: Gated CD8α IFNγ⁺
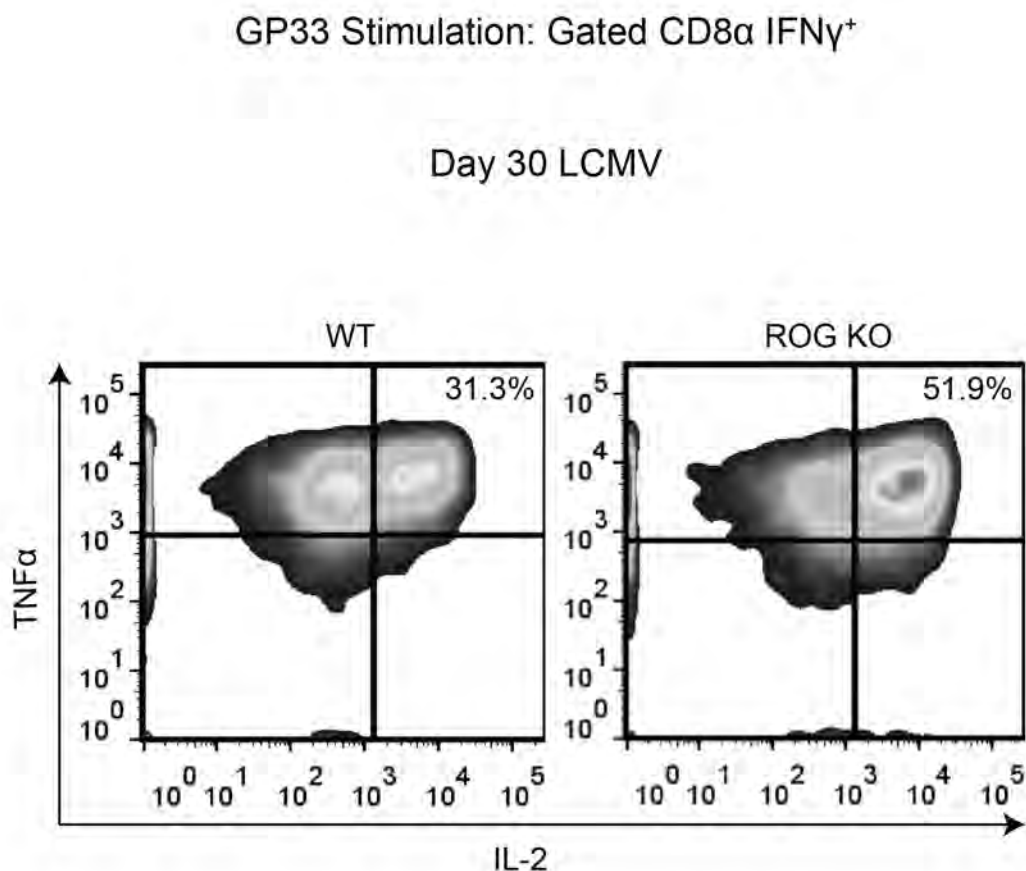
Day 30 LCMV

FIGURE V-17. Higher frequency of triple cytokine producing P14 TCR transgenic ROG-deficient memory T cells. 2×10^5 P14 splenocytes from WT or *Zbtb32⁻/⁻ mice* were adoptively transferred into a C57BL/6 host followed by LCMV infection. Day 30 LCMV-infected splenocytes from WT hosts which were harboring P14 TCR transgenic WT or *Zbtb32⁻/⁻* CD8⁺T cells were cultured for 5 hours in the presence of LCMV-specific GP33 peptide. Plots are gated on virus-specific CD8⁺ IFNγ⁺ T cells. Numbers in top right represent the percentage of TNF⁺ IL-2⁺ virus-specific CD8⁺T cells. Data are representative of three independent experiments with at least three mice in each experiment.
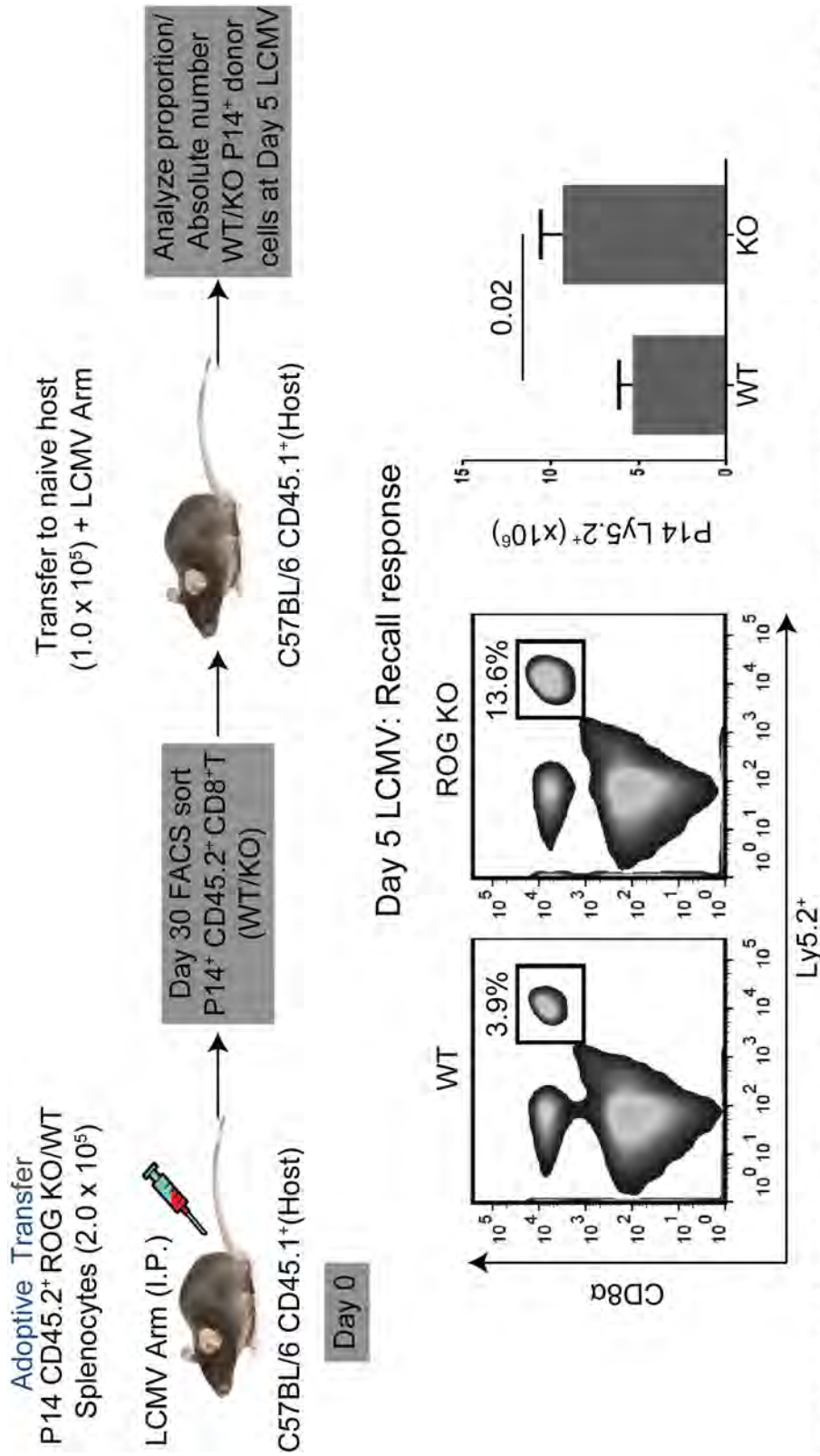
FIGURE V-18. ROG-deficient memory T cells have a superior recall response. Day 30 LCMV-infected 1x10^{5} splenic P14 TCR transgenic WT or *Zbtb32-/-* CD8+ T cells were transferred into a naive host which were then infected with LCMV as shown in the schematic (top). Percentage and absolute number (bottom) of transferred P14 TCR transgenic WT or *Zbtb32-/-* CD8+T cells were analysed in the spleen at day 5 post-LCMV infection (recall response). Data are representative of two independent experiments with at least three mice in each experiment. All error bars represent the SEM. FACS plots are gated on total lymphocytes.

host when compared to WT controls. Therefore, the data show that intrinsic deficiency of ROG in CD8⁺T cells leads to generation of functionally superior memory T cells.

F. Increased immunopathology and decreased survival of ROG-deficient mice in response to high dose LCMV Clone 13 infection

LCMV-variant Clone 13 has been widely used to study T cell responses during persistent infection (11). Due to higher affinity of the viral glycoprotein for its α-dystroglycan receptor expressed on macrophages and dendritic cells Clone 13 can systemically spread and replicate in both lymphoid and non-lymphoid tissues to cause persistent infection as opposed to the parent Armstrong strain which replicates in lymphoid tissues and fails to exhaust the T cell response (11). As shown in Figure V-19, in C57BL/6 mice the outcome of the LCMV Clone 13 infection depends on the dose of viral inoculum. At low dose inoculum a strong T cell response is generated and virus is cleared, on the other end of the spectrum a high dose inoculum causes systemic viral replication leading to T cell exhaustion and persistent infection. Interestingly, in a medium dose response there is systemic viral replication along with strong T cell responses, which are detrimental to the host leading to immunopathology and host mortality (22).

ROG deficient mice generated stronger T cell responses during acute LCMV infection compared to WT controls (Figure V-4). I hypothesized that a strong T cell response to high dose Clone 13 infection by ROG-deficient mice
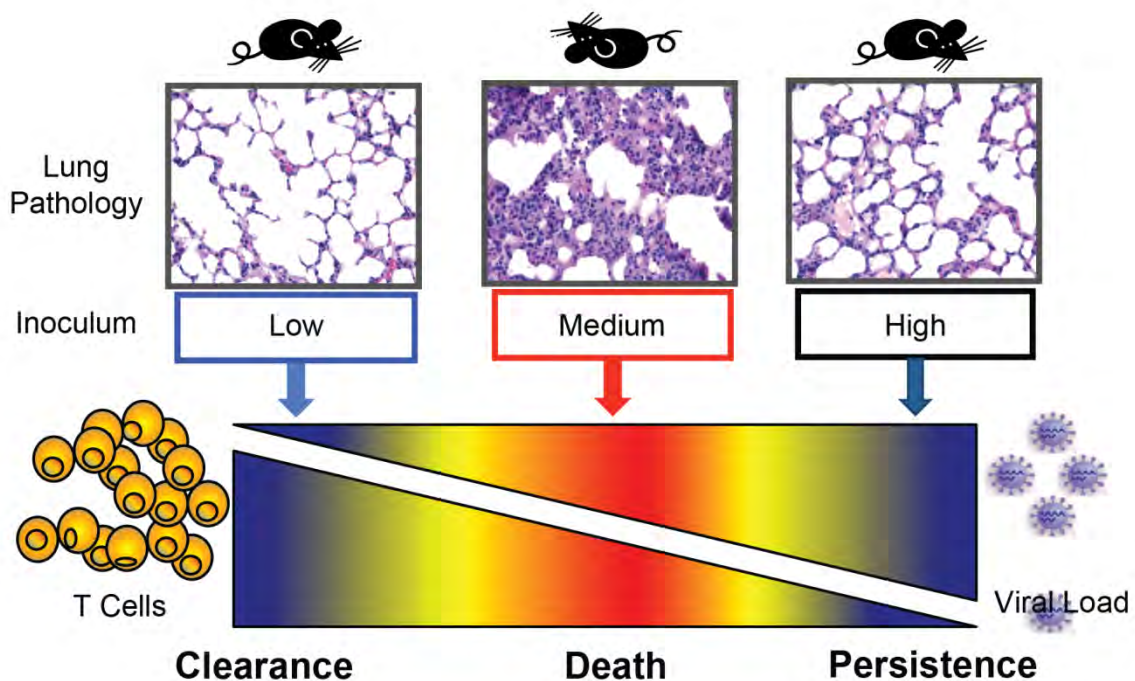
FIGURE V-19. LCMV Clone 13 infection model. The outcome of LCMV Clone 13 infection depends on the dose of viral inoculum. At low dose inoculum strong T cell response is generated and virus is cleared, whereas a high dose inoculum causes systemic viral replication leading to T cell exhaustion and persistent infection. During a medium dose response there is systemic viral replication along with strong T cell responses which is detrimental to the host leading to immunopathology and host mortality. Lung pathology as identified by H&E staining during LCMV Clone 13 infection is shown. (Slide Courtesy: Dr. Stephen N.Waggoner)

might emulate a medium dose response leading to immunopathology and host mortality. To test the hypothesis, WT or *Zbtb32*$^{-/-}$ mice were intravenously injected with high dose inoculum of LCMV Clone 13. Consistent with the hypothesis, I found that between days 9 and 15 post infection approximately 70% of the ROG-deficient mice succumbed to the high dose Clone 13 infection (Figure V-20). Importantly, the kinetics of the death curve coincided with the peak of the T cell response suggesting that the mortality was T cell dependent. As shown in Figure V-21, there was also a precipitous drop in body weight of the ROG deficient mice around day 8 post infection, correlating with their decreased survival compared to WT controls. Previous studies in our lab have shown that mortality during high dose LCMV Clone 13 infection can be associated with increased lung pathology (22). To test whether these ROG-deficient mice had increased lung pathology, lung sections from day 10 LCMV Clone 13 infected ROG-deficient and WT mice were stained with hematoxylin and eosin (H&E). In a blind study, Dr. Liisa Selin at UMMS scored the lung histology on an arbitrary scale from 1-5 (1 being the best and 5 being the worst). As shown in Figure V-22, compared to the WT mice, lungs of ROG-deficient mice had severe pulmonary oedema, interstitial mononuclear infiltration, and presence of bronchus associated lymphoid tissue (BALT) and necrotizing bronchiolitis. In accordance with this observation the lung histology scores of ROG deficient mice were much worse when compared to WT controls (Figure V-22). Since the kinetics of death seen in the ROG-deficient mice suggested that mortality was T cell dependent, I
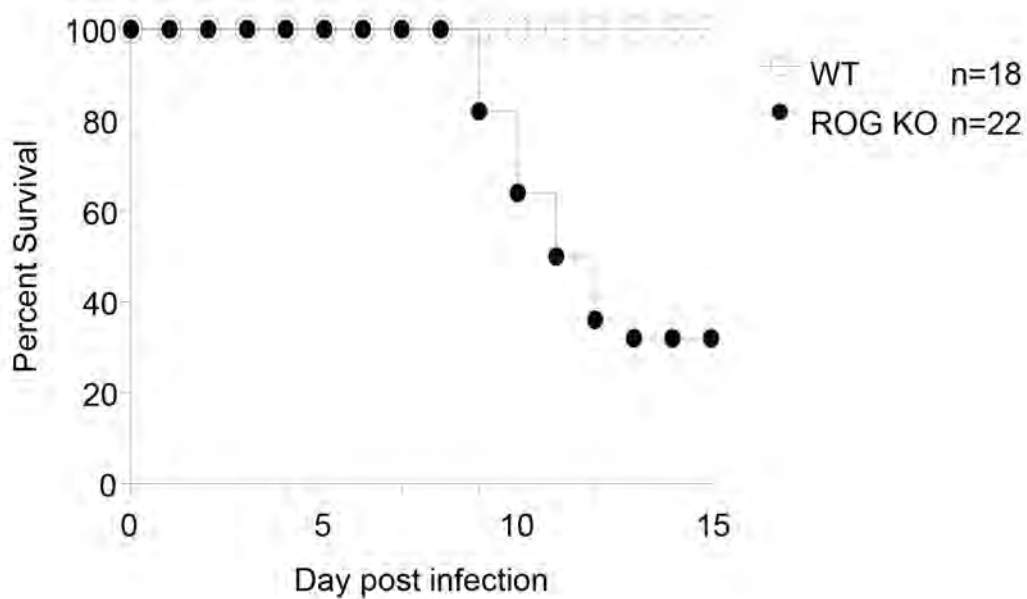
FIGURE V-20. Decreased survival of ROG-deficient mice during high dose LCMV Clone 13 infection. WT or ROG-deficient mice were infected intravenously with high dose LCMV Clone 13. The percent of surviving mice was recorded daily until day 15 post infection. The survival curve represented above is a compilation of three independent experiments.
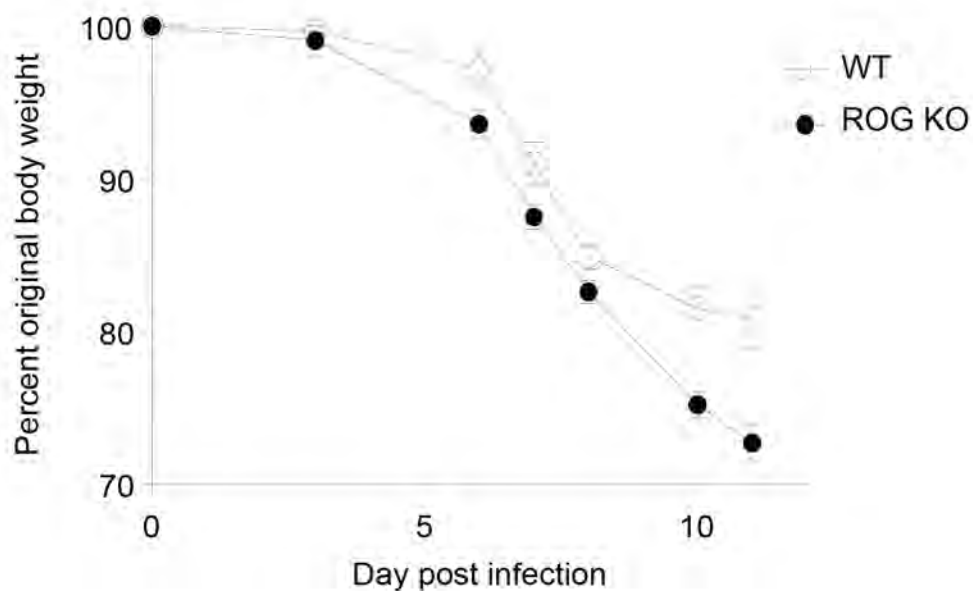
FIGURE V-21. Increased body weight loss of ROG-deficient mice during high dose LCMV Clone 13 infection. WT or ROG-deficient mice were infected intravenously with high dose LCMV Clone 13. The body weight of mice was recorded daily until day 12 post infection. The curve represents percent of the original body weight. The graph above is a representative of three independent experiment with at least four mice in each experiment.
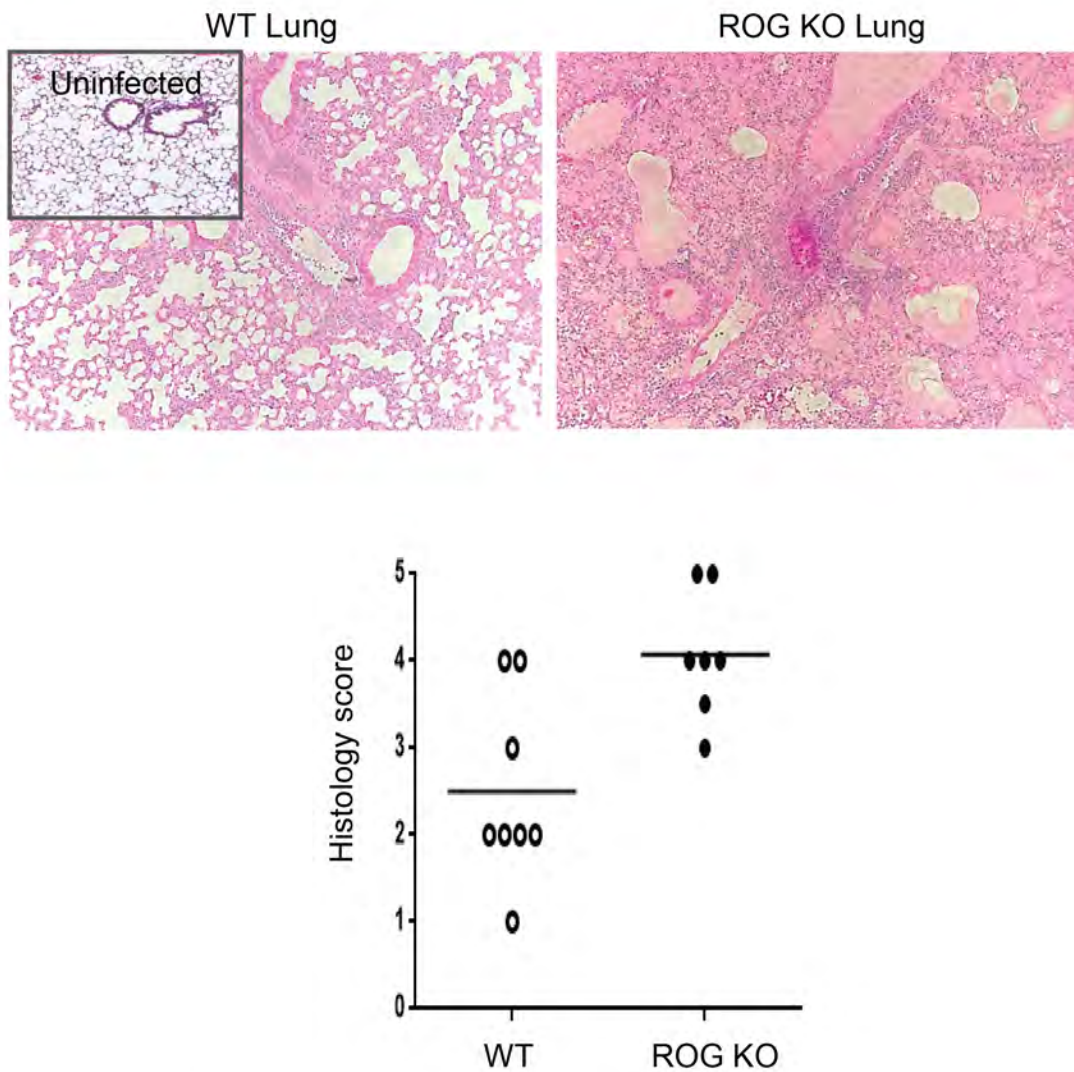
FIGURE V-22. Increased lung pathology in ROG-deficient mice during high dose LCMV Clone 13 infection. WT or ROG-deficient mice were infected intravenously with high dose LCMV Clone 13. Lung sections from WT or ROG-deficient mice were stained with hematoxylin and eosin (H&E) (top). WT uninfected lung is shown in the inset for comparison. Histology was scored (bottom) in a blind study on an arbitrary scale from 1-5 (1 being the best and 5 being the worst). The histology slides are a representative of two independent experiment with at least three mice in each experiment. The graph is a compilation of two independent experiments.

questioned whether ROG-deficient mice were generating a stronger T cell response to Clone 13 infection. To address this question, splenocytes and lung leukocytes were cultured directly *ex-vivo* for 5 hours in the presence of LCMV peptide epitopes NP396 and GP33. As detected by IFNγ production in response to cognate peptide, approximately twice as many NP396- and GP33-specific CD8⁺T cells were present in the spleen and lungs of *Zbtb32*⁻/⁻ mice at day 10 post infection when compared to WT controls (Figure V-23). A stronger CD8⁺T cell response might also lead to enhanced viral clearance and to test this idea I enumerated LCMV Clone 13 viral-titers in the spleen and liver of *Zbtb32*⁻/⁻ and WT mice at day 10 post infection. As shown in Figure V-24, viral clearance in the spleen of *Zbtb32*⁻/⁻ mice was enhanced compared to WT controls, though no significant differences were observed in the liver. Overall the data suggest that higher frequencies of IFNγ producing virus-specific CD8⁺T cells in *Zbtb32*⁻/⁻ mice also resulted in enhanced viral control following LCMV Clone 13 infection. However, systemic viral replication along with strong T cell responses in the ROG-deficient mice led to lung immunopathology and increased host mortality.

G. Discussion to Chapter V.

Transcription factors of the POZ family of proteins have been shown to play a role in T cell function and differentiation. This study was performed in collaboration with Dr. HyunMu Shin and Dr. Leslie J. Berg at UMMS. In this chapter I have shown that the transcription factor ROG plays a non-redundant
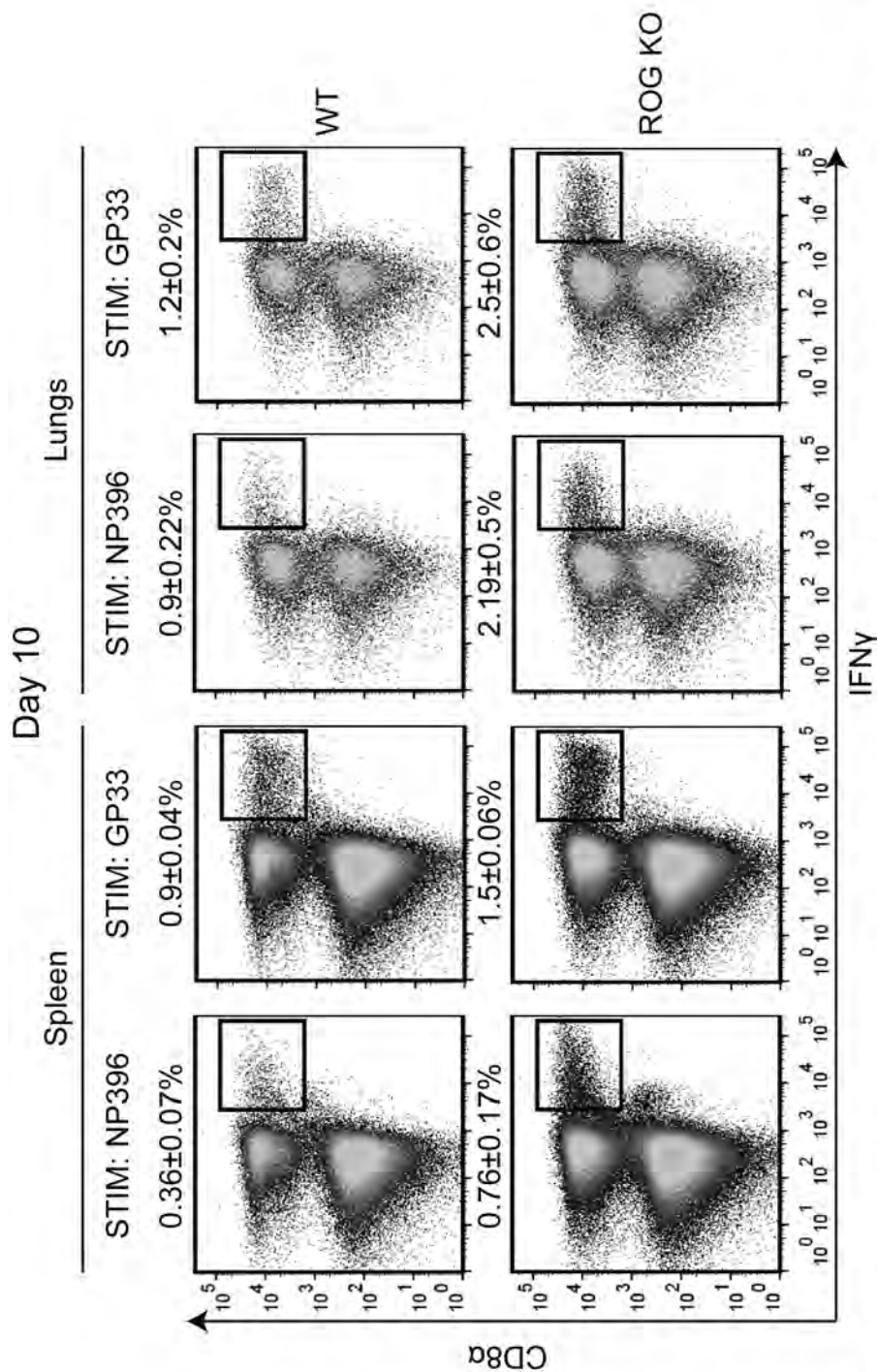
FIGURE V-23. Increased virus-specific T cell response in ROG-deficient mice during high dose LCMV Clone 13 infection. WT or ROG deficient mice were infected intravenously with high dose LCMV Clone 13. Day 10 LCMV-infected lymphocytes from spleen and lungs of WT and ROG-deficient mice were cultured for 5 hours in the presence of LCMV-specific NP396 and GP33 peptides followed by intracellular cytokine staining. Numbers in plots represent the percentage ± SEM of virus-specific CD8⁺ IFNγ⁺ T cells. Data are representative of two independent experiments with at least three mice in each experiment. FACS plots are gated on total lymphocytes.
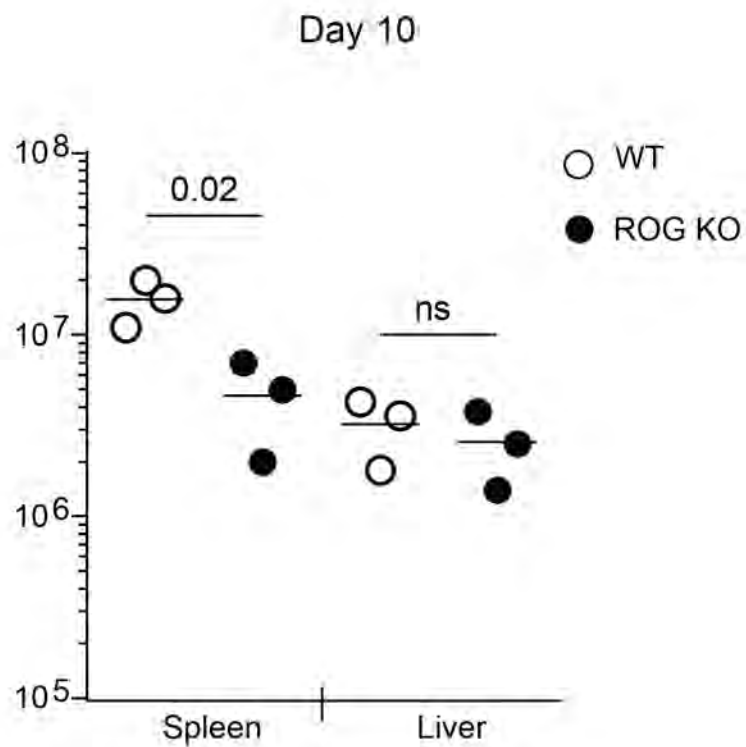
FIGURE V-24. Enhanced viral clearance in the spleen of ROG-deficient mice during high dose LCMV Clone 13 infection. LCMV Clone 13 titers in spleen and liver from infected WT and *Zbtb32*-/- mice were determined by plaque assay at day 10 post infection. Data are representative of two independent experiments with at least three mice in each experiment.

role in regulating T cell responses and memory generation during acute and chronic LCMV infection.

Initial studies done by Dr. HyunMu Shin had shown that ROG was induced by IL-2 signaling. A recently published study showed by ChIP-seq analysis that ROG is a direct target of STAT5 [199], consistent with our data that ROG is an IL-2 induced gene. Furthermore, by performing Q-PCR analysis to quantitate *Zbtb32* mRNA we found that IFNβ and IL-12 can also induce ROG in addition to IL-2 (data not shown). IL-2, IFNβ and IL-12 cytokines are induced early during an anti-viral response, consistent with the idea of ROG being an early induced gene.

In this study I have shown that ROG deficiency in CD8$^+$T cells leads to an increased magnitude of CD8$^+$T cell response. This could have been due to increased proliferation or decreased apoptosis of ROG-deficient T cells. I did not find any differences in active Caspase-3 staining between WT and *Zbtb32*$^{-/-}$ P14 CD8$^+$T cells at day 9 and day 15 post-LCMV infection (data not shown). Therefore, differences in T cell apoptosis do not account for increased proportion and absolute number of ROG-deficient T cells. To test whether ROG deficiency leads to increased T cell proliferation *in vivo*, I compared BrdU incorporation between day 7 LCMV-infected P14 TCR transgenic *Zbtb32*$^{-/-}$ or WT CD8$^+$T cells (data not shown). I did not find any differences in BrdU incorporation between WT or *Zbtb32*$^{-/-}$ P14 CD8$^+$T cells suggesting that there were no differences in

their proliferative capacities at the time point which was tested. Previous studies have shown that ROG-deficient T cells are more proliferative *in vitro* (154, 156). BrdU labels cells which are actively proliferating and undergoing DNA synthesis and is not an indicator of the number of divisions a cell has gone through. Therefore, due to this limitation of the BrdU assay I cannot conclude that there were no differences in proliferation between WT and ROG-deficient CD8^{+}T cells.

In response to a high dose LCMV Clone 13 infection ROG-deficient mice generated stronger T cell responses and had enhanced viral clearance compared to WT mice. However, I also saw increased lung pathology and mortality in the ROG-deficient mice. Since ROG-deficient mice had stronger T cell responses during LCMV Clone 13 infection, I questioned whether the pathology was CD4^{+}T cell dependent. I found no differences in survival of LCMV Clone 13 infected ROG-deficient mice in which CD4^{+}T cells had been depleted by an antibody treatment when compared to control-treated mice (data not shown), suggesting that CD4^{+}T cells did not contribute to the pathology. Though we have not directly tested, the immunopathology seen in ROG-deficient mice during persistent LCMV infection could be directly due to the increased perforin cytotoxicity or inflammatory cytokines being produced by CD8^{+}T cells and potentially other cell types.

To further elucidate the downstream mechanism by which ROG regulates CD8^{+}T cell responses, microarray analysis was done to compare gene

expression profiles in WT or *Zbtb32*^{-/-} P14 CD8^{+}T cells at day 6 and day 8 post-LCMV infection. Surprisingly, very few genes with a known function were found to be differentially regulated between *Zbtb32*^{-/-} and WT P14 CD8^{+}T cells at day 6 post-infection (Table V-1) and similar results were obtained at day 8 post-infection (data not shown). PVR/CD155 (polio virus receptor) is a member of the nectin family and is known to interact with activating T and NK cell receptor CD226 (200). The co-stimulatory CD155-CD226 axis is known to regulate pro-inflammatory balance in humans (201), and CD155 was shown to induce increased proliferation in Ras mutated cells (202). Consistent with our microarray analysis I found that CD155 was upregulated at the protein level on *Zbtb32*^{-/-} P14 CD8^{+}T at day 6 post-LCMV infection compared to WT controls (data not shown). Dr. HyunMu Shin also found CD7 mRNA to be upregulated on *Zbtb32*^{-/-} T cells compared to WT controls, and expression of CD7 on human CD8^{+}T cells has been shown to correlate with memory subsets (203). The transcription factor EOMES promotes persistence of memory CD8^{+}T cells (110). EOMES was not differentially expressed between *Zbtb32*^{-/-} and WT T cells in our microarray analysis, but, at the protein level higher proportion of EOMES-expressing CD8^{+}T cells were present in the ROG-deficient mice at day 8 (Figure V-15) and day 15 (data not shown) post-LCMV infection. Our microarray analysis has revealed some potentially interesting downstream targets of ROG, and if any of these genes contribute to the ROG phenotype and are directly regulated by ROG remains to be tested.

Gene Symbol	Fold Change $Zbtb32^{-/-}$ / WT	Description
Pglyrp1	3.6	peptidoglycan recognition protein 1
Klk1b11	2.7	kallikrein 1 - related peptidase b11
Ccr9	2.2	chemokine (C-C motif) receptor 9
Pvr	2.0	poliovirus receptor
Tigit	1.7	T cell immunoreceptor with Ig and ITIM domains
Trerf1	1.7	transcriptional regulating factor 1
Cd7	1.7	CD7 antigen
Tnfrsf8	1.4	tumor necrosis factor receptor superfamily, member 8
Gzma	-1.6	granzyme A
Ccr2	-2.3	chemokine (C-C motif) receptor 2
Zbtb32	-11.1	zinc finger and BTB domain containing 32

Table V-1. Microarray analysis to compare differentially regulated genes between ROG-deficient and WT T cells at day 6 post-LCMV infection. Day 6 LCMV-infected P14 TCR transgenic WT or $Zbtb32^{-/-}$ CD8$^+$T cells were sorted and total RNA was isolated. Affymetrix GeneChip Mouse Gene 1.0 ST array was used, and data were analysed on Affymetrix Transcriptome Analysis Console (TAC) software. Fold change of genes with a known function expressed in $Zbtb32^{-/-}$ CD8$^+$T cells relative to WT is listed in the table. Data are compilation of two independent experiments. This table was generated by Dr. HyunMu Shin.

Overall in the studies highlighted in this chapter I show that ROG plays a non-redundant role in regulating CD8$^+$T cell response during acute and chronic viral infections. Interestingly, ROG deficiency was beneficial to the host in an acute infection setting and led to generation of a stronger T cell response and better memory population compared to WT controls. Contrary to an acute infection setting, ROG deficiency during a chronic LCMV infection led to immunopathology and host mortality. Therefore these studies highlight a unique system where deficiency of a transcription factor can have both beneficial and detrimental effects depending on the infection model used (Figure V-25). Furthermore, it also highlights the importance of carefully considering the effects of modulating the transcriptional machinery to regulate the balance between effector and memory T cell generation during different infections, as it might lead to different potential outcomes.
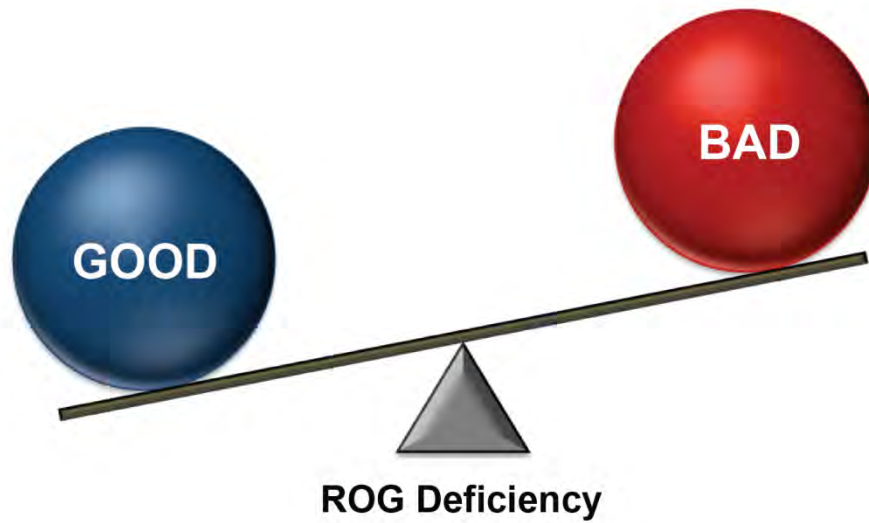
Acute LCMV Armstrong Infection

↑ CD8+T cell response

↑ Quantity and quality of memory CD8+T cells

High Dose LCMV Clone 13 Infection

↑ Immunopathology

↑ Mortality

Figure V-25. Differential effects of ROG deficiency during acute and chronic LCMV infection.

CHAPTER VI: DISCUSSION

For a successful immune response, it is essential that T cells eradicate the pathogen and form a population of long-lived memory T cells to prevent re-infection by the same pathogen. During an immune response the effector CD8$^+$T cell pool is heterogeneous, and comprises of short-lived effector cells and memory precursor cells. Several surface antigenic markers can be used to identify T cells with a memory potential (68), but we are only beginning to elucidate the mechanisms underlying transcriptional regulation of effector-memory CD8$^+$T cell differentiation (68). Understanding the mechanisms that govern memory CD8$^+$T cell differentiation and maintenance is essential to aid both preventative and therapeutic vaccine designs.

T cells activated in the secondary lymphoid organs migrate to non-lymphoid tissues to eliminate the foreign antigen (50, 128-130, 204). Since most of the infections are initiated in the non-lymphoid tissues, the presence of memory T cells in the peripheral non-lymphoid tissues is critical for protective immunity during secondary infections (133, 137, 138). Our lab has previously shown that during and after resolution of the immune response, LCMV-specific CD8$^+$T cells in the non-lymphoid tissues are more resistant to apoptosis than those in the lymphoid organs (139). Understanding the mechanisms which regulate T cell survival in non-lymphoid tissue environments is important, as this stability of T cells in the peripheral tissues bolsters protective immunity to secondary

infections. This thesis specifically studies mechanisms regulating survival of T cells in non-lymphoid tissues and transcription-factor mediated regulation of effector-memory T cell generation.

In chapter III, I have examined the factors contributing to decreased T cell apoptosis in the non-lymphoid tissues. As a representative of non-lymphoid tissue, I have primarily looked at the peritoneal cavity, which is also the site of antigen delivery in my experiments. The development of immune responses and dynamics of T cell migration into the peritoneal environment are not fully understood. The peritoneal cavity is unique from other non-lymphoid tissues as it lacks a defined architecture and comprises of peritoneum wall, omentum, mesentery and free cells present in the peritoneal fluid. Although not considered as a true secondary lymphoid organ, the milky spots consists of lymphocytes embedded in the omentum and can drive T and B cell responses in response to peritoneal antigens (205). Omentum was also shown to be a site for generation of NK and T cell dependent anti-tumor response following delivery of tumor cells to the peritoneal cavity (206). However, one study identified mediastinal lymph nodes as a primary site for initial T cell responses to antigens delivered into the peritoneal cavity (207). Since the kinetics of T cell response in the mediastinal lymph nodes are faster when compared to other lymphoid and non-lymphoid tissues during an immune response to intra-peritoneally delivered LCMV (207), I chose not to look at mediastinal lymph nodes while comparing tissue-dependent T cell apoptosis in my studies. Several studies have shown that following

activation in the secondary lymphoid organs T cells need to express appropriate chemokine receptors or integrins to gain entry into the non-lymphoid tissues (208). For instance, migration into the small intestine epithelium requires expression of CCR9 and α4β7 integrin (209), whereas migration to the skin requires expression of cutaneous lymphocyte antigen (CLA) (210). T cell recruitment to the peritoneum can be mediated by P and E selectins (211), and in this thesis I have shown that CXCR3 may play a role in driving T cell migration into the peritoneal cavity.

Recent studies have identified a subset of cells called the resident memory T cells (Trm), which specifically reside and persist in the non-lymphoid tissues without recirculating (212-215). Trm cells express CD103 and CD69, and are maintained by action of the cytokines TGFβ and IL-15 (213, 215). Our lab has previously shown that TGFβ may be one of the factors promoting T cell survival in the peritoneal environment (139). Furthermore, I have found that virus-specific CD8$^+$T cells in the PEC have increased expression of CD122 (Figure III-34), which may allow for IL-15 mediated T cell survival. Peritoneal cavity is considered an 'effector permissive' tissue, as it is accessible to effector T cells during an immune response after which it becomes inaccessible to circulating memory T cells (208, 216). Parabiosis experiments have shown that non-lymphoid tissues can differ in their permissiveness to memory T cells (210). Unlike lungs and liver, the entry into peritoneal cavity, brain and lamnia propria by memory T cells is restricted (210). Thus it is possible that majority of the memory T cells in

the peritoneal cavity would be generated from the effector T cells which are seeded during the primary immune response. Therefore, as elucidated in this thesis, factors promoting survival of T cells in the peritoneal cavity during resolution of the immune response will be critical in ensuring maintenance and survival of appropriate numbers of functional memory T cells to provide protection to secondary challenges. Furthermore, analogous mechanisms might also regulate T cell survival and memory development in other restricted non-lymphoid tissues.

Similar transcriptional programs might regulate generation and survival of memory T cells in the lymphoid and non-lymphoid tissues. Consistent with this idea, I have several lines of preliminary evidence indicating that differential expression of Blimp-1 might contribute to tissue-dependent apoptotic differences: a) in a single experiment I found that decreased proportions of Blimp-1-expressing LCMV-specific GP33$^+$CD8$^+$T cells were present in the PEC compared to spleen at day 10 (49±4.1% in the PEC vs 66.3±3.3% in the spleen) and day 15 (32.3±3.3% in the PEC vs 59.1±4.8% in the spleen) post-LCMV infection, b) microarray analysis comparing virus-specific CD8$^+$T cells in the PEC and spleen at day 9 post-LCMV infection revealed a 2 fold up-regulation of Blimp-1 message in the spleen compared to the PEC, c) higher proportions of CD27 expressing T cells were present in the PEC compared to spleen (Figure III-9), and we have identified CD27 as a Blimp-1 regulated gene in chapter IV, d) approximately 100% of virus-specific Blimp-1-deficient T cells were found to be expressing

TCF-1 at day 8 post-LCMV infection (data not shown), suggesting that Blimp-1 might be a master regulator of TCF-1, and I have shown that TCF-1 is important in promoting survival of T cells in the non-lymphoid tissues during resolution of the immune response (Figure III-20), e) decreased Blimp-1 expression in the PEC further supports the idea of increased IL-2 being produced in the PEC, since Blimp-1 is known to suppress IL-2 production in T cells (193). Future studies will need to address whether differential expression of Blimp-1 might also contribute to tissue dependent apoptotic differences.

The studies highlighted in chapter IV, clearly demonstrate that Blimp-1 negatively regulates CD25 (IL-2Rα) and CD27 expression to impact the balance of short-lived and memory precursor effector CD8$^+$T cells responding to an acute virus challenge. One of the important findings of this study is the differential function of IL-2Rα signaling at T cell priming and contraction. IL-2Rα signaling during T cell priming promotes generation of short-lived effectors (80, 91), but during the peak and resolution of the immune response IL-2Rα signals promote generation of memory precursors, and this change is dependent on Blimp-1. In accordance with our results, timing of IL-2 therapy delivered in form of IL-2 complexes to IL-2-deficient CD4$^+$T cells during priming phase of the immune response to Influenza virus infection leads to generation of effector-like CD4$^+$T cells, whereas IL-2 therapy during peak of the response promotes generation of memory like CD4$^+$T cells (unpublished observation, personal communication Dr. Kai Mckinstry and Dr. Tara Strutt). Interestingly, Blimp-1 is itself an IL-2-induced

gene and, in an auto-regulatory feedback loop, shuts down IL-2 production to regulate the effector T cell pool (193). Genome wide ChIP-sequencing analysis done by our collaborating group revealed a large number Blimp-1 target genes and these studies will provide a starting ground to identify other mechanisms by which Blimp-1 suppresses memory CD8$^+$T cell differentiation.

In the studies presented in Chapter V, I have shown that the transcription factor ROG negatively regulates CD8$^+$T cell responses and memory generation during viral infections. In collaboration with Dr. HyunMu Shin and Dr. Leslie J. Berg we are trying to elucidate upstream and downstream mechanisms by which ROG regulates *in vivo* T cell responses. Similar to Blimp-1, we found that ROG is an IL-2 induced gene. A recent study showed that ROG is a STAT5 target gene (199), consistent with ROG being an IL-2 regulated gene. Interestingly, in B cells ROG was shown to negatively regulate CIITA and MHC-II gene expression (157), a process also regulated by Blimp-1. Moreover, in this study Blimp-1 was shown to interact with ROG. Blimp-1 and ROG are transcriptional repressors, and both of them can suppress memory T cell generation as shown in Chapter IV and V. Therefore, it is possible that ROG and Blimp-1 might negatively regulate similar genes in T cells. Only a few genes were shown to be differentially regulated between WT and *Zbtb32*$^{-/-}$ T cells in our microarray analysis at day 6 and day 8 post-LCMV infection (Table V-1). This could have been due to an overlapping function of ROG and Blimp-1 in T cells, as Blimp-1 is also expressed at high levels at these time points and Blimp-1 is still active in *Zbtb32*$^{-/-}$ T cells. However,

the T cell phenotype seen during ROG deficiency indicates that ROG also plays a non-redundant role in regulating *in vivo* T cell responses. It is tempting to speculate that Blimp-1 and ROG might co-operate to negatively regulate T cell responses and memory generation during viral infections.

Recent studies based on kinetic analysis of gene expression changes in CD8^{+}T cells during an immune response have revealed that a network of genes work in coordinated fashion to regulate CD8^{+}T cell differentiation [127, 217]. These studies show that during an immune response transcription factors can regulate a large cluster of genes which are induced or suppressed in a concerted fashion and their kinetic regulation can give us insights into the biological process they control. In those studies Blimp-1 was shown to be in a cluster of genes which regulate effector-memory differentiation [127], and our studies have revealed that Blimp-1 can directly negatively regulate a number of transcription factors known to be associated with memory CD8^{+}T cell differentiation [81]. Analysis of transcriptional networks did not identify ROG as one of the signature genes involved in effector-memory transition [127, 217]. However, gene expression analysis of Zn finger containing transcription factors during both acute and chronic LCMV infection [89] have shown that kinetics of ROG expression are similar to Blimp-1, although ROG expression declines much faster than Blimp-1 once the immune response has resolved. Thus, the expression kinetics and function of ROG as presented in this thesis suggest that ROG might be a part of

the same cluster of genes as Blimp-1 and forms a part of a network to promote terminal effector fate in CD8$^+$T cells.

Understanding the dynamics which regulate the fate of a T cell on a single cell level might also help us gain insights into how transcriptional programs are imprinted during course of the immune response. Some elegant studies have been done to track the fate of individual CD8$^+$T cell precursor during an infection (218-220). It was shown that diverse types of effector CD8$^+$T cell progeny are generated from a single cell clone and inflammatory microenvironment and tissue specific factors can influence this heterogeneity on a populational level (218). Interestingly, it has been shown that SLEC and MPEC populations reside in different areas in the spleen supporting the idea that microenvironment can influence T cell fate (221). SLECs localize to red pulp areas whereas MPECs localize to white pulp and T cell zones where they interact with stromal cells which produce IL-7. The studies highlighted in Chapter III show that tissue environment can also influence T cell fate and a higher proportion of T cells with an antigenic and transcription factor phenotype correlating with enhanced memory and survival were found in the non-lymphoid tissues when compared to lymphoid organs. Therefore, individual T cells can receive cues from the tissue microenvironment to induce an antigenic and transcriptional profile governing their fate.

For an optimal response, CD8^{+}T cells require antigenic stimulation through the TCR, as well as co-stimulation and cytokine stimulation via inflammatory cytokines. The quantity and quality of these three signals inscript a transcriptional program governing the fate of effector T cells. It is now evident that several transcription factors act in concert to regulate the balance between short-lived effector and memory T cell generation. In this thesis we have identified one of the mechanism by which transcription factor Blimp-1 regulates the balance between effector and memory T cell generation. We also found that the transcription factor ROG negatively regulates CD8^{+}T cell responses and memory development. Stability of memory T cells at non-lymphoid tissues may be critical for immunity to secondary challenges and we found that decreased T cell apoptosis in the non-lymphoid tissues was due to a combination of several factors. As shown in Figure VI-1, several pathways act in conjunction to restore homeostasis and promote development of memory T cells in lymphoid and non-lymphoid tissues to provide protection to secondary challenges. This thesis will contribute to our understanding of the mechanisms promoting stability of T cells in non-lymphoid tissues and transcriptional regulation of memory CD8^{+}T cell differentiation. The knowledge of these processes could eventually allow us to manipulate T cells and their environment to improve treatment for infectious diseases and generate better vaccine designs.
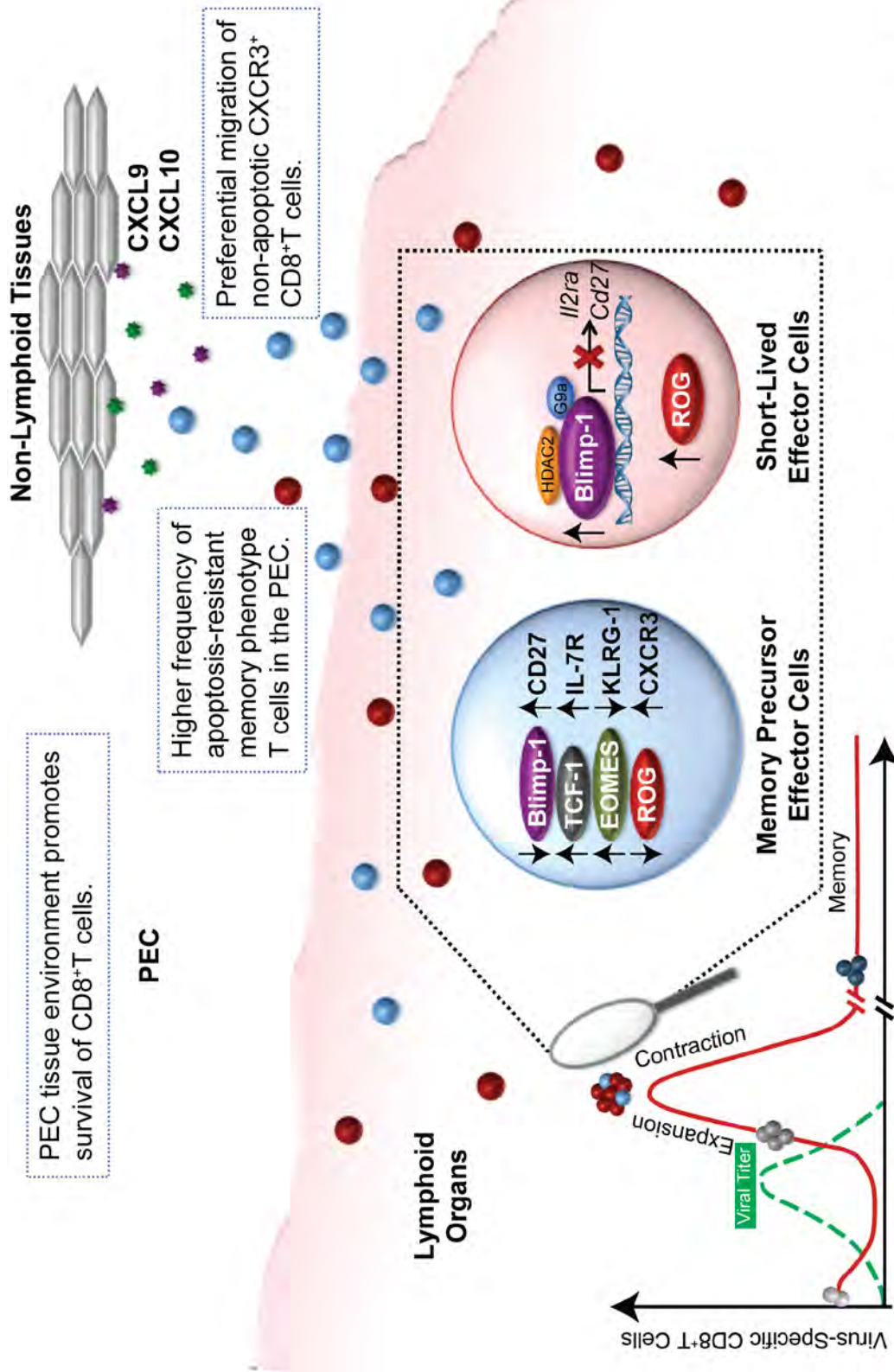
Figure VI-1. Mechanism regulating tissue-dependent T cell apoptosis and transcriptional regulation of memory CD8⁺T cell differentiation during viral infections.

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