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Attrition of CD8 T Cells During The Early Stages of Viral Infections

A Dissertation Presented

By

Kapil Bahl

Submitted to the Faculty of the University of Massachusetts Graduate

School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

January 9, 2008

Immunology and Virology

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A Dissertation Presented
By

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Abstract

Profound lymphopenia has been observed during many acute viral infections, and our laboratory has previously documented a type 1 IFN-dependent loss of most memory ($CD44^{hi}$) and some naïve ($CD44^{lo}$) CD8 T cells immediately preceding the development of the antiviral T cell response at days 2-4 following lymphocytic choriomeningitis virus (LCMV) infection. In this thesis, I will examine additional mechanisms involved in the early attrition of CD8 T cells and evaluate whether antigen-specific and non-specific CD8 T cells are equally susceptible. Lastly, I will examine whether the early attrition of CD8 T cells contributes to the generation of an effective immune response.

Poly(I:C), a potent inducer of type 1 IFN, was previously shown to cause the attrition and apoptosis of $CD8\alpha^+CD44^{hi}$ cells in normal mice, but not in type 1 IFN receptor-deficient mice (IFN1-R KO). I questioned whether additional molecule(s) might contribute to the type 1 IFN-induced apoptosis of $CD8\alpha^+CD44^{hi}$ cells. I used a PCR array to determine the expression of 84 apoptosis-related genes at 6 hours post-poly(I:C) treatment, relative to an untreated control. There was an 11-fold increase in CD40 RNA expression in $CD8\alpha^+CD44^{hi}$ cells isolated from poly(I:C)-treated mice. CD40 protein expression was also increased on $CD8\alpha^+CD44^{hi}$ cells, peaking between 9 and 12 hours following poly(I:C) treatment, before declining thereafter. This increase in CD40 protein expression directly correlated with an increase in Annexin V reactivity, an indicator of early apoptosis. Nevertheless, CD40 was not required for the loss of $CD8\alpha^+CD44^{hi}$ cells, as both wildtype and CD40-deficient mice were equally

susceptible to the poly(I:C)-induced attrition. Upon further characterization, I found this population of $CD40^+CD8\alpha^+CD44^{hi}$ cells to be $CD11c^+B220^-Thy1.2^-MHCII^{hi}$, which is consistent with a “lymphoid” $CD8\alpha^+$ DC phenotype. Kinetic analysis revealed a type 1 IFN-dependent increase in this $CD8\alpha^+$ DC population at 12 hours post-poly(I:C) treatment. This increase was only observed in the spleen, as no increase in percentage was observed in the peritoneal cavity (PEC), lungs, inguinal lymph nodes (iLN), or peripheral blood. Collectively, these results suggest that the type 1 IFN-dependent increase in splenic $CD8\alpha^+$ DCs accounts for the observed increase in Annexin V reactive cells following poly(I:C) treatment.

These findings required a re-evaluation of the type 1 IFN-induced attrition of $CD8^+CD44^{hi}$ T cells with an anti- $CD8\beta$ antibody, which is a more exclusive marker for T cells than the anti- $CD8\alpha$ antibody. Kinetic analysis revealed a significant decrease in splenic $CD8\beta^+CD44^{hi}$ T cells at 12 hours post-poly(I:C) treatment. This reduction in splenic $CD8\beta^+CD44^{hi}$ T cells was not due to trafficking to other organs, as the PECs, lungs, iLN, lungs, and peripheral blood all exhibited significant, although varying, decreases in the percentage of $CD8\beta^+CD44^{hi}$ T cells at 12 hour following poly(I:C) treatment. These data support the notion that the type 1 IFN-induced attrition of $CD8\beta^+CD44^{hi}$ T cells was a “global” phenomenon and could not be completely due to migration out of the spleen.

The attrition of $CD8\beta^+CD44^{hi}$ T cells was also dependent upon type 1 IFN at 3 days post-LCMV infection, as there was no significant reduction of this

population in IFN1-R KO mice. The loss of wildtype $CD8\beta^+CD44^{hi}$ T cells correlated with an increased activation of caspases 3 and 8, which are enzymes that play essential roles in apoptosis and inflammation. A significant loss of $CD4^+CD44^{hi}$ T cells, which also correlated with an increased activation of caspases 3 and 8, was observed at 3 days post-LCMV infection. Collectively, these results suggest that attrition of both $CD4^+CD44^{hi}$ and $CD8\beta^+CD44^{hi}$ T cell populations is type 1 IFN-dependent and associated with the activation of caspases following LCMV infection.

At 3 days post-LCMV infection, both wildtype $CD8\beta^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cell populations had a higher frequency of cells with fragmented DNA, a hallmark characteristic of the late stages of apoptosis, as revealed by terminal transferase dUTP nick end labeling (TUNEL), relative to uninfected controls. This suggests that the loss of both populations was due to apoptosis. Therefore, I questioned whether the LCMV-induced apoptosis of both $CD4^+CD44^{hi}$ and $CD8\beta^+CD44^{hi}$ T cell populations occurred through a mitochondrial-induced pathway involving the pro-apoptotic molecule Bim. The attrition of both $CD4^+CD44^{hi}$ and $CD8\beta^+CD44^{hi}$ T cells was significantly higher in wildtype mice compared to Bim KO mice at 3 days post-LCMV infection. Moreover, both wildtype $CD8\beta^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cell populations had higher frequency of TUNEL⁺ cells, relative to Bim KO populations. These results suggest that the apoptosis of $CD8\beta^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cells, following LCMV infection, might occur through a mitochondrial-induced pathway involving Bim.

Studies have shown “lymphoid” CD8 α^+ DCs to be involved in the phagocytosis of apoptotic lymphocytes. Therefore, I evaluated whether host CD8 α^+ DCs are capable of phagocytosing apoptotic lymphocytes by adoptively transferring CFSE-labeled wildtype donor splenocytes (Ly5.1) into congenic wildtype hosts (Ly5.2), followed by inoculation with poly(I:C). There was an increased frequency of donor cells (Ly5.1, CFSE $^+$) within the host CD8 α^+ CD11c $^+$ gate at 9 and 12 hours post-poly(I:C) treatment. The results suggest that type 1 IFN-activated CD8 α^+ DCs might aid in the rapid clearance of apoptotic cells during the type 1 IFN-induced attrition associated with viral infections.

I next questioned whether TCR engagement by antigen would render CD8 T cells resistant to attrition. I tested whether a high concentration of antigen (GP33 peptide) would protect LCMV-specific naïve TCR transgenic P14 cells specific for the GP33 epitope of LCMV and GP33-specific LCMV-immune cells from depletion. Both naïve P14 and memory GP33-specific donor CD8 T cells decreased substantially 16 hours after inoculation poly(I:C), regardless of whether a high concentration of GP33 peptide was administered to host mice beforehand. The increased activation status of naïve antigen-specific cells via peptide inoculation did not confer resistance to type 1 IFN-induced depletion. Donor naïve P14 and LCMV-specific memory cells were also depleted from day 2 LCMV-infected (Clone 13) hosts by 16 hours post-transfer. These results indicate that antigen engagement does not protect CD8 T cells from the type 1 IFN-induced attrition associated with viral infections.

Computer models indicated that early depletion of memory T cells may allow for the generation for a more diverse T cell response to infection by reducing the immunodomination caused by cross-reactive T cells. To test this in a biological system, I questioned whether the reduced apoptosis of the cross-reactive memory CD8 population (NP205), in aged LCMV-immune mice (18-22 months), following heterologous virus challenge (PV), would allow it to dominate the immune response. At day 8 post-PV infection, the cross-reactive memory CD8 T cell response (NP205) was more immunodominating in aged LCMV-immune mice relative to younger LCMV-immune mice. This was indicated by the increased ratio of the cross-reactive NP205 response to the newly arising non-cross-reactive, PV-specific NP38 response in older LCMV-mice relative to younger LCMV immune-mice, at day 8 post-PV infection. These data suggest that the early attrition of T cells allows for the generation of a more diverse T cell response to infection by reducing the immunodomination caused by cross-reactive T cells. Collectively, these findings offer further insight into the early attrition of T cells associated with viral infections.

Table of Contents

	Page
Copyright Notice	ii
Signature Page	iii
Acknowledgements	iv
Abstract	v
Table of Contents	x
List of Tables	xiv
List of Figures	xv
Abbreviations	xviii
Chapter I: Introduction	1
A. Lymphocytic choriomeningitis virus (LCMV).....	1
B. Immune Response to LCMV.....	3
C. Type 1 IFN Response to Viral Infection.....	6
D. Apoptotic Signaling Pathways.....	11
E. Type 1 IFN and Apoptosis.....	15
F. Dendritic Cells.....	18
G. Attrition of CD8 ⁺ T Cells and Heterologous Immunity.....	20
H. Thesis Objectives.....	23
Chapter II: Materials and Methods	24
A. Virus stocks and inoculation.....	24

B. Mice.....	24
C. RNA Isolation and Gene Expression Profiling.....	25
D. Apoptosis Assays (Caspase Activation, Annexin V Staining, TUNEL Staining).....	26
E. Synthetic peptides.....	27
F. Adoptive transfers.....	28
G. Inoculations.....	29
H. Intracellular IFN- γ staining.....	29
I. Computer modeling.....	30
J. Statistical analyses.....	30

Chapter III: Apoptotic Properties of Cells During the Early Stage of the Immune Response to LCMV Infection.....	32
A. Type 1 IFN-induced attrition of CD8 α^+ CD44 ^{hi} cells.....	33
B. An increase in “lymphoid” CD8 α^+ DCs accounts for the observed increase in Annexin V reactive CD8 α^+ CD44 ^{hi} cells following poly(I:C) treatment.....	37
C. Re-evaluation of the type 1 IFN-Induced attrition of CD8 T Cells.....	52
D. Re-evaluation of the type 1 IFN-Induced attrition of CD8 T cells associated with viral infection.....	55
E. The type 1 IFN-induced attrition of CD4 T cells associated with viral infection.....	59
F. Apoptosis of T Cells During the Early Immune Response to LCMV.....	61

G. Bim-Mediated Apoptosis of T Cells During the Early Immune Response to LCMV.....	63
H. The Role of “lymphoid” CD8 α^+ Dendritic Cells During type 1 IFN-Induced Apoptosis of T Cells.....	68

Chapter IV: Type 1 IFN-Induced Attrition of CD8 T Cells in the Presence or Absence of Cognate Antigen During the Early Stages of LCMV Infection.....70

A. GP33-45 peptide does not protect naïve antigen-specific CD8 T cells from depletion induced by poly(I:C).....	71
B. Antigen-specific naïve CD8 T cells are not protected from depletion during the early phase of an acute viral infection.....	76
C. GP33-45 peptide does not protect antigen-specific memory CD8 T cells from depletion by poly (I:C) treatment.....	79
D. Antigen-specific memory CD8 T cells are not protected from depletion during the early phase of an acute viral infection.....	82

Chapter V: Early CD8 T Cell Attrition Contributes to the Development of a Diverse Immune Response Following Viral Infection.....84

A. Computer modeling predicts that early attrition of memory CD8 T cells allows for more diversity in newly arising T cell responses.....	85
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B. Reduced attrition of cross-reactive memory cells in old LCMV-immune mice limits the diversity of the ensuing immune response to heterologous Pichinde infection.....88

Chapter VI: Discussion.....94

A. A re-assessment of the type 1 IFN-induced apoptosis of T cells.....94

B. A role for “lymphoid” CD8 α^+ DCs during the type 1 IFN-induced early lymphopenia.....105

C. Antigen engagement does not protect CD8 T cells from the type 1 IFN-induced attrition associated with viral infections.....107

D. Early attrition of CD8 T cells allows for greater diversity during challenge with a heterologous virus.....110

Chapter VII: References.....114

List of Tables

	Page
Table 1. Parameters of type 1 IFN-induced attrition.....	36
Table 2. Description of Bim-related mutant and knockout mice.....	101

List of Figures

	Page
Figure 1. The type 1 IFN-induced attrition of CD8 α ⁺ CD44 ^{hi} cells is associated with an increase in Annexin V reactivity.....	34
Figure 2. Increase in Annexin V reactivity correlates with an increase in CD40 expression on CD8 α ⁺ CD44 ^{hi} cells at multiple timepoints following poly(I:C) treatment.	39
Figure 3. Both wildtype and CD40 KO CD8 α ⁺ CD44 ^{hi} cells are equally susceptible to type 1 IFN-induced attrition.	41
Figure 4. The CD8 α ⁺ CD44 ^{hi} population contains a non-T cell, Annexin V reactive sub-population following poly(I:C) treatment.	44
Figure 5. The Thy1.2 ⁻ CD40 ⁺ CD8 α ⁺ CD44 ^{hi} population displays a “lymphoid” DC phenotype.....	46
Figure 6. CD8 α ⁺ DCs increase in number following poly(I:C) treatment.....	48
Figure 7. The increased percentage of the CD8 α ⁺ DCs in the spleen is not observed in other compartments following poly(I:C) treatment.....	49
Figure 8. The increase in the percentage of the CD8 α ⁺ DCs in the spleen is type 1 IFN-dependent.....	51
Figure 9. CD8 β ⁺ CD44 ^{hi} T cells undergo attrition following poly(I:C) treatment.....	53
Figure 10. Trafficking to other compartments does not account for the loss of splenic CD8 β ⁺ CD44 ^{hi} T cells following poly(I:C) treatment.....	54

Figure 11. Increase in Annexin V reactivity correlates with an increase in CD40 expression on CD8 α ⁺ CD44 ^{hi} , but not CD8 β ⁺ CD44 ^{hi} cells, following LCMV infection.....	56
Figure 12. The attrition of CD8 β ⁺ CD44 ^{hi} T cells, following LCMV infection, is type 1 IFN-dependent and correlates with an increase in caspase activation.....	58
Figure 13. The attrition of CD4 ⁺ CD44 ^{hi} T cells, following LCMV infection, is type 1 IFN-dependent and correlates with an increase in caspase activation.....	60
Figure 14. The LCMV-induced attrition of CD8 β ⁺ CD44 ^{hi} and CD4 ⁺ CD44 ^{hi} T cells is due to apoptosis.....	62
Figure 15. The LCMV-induced apoptosis of CD8 β ⁺ CD44 ^{hi} T cells is partially dependent on Bim.....	65
Figure 16. The LCMV-induced apoptosis of CD4 ⁺ CD44 ^{hi} T cells is partially dependent on Bim.....	67
Figure 17. CD8 α ⁺ CD11c ⁺ DCs may contribute to the rapid clearance of apoptotic cells.....	69
Figure 18. A high dose of antigen does not protect naïve antigen-specific CD8 T cells from depletion.....	75
Figure 19. High viral load does not protect antigen-specific naïve CD8 T cells from depletion during the early phase of an acute viral infection.....	78
Figure 20. A high dose of antigen does not protect antigen-specific memory CD8 T cells from depletion.....	81

Figure 21. High viral load does not protect antigen-specific memory CD8 T cells from depletion during the early phase of an acute viral infection.....	83
Figure 22. Computer modelling indicates that the early apoptosis of memory cells allows for more diversity in arising T cell responses.....	87
Figure 23. Reduced attrition in old LCMV-immune mice limits the diversity in the arising T cell response following heterologous Pichinde viral challenge.....	91-93
Figure 24. Donor IFN1-R KO memory CD8 T cells undergo attrition in wildtype hosts following poly(I:C) treatment.....	104

Abbreviations

AAD	amino-actinomycin D
AAF	IFN- α activated factor
AICD	activation-induced cell death
APC	antigen presenting cells
APC	allophycocyanin
BAD	BCL2/BCLXL associated death promoter
BAX	BCL2 associated X protein
BAK	BCL2 antagonist killer
BH3	BCL2 homology 3
BID	BH3 interacting Domain
BIK	BCL2 interacting Killer
BIM	BCL2 interacting molecule
BMF	BCL2 modifying factor
BRDU	bromodeoxyuridine
CARD	caspase recruitment domains
CFSE	carboxyfluoroscein succinimidyl ester
C _T	threshold cycle
CTL	cytotoxic T lymphocyte
CY	cyanine
CYT	cytochrome
DC	dendritic cells
DD	death domain

DED	death effector domain
DISC	death-inducing signaling complex
DN	double negative
DNA	deoxyribonucleic acid
DR	death receptor
DTR	diphtheria toxin receptor
dUTP	2'-deoxyuridine 5'-triphosphate
FADD	fas-associated death domain
FLICE	caspase-8/FADD-like interleukin-1beta converting enzyme
FLIP	FLICE-like inhibitory protein
GAS	IFN γ activated site
GLD	generalized lymphoproliferative disease
GP	glycoprotein
HBSS	Hanks balanced salt solution
HSV	herpes simplex virus
IFN	interferon
iLN	inguinal lymph nodes
IP	intraperitoneal
IRF	IFN regulatory 9
ISGF3	IFN stimulated gene factor
ISRE	IFN stimulated response element
IV	intravenous

JAK	Janus kinase
KO	knockout
LC8	dynein light chain 8
LCMV	lymphocytic choriomeningitis virus
LPR	lymphoproliferation
MCL1	myeloid cell leukemia 1
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation antigen 5
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
NFkB	nuclear factor kappa B
NK	natural killer
NP	nucleoprotein
PCR	polymerase chain reaction
PE	phycoerytherin
PEC	peritoneal exudate cells
PERCP	peridinin-chlorophyll-protein complex
PKR	double-stranded RNA-activated protein kinase
POLY(I:C)	polyinosine:polycytidylic acid
PRR	pattern-recognition receptor
PS	phosphatidylserine
PV	Pichinde virus
RAG	recombinase-activating gene

RIGI	RNA helicases retinoic acid inducible gene-I
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	real-time
STAT	signal transducers and activators of transcription
TCR	T cell receptor
TIM4	T-cell immunoglobulin- and mucin-domain-containing molecule
TLR	toll-like receptor
TNFRSF	tumor necrosis factor receptor super family
TNFR	tumor necrosis factor receptor
TRADD	tumor necrosis factor receptor-associated death domain
TRAIL	tumor necrosis factor related apoptosis inducing ligand
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
TYK	tyrosine kinase
VSV	vesicular stomatitis virus
VV	vaccinia virus

Chapter I: Introduction

A. Lymphocytic Choriomeningitis Virus (LCMV)

LCMV is the prototypic virus of the arenavirus group, which consists of ambisense, enveloped RNA viruses that cause persistent infections in a variety of rodent populations and occasionally cause human infections. The arenavirus group includes a variety of Old World and New World viruses. LCMV is an Old World virus whose natural host is *Mus musculus*. The LCMV virion contains two ambisense RNAs, each encoding two genes of opposite polarity separated by a double-stranded hairpin structure. The large (L) RNA encodes (in a 5' to 3' direction) both the small zinc-binding protein (Z), which facilitates virus budding from the plasma membrane (Perez, Craven et al. 2003), and the RNA-dependent RNA polymerase, which is required for transcription and replication of the viral genome. The short (S) RNA contains the genes that encode (in a 5' to 3' direction) both the glycoprotein (GP) precursor polypeptide and the nucleoprotein (NP), which encapsidates the genome segments and represents the most abundant protein in the virion. The GP precursor polypeptide is post-translationally processed into GP-1 and GP-2. Linked through a non-covalent association, the GP-1 and GP-2 protein form tetrameric spikes, which mediate cell entry. Productive infection of cells requires the interaction of the GP-1 protein with its receptor, α -dystroglycan, which is expressed ubiquitously. Upon cell entry, fusion of the virus with the phagolysosome membrane is mediated by GP-2. Ribonucleoprotein (RNP) complexes, containing the viral RNA complexed to

NP and the viral polymerase, are then delivered into the cytoplasm, and replication of the virus is initiated. Once assembled, the virus leaves the cell by budding from the plasma membrane.

Currently, there are 3 independently isolated LCMV strains, Armstrong, Traub, and WE, commonly used in the laboratory today. In 1933, the Armstrong strain of LCMV was isolated from a monkey that received a serial passage of cerebrospinal fluid from a suspected human case of St. Louis encephalitis virus (Armstrong and Lillie 1934). This monkey ultimately developed a severe lymphocytic choriomeningitis, a symptom for which the virus was named. In 1935, Eric Traub reported, in *Science*, of a virus (Traub strain) serologically identical to the Armstrong strain, and this caused a relatively asymptomatic persistent infection of his mouse colony (Traub 1935). In 1936, a third strain of LCMV, WE, was isolated from a patient who had contact with Traub's mouse colony and eventually died from a hemorrhagic disease (Scott and Rivers 1936). In the 1980s, it was discovered that certain LCMV variants, such as the Clone 13 variant of the Armstrong strain and the Docile variant of WE, could initiate immunosuppression in immunocompetent adult mice (Ahmed, Salmi et al. 1984; Ahmed and Oldstone 1988). Sequence analysis of Clone 13 revealed a five-nucleotide change from the parental LCMV Armstrong strain. While three mutations were silent, two of these nucleotide changes led to amino acid changes in the viral polymerase (lysine to glutamine at amino acid 1079) or the GP, specifically GP260, which became a leucine instead of phenylalanine. This mutation occurred in GP-1, resulting in a 2-3 log increase in binding affinity of

Clone 13, relative to the parental Armstrong strain, to α -dystroglycan (Sevilla, Kunz et al. 2000; Kunz, Sevilla et al. 2001; Smelt, Borrow et al. 2001). Both WE and Traub strains of LCMV bind to α -dystroglycan with an affinity similar to that of Clone 13. α -Dystroglycan is expressed primarily on dendritic cells (CD11c⁺ and DEC205⁺). Therefore, strains that bind with high affinity to α -dystroglycan (Clone 13, WE, and Traub) replicate predominantly in the splenic white pulp and in marginal zone DCs (70% of DCs infected), causing immunosuppression and the establishment of viral persistence. In contrast, LCMV strains that bind α -dystroglycan with low affinity (parental Armstrong strain), replicate primarily in the splenic red pulp, generating a robust anti-LCMV response that ultimately results in viral clearance. The Armstrong and Armstrong-derived Clone 13 will be the two strains of LCMV used for this study.

B. Immune Response to LCMV

The LCMV model system has been of great importance for developing knowledge of the immune response to viruses. LCMV is a relatively non-cytopathic virus that does not down-regulate MHC expression (Bukowski and Welsh 1985), giving it the capacity to induce very strong T cell responses. The host response to an acute LCMV infection is dependent upon a number of variables, including mouse strain, viral strain, viral dose and route of inoculation. Nevertheless, there are similarities in the dynamics of the immune responses to most strains of LCMV in mice inoculated with low to moderate doses (Welsh 2000). Upon intraperitoneal (i.p.) or intravenous (i.v.) infection with LCMV, there

is a strong sterilizing host immune response leading to clearance of the virus and long term immunological memory. During the early stages of viral replication, type 1 interferon (IFN) is induced, and this stimulates the activation and proliferation of NK cells during the first 4 days following infection (Merigan, Oldstone et al. 1977; Welsh 1978; Biron, Turgiss et al. 1983; Biron, Sonnenfeld et al. 1984). Although the innate response to LCMV is quite robust, it is the adaptive immune response that is responsible for viral clearance. Viral titers decline after day 5 due the sensitivity of the virus to cytotoxic lymphocytes (CTLs), which are detected as early as 6 days post-infection, *ex vivo*. As with most viral infections, LCMV infection is a potent stimulator of CD8 T cells, which recognize viral peptides in association with Class I MHC molecules on virus-infected antigen-presenting cells. CD8 T cell stimulation is so profound that the CD4/CD8 ratio changes from 2:1 to about 1:3 at the peak of the response (around day 8 or 9) (McFarland, Nahill et al. 1992; Lau, Jamieson et al. 1994; Selin, Vergilis et al. 1996; Varga and Welsh 1998). High numbers of activated macrophages and anti-LCMV-antibody producing splenic B cells also peak at this timepoint (Welsh and Doe 1980; Moskophidis and Lehmann-Grube 1984), presumably recruited by the increased amount of T-cell-dependent cytokines (IL-2 and IFN- γ) produced by the expanded CTL population (Kasaian and Biron 1989; Gessner, Drjupin et al. 1990). These proliferating T cells are mostly specific to viral peptides, but their fate and the fate of bystander T cells are highly regulated by apoptotic events that come in two waves during an acute infection. The first wave, which is the topic of this study, is associated with a transient

lymphopenia (2-4 days post-infection), occurring prior to the development of the anti-viral T cell response (McNally, Zarozinski et al. 2001). There is a substantial loss in many types of leukocytes during this early lymphopenia, but bona fide antigen-specific memory and “memory phenotype” (CD44^{hi}) CD8 T cells are among the most susceptible. This loss in CD8 T cells occurs throughout the body, including lymphoid and non-lymphoid tissues, and cannot be accounted for simply by lymphocyte migration. The loss of memory CD8 T cells was, at least in part, thought to be due to apoptosis, as they were reported positive for early apoptotic markers (Razvi, Jiang et al. 1995; McNally, Zarozinski et al. 2001; Jiang, Lau et al. 2003). Although the dynamics of CD8⁺ T cell lymphopenia have been established in mice infected with LCMV, the early stages of infections by many human viruses, including influenza, measles, West Nile, Ebola, Lassa, and SARS corona viruses, are also characterized by a severe lymphopenia (McNally, Zarozinski et al. 2001; Nabeshima, Murata et al. 2002; Peacock, Kim et al. 2003; Wong, Wu et al. 2003). Similar lymphopenias are seen in viral infections of domestic animals (Tompkins, Nelson et al. 1991; Harder, Kenter et al. 1996; Vallee, Tait et al. 2001; Zitzow, Rowe et al. 2002).

The second wave of apoptosis occurs following the peak of the T cell response, resulting in a significant loss of activated effector T cells. Those cells that survive this contraction phase, presumably through increased expression of the interleukin 7 receptor alpha-chain (Kaech, Tan et al. 2003), enter an immune or memory state and respond rapidly upon homologous re-challenge. CD8 $\alpha\alpha$, which is transiently expressed on a subset of CD8 $\alpha\beta$ T cells upon antigenic

stimulation, has been suggested to promote the survival and differentiation of activated lymphocytes into memory CD8⁺ T cells (Madakamutil, Christen et al. 2004). While much research has focused on the contraction phase, still little is known of the first wave of apoptosis, including the underlying mechanisms and its contribution, if any, to the generation of an effective immune response.

C. Type 1 IFN Response to Viral Infection

Upon infection with a pathogen, the initial host response is to secrete inflammatory cytokines, which can activate the innate arm of the immune response. The recruitment of innate immune cells such as NK cells, neutrophils, macrophages, and dendritic cells to the site of infection can either completely eliminate or prevent the spread of infection until the adaptive arm of the immune response is activated. One of the most prominent inflammatory cytokines released early after viral infection is type 1 IFN, one of two main classes of related cytokines (the other being type 2 IFN, or IFN- γ). The type 1 IFN family includes many members, but IFN- α and IFN- β are the two predominant forms. Type 1 IFN is produced from multiple IFN- α genes and a single IFN- β gene, clustered on murine chromosome 14. Each IFN- α subtype is encoded by its own gene, containing no introns, and regulated by its own promoter sequence (Braganca and Civas 1998). Once type 1 IFN is induced and secreted from an infected cell, it acts in both an autocrine and paracrine fashion, stimulating its receptor expression on the infected cell and those cells that neighbor it. This process, termed “priming,” results in the increased production of type 1 IFN after

its initial induction. This positive feedback loop occurs in two steps. First, IFN regulatory factor 3 (IRF-3), which is produced constitutively in most cell types, induces the early expressed type 1 IFN subtypes, IFN- β and IFN- α 4. These subtypes subsequently signal through the type 1 IFN-receptor to induce IFN regulatory factor 7 (IRF-7) expression, leading to the induction of the other IFN- α subtypes (Marie, Durbin et al. 1998; Sato, Hata et al. 1998; Sato, Suemori et al. 2000).

The receptor for all type 1 IFNs is composed of two chains, IFN α R1 and IFN α R2 (Novick, Cohen et al. 1994). Each chain of the receptor is constitutively bound to a distinct member of the Janus kinase family: IFN α R1 to tyrosine kinase 2 (TYK2) and IFN α R2 to Janus kinase 1 (JAK1). Ligand binding induces the dimerization of both receptor chains and the phosphorylation of TYK2, JAK1, and the intracellular tyrosine residues of each receptor chain (Colamonici, Porterfield et al. 1994; van Boxel-Dezaire, Rani et al. 2006). The transphosphorylation of both chains by these receptor-associated tyrosine kinases results in the activation of signal transducers and activators of transcription (STATs) 1 and 2, which are released from the IFN α R2 chain upon its phosphorylation (Stark, Kerr et al. 1998). The phosphorylation of the IFN α R1 creates a binding site for STAT2 and, upon STAT2 phosphorylation, STAT1 is able to bind (Leung, Qureshi et al. 1995). This results in dimerization of STAT1 and STAT2 and their subsequent dissociation from the receptor. Before translocation into the nucleus, however, the heterodimer forms the transcriptional-activator heterotrimeric complex, IFN-stimulated gene factor 3 (ISGF3), consisting of STAT1, STAT2, and IFN

regulatory factor 9 (IRF-9) (Darnell, Kerr et al. 1994; Haque and Williams 1994; Bluysen, Durbin et al. 1996). Once ISGF3 enters the nucleus, it can induce the expression of over 300 type 1 IFN-stimulated genes containing IFN-stimulated response elements (ISRE) (Kessler, Levy et al. 1988; Williams 1991). In addition to forming a heterodimer with STAT2, STAT1 can homodimerize, creating a complex termed IFN- α -activated factor (AAF) (Decker, Lew et al. 1991), which can induce the expression of genes containing a IFN- γ -activated site (GAS) upon entry into the nucleus (Decker, Lew et al. 1991; Lew, Decker et al. 1991). Type 1 IFNs fail to elicit an antiviral or an anti-proliferative response in cells lacking STAT1 or STAT2 (Muller, Laxton et al. 1993). Moreover, STAT1-deficient mice have an increased susceptibility to viral infections, as type 1 IFN is unable to induce expression of genes necessary to establish an antiviral state (Durbin, Hackenmiller et al. 1996; Meraz, White et al. 1996). Nevertheless, type 1 IFNs can activate additional STAT family members, including STATs 3 and 5, which mediate the potent anti-apoptotic and mitogenic effects in T cells lacking STAT1 upon IFN- β signaling (Tanabe, Nishibori et al. 2005; van Boxel-Dezaire, Rani et al. 2006). While type 1 IFN can limit non-specific CD8 T cell expansion in a STAT1-dependent manner, it can promote antigen-specific expansion and IFN- γ production by CTLs in a STAT4-dependent fashion (Nguyen, Watford et al. 2002).

The initial induction of type 1 IFN is generally thought to occur by two complementary receptor systems that account for most virus detection. One class of receptors is expressed ubiquitously and is localized to the cell's cytosol,

where it detects viral nucleic acids upon infection. This class of receptors includes the RNA helicases retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation antigen 5 (MDA5), which is the primary cytosolic receptor for poly(I:C), a potent inducer of type 1 IFN (Gitlin, Barchet et al. 2006; Kato, Takeuchi et al. 2006). The double-stranded RNA-activated protein kinase (PKR) is also involved in the cytosolic induction of type 1 IFN (Zinn, Keller et al. 1988), but it is unclear whether PKR itself is the pattern-recognition receptor (PRR) (Alexopoulou, Holt et al. 2001). The second class of receptors detects viral nucleic acids in endosomes (Stetson and Medzhitov 2006). This class of receptors includes the toll-like receptors, TLR3, TLR7, TLR8, and TLR9, which all can lead to type 1 IFN signaling upon detection of viral nucleic acids. TLR3 recognizes double-stranded RNA (Alexopoulou, Holt et al. 2001), TLR 7 and 8 recognize single-stranded RNA (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004; Lund, Alexopoulou et al. 2004), and TLR9 detects unmethylated CpG motifs in DNA. Although a potent inducer of type 1 IFN, TLR4 detects lipopolysaccharides derived from gram-negative bacteria.

Most cell types, with the appropriate stimulation, are capable of producing type 1 IFN *in vitro*, but it has been difficult to identify the principle producers of virus-induced type 1 IFN *in vivo* (Malmgaard 2004). Murine plasmacytoid DCs have been shown to be a major producer of type 1 IFN during *in vivo* infections with MCMV, VSV, and HSV-1 (Asselin-Paturel, Boonstra et al. 2001; Barchet, Cella et al. 2002; Dalod, Salazar-Mather et al. 2002), but not during infections with LCMV Armstrong or with the synthetic dsRNA analog, poly(I:C) (Diebold,

Montoya et al. 2003; Diebold, Kaisho et al. 2004). Interestingly, cultured non-plasmacytoid dendritic cells produce large amounts of type 1 IFN after *in vivo* infection with LCMV Clone 13 but not after infections with the Armstrong strain (Diebold, Montoya et al. 2003). Recently, it has been shown that marginal zone organization, and marginal zone macrophages, specifically, are critically involved in the production of type 1 IFN against LCMV Armstrong (Louten, van Rooijen et al. 2006).

As mentioned previously, type 1 IFNs activate innate immune cells (i.e. macrophages and NK cells) and induce a variety of target genes that exhibit immediate antiviral activity (Muller, Steinhoff et al. 1994; van den Broek, Muller et al. 1995; Biron 2001). The immunomodulatory role of type 1 IFN has gained noticeable attention over the last few years. For instance, infection with LCMV has been shown to induce CD8 T cell responses against antigens that are not expressed directly within antigen presenting cells (APCs), termed cross-priming, by a type 1 IFN-dependent mechanism (Le Bon, Etchart et al. 2003). Also, it has been shown that CD8 T cells lacking type 1 IFN receptor have a reduced ability to expand and differentiate into effector CTL's following infection with LCMV (Kolumam, Thomas et al. 2005; Aichele, Unsoeld et al. 2006; Thompson, Kolumam et al. 2006).

Although the underlying mechanisms leading to the early attrition of memory CD8 T cells following LCMV infection are still under investigation, kinetic analyses revealed that type 1 IFN induction immediately preceded this early attrition (McNally, Zarozinski et al. 2001). Experiments using the potent type 1

IFN inducer, poly(I:C), caused a similar reduction in total lymphocytes and memory CD8 T cells compared to an LCMV infection, though with more rapid kinetics, in parallel with the type 1 IFN response. Also, type 1 IFN receptor-deficient mice were resistant to the early attrition, with little reduction in the total number of lymphocytes or memory phenotype CD8 T cells after either LCMV infection or poly(I:C) treatment (McNally, Zarozinski et al. 2001). This loss in T cells can be induced by inoculation with recombinant IFN- β (McNally, Zarozinski et al. 2001) and blocked by antibody to type 1 IFN (Jiang, Gross et al. 2005). Interestingly, type 1 IFN has also been implicated in the splenic apoptosis early during infection with the gram-positive bacteria *Listeria monocytogenes*, although this appears to have a negative effect on the bacterial handling in the mouse (Carrero, Calderon et al. 2004; O'Connell, Saha et al. 2004). While ample evidence implicates type 1 IFN in the early attrition of memory CD8 T cells following LCMV infection, it is not yet known whether these effects are direct or indirect.

D. Apoptotic Signaling Pathways

There are two major pathways that can initiate apoptosis in cells: the death receptor pathway (extrinsic) and the mitochondrial-mediated pathway (intrinsic). Both pathways, for the most part, rely on the activation of multiple cysteine aspartate-specific proteases called caspases. The primary function of caspases is to cleave cellular substrates resulting in the amplification of apoptosis process and the stepwise dismantling of the cell (Best and Bloom

2004). Caspases contain a N-terminal prodomains that consist of one or more binding domains (Lavrik, Golks et al. 2005). Caspases 8 and 9 serve as key initiator caspases as their long prodomains contain multiple binding domains, such as death effector domains (DED) or caspase recruitment domains (CARD) that allow for homotypic interactions with specific adaptor proteins and their subsequent autocatalytic processing upon aggregation at these sites (Fesik 2000). Once activated, initiator caspases can then activate the small prodomains of downstream executioner caspases, such as caspase 3, ultimately resulting in the cleavage of cellular substrates (Hengartner 2000). This process leads to the morphological changes characteristic of apoptosis: cell shrinkage, membrane blebbing, chromatin condensation, and the appearance of apoptotic bodies that will be phagocytosed by macrophages and dendritic cells (Pokrovskaja, Panaretakis et al. 2005). Death receptors are members of the tumor necrosis factor receptor super family (TNFRSF), which is characterized by a specific intracellular domain, known as the death domain (DD). Members of the TNFRSF include Fas, tumor necrosis factor receptor 1 and 2 (TNFR1/TNFR2), and the TNF-related apoptosis-inducing ligand (TRAILR2/DR5). Upon ligand/receptor binding, intracellular adaptor proteins (FADD or TRADD) are recruited via their DD. Caspase 8 is recruited to the complex via its DED, thus contributing to the formation of the death-inducing signaling complex (DISC). Caspase 8 is activated by autocatalytic processing and functions as a key initiator caspase, leading to the activation of downstream effector caspases such as caspase 3. In some cell types, the apoptotic signal is further amplified by the caspase 8-mediated

cleavage of Bid. This truncated form of Bid translocates to the mitochondria, where it binds to Bak and Bax, leading to activation of the intrinsic mitochondrial pathway (Gross, McDonnell et al. 1999; Wang 2001). TNFRSF-mediated cell death can be inhibited by the caspase-8 (FLICE)-like inhibitory protein (FLIP), which consists of many splice variants and cleavage products and elicits its effects at the DISC (Krammer, Arnold et al. 2007).

Multiple stress/death signals, however, can lead to the direct activation of the mitochondrial apoptotic pathway without death receptor signaling. Such stress signals are produced by cytotoxic drugs, such as DNA-damaging agents and γ -irradiation, oxidative stress, or growth factor deprivation. Cell death at the mitochondrial level is initiated by perturbation of the mitochondrial membrane and proceeds via the release of cytochrome c (cyt c) from the intermembrane space of the mitochondria via pores formed by homooligomers and heterooligomers of the proapoptotic Bcl-2 family membranes, Bak and Bax. Once released into the cytosol, cytochrome c forms a complex, called the apoptosome, with apoptotic protease-activating factor-1 (Apaf-1), dATP, and the inactive form of Caspase 9. Upon activation, caspase 9 can activate downstream effector caspases, including caspase 3.

The susceptibility of cells to apoptosis via the intrinsic pathway is dependent on the balance between pro-apoptotic and anti-apoptotic Bcl-2 family members (Gross, McDonnell et al. 1999; Strasser, O'Connor et al. 2000; Wang 2001). These Bcl-2 family members are classified into 3 main categories: the anti-apoptotic, such as Bcl-2, Bcl-xl, Bcl-w, and Mcl-1, which share between one

and four regions of Bcl-2 homology (BH) domains; the Bax-like pro-apoptotic: Bax, Bak, Bok, and Diva, which contain two or three BH regions; and the BH3-only pro-apoptotic proteins, Bik, Bid, Bim, BMF, and Bad (Gross, McDonnell et al. 1999; Strasser, O'Connor et al. 2000; Wang 2001). BH3-only proteins are required for the initiation of apoptosis, whereas the Bax/Bak-like proteins play an essential role in downstream signaling events (Huang and Strasser 2000). In addition to physical levels, post-translational modifications also play important roles in regulating the effects of both pro- and anti-apoptotic members of the Bcl-2 family.

Bcl-2-interacting molecule (Bim) is involved in apoptosis induced by cytokine withdrawal or calcium flux (Bouillet, Metcalf et al. 1999) and mediates the programmed death of T lymphocytes during the shutdown of an immune response following viral infection (Strasser and Pellegrini 2004). Loss of Bim protects self-antigen-specific thymocytes (Bouillet, Purton et al. 2002) and B cells (Enders, Bouillet et al. 2003) against negative selection and prolongs the *in vivo* survival of T cells activated by the superantigen staphylococcal enterotoxin B (SEB) (Hildeman, Zhu et al. 2002) or cross-presented antigen (Davey, Kurts et al. 2002). Bim also mediates the apoptosis of IL7R α ^{lo} effector T cells and limits T cell memory generation following LCMV infection (Wojciechowski, Jordan et al. 2006). Likewise, the overall survival of peripheral T cells requires suppression of Bim function either directly by IL-7 or by IL-7-driven induction of Bcl-2 (Wojciechowski, Tripathi et al. 2007). There are 3 splice forms of Bim: Bim_S, Bim_L, and Bim_{EL}. Bim_S is a much more potent inducer of apoptosis than Bim_L and

Bim_{EL}, suggesting that the extra region present near the N-terminus of these two splice forms attenuates apoptotic activity. Bim_S, however, has not been detected in any cell types (O'Connor, Strasser et al. 1998). The pro-apoptotic activity of Bim_L and Bim_{EL} is regulated at the post-translational level by close interaction with the dynein light chain 8 (LC8) via the microtubule-associated dynein motor complex. Apoptotic stimuli disrupt this interaction, allowing Bim_L and Bim_{EL} to dissociate from LC8 and neutralize the anti-apoptotic activity of Bcl-2 (Puthalakath, Huang et al. 1999). Bim can also bind and directly activate Bak and Bax, which are required for mitochondrial cell death in response to diverse stimuli (Wei, Zong et al. 2001). Bak is a mitochondrial integral protein, whereas Bax is sequestered in the cytoplasm by 14-3-3 protein, under normal conditions and is released upon the initiation of apoptosis (Nomura, Shimizu et al. 2003).

E. Type 1 IFN and Apoptosis

Although type 1 IFN has been used to induce apoptosis in certain multiple melanoma cell lines for many years, the precise mechanisms have remained elusive (Otsuki, Yamada et al. 1998; Chen, Gong et al. 2001). In certain cancer cells, however, type 1 IFN was found to induce apoptosis through the mitochondrial route, specifically, involving Bim up-regulation, Bak conformational change and caspase activation. In addition, certain cancer cell lines resistant to type 1 IFN-induced apoptosis do not express Bim, even though they express significant levels of type 1 IFN receptor, supporting a role for type 1 IFN in the regulation of Bim expression levels (Gomez-Benito, Balsas et al. 2007).

Interestingly, the signaling adaptor for MDA5, which is involved in poly(I:C)-induced type 1 IFN production, is localized to the outer membrane of mitochondria, suggesting that this pathway may interact with Bcl-2 family members, such as Bim (Balachandran, Roberts et al. 2000; Seth, Sun et al. 2005). Type 1 IFN can also induce the activation of several caspases in various tumor cell lines, including caspase 3 and 9, both associated with mitochondrial-induced apoptosis. Caspase 8, which is typically associated with death receptor-mediated apoptosis, was also activated in certain cells lines, but to a lesser extent (Thyrell, Erickson et al. 2002; Panaretakis, Pokrovskaja et al. 2003). Collectively, these results suggest that type 1 IFN-stimulated signaling might interfere with other growth factor-induced or cytokine-induced pathways characteristic of mitochondrial-induced apoptosis (Pokrovskaja, Panaretakis et al. 2005).

Nevertheless, there are over 300 genes that can be induced by type 1 interferon (Kessler, Levy et al. 1988; Williams 1991; Maher, Romero-Weaver et al. 2007). Therefore, there is the potential for numerous molecules that may indirectly or directly contribute to apoptosis of memory CD8 T cells downstream of type 1 IFN-signaling. Since type 1 IFN initiates the activation of NK cells during the early host immune response, it was hypothesized that NK cells may be contributing to the early attrition of T cells. Mice depleted of NK cells and subsequently inoculated with poly(I:C), however, showed similar amounts of memory CD8 T cell attrition to that of non-depleted, untreated wildtype hosts (McNally, Zarozinski et al. 2001). Likewise, mice deficient in perforin showed a

similar phenotype to that of wildtype mice following poly(I:C) treatment, suggesting that this key mediator of cell lysis was not involved in the early attrition of memory CD8 T cells (McNally, Zarozinski et al. 2001). Type 1 interferon also stimulates the synthesis of IFN- γ , which can duplicate many of the effects of type 1 IFN (Sen and Ransohoff 1993). Poly(I:C), however, induced the apoptosis and attrition of memory CD8 T cells in mice lacking IFN- γ receptors (McNally, Zarozinski et al. 2001). Many TNFSF members are affected by type 1 IFN and, therefore, could potentially contribute to the apoptosis of memory CD8 T cells. Elevated levels of Fas were detected on poly(I:C)-treated memory CD8 T cells, but poly(I:C)-treated *gld* mice (deficient in FasL) showed a similar amount of memory CD8 T cell apoptosis relative to that of the wildtype (McNally, Zarozinski et al. 2001). Poly(I:C) also induces potentially cytotoxic cytokines such as TNF and TRAIL, so named because of its high homology to other members of the TNF family, particularly FasL, is also induced by type 1 IFN (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996; Sato, Hida et al. 2001). TRAIL is expressed by many cells of the immune system and has one known murine death receptor, TRAIL-R2 (also known as DR5 or Killer) (Wu, Burns et al. 1999). This can activate both caspases and NF- κ B and at least two decoy receptors that lack intracellular signaling domains (Wu, Burns et al. 1999). TRAIL was initially shown to induce apoptosis on tumor cells but not most normal cells (Wiley, Schooley et al. 1995; Pan, O'Rourke et al. 1997), but has since been implicated in the death of hepatocytes, thymocytes, and neurons (Martin-Villalba, Herr et al. 1999; Lamhamedi-Cherradi, Zheng et al. 2003; Mundt, Kuhnel et al.

2003). Interestingly, TRAIL plays a critical role in regulating the generation of CD8⁺ T cell memory through activation-induced cell death (AICD) (Janssen, Droin et al. 2005) and in the apoptosis associated with listeriosis upon infection with *Listeria monocytogenes* (Zheng, Jiang et al. 2004). It is not known, however, whether either TNF or TRAIL might play a role in the early attrition of memory CD8⁺ T cells during infection with LCMV.

F. Dendritic Cells

DCs have a central immunoregulatory role in maintaining peripheral tolerance and initiating antigen-specific T cell responses. Three subpopulations of CD11c⁺, MHC class II⁺ DCs are found in a murine spleen. They are distinguished based upon their differential expression of CD4 and CD8 α homodimers: CD4⁺CD8 α ⁻ (CD4⁺), CD4⁻CD8 α ⁺ (CD8⁺), and CD4⁻CD8 α ⁻ double negative (DN) DCs (Vremec, Pooley et al. 2000; Montoya, Edwards et al. 2005). A fourth subpopulation of splenic DCs, the plasmacytoid DC (pDC), is phenotypically characterized as being CD11c^{low}, MHC class II^{low}, and B220⁺ (Montoya, Edwards et al. 2005). CD4⁺, CD8 α ⁺, and DN DCs have the capacity to efficiently present antigen and stimulate the proliferation of both CD4 and CD8 T cells *in vivo*, but differ in their capacity to secrete cytokines. The CD8 α ⁺ DC population produces higher levels of IL-12 compared to both CD4⁺ and DN DC populations under many experiment conditions (Hochrein, Shortman et al. 2001). Although pDCs are capable of stimulating T cell responses (O'Keeffe, Hochrein et al. 2002), they are relatively poor APCs. They are, however, a major source of

type 1 IFN during many viral infections (Asselin-Paturel, Boonstra et al. 2001; Dalod, Salazar-Mather et al. 2002). Of all the splenic DC populations, immature CD8 α ⁺ DCs appear to be specialized for the uptake and cross-presentation of exogenous antigens on MHC class I (den Haan, Lehar et al. 2000; Pooley, Heath et al. 2001). One way these exogenous antigens gain access to the cross-presentation pathway is via the capture of apoptotic cells (Albert, Pearce et al. 1998; Albert, Sauter et al. 1998). Phagocytosis of these apoptotic cells followed by toll-like receptor signaling can activate the uninfected DC, resulting in subsequent cross-priming of CTLs against viruses that do not directly infect DCs (Schulz, Diebold et al. 2005). With certain viruses, such as LCMV-Armstrong, the cross-priming of CD8⁺ T cells occurs by a type 1 IFN-dependent mechanism (Le Bon, Etchart et al. 2003). Type 1 IFN, however, induces opposing effects on cross-presentation, depending on the maturation status of the DC. Type 1 IFN treatment of immature DCs results in a significant inhibition of CD40L-induced IL-12 production, resulting in an inhibition of CD8 T cell activation, whereas exposure of mature DCs to type 1 IFN results in high levels of CD40L-induced IL-12 production and the subsequent activation of CD8 T cells. These effects occur via a STAT1-dependent and STAT4-dependent mechanism, respectively (Longman, Braun et al. 2007).

Apoptotic bodies are engulfed by phagocytes through the recognition of phosphatidylserine (PS). Normal cells generally maintain an asymmetric distribution of phospholipids across the plasma membrane, restricting phosphatidylserine to the inner leaflet of the plasma membrane. When cells

undergo apoptosis, this asymmetric distribution is lost, as PS equilibrates between the outer and inner leaflet (Callahan, Williamson et al. 2000). The increased expression of PS on the outer leaflet of apoptotic or necrotic cells acts as an “eat me” sign for phagocytes (Fadok, Savill et al. 1992). Normally, the presence of apoptotic cells is difficult to detect *in vivo*, due to the rapid clearance by phagocytes. Certain viruses, however, can have an inhibitory effect on DCs, causing them to inefficiently phagocytose apoptotic lymphocytes. Recent studies have linked the early type 1 IFN response to impairment in DC differentiation and function early during the LCMV infection through a STAT2-dependent, but STAT1-independent, pathway (Hahm, Trifilo et al. 2005). Although both occur by a type 1 IFN-dependent mechanism, it is not known whether the impairment in DC differentiation and function is related to the early attrition of memory CD8 following LCMV.

G. Attrition of CD8⁺ T Cells and Heterologous Immunity

Many of the viruses associated with profound lymphopenia can also induce remarkably strong immune responses, provided that innate defenses keep the infection from overwhelming the host. This, plus the fact that partial lymphopenia induced by sublethal irradiation or immunosuppressive drug treatment can enhance immune responses to tumor (Ma, Urba et al. 2003) and viral (Pfizenmaier, Jung et al. 1977) antigens, have led us to speculate that the virus-induced lymphopenia might facilitate the mounting of a strong antiviral T cell response (McNally, Zarozinski et al. 2001; Peacock, Kim et al. 2003).

Consistent with this hypothesis is the fact that older mice develop less initial lymphopenia and mount a weaker CD8 T cell response to LCMV than do younger mice (Kapasi, Murali-Krishna et al. 2002; Jiang, Anaraki et al. 2003).

Studies have also shown that engagement of naïve T cells with their ligands, under conditions of appropriate co-stimulation, causes the upregulation of the anti-apoptosis protein Bcl-X_L, thereby giving them a survival advantage (Boise, Minn et al. 1995). One might, therefore, predict that antigen-specific T cells engaging their cognate ligand would preferentially resist the type 1 IFN-induced apoptosis. It was reported that depletion of both naïve and memory CD8 T cells during the early stages of the immune response to infection is selective (Jiang, Lau et al. 2003). Most T cells, regardless of specificity, were induced to express early activation markers upon infection, but nonspecific T cells were depleted, while those T cells specific for the pathogen expressed late activation markers and expanded in number (Jiang, Lau et al. 2003). However, it is unclear whether there was a transient apoptotic loss before the rapid antigen-specific proliferation. This resistance to early attrition, upon antigen engagement, may influence the immunodominance hierarchy of CD8⁺ T cells in the context of an infection with a heterologous pathogen, which can cross-react with T cells specific for a previously encountered pathogen.

Effector CD8 T cells that survive the contraction phase following viral infection create a memory CD8⁺ T cell pool with a distinct hierarchy of epitope-specific responses in a naïve host. Some of these viral epitopes are dominant and stimulate strong T cell responses, while others are subdominant and

stimulate weaker or barely detectable T cell responses. Immunodominance is regulated by various parameters, including the efficiency of processing and presentation of the peptide, the affinity between peptide and the MHC-I, the availability of T cells with TCR that recognize the peptide–MHC complex, and the competition between T cells for binding domains on the APC. When this memory CD8 T cell pool encounters a cross-reactive antigen, a preferential expansion of cross-reactive memory CD8 T cells may occur, due to the high frequency of these cells and their ability to rapidly proliferate compared to naïve T cells (Selin, Cornberg et al. 2004). Brehm et al demonstrated this concept using two distantly related arenaviruses, LCMV and Pichinde virus (PV). These viruses encode nucleoprotein epitopes (NP205), sharing 6 of 8 amino acids. In a naïve host, NP205 is normally a subdominant epitope for both viruses, but due to a selective expansion of NP205-specific cross-reactive memory CD8 T cells, the NP205-specific T cell response became dominant when LCMV-immune mice were infected with PV, or when PV-immune mice were infected with LCMV. This demonstrates how cross-reactive expansion can alter the hierarchy of T cell responses (Brehm, Pinto et al. 2002).

In some cases, memory T cells can cross-react with a heterologous virus and provide partial protective immunity (Brehm, Pinto et al. 2002), but in other cases, cross-reactive memory responses may cause a deviation of the immune response and result in unusual immunopathology (Chen, Fraire et al. 2001). Due to the competition between T cells that gives rise to immunodominance, low affinity cross-reactive memory T cells might prevent the development of more

effective high-affinity T cells responding to the immunodominant epitopes that normally dominate during a homologous infection. Therefore, since memory CD8 T cells are more susceptible than naïve CD8 T cells to type 1 IFN-dependent attrition, the loss of cross-reactive memory CD8 T cells may reduce their immunodominance and allow for a more diverse immune response. Alternatively, if a cross-reactive memory CD8 T cell population was protected from depletion by antigen-engagement, it could dominate the immune response and prevent the expansion antigen-specific naïve CD8 T cells.

H. Thesis Objectives

The goal of this thesis was to further elucidate the mechanism(s) by which the attrition of T cells occurs and evaluate whether it contributes to the generation of a diverse and effective immune response. Therefore, I ask the following 3 important fundamental questions.

1. What downstream molecule(s) contribute to the type 1 IFN-induced attrition of T cells during the early immune response to viral infection?
2. Are antigen-specific and non-specific T cells equally susceptible to the type 1 IFN-induced attrition associated with early immune response to viral infection?
3. Does the early attrition of memory T cells facilitate the development of a diverse naïve T cell response?

Chapter II: Materials and Methods

A. *Virus stocks and inoculation*

LCMV Armstrong strain and its highly disseminating variant, clone 13, are ambisense RNA viruses in the Old World arenavirus family and were propagated in BHK21 baby hamster kidney cells (Welsh, Lampert et al. 1976; Yang, Dundon et al. 1989). Pichinde virus, strain AN3739, a New World arenavirus only distantly related to LCMV, was propagated in BHK21 cells (Yang, Dundon et al. 1989). LCMV and PV were titrated by plaque assay on Vero cells.

B. *Mice*

Male C57BL/6 (B6) mice and IL-7R KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.SJL-Ptprc^a (Ly5.1), (C57BL/6 x C57BL/10SgSnAi)—[Tg] TCR-LCMV P14-[KO]rag2 (P14), and 129SVE (129) mice were purchased from Taconic Laboratories (Germantown, NY). All mice were purchased at 5-6 weeks of age and maintained under specific pathogen-free conditions within the Department of Animal Medicine at the University of Massachusetts Medical School. IFN- α/β receptor knockout mice (strain 129SVE and C57BL/6) (IFN1-R KO) (Muller, Steinhoff et al. 1994) were bred in-house. C57BL/6-Tg(TcraTcrb)1100Mjb (OT-1) (Barnden, Heath et al. 1994) crossed to B6.PL-*Thy1^a*/Cy (Thy1.1) mice and Granzyme B KO mice were bred by Dr. Kenneth L. Rock (University of Massachusetts Medical School). C57BL/6-Bim knockout mice (Bim KO) were bred by Dr. Roger J. Davis (University of

Massachusetts Medical School). C57BL/6-CD40 knockout mice (CD40 KO) were bred by Dr. Dale L. Greiner (University of Massachusetts Medical School). TNFR1 KO, TNFR2 KO, and vFLIP transgenic (tg) mice were bred by Dr. Francis Chan (University of Massachusetts Medical School). STAT1 KO and STAT3 dominant negative (DN) mice were bred by Dr. Joonsoo Kang (University of Massachusetts Medical School). TRAIL KO mice were bred by Dr. Stephen P. Schoenberger (La Jolla Institute for Allergy & Immunology). To generate LCMV-immune mice, B6 mice were infected i.p. with 5×10^4 pfu of the Armstrong strain of LCMV and were considered immune 6 weeks or longer after infection. To generate PV-immune mice, B6 mice were infected i.p. with 2×10^7 pfu of PV and were considered immune 6 weeks or longer after infection. All experiments were done with institutional guidelines as approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

C. RNA Isolation and Gene Expression Profiling

RNA was isolated from splenocytes using an RNeasy kit (Qiagen) and evaluated spectrophotometrically at 260 nm to determine concentration. First strand synthesis was done using the ReactionReady™ First Strand cDNA Synthesis Kit (Superarray) on a PTC-200 Thermo Cycler (MJ Research) using the following program: 37°C for 60 min, followed by 95°C for 5 min.

A mouse apoptosis RT² Profiler™ PCR Array was used to determine relative increases or decreases in the expression 84 key genes involved in apoptosis, or programmed cell death. The SYBR Green PCR master mix was

made using the RT² Real-Time[™] SYBR Green Master Mix. Real-Time PCR was performed using Bio-Rad MyIQ in combination with continuous SYBR Green detection (Superarray). The reaction was performed in a 25 μ L reaction volume containing 3 μ L cDNA and 22 μ L of RT² Real-Time[™] SYBR Green Master Mix (Superarray). The general PCR condition profile was as follows: polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Fold changes in expression were determined by the $\Delta\Delta C_T$ method, using a provided Excel-based PCR array data analysis template.

D. Apoptosis Assays (Caspase Activation, Annexin V Staining, TUNEL Staining)

Caspase activity was determined via CaspGLOW[™] fluorescein active stains specific for caspase-3 (FITC-DEVD-FMK) and caspase-8 (FITC-IETD-FMK). All caspase stains were purchased from BioVision (Mountain View, CA). Upon the completion of surface staining, each active caspase stain was added at 0.3 μ l per sample and incubated for 1 hour at 37°C with 5% CO₂. Cells were washed and resuspended in Wash buffer[™] prior to analysis via flow cytometry. For some experiments, apoptosis was evaluated via flow cytometry using fluorescently-conjugated Annexin V (BD Pharmingen). Upon the completion of surface staining, the cells were washed and incubated in Annexin V binding buffer with Annexin V at a 1:20 dilution for 15 minutes at room temperature. The cells were then washed, resuspended in Annexin V binding buffer, and analyzed by flow cytometry immediately. Annexin staining was done in conjunction with the

vital dye 7-amino-actinomycin D (7-AAD) to differentiate early apoptosis (Annexin V⁺ 7-AAD⁻) from late apoptosis/necrosis (Annexin V⁺ 7-AAD⁺).

Apoptosis was also evaluated via flow cytometry using terminal transferase dUTP nick end labeling (TUNEL). Erythrocytes were removed from harvested splenocytes using a 0.84% NH₄Cl solution. Splenocytes were added to a 48 well plate at 1 x 10⁶ cells per well and incubated at either 37°C (5% CO₂) or 4°C for 5 hours. Cells were then harvested and stained with the appropriate fluorescent surface antibodies. Upon the completion of surface staining, the cells were fixed according to the manufacturer's protocol. TUNEL⁺ cells were detected using an ApoDIRECT In Situ DNA Fragmentation Assay Kit (BioVision).

E. Synthetic peptides

Several previously defined T cell epitopes encoded by LCMV were used in this study (Whitton, Southern et al. 1988; van der Most, Murali-Krishna et al. 1998). LCMV-specific epitopes include NP396-404 (FQPQNGQFI), GP33-41 (KAVYNFATC), and GP276-286 (SGVENPGGYCL). A previously defined T cell epitope encoded by PV, NP38-45 (SALDFHKV), was also used in this study (Brehm, Pinto et al. 2002). All peptides listed above were purchased from Biosource International and were purified with reverse phase-HPLC to 90% purity. The immunizing peptide, GP33-45 (KAVYNFATCGIFA), was donated by Dr. Kenneth L. Rock (University of Massachusetts Medical School) (Ciupitu, Petersson et al. 1998).

F. Adoptive transfers

Single-cell suspensions were prepared from the spleens of C57BL/6 (wildtype), IFN1-R, P14, OT-1, LCMV-immune, or PV-immune mice, and erythrocytes were removed by lysis, using a 0.84% NH₄Cl solution. Prior to some transfers, donor splenocytes were labeled with the fluorescent dye CFSE (2 μM) for 15 minutes at 37°C (5% CO₂) (Molecular Probes, Eugene, OR) (Oehen, Brduscha-Riem et al. 1997).

For P14 and OT-1 co-transfer experiments, 3-4 x 10⁶ P14 cells (Ly5.2, Thy1.2) and OT-1 cells (Ly5.2, Thy1.1) were adoptively transferred, via tail vein injection (i.v.), into Ly5.1 recipient mice in a 200 μl volume of HBBS (Life Technologies, Grand Island, NY). For immune transfers, 4 x 10⁷ LCMV-immune or PV-immune splenocytes were also adoptively transferred, i.v., into Ly5.1 recipient mice in a 200 μl volume of HBBS. GP33-45 peptide immunizations were done 1 day post-transfer. OT-1 and P14 donor cells were visualized with fluorescently labeled Vα2 (B20.1) (BD Pharmingen). P14 donor cells were differentiated from OT-1 donor cells via fluorescently labeled Thy1.2 (53-2.1) antibody (BD Pharmingen). LCMV- and PV-immune donor cells were visualized with fluorescently labeled Ly5.2 (104) antibody (eBioscience).

To evaluate whether CD8α⁺ DCs were capable of phagocytosing apoptotic cells, 2 x 10⁷ CFSE-labeled wildtype (Ly5.1) cells were adoptive transferred, i.v., into congenic wildtype (Ly5.2) hosts. Host DCs (Ly5.2) were visualized with fluorescently labeled antibody to CD11c (HL3) antibody (eBioscience) and Ly5.2 (104) antibody (eBioscience).

G. Inoculations

For experiments involving *in vivo* peptide administration, mice were inoculated i.v. with 5 μ g of GP33-45 peptide (in 200 μ l of Hanks balanced salt solution (HBSS)) per mouse. Poly(I:C) (Amersham Pharmacia) was administered at a dose of 100 μ g/200 μ l in HBSS per mouse 5 hours later. For the induction of an acute LCMV-Clone 13 or LCMV-Armstrong infection, mice were injected i.v. or i.p. with 5×10^4 pfu of virus in 0.1 ml of PBS. In some experiments, CFSE-labeled donor splenocytes were then transferred i.v. into LCMV-infected hosts at 2 days post-infection. Splenocytes and leukocytes from other tissues were harvested 16 hours after donor cell transfer.

H. Intracellular IFN- γ staining

LCMV-specific memory CD8 T cells were detected by measuring IFN- γ secretion in response to stimulation with LCMV peptides using the Cytotfix/Cytoperm Kit Plus (with GolgiPlug; BD Pharmingen), as described previously (Varga and Welsh 1998). Splenocytes (2×10^6) were incubated in 96-well plates (5 h; 37°C) with 5 μ M synthetic peptide, 10 U/ml human rIL-2 (BD Pharmingen) and 0.2 μ l of GolgiPlug. Cells were then washed in Flow Cytometry Buffer (HBBS, 2% FCS, and 0.1% NaN₃), blocked with α -Fc (2.4G2), and incubated (30 min; 4°C) with a combination of fluorescently labeled mAbs specific for CD8 α (53-6.7, APC), CD69 (H1.2F3, PerCP-Cy5.5) (BD Pharmingen), CD45.2 (104, PE), and CD44 (IM7, APC-Cy7) (eBioscience).

Subsequent fixation and permeabilization of the cells was performed to allow intracellular access of mAb to IFN- γ (XMG1.2; PE-Cy7) (eBioscience). Freshly stained samples were analyzed on a BD Biosciences LSR II and FlowJo software.

I. Computer modeling

For computer simulations we used IMMSIM, an agent-based model of the immune system governed by probabilistic events. This program can be found at www.immsim.org and can be downloaded for the purpose of research and education. This model's past applications are found in several previous reports (Celada and Seiden 1992; Seiden and Celada 1992; Celada and Seiden 1996; Kohler, Puzone et al. 2000; Behn, Celada et al. 2001). The IMMSIM model consists of epithelial cells in a grid of discrete "interaction sites," where T cells, B cells, and APCs of the immune system encounter each other and antigens, and mount cellular and humoral responses whenever a virus infects and expresses antigens in the target epithelial cells. The interactions are governed by affinity and chance encounters (via computer generated random numbers, RNs). Different RNs result in responses different in repertoire and events, simulating the variability within individual mice *in vivo*. IMMSIM computer simulations were conducted by Claudio Calcagno, Dario Gherzi, Roberto Puzone, and Franco Celada at the University of Genoa, Genoa, Italy.

J. Statistical analyses

Student's t test was calculated using GraphPad InStat and used for data analysis where appropriate. Results are expressed as the mean \pm standard deviation.

Chapter III: Apoptotic Properties of Cells During the Early Stage of the Immune Response to LCMV Infection

Our lab has previously documented a type 1 IFN-dependent attrition of $CD8\alpha^+CD44^{hi}$ cells, immediately preceding the development of the antiviral T cell response to LCMV (McNally, Zarozinski et al. 2001). This loss was attributed to apoptosis, since the early attrition correlated with an increase in Annexin V reactivity. In this chapter, I show that a population of “lymphoid” $CD8\alpha^+$ DCs, found within the $CD8\alpha^+CD44^{hi}$ gate, increases upon poly(I:C) treatment and accounts for the increase in Annexin V reactivity. Since Annexin V reactivity was previously thought to be associated with the apoptosis of $CD8^+$ T cells, I re-examined the early attrition of T cells associated with viral infections. I show that the loss of $CD8\beta^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cells is type 1 IFN-dependent and correlates with an increase in caspase activation following LCMV infection. Furthermore, this loss is associated with increased DNA fragmentation, which is a hallmark characteristic of the late stages of apoptosis. I also show that the apoptosis of $CD8\beta^+CD44^{hi}$ and $CD4^+CD44^{hi}$ T cells is reduced in Bim KO mice following LCMV infection, thereby suggesting that the death occurs through a mitochondrial-induced pathway involving Bim. Finally, I show that the “lymphoid” $CD8\alpha^+$ DC population may aid in the rapid clearance of apoptotic cells during the early type 1 IFN-induced lymphopenia.

A. Type 1 IFN-induced attrition of CD8 α ⁺CD44^{hi} cells

It has been previously demonstrated that CD8 T cells undergo apoptosis and decline in number in response to type 1 IFN. This apoptotic loss, however, is more pronounced in the bona fide antigen-specific memory and the “memory phenotype” CD8 T cell population (CD44^{hi}) than in the naïve T cell population (CD44^{lo}) (McNally, Zarozinski et al. 2001). A greater than 75% reduction in the number of CD8 α ⁺CD44^{hi} cells in the spleen occurred 24 hours following treatment with the type 1 IFN-inducer poly(I:C) (Figure 1A), while a smaller (58%), though significant, reduction in naïve CD8 α ⁺CD44^{lo} cell number was also observed (Figure 1C). Although both populations underwent a decline in cell number 12 hours following poly(I:C) treatment, the CD8 α ⁺CD44^{hi} cell population exhibited a greater increase in Annexin V reactivity, relative to the CD8 α ⁺CD44^{lo} cell population (Figure 1B and 1D). Type 1 IFN-receptor knockout (IFN1-R KO) mice exhibited a slight, but not statistically significant, decline in CD8 α ⁺CD44^{hi} and CD8 α ⁺CD44^{lo} cell number 24 hours following poly(I:C) treatment (Figure 1A and 1C). Neither IFN1-R KO population (CD8 α ⁺CD44^{hi} or CD8 α ⁺CD44^{lo}) had an increase in Annexin V reactivity 12 hours following poly(I:C) treatment, further supporting the involvement of type 1 IFN in the apoptotic loss of CD8 α cells, particularly in the CD8 α ⁺CD44^{hi} population (Figure 1C and 1D).

Although these data support the involvement of type 1 IFN, I questioned whether additional molecule(s) might contribute the loss of CD8 T cells. I compared the poly(I:C)-induced attrition of CD8 α ⁺CD44^{hi} cells, the more susceptible population, in multiple knockout and mutant mice, such as Granzyme

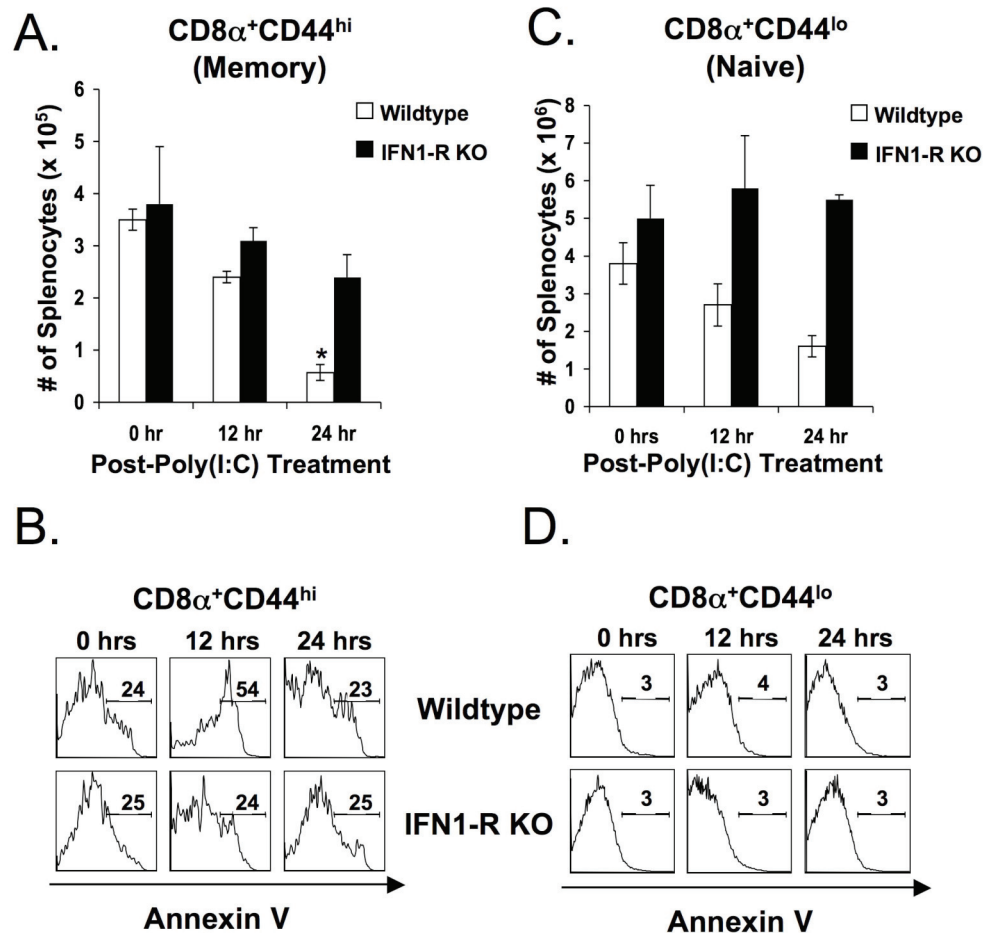


Figure 1 – The type 1 IFN-induced attrition of CD8 α ⁺CD44^{hi} cells is associated with an increase in Annexin V reactivity. Wildtype 129SVE (wildtype) and IFN α β -receptor KO (IFN1-R KO) mice were inoculated with poly(I:C) i.p. Splenocytes were harvested at 0, 12 and 24 hours post-inoculation. **A**, absolute numbers of CD8 α ⁺CD44^{hi} splenocytes present 0, 12, and 24 hours post-poly(I:C) treatment. *, $P < 0.05$, wildtype at 24 hrs post-poly(I:C) treatment compared to 0 hour timepoint. **B**, percentage of wildtype and IFN1-R KO Annexin V⁺CD8 α ⁺CD44^{hi}, post-poly(I:C) treatment. Gates were set upon 7-AAD⁻, CD8 α ⁺ cells to exclude dead/necrotic cells. **C**, absolute numbers of CD8 α ⁺CD44^{lo} splenocytes present 0, 12, and 24 hours post-poly(I:C) treatment. **D**, percentages of wildtype and IFN1-R KO Annexin V⁺CD8 α ⁺CD44^{lo}, post-poly(I:C) treatment. Numbers were calculated based on percentages obtained via flow cytometry. Representative data with n=3 per group. FACS plots represent 1 of 3 mice. Gates were set upon 7-AAD⁻, CD8 α ⁺ cells to exclude dead/necrotic cells. The data is representative of 2 independent experiments.

B KO, IL-7R KO, TRAIL KO, TNFR1 KO, TNFR2 KO, STAT1 KO, STAT3 dominant negative (DN) and vFLIP transgenic (Tg) mice along with their relevant controls. All mice underwent a comparable loss of CD8 α ⁺CD44^{hi} cells compared to their wildtype controls at 12 hours post-poly(I:C) treatment, suggesting that none of these molecules contribute to the type 1 IFN-induced attrition. Table 1 summarizes these results.

Table 1 - Parameters of Type 1 IFN-induced T cell attrition

Mouse Type	Time and treatment	n	# of CD8 α^+ CD44 ^{hi} cells (10 ⁵) (% change)
C57BL6/J	Untreated	3	12 \pm 7.5
	12 hours poly(I:C)	3	4.4 \pm 1.5 (-63) ^b
Granzyme B KO	Untreated	3	10 \pm 4.6
	12 hours poly(I:C)	3	4.4 \pm 0.1 (-56) ^b
C57BL6/J	Untreated	3	12 \pm 7.5
	12 hours poly(I:C)	3	4.4 \pm 1.5 (-63) ^b
IL7-R KO	Untreated	3	2.6 \pm 1.3
	12 hours poly(I:C)	3	1.3 \pm 0.1 (-50) ^b
C57BL6/J	Untreated	3	14.6 \pm 1.0
	12 hours poly(I:C)	3	3.1 \pm 0.5 (-79) ^b
TRAIL KO	Untreated	3	25.4 \pm 0.6
	12 hours poly(I:C)	3	4.8 \pm 0.4 (-81) ^b
C57BL6/J	Untreated	3	12 \pm 7.5
	12 hours poly(I:C)	3	4.4 \pm 1.5 (-63) ^b
TNFR1 KO	Untreated	3	11 \pm 2.7
	12 hours poly(I:C)	3	4.0 \pm 1.8 (-64) ^b
C57BL6/J	Untreated	3	26 \pm 2.6
	12 hours poly(I:C)	3	4.9 \pm 0.5 (-81) ^b
TNFR2 KO	Untreated	3	18 \pm 3.8
	12 hours poly(I:C)	3	2.7 \pm 0.8 (-85) ^b
C57BL6/J	Untreated	3	30.7 \pm 9.9
	12 hours poly(I:C)	3	8.6 \pm 2.8 (-72) ^b
vFLIP Tg	Untreated	3	21.4 \pm 6.4
	12 hours poly(I:C)	3	8.0 \pm 0.7 (-63) ^b
129SVE	Untreated	3	5.0 \pm 1.6
	12 hours poly(I:C)	3	2.4 \pm 0.8 (-52) ^b
STAT1 KO	Untreated	3	5.6 \pm 2.6
	12 hours poly(I:C)	3	3.3 \pm 0.5 (-41) ^b
C57BL6/J	Untreated	3	8.9 \pm 0.6
	12 hours poly(I:C)	3	4.1 \pm 0.4 (-53) ^b
STAT3 DN	Untreated	3	12.7 \pm 1.4
	12 hours poly(I:C)	3	4.8 \pm 1.1 (-63) ^b

^aMice were inoculated with 200 μ g of poly(I:C) i.p. and were sacrificed after 12 hours. Spleens were harvested and analyzed using flow cytometry (see Materials and Methods)

^bP < 0.05 compared to untreated control

B. An increase in “lymphoid” CD8 α ⁺ DCs accounts for the observed increase in Annexin V reactive CD8 α ⁺CD44^{hi} cells following poly(I:C) treatment

To determine whether additional pro-apoptotic molecule(s) may contribute to the type 1 IFN-induced apoptosis of CD8 α ⁺CD44^{hi} cells, I first used a mouse apoptosis RT² Profiler™ PCR Array to determine relative increases or decreases in the expression 84 key apoptotic genes. There was an 11-fold increase in CD40 RNA isolated from CD8 α ⁺CD44^{hi} cells 6 hours post-poly(I:C) relative to RNA isolated from untreated CD8 α ⁺CD44^{hi} cells. Six hours post-poly(I:C) was chosen for RNA isolation, since this was the timepoint that immediately preceded a significant loss of CD8 α ⁺CD44^{hi} cells and the corresponding increase in Annexin V reactivity. No significant increase in CD40 RNA expression was observed in the CD8 α ⁺CD44^{lo} population 6 hours post-poly(I:C) treatment. Typically, CD40 surface protein expression is thought to be limited to B cells, DCs, and macrophages (Banchereau, Bazan et al. 1994), but CD8 T cells, under certain conditions, have also been shown to transiently express CD40 (Bourgeois, Rocha et al. 2002). Gating on the CD8 α ⁺CD44^{hi} population at multiple timepoints following poly(I:C) revealed an increase in CD40 surface protein expression that peaked around 9 and 12 hours following poly(I:C) treatment (30% of the CD8 α ⁺CD44^{hi} population) and subsequently declined thereafter, reaching untreated levels by 72 hours (around 2% of the CD8 α ⁺CD44^{hi} population) (Figures 2A and 2B). The expression of CD40 directly correlated with increased Annexin V reactivity. There was little increase in

Annexin V reactivity in the CD40⁻CD8 α ⁺CD44^{hi} population at all timepoints following poly(I:C) treatment (Figure 2A and 2B).

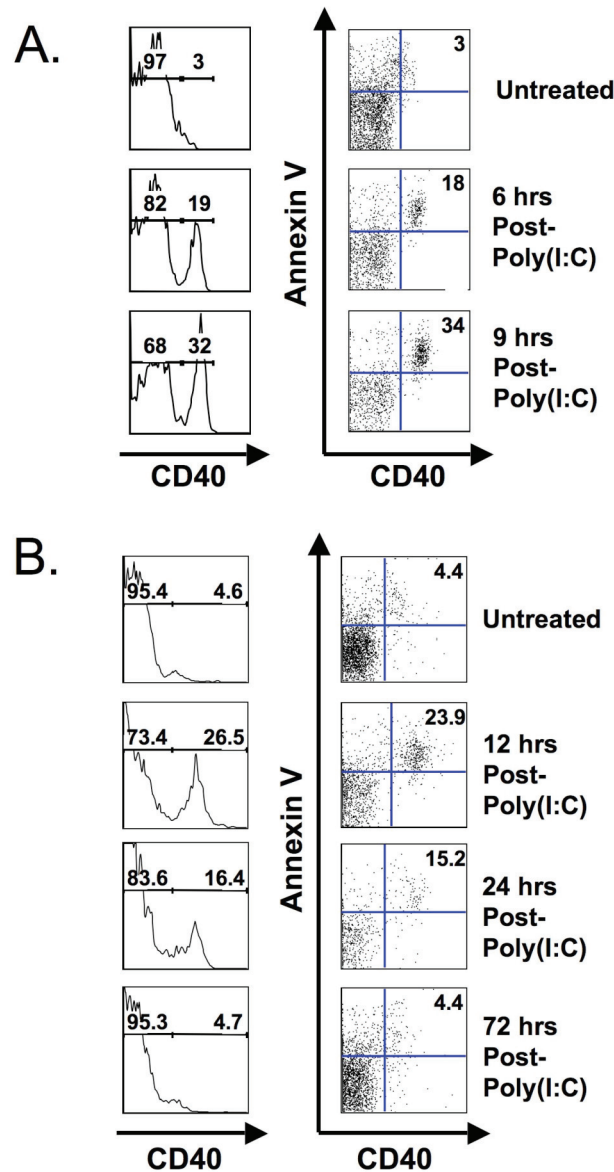


Figure 2 – Increase in Annexin V reactivity correlates with an increase in CD40 expression on CD8 α ⁺CD44^{hi} cells at multiple timepoints following poly(I:C) treatment. C57BL/6 (wildtype) mice were inoculated with poly(I:C) i.p. Splenocytes were harvested at 0 (untreated), 3, 6, 9, 12, 24, and 72 hours post-inoculation. *A*, percentage of CD40⁺CD8 α ⁺CD44^{hi} cells (left column) and Annexin V⁺CD40⁺CD8 α ⁺CD44^{hi} cells (right column) at 0 (NT), 6, and 9 hours post-poly(I:C). *B*, percentage of CD40⁺CD8 α ⁺CD44^{hi} cells (left column) and Annexin V⁺CD40⁺CD8 α ⁺CD44^{hi} cells (right column) at 0 (NT), 12, 24, and 72 hours post-poly(I:C). Gates were set on 7-AAD⁻ cells. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

Because increased CD40 expression directly correlated with increased Annexin V reactivity, I questioned whether CD40 expression was necessary for the poly(I:C)-induced attrition and tested CD40 KO mice. Both wildtype and CD40 KO mice, however, underwent a similar decrease in both percentage and number of CD8 α^+ CD44^{hi} cells, relative to the untreated control (Figure 3A and 3B), suggesting that the attrition of CD8 α^+ CD44^{hi} cells is not dependent on the expression of CD40.

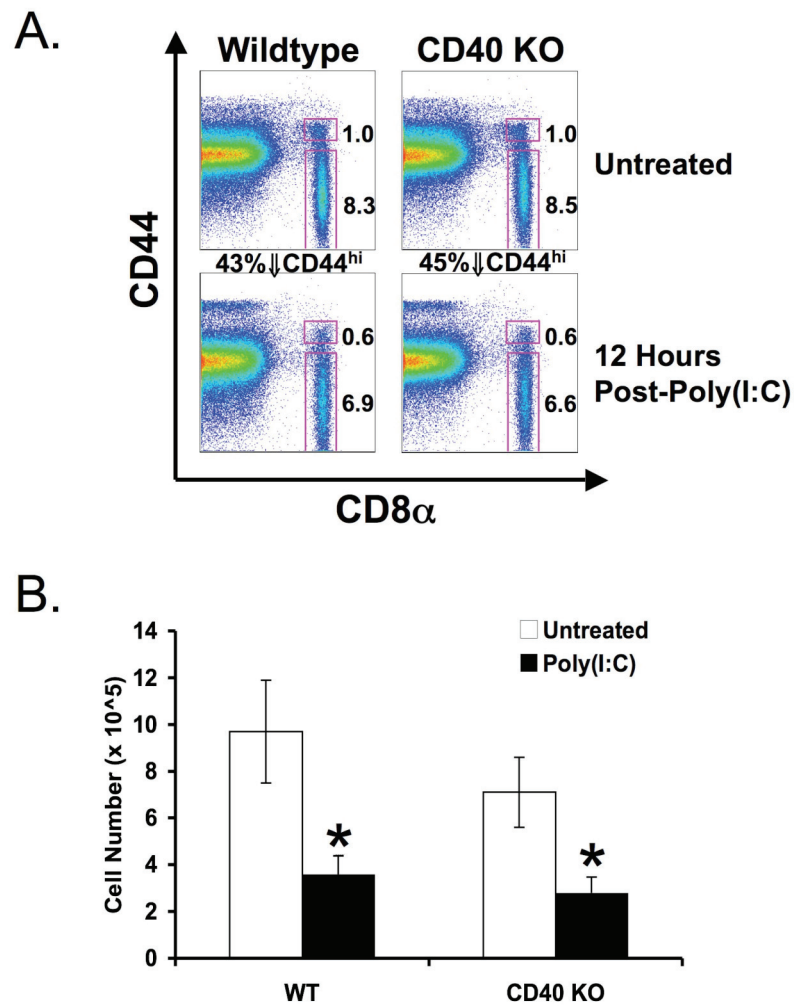


Figure 3 – Both wildtype and CD40 KO CD8 α ⁺CD44^{hi} cells are equally susceptible to type 1 IFN-induced attrition. C57BL/6 (wildtype) and CD40 KO mice were inoculated with poly(I:C) i.p. Splenocytes were harvested at 0 (untreated) and 12 hours post-inoculation. *A*, percentages of wildtype and CD40 KO CD8 α ⁺CD44^{hi/lo} cells. *B*, absolute numbers of both wildtype and CD40 KO CD8 α ⁺CD44^{hi} cells. Gates were set upon 7-AAD⁻ cells. Representative data with n=3 per group FACS plots represent 1 of 3 mice. Numbers were calculated based on percentages obtained via flow cytometry. *, $P < 0.05$. The data is representative of 2 independent experiments.

I next questioned whether this population of CD40⁺CD8 α ⁺CD44^{hi} cells expressed Thy1.2, a pan T cell marker, following poly(I:C) treatment. While the CD40⁻CD8 α ⁺CD44^{hi} population was Thy1.2⁺, the CD40⁺CD8 α ⁺CD44^{hi} population was Thy1.2⁻, suggesting that this sub-population does not consist of T cells (Figure 4A). For further confirmation, I also stained the CD8 α ⁺CD44^{hi} population for CD8 β expression following poly(I:C) treatment. 87% of the untreated CD8 α ⁺CD44^{hi} population co-expressed CD8 β , while only 63% of the CD8 α ⁺CD44^{hi} population co-expressed CD8 β at 12 hours post-poly(I:C) treatment, further suggesting there is a non-T cell population within the CD8 α ⁺CD44^{hi} gate (Figure 4B). I next compared the poly(I:C)-induced attrition of both CD8 α ⁺CD44^{hi} and CD8 β ⁺CD44^{hi} populations. There was a 53% reduction in the percentage of CD8 α ⁺CD44^{hi} cells at 12 hours post-poly(I:C), relative to the untreated control (Figure 4C), and this correlated with a 3-fold increase in Annexin V⁺ cells and a 2-fold increase in the mean fluorescent intensity (MFI) of Annexin V reactivity (Figure 4D). There was a more pronounced decrease in percentage of CD8 β ⁺CD44^{hi} cells (73%) relative to that of the CD8 α ⁺CD44^{hi} population. This decrease correlated with only a modest increase in both the percentage and MFI of Annexin V⁺ CD8 β ⁺CD44^{hi} cells, relative to the untreated control (Figure 4D). CD40 surface protein expression was not detected on the CD8 β ⁺CD44^{hi} population at any timepoint following poly(I:C) treatment (data not shown).

Based upon these findings, multiple conclusions can be made. First, the assessment of the poly(I:C)-induced attrition of CD8⁺CD44^{hi} T cells was

underestimated using the anti-CD8 α antibody. This was due to the poly(I:C)-induced increase in Thy1.2⁻CD40⁺CD8 α ⁺CD44^{hi} cells, which also accounted for the observed increase in Annexin V reactivity. Thus, the use of an anti-CD8 β antibody is a more reliable indicator of the attrition of CD8⁺CD44^{hi} T cells. Nevertheless, the attrition did not correlate with an increase in Annexin V reactivity, thereby leaving it unclear as to whether the loss of CD8 β ⁺CD44^{hi} T cells was due to apoptosis. This issue will be addressed in this thesis.

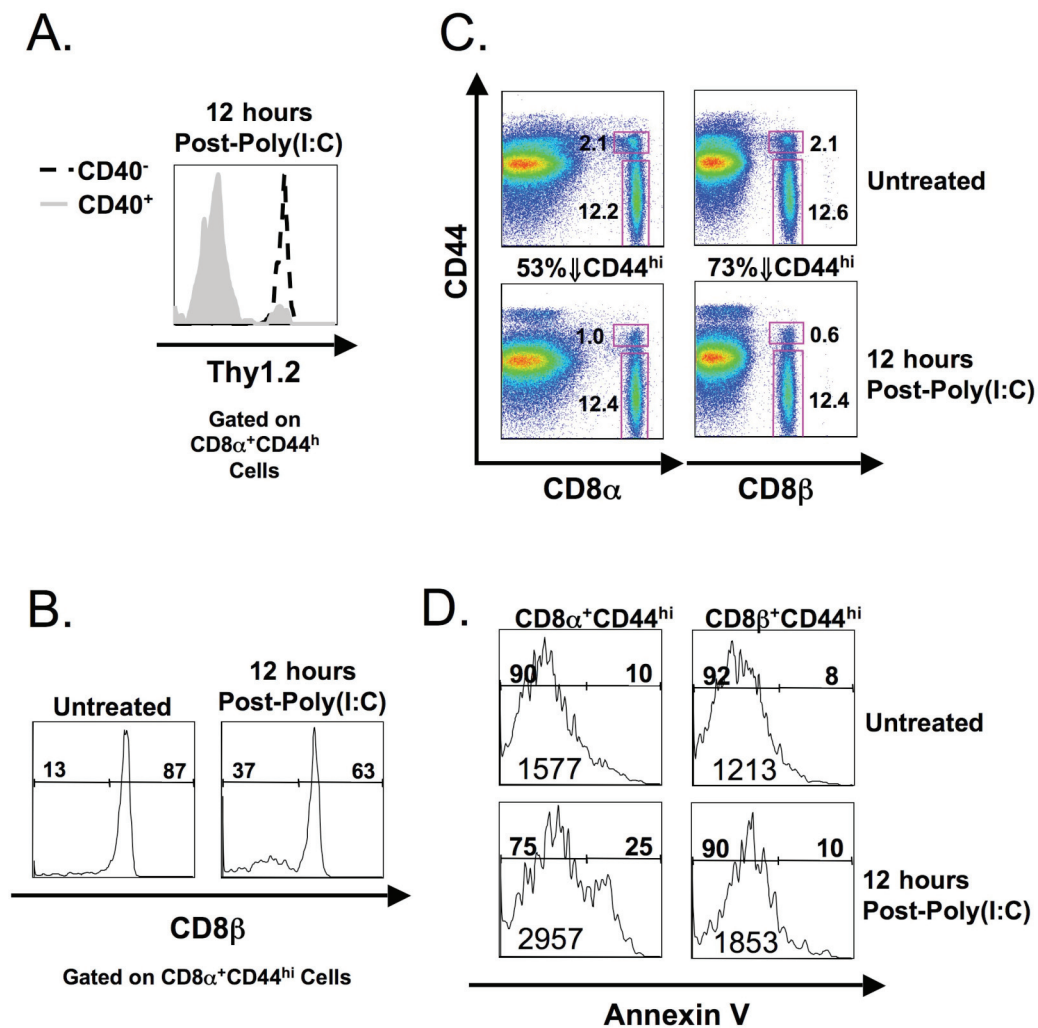


Figure 4 – The CD8 α ⁺CD44^{hi} population contains a non T-cell, Annexin V reactive population following poly(I:C) treatment. C57BL/6 (wildtype) mice were inoculated with poly(I:C) i.p. Splenocytes were harvested at 0 (untreated) and 12 hours post-inoculation. *A*, Thy1.2 expression on CD40⁺CD8 α ⁺CD44^{hi} and CD40⁻CD8 α ⁺CD44^{hi} cells. Gates were set on CD8 α ⁺CD44^{hi} cells. *B*, CD8 β expression on CD8 α ⁺CD44^{hi} cells. Gates were set on CD8 α ⁺CD44^{hi} cells. *C*, percentages of wildtype CD8 α ⁺CD44^{hi/lo} and CD8 β ⁺CD44^{hi/lo} cells. *D*, percentage of Annexin V⁺CD8 α ⁺CD44^{hi} and Annexin V⁺CD8 β ⁺CD44^{hi} cells. Numbers on the lower left corner of each plot indicate Annexin V mean fluorescent activity (MFI). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

To further phenotype the $\text{Thy1.2}^- \text{CD40}^+ \text{CD8}\alpha^+ \text{CD44}^{\text{hi}}$ population that increases upon poly(I:C) treatment, I next questioned whether they may be a subset of DCs, since certain DCs can express a $\text{CD8}\alpha\alpha$ -homodimer (Vremec, Pooley et al. 2000; Montoya, Edwards et al. 2005). I used the DC marker, CD11c, which can also be found on CD8 T cells, but only at much later timepoints following viral infection (Day 8) (Lin, Roberts et al. 2003). CD11c expression was high in the $\text{CD8}\alpha^+ \text{CD44}^{\text{hi}}$ population, 12 hours following poly(I:C) treatment (26.4%), while no significant increase was observed in the $\text{CD8}\beta^+ \text{CD44}^{\text{hi}}$ population (Figure 5A). This result suggests that this sub-population of $\text{CD8}\alpha^+ \text{CD44}^{\text{hi}} \text{CD11c}^+$ cells has a DC phenotype and increases in frequency in the spleen at 12 hours post-poly(I:C). I further characterized this population of $\text{CD8}\alpha^+ \text{CD44}^{\text{hi}} \text{CD11c}^+$ DCs at 12 hours post-poly(I:C) as class II^{hi} (I-A^B), CD40^+ (Figure 5B), and B220^- (Figure 5C). Collectively, these results suggest that this population displays a “lymphoid” DC phenotype.

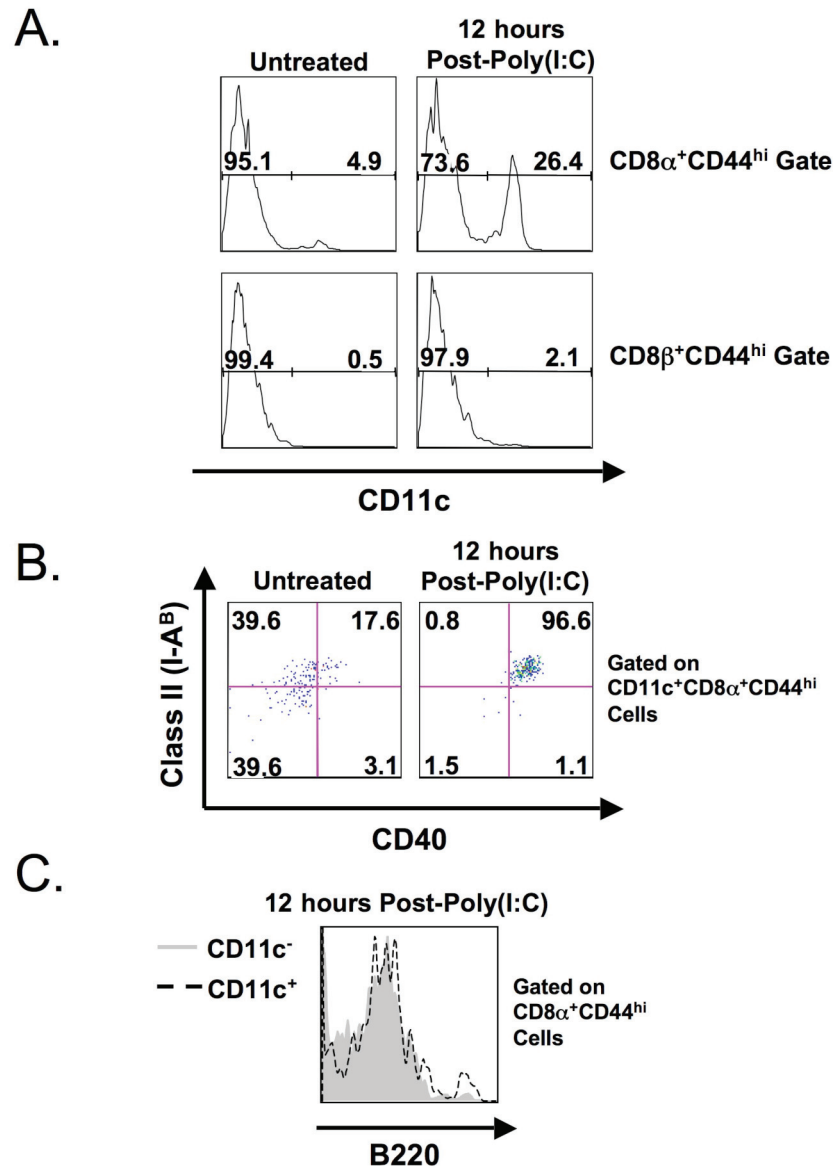


Figure 5 – The Thy1.2-CD40⁺CD8 α ⁺CD44^{hi} population displays a “lymphoid” DC phenotype. C57BL/6 (wildtype) mice were inoculated with poly(I:C) i.p. Splenocytes were harvested at 0 (untreated) and 12 hours post-inoculation. A, CD11c expression on CD8 α ⁺CD44^{hi} and CD8 β ⁺CD44^{hi} cells. CD8 α ⁺CD44^{hi} and CD8 β ⁺CD44^{hi} gates were set on 7-AAD⁻ cells. B, percentage of Class II (I-A^B) and CD40 expression on CD11c⁺CD8 α ⁺CD44^{hi} cells at 0 (untreated) and 12 hours post-poly(I:C) treatment. Representative data with n=3 per group. C, B220 expression on both CD11c⁺ and CD11c⁻CD8 α ⁺CD44^{hi} populations at 12 hours post-poly(I:C). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

Kinetic analysis of this poly(I:C)-induced increase in CD8 α^+ DCs revealed a 2-fold increase in the percentage of CD8 α^+ DCs in the spleen as early as 9 hours and a 3-fold increase at 12 hours post-poly(I:C) treatment (Figure 6A). This increase in percentage corresponded to a 2-fold increase in total CD8 α^+ DC number at 9 and 12 hours post-poly(I:C) treatment (Figure 6B). This increase was only observed in the spleen, as no increase in percentage was observed in the peritoneal cavity (PEC), lungs, inguinal lymph nodes (iLN), or peripheral blood (Figure 7).

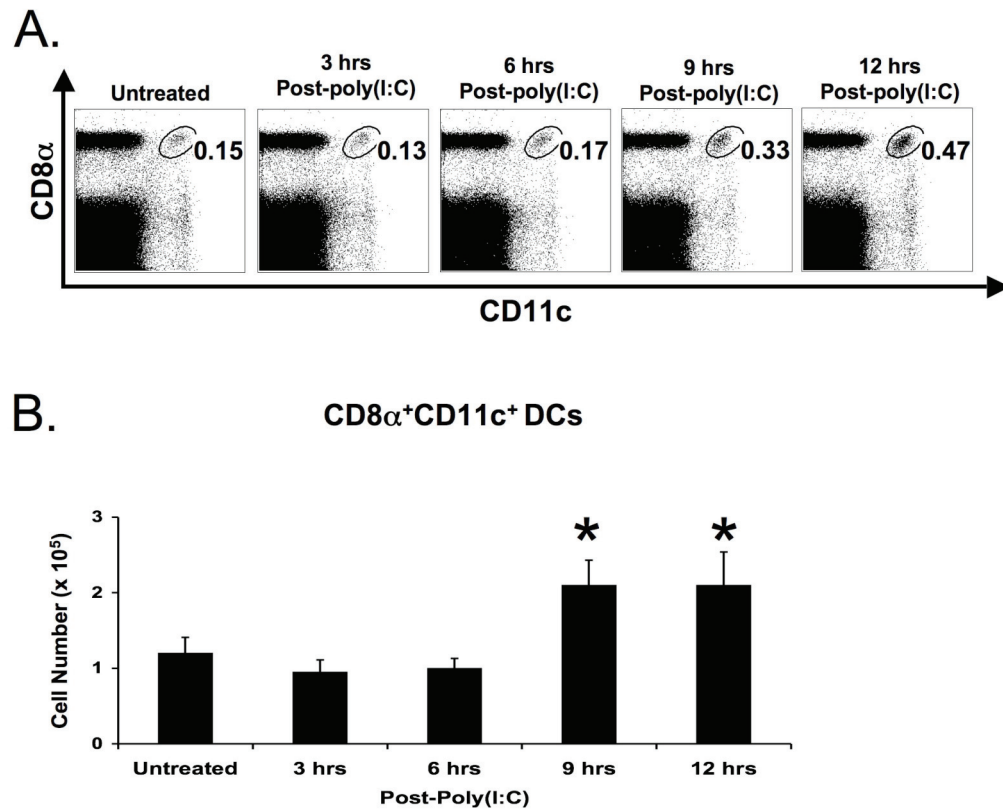


Figure 6 – CD8 α^+ DCs increase in number following poly(I:C) treatment. Wildtype mice were inoculated with poly(I:C). Splenocytes were harvested at 0 (untreated), 3, 6, 9, and 12 hours post-poly(I:C). *A*, the percentage of CD8 α^+ DCs. Gates were set on 7-AAD $^-$ cells. *B*, absolute numbers of CD8 α^+ DCs. Numbers were calculated based on percentages obtained via flow cytometry. Representative data with $n=3$ per group. *, $P < 0.05$, cell number at 9 and 12 hours post-poly(I:C) treatment compared to 0 hours (untreated). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

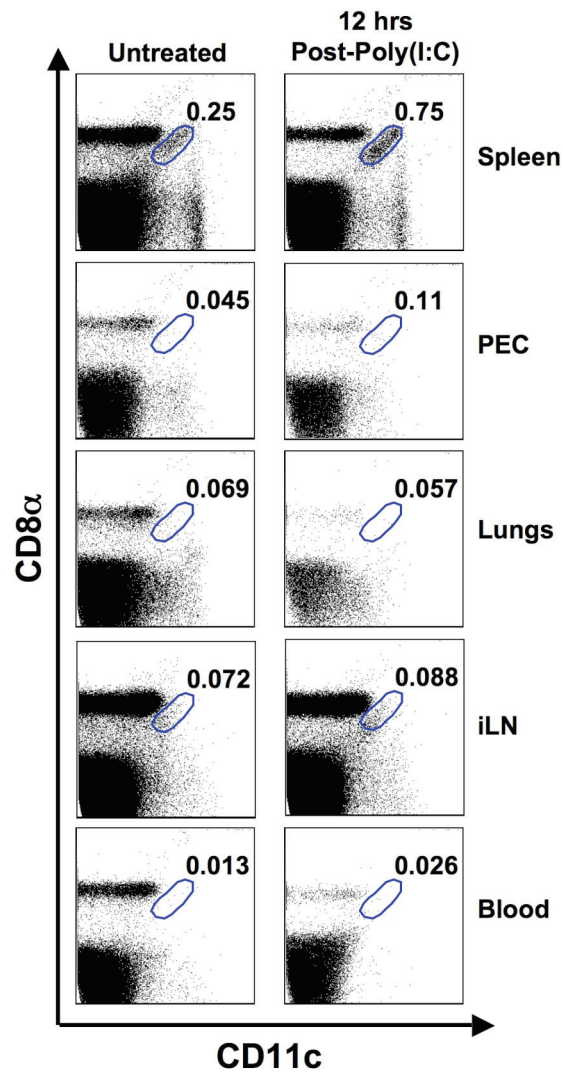


Figure 7 – The increased percentage of the CD8 α ⁺ DCs in the spleen is not observed in other compartments following poly(I:C) treatment. C57BL/6 (wildtype) mice were inoculated with poly(I:C) and spleens, PECs, lungs, iLN, and peripheral blood were harvested 12 hours later. The percentage of CD8 α ⁺ DCs at 0 (untreated) and 12 hours post-poly(I:C). Gates were set upon 7-AAD⁻ cells. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

I also questioned whether this increase in splenic CD8 α ⁺ DCs was type 1 IFN-dependent. Both wildtype and IFN1-R KO mice were inoculated with poly(I:C) and splenocytes were harvested at 12 hours post-treatment. I observed

a 3-fold increase in the percentage of wildtype CD8 α^+ DCs (0.6% to 1.5%, $p = 0.0073$), compared to a 1.5-fold increase in the percentage of IFN1-R KO CD8 α^+ DCs (0.6% to 0.8%, $p = 0.0373$) at 12 hours post-poly(I:C) (Figure 8), suggesting that this increase was partially type 1 IFN-dependent. Collectively, these results indicate that the increase in CD8 α^+ DCs is partially type 1 IFN-dependent and specific to the spleen. Furthermore, this increase in CD8 α^+ DCs accounts for the observed increase in Annexin V reactive cells following poly(I:C) treatment.

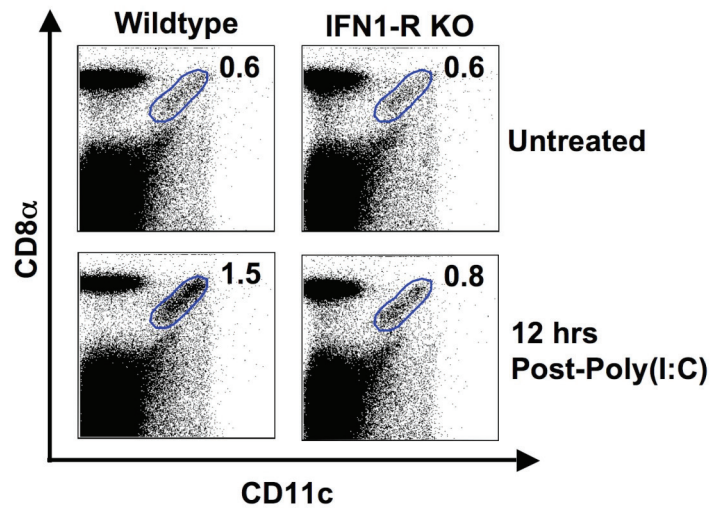


Figure 8 – The increase in the percentage of the $CD8\alpha^+$ DCs in the spleen is type 1 IFN-dependent. C57BL/6 (wildtype) mice and IFN1-R KO mice were inoculated with poly(I:C) and spleens were harvested 12 hours later. The percentage of wildtype and IFN1-R $CD8\alpha^+$ DCs at 0 (untreated) and 12 hours post-poly(I:C). Gates were set upon 7-AAD⁻ cells. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

C. Re-evaluation of the type 1 IFN-Induced attrition of CD8 T Cells

In light of these findings, it was necessary to re-evaluate the early attrition of CD8⁺ T cells using the anti-CD8 β antibody, which is a more exclusive marker for T cells than the anti-CD8 α antibody. I first conducted a kinetic analysis of the poly(I:C)-induced attrition of CD8 β ⁺CD44^{hi} T cells in the spleen. This time course study revealed a 27% decrease in the percentage of CD8 β ⁺CD44^{hi} T cells as early as 3 hours following poly(I:C) treatment and a 51% decline at 12 hours (Figure 9A). This was reflected in a statistically significant decrease in CD8 β ⁺CD44^{hi} T cell number at 12 hours post-poly(I:C) treatment (Figure 9B). This reduction in splenic CD8 β ⁺CD44^{hi} T cells was not due to trafficking to other organs, as the PECs, lungs, iLN, lungs, and peripheral blood all exhibited significant, although varying, decreases in the percentage of CD8 β ⁺CD44^{hi} T cells at 12 hour following poly(I:C) treatment (Figure 10). These data support the notion that the type 1 IFN-induced attrition of CD8 β ⁺CD44^{hi} T cells was a “global” phenomenon and could not be due to migration out of the spleen, although a small re-distribution of cells cannot be completely ruled out.

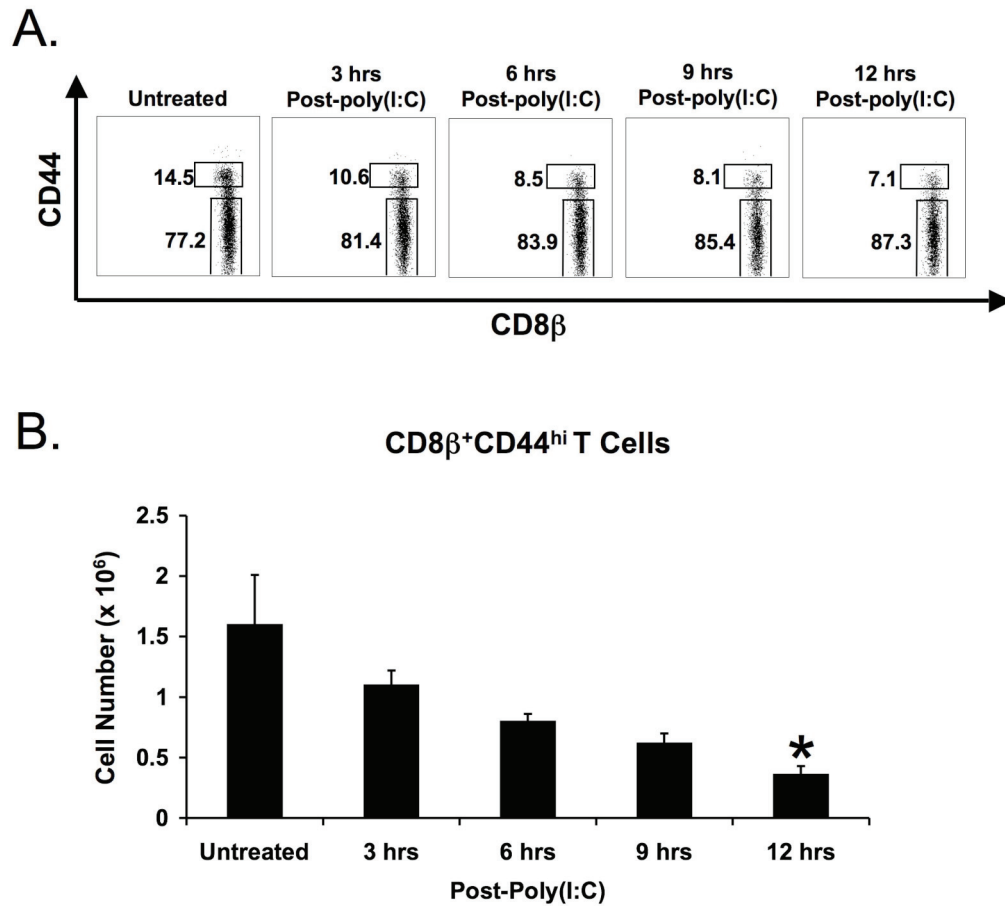


Figure 9 – CD8 β^+ CD44^{hi} T cells undergo attrition following poly(I:C) treatment. Wildtype mice were inoculated with poly(I:C). Splenocytes were harvested at 0 (untreated), 3, 6, 9, and 12 hours post-poly(I:C). *A*, the percentage of CD8 β^+ CD44^{hi/lo} T cells. Gates were set on 7-AAD⁻ cells. *B*, absolute numbers of CD8 β^+ CD44^{hi} T cells. Numbers were calculated based on percentages obtained via flow cytometry. Representative data with $n=3$ per group. *, $P < 0.05$, cell number at 12 hours post-poly(I:C) treatment compared to 0 hours (untreated). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

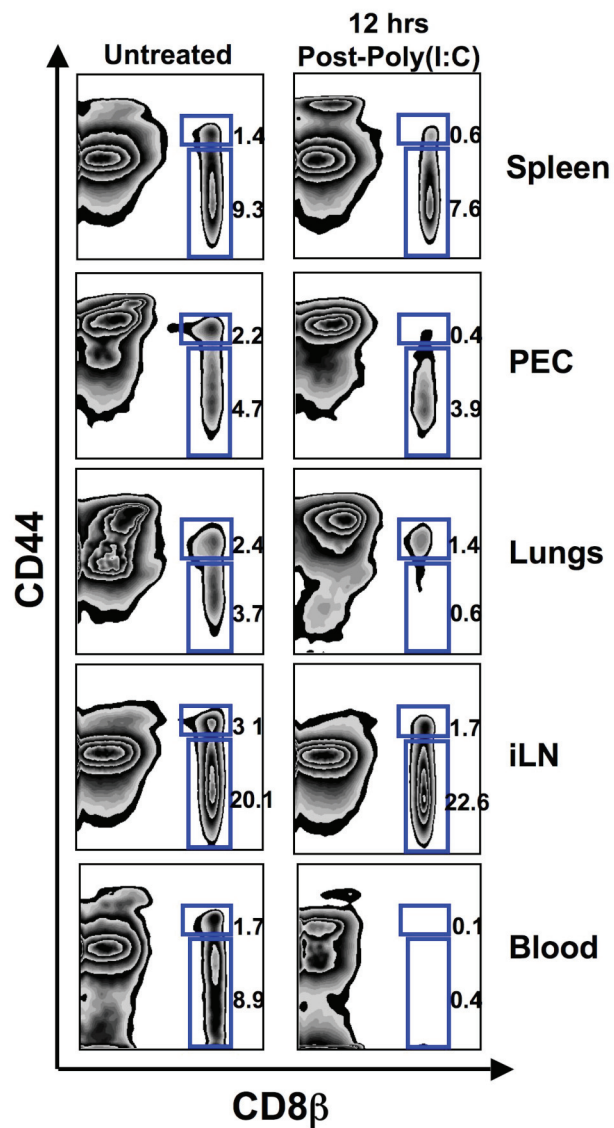


Figure 10 – Trafficking to other compartments does not account for the loss of splenic CD8 β ⁺CD44^{hi} T cells following poly(I:C) treatment. C57BL/6 (wildtype) mice were inoculated with poly(I:C) i.p. Splenocytes, PECs, Lungs, iLN, and peripheral blood were harvested at 0 (untreated) and 12 hours post-inoculation. Plots display the percentage of CD8 β ⁺CD44^{hi/lo} at 0 (Untreated) and 12 hours post-poly(I:C) treatment. Gates were set upon 7-AAD⁻ cells. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

D. Re-evaluation of the type 1 IFN-Induced attrition of CD8 T cells associated with viral infection

I next re-evaluated the attrition of CD8 T cells following LCMV infection. There was a greater increase in the percentage CD40⁺Annexin V⁺ cells in the CD8 α ⁺CD44^{hi} gate compared to the CD8 β ⁺CD44^{hi} gate, suggesting that the CD8 α ⁺ DC population was present following LCMV infection (Figure 11). Therefore, I re-evaluated whether attrition of CD8 β ⁺CD44^{hi} T cells was dependent on type 1 IFN induction following LCMV infection. Both wildtype and type 1 IFN1-R knockout mice were infected with LCMV. Spleens were harvested 3 days post-infection. There was an 80% decrease in the percentage of wildtype CD8 β ⁺CD44^{hi} T cells relative to the untreated control, and an 80% decrease in overall wildtype CD8 β ⁺CD44^{hi} T cell number (Figure 12A and 12B). There was a statistically significant decrease in the percentage of IFN1-R KO CD8 β ⁺CD44^{hi} T cells ($p < 0.05$), but no corresponding decrease was observed in IFN1-R KO CD8 β ⁺CD44^{hi} T cell number (Figure 12A and 12B). These data suggest that the early attrition of CD8 β ⁺CD44^{hi} T cells following LCMV infection is type 1 IFN-dependent.

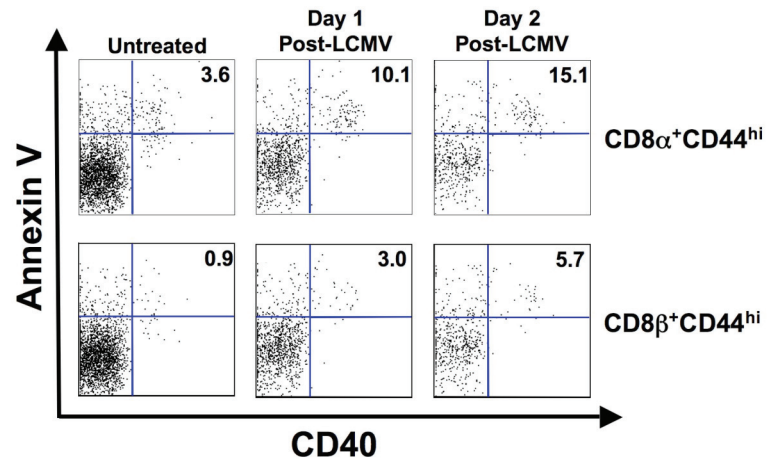


Figure 11 - Increase in Annexin V reactivity correlates with an increase in CD40 expression on CD8 α ⁺CD44^{hi}, but not CD8 β ⁺CD44^{hi} cells, following LCMV infection. C57BL/6 (wildtype) mice were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated), 1, and 2 days post-infection. Percentage of Annexin V⁺CD40⁺CD8 α ⁺CD44^{hi} and Annexin V⁺CD40⁺CD8 β ⁺CD44^{hi} cells. Gates were set on 7-AAD⁻ cells. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

Although I have shown that the attrition of CD8 β ⁺CD44^{hi} T cells is type 1 IFN-dependent, it was now unclear whether this loss is due to apoptosis. Previous studies have shown that the IFN- α -induced apoptosis of some malignant cell lines occurs by a caspase-dependent mechanism (Thyrell, Erickson et al. 2002). I, therefore, compared the level of caspase 3 and 8 activation between CD8 β ⁺CD44^{hi} T cells from both wildtype and IFN1-R KO mice, 3 days post-LCMV infection. Both wildtype and IFN1-R KO CD8 β ⁺CD44^{hi} T cells had small but significant increases in the MFI of caspase 8 activity 3 days post-LCMV infection (Figure 12C). Both caspase 8 (Figure 12C) and caspase 3 (Figure 12D) activity was significantly increased in the wildtype, but not the IFN1-R KO CD8 β ⁺CD44^{hi} T cell population, suggesting that the activation of caspases might contribute to the type 1 IFN-induced apoptosis of this population.

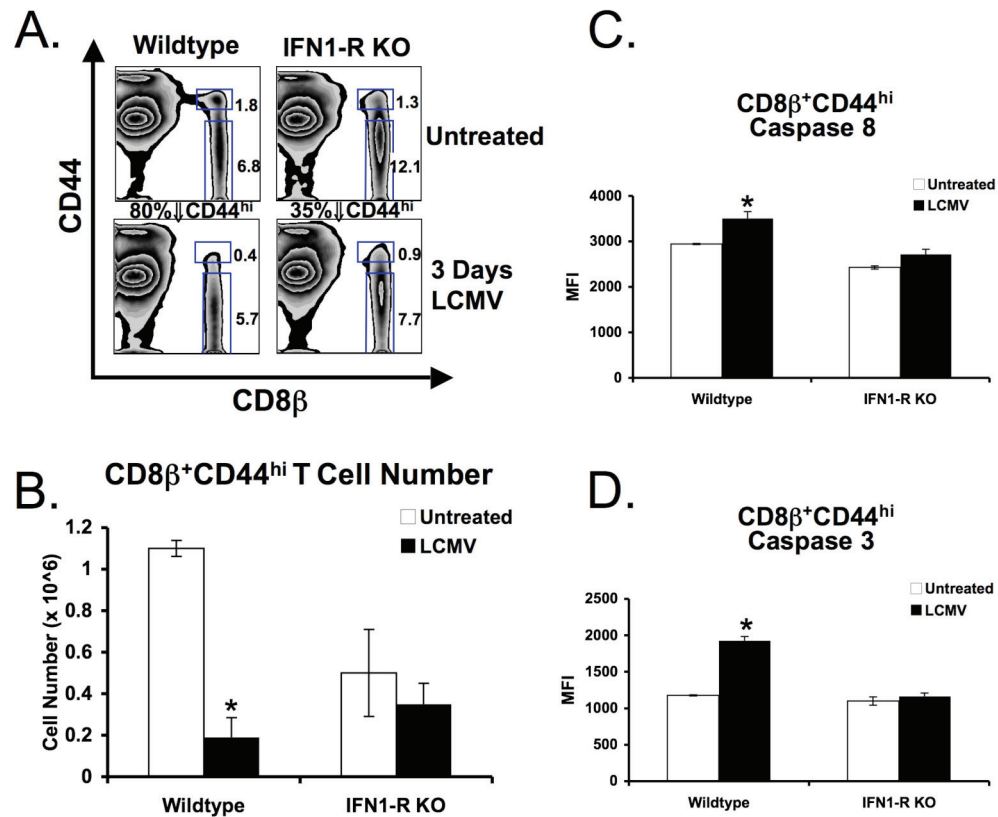


Figure 12 – The attrition of CD8 β ⁺CD44^{hi} T cells, following LCMV infection, is type 1 IFN-dependent and correlates with an increase in caspase activation. C57BL/6 (wildtype) and IFN1-R KO were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated) and 3 days post-infection. **A**, plots show the percentage of both wildtype and IFN1-R KO CD8 β ⁺CD44^{hi/lo} T cells. **B**, absolute numbers of wildtype and IFN1-R KO CD8 β ⁺CD44^{hi} T cells. Numbers were calculated based on percentages obtained via flow cytometry. **C**, MFI of caspase 8 activation of wildtype and IFN1-R KO CD8 β ⁺CD44^{hi} T cells. **D**, MFI of caspase 3 activation of wildtype and IFN1-R KO CD8 β ⁺CD44^{hi} T cells. *, $P < 0.05$, MFI number at 3 days post-LCMV infection compared to day 0 (Untreated Control). Gates were set upon 7-AAD⁻ cells. Representative data with $n=3$ per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

E. The type 1 IFN-induced attrition of CD4 T cells associated with viral infection

It was shown previously that CD4 T cell memory remains remarkably stable under conditions of CD8 memory T cells loss (Varga, Selin et al. 2001), but I questioned whether CD4⁺CD44^{hi} T cells were as susceptible as CD8 β ⁺CD44^{hi} T cells to the type 1 IFN-induced attrition following LCMV infection. There was a 65% decrease in the percentage of wildtype CD4⁺CD44^{hi} T cells, 3 days post-LCMV infection, relative to the uninfected control (Figure 13A). This decrease correlated with a greater than 50% reduction in the overall number of CD4⁺CD44^{hi} T cells (Figure 13B). No corresponding decrease was observed in either the percentage or number of CD4⁺CD44^{hi} T cells from IFN1-R KO mice, suggesting that the loss of CD4⁺CD44^{hi} T cells is type 1 IFN-dependent (Figure 13A and 13B). Wildtype CD4⁺CD44^{hi} T cells had significant increases in caspase 3 and 8 activity, 3 days post-LCMV infection, relative to the untreated control (Figure 13C and 13D). IFN1-R KO CD4⁺CD44^{hi} T cells, however, had no significant increase in either caspase 3 or 8 activity, relative to the untreated control (Figure 13C and 13D), suggesting that both caspase 3 and 8 may be involved in the type 1 IFN-induced attrition of CD4⁺CD44^{hi} T cells following LCMV infection.

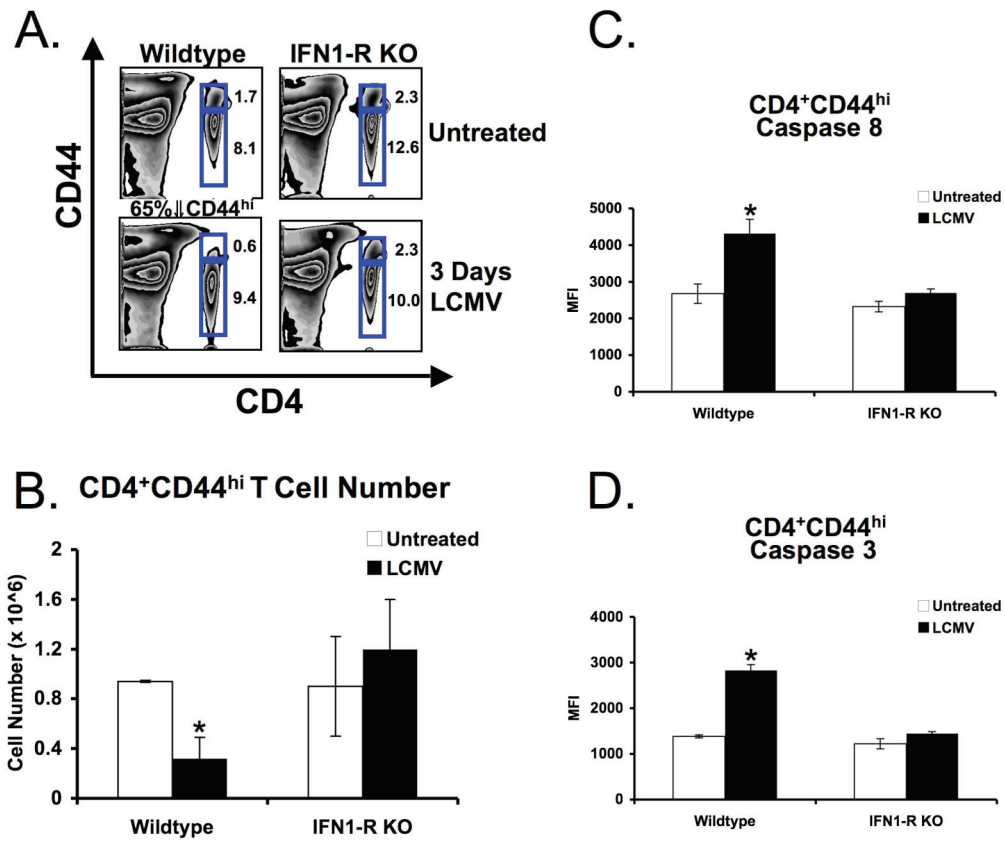


Figure 13 – The attrition of CD4⁺CD44^{hi} T cells, following LCMV infection, is type 1 IFN-dependent and correlates with an increase in caspase activation. C57BL/6 (wildtype) IFN1-R KO were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated) and 3 days post-infection. **A**, plots show the percentage of both wildtype and IFN1-R KO CD4⁺CD44^{hi/lo} T cells. **B**, absolute numbers of wildtype and IFN1-R KO CD4⁺CD44^{hi} T cells. Numbers were calculated based on percentages obtained via flow cytometry. **C**, MFI of caspase 8 activation of wildtype and IFN1-R KO CD4⁺CD44^{hi} T cells. **D**, MFI of caspase 3 activation of wildtype and IFN1-R KO CD4⁺CD44^{hi} T cells. *, $P < 0.05$, MFI number at 3 days post-LCMV infection compared to day 0 (Untreated Control). Gates were set upon 7-AAD⁻ cells. Representative data with $n=3$ per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

F. Apoptosis of T Cells During the Early Immune Response to LCMV

The analysis of apoptosis *in vivo* has proven difficult, because dying cells are rapidly scavenged by phagocytic cells bearing receptors for PS, which is expressed on the surface of apoptotic cells (van Engeland, Nieland et al. 1998). The TUNEL assay relies on the presence of nicks in DNA, which can be identified by terminal transferase, an enzyme that will catalyze the addition of fluorescently labeled dUTPs. Splenocytes isolated from either uninfected or LCMV-infected (Day 3) wildtype mice were incubated at 37°C for 5 hours. This short *in vitro* incubation allows for the detection of DNA fragmentation in cells via TUNEL staining. As a control, splenocytes were also incubated at 4°C for 5 hours. Incubation at this non-physiological temperature would result in a decrease in DNA fragmentation, since metabolic processes would be hindered. After brief incubation at 37°C, 16.3% of uninfected CD8 β ⁺CD44^{hi} T cells were TUNEL⁺, compared to 36.8% of LCMV-infected CD8 β ⁺CD44^{hi} T cells (6.2% and 12% at 4°C, respectively) (Figure 14A). Similarly, 26.7% of uninfected CD4⁺CD44^{hi} T cells were TUNEL⁺, compared to 39.6% of LCMV-infected CD4⁺CD44^{hi} T cells at 37°C (9.2% and 18.7% at 4°C, respectively) (Figure 14B). These results suggest that the attrition of both CD8 β ⁺CD44^{hi} and CD4⁺CD44^{hi} T cell populations, following LCMV infection, occurs through an apoptotic mechanism due to the increased amounts of DNA fragmentation, as detected by TUNEL.

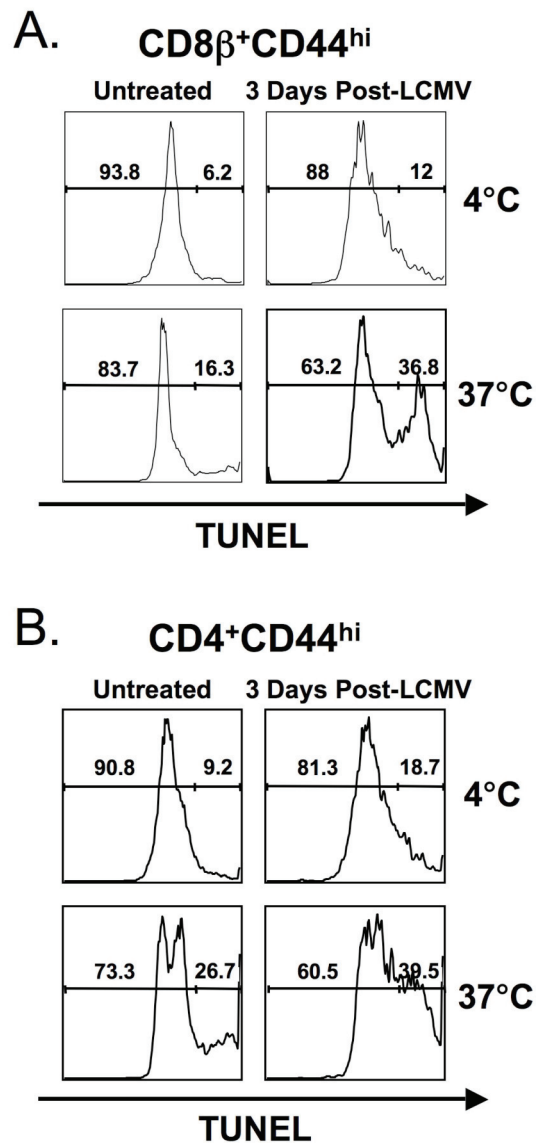


Figure 14 – The LCMV-induced attrition of CD8 β ⁺CD44^{hi} and CD4⁺CD44^{hi} T cells is due to apoptosis. C57BL/6 mice were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated) and 3 days post-infection. DNA fragmentation was assessed via TUNEL staining after a brief *in vitro* culture of splenocytes for 5 hours at either 37°C or 4°C (see materials and methods). *A*, DNA fragmentation, as measured by TUNEL, of wildtype CD8 β ⁺CD44^{hi} T cells (37°C and 4°C). *B*, DNA fragmentation, as measured by TUNEL, of wildtype CD4⁺CD44^{hi} T cells (37°C and 4°C). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

G. Bim-Mediated Apoptosis of T Cells During the Early Immune Response to LCMV

Bim is a pro-apoptotic molecule involved in mitochondrial-induced apoptosis and type 1 IFN was found to induce apoptosis in certain cancer cell lines through the upregulation of Bim (Gomez-Benito, Balsas et al. 2007). Therefore, I questioned whether the apoptosis of T cells would be reduced in Bim KO mice, relative to wildtype mice at 3 days post-LCMV infection. Over 70% of wildtype CD8 β ⁺CD44^{hi} cells were depleted in percentage and number at 3 days post-LCMV infection compared to only 40% of Bim KO CD8 β ⁺CD44^{hi} cells, relative to the untreated control (Figure 15A and 15B). The decrease in Bim KO CD8 β ⁺CD44^{hi} cell number, however, was not significant (Figure 15B).

Others have suggested that decreased induction of functional type 1 IFN may be involved in the reduced apoptosis of T cells (Jiang, Gross et al. 2005). Serum from wildtype and Bim KO mice, at 3 days post-LCMV, had comparable type 1 IFN endpoint dilution titers in a standard type 1 IFN bioassay using microtiter plate wells of L-929 cells challenged with vesicular stomatitis virus (1/2560 \pm 0, n=3). Therefore, differences in the apoptosis between wildtype and Bim KO CD8 β ⁺CD44^{hi} cells cannot be attributed to differences in type 1 IFN induction.

After brief incubation at 37°C, 19.2% of untreated wildtype CD8 β ⁺CD44^{hi} T cells were TUNEL⁺, compared to 12.3% of Bim KO CD8 β ⁺CD44^{hi} cells (5.6% and 5.0% at 4°C, respectively) (Figure 15C). At 3 days post-LCMV infection, 48.3% of wildtype CD8 β ⁺CD44^{hi} T cells were

TUNEL⁺, compared to only 15% of Bim KO CD8 β ⁺CD44^{hi} T cells at 37°C (18.1% and 7.0% at 4°C, respectively) (Figure 15C). The reduced attrition coupled with the reduction in DNA fragmentation in Bim KO mice suggests that Bim may contribute to the apoptosis of CD8 β ⁺CD44^{hi} T cells following LCMV infection.

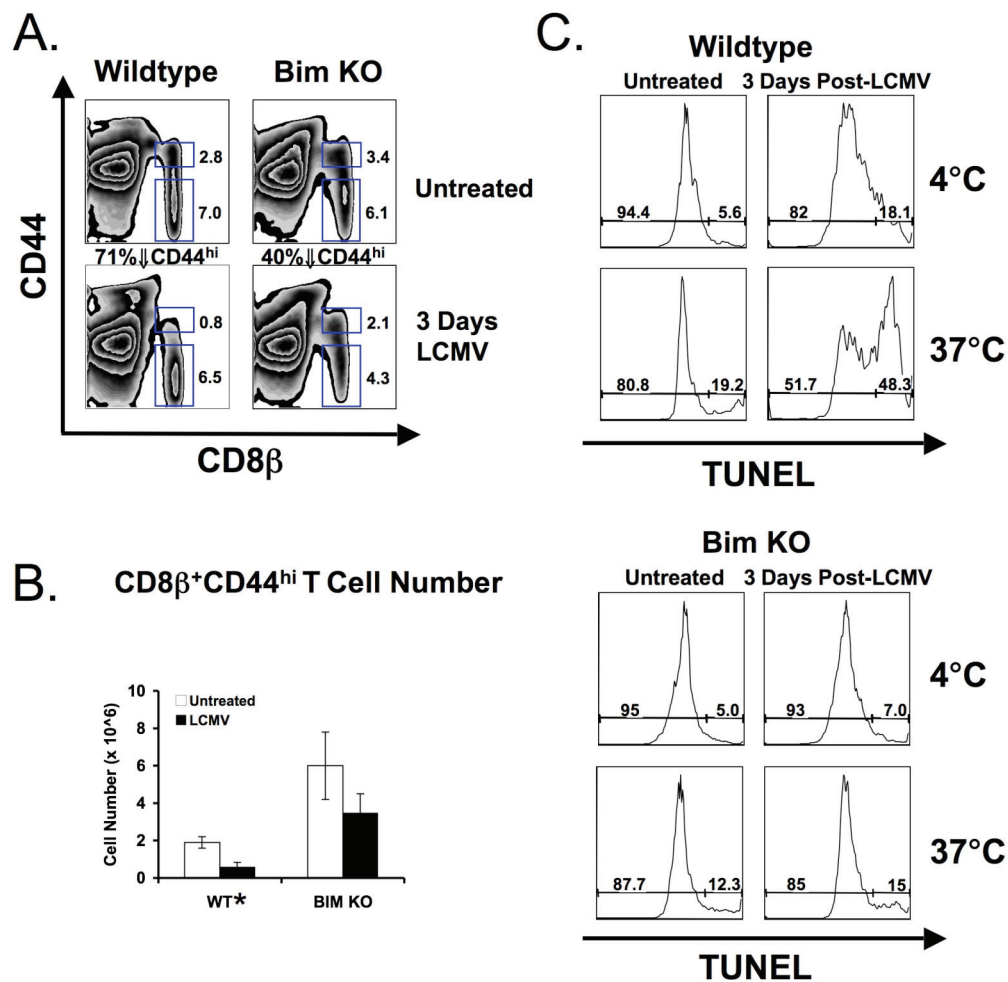


Figure 15 – The LCMV-induced apoptosis of CD8 β ⁺CD44^{hi} T cells is partially dependent on Bim. C57BL/6 (wildtype) and Bim-deficient mice (Bim KO) were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated) and 3 days post-infection. DNA fragmentation was assessed via TUNEL staining after a brief *in vitro* culture of splenocytes for 5 hours at either 37°C or 4°C (see materials and methods). A, plots show the percentage of both wildtype and Bim KO CD8 β ⁺CD44^{hi/lo} cells. B, absolute numbers of wildtype and Bim KO CD8 β ⁺CD44^{hi} cells. Numbers were calculated based on percentages obtained via flow cytometry. *, $P < 0.05$, cells number at 3 days post-LCMV infection compared to day 0 (untreated). C, DNA fragmentation, as measured by TUNEL, of both wildtype and Bim KO CD8 β ⁺CD44^{hi} T cells (37°C and 4°C). Representative data with n=3 per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

The attrition of CD4⁺CD44^{hi} T cells in wildtype and Bim KO mice at 3 days following LCMV infection was also examined. At 3 days post-LCMV infection, there was a 69% decrease in both the percentage and number of wildtype CD4⁺CD44^{hi} T cells compared to only a 44% decrease in Bim KO CD4⁺CD44^{hi} T cells, relative to the untreated control (Figure 16A). The decrease in Bim KO CD4⁺CD44^{hi} T cell number, however, was not significant (Figure 16B). After brief incubation at 37°C, 27.3% of untreated wildtype CD4⁺CD44^{hi} T cells were TUNEL⁺, compared to 15.8% of Bim KO CD4⁺CD44^{hi} T cells (10.9% and 6.5% at 4°C, respectively) (Figure 16C). At 3 days post-LCMV infection, 45.5% of wildtype CD4⁺CD44^{hi} T cells were TUNEL⁺, compared to only 28% of Bim KO CD4⁺CD44^{hi} T cells at 37°C (22.2% and 11.2% at 4°C, respectively) (Figure 16C). Collectively, these results suggest that Bim may be involved in apoptosis of CD4⁺CD44^{hi} T cells following LCMV infection. Moreover, these results suggest a common mechanism in the apoptosis of CD8β⁺CD44^{hi} and CD4⁺CD44^{hi} T cells following LCMV infection.

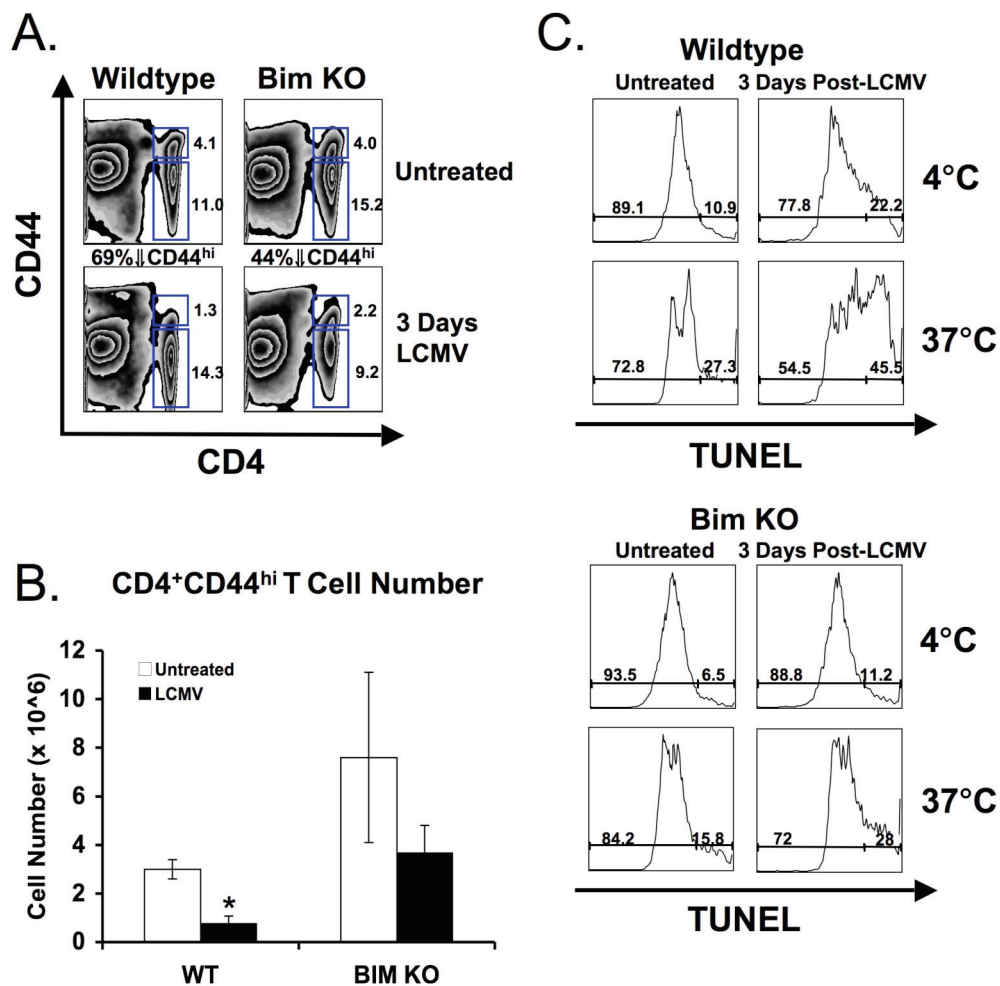


Figure 16 – The LCMV-induced apoptosis of CD4⁺CD44^{hi} T cells is partially dependent on Bim. C57BL/6 (wildtype) and Bim-deficient mice (Bim KO) were infected with LCMV-Armstrong i.p. Splenocytes were harvested at 0 (untreated) and 3 days post-infection. DNA fragmentation was assessed via TUNEL staining after a brief *in vitro* culture of splenocytes for 5 hours at either 37°C or 4°C (see materials and methods). **A**, plots show the percentage of both wildtype and Bim KO CD4⁺CD44^{hi/lo} cells. **B**, absolute numbers of wildtype and Bim KO CD4⁺CD44^{hi} cells. Numbers were calculated based on percentages obtained via flow cytometry*, $P < 0.05$, cells number at 3 days post-LCMV infection compared to day 0 (untreated). **C**, DNA fragmentation, as measured by TUNEL, of both wildtype and Bim KO CD4⁺CD44^{hi} T cells (37°C and 4°C). Representative data with $n=3$ per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

H. The Role of “lymphoid” CD8 α ⁺ Dendritic Cells During type 1 IFN-Induced Apoptosis of T Cells

It has been shown that the “lymphoid” CD8 α ⁺ DC population is capable of phagocytosing apoptotic cells (Albert, Pearce et al. 1998; Albert, Sauter et al. 1998). Monocytes have been shown to phagocytose apoptotic bodies that are shed from the neighboring apoptotic cell and, in the process, become “false positive” by the Annexin V assay (Marguet, Luciani et al. 1999). This suggests that CD8 α ⁺ DCs might become Annexin V⁺ upon engulfing apoptotic lymphocytes. Therefore, I examined whether this CD8 α ⁺ DC population might be involved in the clearance of apoptotic cells following poly(I:C) treatment. To do this, I adoptively transferred CFSE-labeled wildtype splenocytes (Ly5.1) into congenic wildtype hosts (Ly5.2) and harvested splenocytes at multiple timepoints following poly(I:C) treatment. The CD8 α ⁺CD11c⁺ DC population assimilated CFSE⁺ donor cells at 9 and 12 hours post-poly(I:C), while the CD8 α ⁻CD11c⁺ DC population had a relatively lower capacity for the uptake of CFSE⁺ donor cells at similar timepoints (Figure 17). Collectively, these results suggest that the CD8 α ⁺CD11c⁺ DC population may aid in the rapid clearance of apoptotic cells following type 1 IFN-induced attrition and in turn, become reactive with Annexin V in the process.

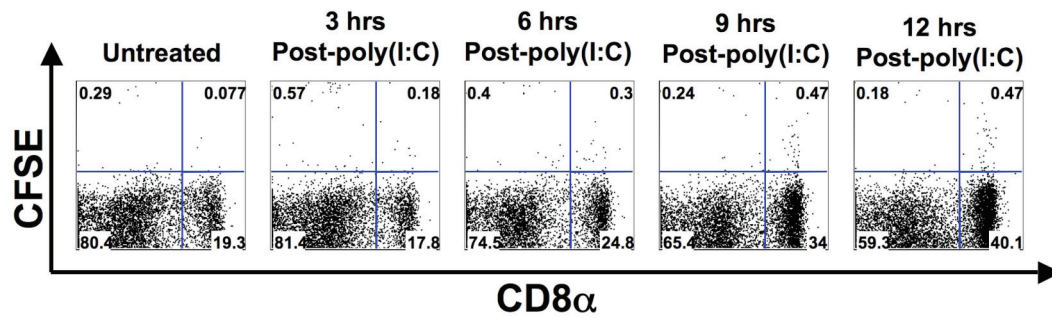


Figure 17 – CD8 α ⁺CD11c⁺ DCs may contribute to the rapid clearance of apoptotic cells. Two x 10⁷ CFSE-labeled wildtype splenocytes (Ly5.1) were adoptively transferred into congenic wildtype recipients (Ly5.2). Splenocytes were harvested at 0 (untreated), 3, 6, 9, and 12 hours post-poly(I:C). The capacity of host (Ly5.2) CD8 α ⁺CD11c⁺ and CD8 α -CD11c⁺ DCs to take up CFSE⁺ donors cells (Ly5.1). Gates were set upon CD11c⁺7-AAD⁻ host cells (Ly5.2). FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

Chapter IV: Type 1 IFN-Induced Attrition of CD8 T Cells in the Presence or Absence of Cognate Antigen During the Early Stages of LCMV Infection

In the previous chapter, I investigated potential mechanisms involved in the type 1 IFN-induced attrition of T cells associated with viral infection. It was still unclear, however, whether T cells specific to the virus were as susceptible as non-specific T cells to this early attrition phase. Studies have shown that engagement of naïve T cells with their ligand, under conditions or appropriate co-stimulation, causes the upregulation of anti-apoptotic molecules, thereby giving them a survival advantage over non-specific cells (Boise, Minn et al. 1995). Moreover, a previous study reported that adoptively transferred P14 transgenic T cells, specific for the GP33 epitope of LCMV, were protected from depletion when measured 3 days after LCMV infection, whereas OT-1 transgenic T cells, specific for OVA, were significantly depleted, suggesting that TCR engagement by antigen may protect antigen-specific cells depletion (Jiang, Lau et al. 2003). It was unclear, however, whether this reflected a resistance to early depletion or a compensatory proliferation after the depletion event had occurred. I therefore designed two adoptive transfer models to examine whether a high concentration of antigen protects antigen-specific T cells from the early type 1 IFN-induced attrition associated with viral infections. In the first model, I examine whether naïve P14 cells and GP33-specific memory cells are protected from depletion by administering a high concentration of GP33-45 peptide, prior to the induction of cytokine-induced lymphopenia. In the second model, I examine whether naïve

P14 cells and GP33-specific memory cells are protected from depletion during a highly disseminating viral infection (Clone 13). Using both of these adoptive transfer model systems, I show that antigen engagement does not protect CD8 T cells from the type 1 IFN-induced attrition associated with viral infections.

A. GP33-45 peptide does not protect naïve antigen-specific CD8 T cells from depletion induced by poly(I:C)

To determine whether antigen engagement protects antigen-specific T cells from depletion, P14 splenocytes (Ly5.2) and OT-1 splenocytes (Thy1.1) were labeled with CFSE and adoptively transferred into naïve B6 recipient Ly5.1 mice. Ly5.1 recipient mice were then inoculated with 5 µg GP33-45 peptide i.v. or PBS i.v., 1 day after transfer. Immunization with GP33-45 peptide alone elicits CTL responses *in vivo* (Ciupitu, Petersson et al. 1998), making it an appropriate antigen for use in our model system. This concentration of peptide and inoculation procedure for P14 cells had been optimized by others in our department and shown to result in the proliferation of virtually all (>95%) detectable CFSE-labeled P14 cells by 3 days post-peptide inoculation (LuAnn Pozzi, UMass Medical School, Ph.D. Thesis, 2005). In my hands, over 70% of the P14 transgenic cells had up-regulated CD69 expression by 21 hours post-peptide treatment (Figure 18A), and no division had yet occurred, as indicated by the lack of CFSE dilution (Figure 18B). These results indicate that there was sufficient GP33 peptide available to engage the receptors on the P14 T cells. Moreover, this dose of peptide did not result in the proliferation of P14 donor cells

between the time of initial peptide inoculation and splenocyte harvest (21 hours). Both P14 and OT-1 donor populations had a limited number of “memory phenotype” cells, as less than 15% were CD44^{hi}. Five hours after the GP33-45 peptide was administered, recipient mice were inoculated i.p. with either poly(I:C) or PBS. Splenocytes were harvested 16 hours after poly(I:C) inoculation. Previous experiments using BrdU to determine cell division showed no increase in labeling with BrdU by CD8 T cells 24 hours following poly(I:C) treatment, suggesting that there was no cell division immediately following type 1 IFN treatment (McNally, Zarozinski et al. 2001). Therefore, this system allows me to study whether TCR engagement by antigen (GP33 peptide) may protect antigen-specific cells (P14 cells) from depletion without the potential for compensatory proliferation to occur.

Donor splenocytes (combined P14 and OT-1 cells) represented 0.5% of the untreated host’s splenocyte population. In the experiment shown, OT-1 cells comprised 60%, while P14 cells comprised 35% of the donor population, as distinguished by Thy1.2 and V α 2 staining (Figure 18B). The percentage of donor splenocytes decreased by half after poly(I:C) treatment, to 0.24%, but the ratio of the transgenic T cells in the donor population changed only slightly (OT-1 to P14 ratio was 54% to 41%, respectively), suggesting that both transgenic T cell populations were equally susceptible to depletion. Inoculation with GP33-45 peptide alone had little effect on the percentage of donor splenocytes (0.52%) or on the ratio of OT-1 to P14 donor cells (57% to 38%) in the donor population. After poly(I:C) inoculation of GP33-45 peptide-treated host mice, there was a

substantial loss in the donor splenocyte population (0.35%), which was similar to poly(I:C) treatment without prior GP33-45 peptide treatment (0.24%). Again, the ratio of the two transgenic populations changed slightly (OT-1 to P14 ratio was 61% to 31%, respectively). There was, however, a statistically significant reduction in the percentage of P14 cells in mice treated with GP33-45 peptide followed by poly(I:C) inoculation when compared to poly(I:C) alone, as indicated by the relative decrease in the percentage of P14 cells (31% versus 41%, respectively; $P < 0.01$) (Figure 18B). Moreover, despite the GP33-45 peptide-induced increased activation status (MFI of CD69 expression) of P14 cells relative to OT-1 T cells, this did not correlate with an increased rate of survival after poly(I:C) treatment (19349 vs. 9676, respectively) (Figure 18A).

Figure 18B depicts a representative experiment showing changes in percentage of donor T cell populations within a host's splenocyte population, but this does not take into consideration the global effects of lymphopenia on total (host and donor) cell populations. Fig. 18C depicts the average splenocyte numbers of several mice per group after poly(I:C) treatment. P14 and OT-1 donors were reduced by 50% upon poly(I:C) inoculation. The greater decrease in the number of OT-1 cells relative to the number of P14 cells may be due to different susceptibilities of the transgenic cells to type 1 IFN-induced depletion. When GP33-45 peptide was administered prior to poly(I:C) inoculation, P14 cells were reduced by about 66%, while OT-1 cells were still reduced by about 60%. There was a difference between the number of P14 cells remaining after GP33-45 peptide and poly(I:C) treatment versus poly(I:C) treatment, although not quite

significant ($P < 0.06$) (Figure 18C). Combined, these results suggest that GP33-45 peptide may slightly enhance depletion rather than protect the P14 cells from depletion.

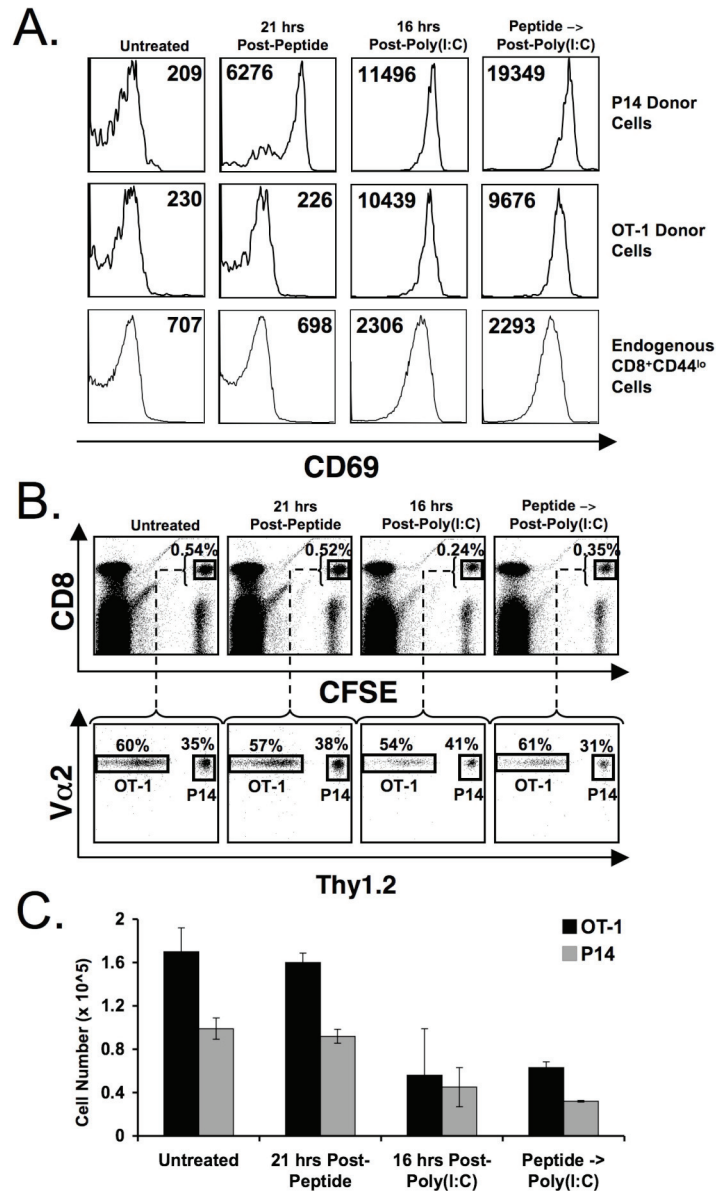


Figure 18 - A high dose of antigen does not protect naïve antigen-specific CD8 T cells from depletion. Two $\times 10^6$ CFSE-labeled P14 cells (Ly5.2) and OT-1 (Thy1.1) cells were co-transferred i.v. into naïve recipients (Ly5.1). Recipient mice were immunized with GP33-45 peptide i.v. 1 day post-transfer. Mice were inoculated with poly(I:C) i.p. 5 hours later. Splenocytes were harvested 16 hours post-poly(I:C) inoculation. *A*, upregulation of CD69 on donor cells donor (P14 and OT-1 cells) and the naïve host CD8 population (CD44^{lo}) post- GP33-45 peptide and/or poly(I:C) inoculation. Numbers in either the upper left/right corners indicate the MFI of CD69 expression. *B*, percentages of P14 and OT-1 donors (gated on CD44^{lo} donor cells) present post- GP33-45 peptide and/or poly(I:C) inoculation. *C*, absolute numbers of P14 and OT-1 donors present post-GP33-45 peptide and/or poly(I:C) inoculation. Numbers were calculated based on percentages obtained from the flow cytometry plots in *B*. Representative data (*B*) with $n=3$ per group (*C*). The data are representative of 2 independent experiments.

B. Antigen-specific naïve CD8 T cells are not protected from depletion during the early phase of an acute viral infection

We next examined whether naïve antigen-specific CD8 T cells are protected from depletion during the early stages of an acute viral infection. To increase the probability of antigen engagement of the T cells, we used the highly disseminating variant of LCMV, Clone 13, which differs from the Armstrong strain by two amino acids, but encodes similar T cell epitopes (Ahmed, Salmi et al. 1984). We chose day 2 post-infection, since both type 1 IFN levels and viral load are high at this time point (Wherry, Blattman et al. 2003), and a decline of CD8 T cell numbers had been detected as early as 2 days post-LCMV Clone 13 infection (Kim and Welsh 2004). CFSE-labeled P14 cells (Ly5.2) were transferred i.v. into either a Day 2 Clone 13-infected mouse or a naïve C57BL/6 mouse (Ly5.1). Splenocytes were harvested 16 hours later. There was a 10-fold loss in the percentage of donor P14 cells when transferred into a Day 2 Clone 13-infected mouse upon harvest (Figure 19A). This loss of donor P14 cells was not specific to the spleen, as the loss also occurred in the lymph nodes (Figure 19A), peripheral blood, bone marrow, peritoneal cavity, and lungs (data not shown), ruling out trafficking to other compartments.

Decreases in the number of P14 donor splenocytes transferred into a day 2 Clone 13-infected mouse were consistent with the decrease in the frequency of total P14 donor cells. Transfer into a Day 2 Clone 13-infected mouse resulted in a >90% reduction in the number of P14 donor cells, relative to transfer into a naïve recipient (Figure 19B). Host CD8 T cells (Ly5.1) underwent a 70%

reduction 2 days post-Clone 13 infection, relative to a naïve Ly5.1 mouse (Figure 19B). These results suggest that the high viral antigen load does not prevent the depletion of antigen-specific naïve P14 cells during the early lymphopenic phase of an acute LCMV infection.

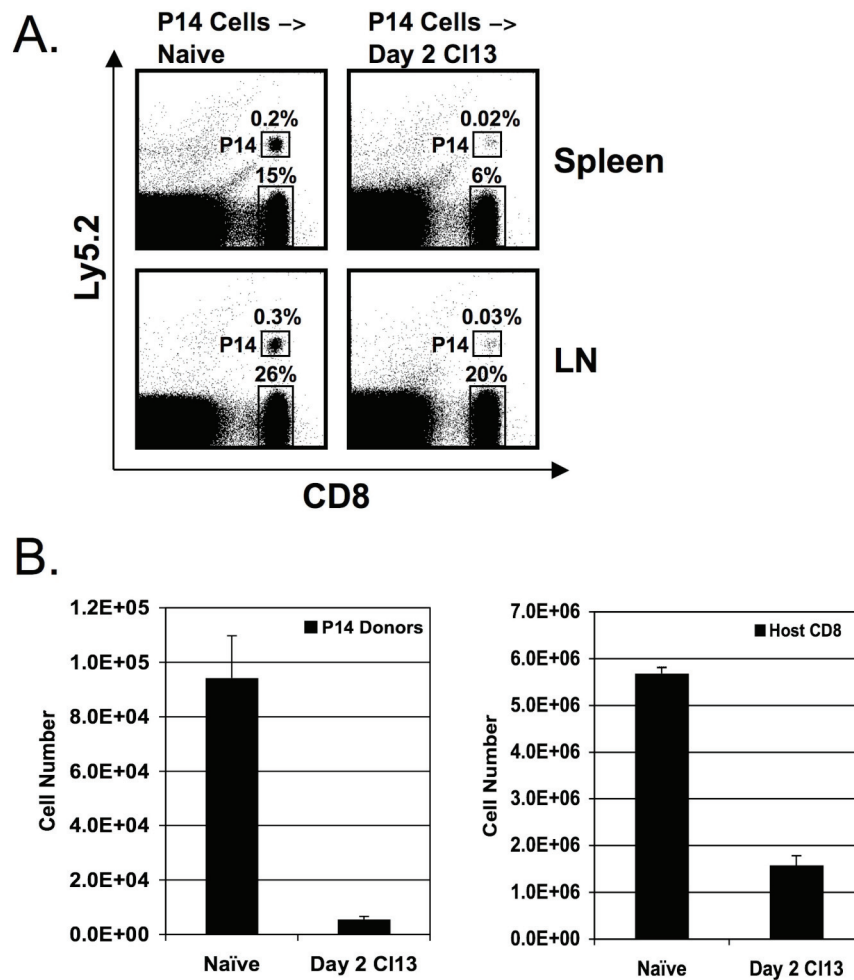


Figure 19 - High viral load does not protect antigen-specific naïve CD8 T cells from depletion during the early phase of an acute viral infection. Four $\times 10^6$ CFSE-labeled P14 transgenic T cells (Ly5.2) were transferred into either a LCMV Clone 13-infected (Day 2) or a naïve C57BL/6 mouse (Ly5.1). Splenocytes were harvested 16 hours post-transfer. *A*, the percentage of P14 donors (Ly5.2) and recipient CD8 T cells (Ly5.1) present in both the pooled lymph nodes (axillary, brachial, cervical, and inguinal lymph nodes) and spleen populations. *B*, absolute numbers of P14 donors splenocytes present 16 hours post-transfer into either a LCMV Clone 13-infected (Day 2) or a naïve C57BL/6 mouse (Ly5.1). Numbers were calculated based on percentages obtained from the flow cytometry plots in *A*. Representative experiment with $n=3$ per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

C. GP33-45 peptide does not protect antigen-specific memory CD8 T cells from depletion by poly (I:C) treatment

Although both naïve and memory CD8 T cells are susceptible to the early apoptosis phenomenon, bona fide antigen-specific memory and “memory phenotype” CD8⁺CD44^{hi} T cells are far more susceptible (Figure 20A) (McNally, Zarozinski et al. 2001). Because memory cells react with ligand in qualitatively and quantitatively different ways than naïve cells (Tanchot, Lemonnier et al. 1997; Zimmermann, Prevost-Blondel et al. 1999; Veiga-Fernandes, Walter et al. 2000; Grayson, Harrington et al. 2002), I also questioned whether antigen engagement would protect them from the early apoptosis. I examined whether treatment with GP33-45 peptide selectively protected GP33-specific, but not other LCMV-specific memory cells, from poly(I:C)-induced depletion. CFSE-labeled LCMV-immune splenocytes (Ly5.2) were adoptively transferred into naïve B6 mice (Ly5.1). Ly5.1 recipient mice were then inoculated with GP33-45 peptide i.v. or PBS i.v. 1 day after transfer. After 5 hours, recipient mice were inoculated with poly(I:C) i.p. Splenocytes were harvested 16 hours post-poly(I:C) inoculation. As expected, both naïve recipient (Ly5.1) and LCMV-immune donor (Ly5.2) CD8 T cell populations underwent attrition upon poly(I:C) inoculation of otherwise untreated mice (92% and 85% reduction, respectively), with a greater decrease in the CD44^{hi} relative to the CD44^{lo} population (Figure 20A). GP33-45 peptide inoculation resulted in a slight reduction in the frequency of LCMV-immune donor (Ly5.2) CD8⁺CD44^{hi} T cells relative to no treatment and did not protect against poly(I:C)-induced attrition. GP33-45 peptide treatment followed by

poly(I:C) inoculation resulted in a similar degree of attrition in both recipient CD44^{hi} (Ly5.1) and LCMV-immune donor (Ly5.2) CD8 T cells compared to poly(I:C) treatment only (Figure 20A and 20B).

Decreases in GP33-specific CD8 T cell numbers upon peptide and/or poly(I:C) treatment were consistent with the decreases in the percentages of total memory T cells (Figure 20B), suggesting that the GP33-specific population was as susceptible as the non-specific memory population. Poly(I:C) treatment resulted in a 67% reduction in the number of GP33-specific CD8 memory T cells and a 51% reduction in the number of total memory CD8 T cells, as determined by α -CD3 stimulation. GP33-45 peptide treatment resulted in almost a 44% decrease in the number of GP33-specific CD8 memory T cells relative to untreated controls and only a marginal 13% decrease in the total number of memory CD8 T cells. GP33-45 peptide treatment followed by poly(I:C) inoculation resulted in an 80% decrease in the number of GP33-specific CD8 memory T cells, relative to untreated controls. The total number of bona fide memory CD8 T cells underwent a similar degree of attrition whether or not the GP33-45 peptide was administered prior to poly(I:C) treatment (Figure 20B). Overall, these findings indicate that GP33-45 peptide was unable to prevent the depletion of GP33-specific CD8 memory T cells during poly(I:C)-induced lymphopenia and may enhance, rather than protect against depletion (Figure 20A and 20B).

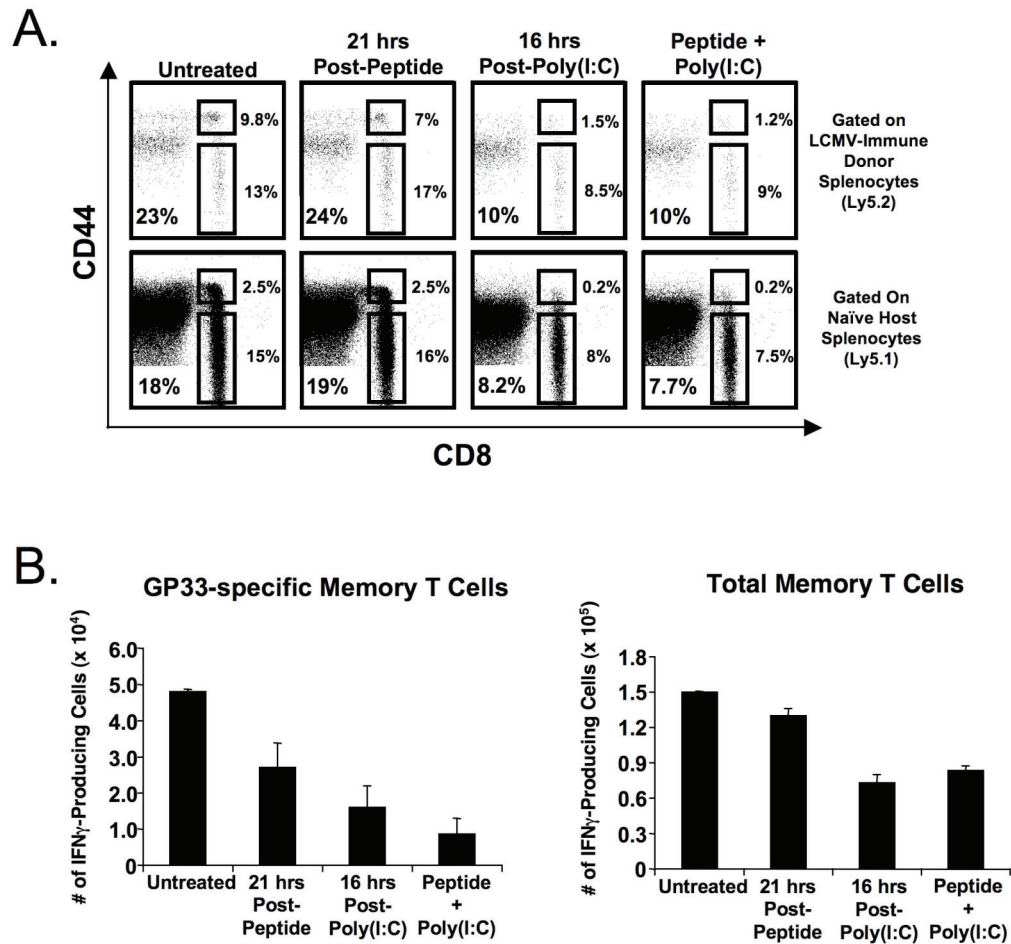


Figure 20 - A high dose of antigen does not protect antigen-specific memory CD8 T cells from depletion. Four $\times 10^7$ CFSE-labeled LCMV-immune cells were transferred i.v. into naïve recipients (Ly5.1). Recipient mice were immunized with GP33-45 peptide (i.v.) 1 day post-transfer. Mice were inoculated with Poly(I:C) (i.p.) 5 hours later. Splenocytes were harvested 16 hours post-Poly(I:C) inoculation. Donor CD8 T cells were visualized via an IFN- γ assay. A, percentages of both CD44^{hi} and CD44^{lo} LCMV donor (Ly5.2) and recipient (Ly5.1) CD8 T cells present post- GP33-45 peptide and/or poly(I:C) inoculation. The percentage of total CD8 T cells is listed in the lower left of each plot. B, absolute numbers of LCMV-specific memory CD8 donor cells present post- GP33-45 peptide and/or poly(I:C) inoculation. Numbers were calculated based on percentages obtained from the flow cytometry plots in A. Representative experiment with n=3 per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

D. Antigen-specific memory CD8 T cells are not protected from depletion during the early phase of an acute viral infection

To test whether LCMV-specific memory CD8 T cells were protected from depletion during the attrition phase of the immune response to a viral infection, CFSE-labeled LCMV-immune cells (Ly5.2) were transferred i.v. into a Day 2 Clone 13-infected host, Day 2 Armstrong-infected host, or a naïve host (Ly5.1). As a non-specific control, PV-immune cells were also transferred under the same conditions. Splenocytes were harvested 16 hours later.

Both LCMV- and PV-immune donor CD8 T cells underwent almost a 50% reduction in both LCMV-Clone 13 and LCMV-Armstrong-infected mice (Figure 21A). There was almost a complete loss of LCMV-specific (NP396, GP33, and GP276) and PV-specific (NP38) CD8 memory T cells after they were transferred into either an Armstrong or Clone 13-infected mouse (2 days post-infection) (Figure 21C). The loss of donor memory cells was not due to trafficking out of the spleen, as other compartments experienced a similar donor cell loss including the lymph nodes (Figure 21B), peripheral blood (0.1% to <0.01%), and lungs (0.1% to 0.04%) 16 hours post-transfer Clone 13-infected mice. These results suggest that the high viral antigen load does not prevent the depletion of LCMV-specific memory CD8 T cells during the early lymphopenic phase of an acute LCMV infection.

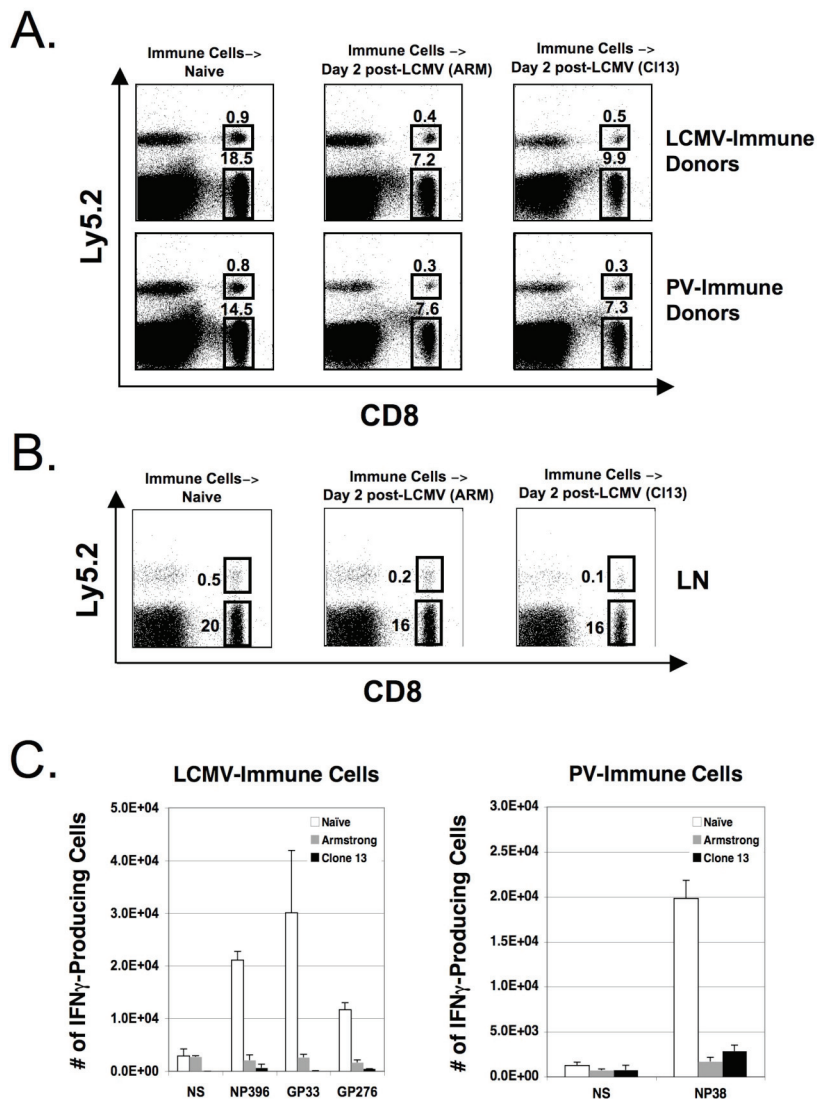


Figure 21 - High viral load does not protect antigen-specific memory CD8 T cells from depletion during the early phase of an acute viral infection. Four $\times 10^7$ CFSE-labeled LCMV-immune cells or PV-immune cells (Ly5.2) were transferred into a LCMV-Armstrong-infected host (Day 2), a LCMV-Clone 13-infected host (Day 2), and a naïve host (Ly5.1). Splenocytes were harvested 16 hours post-transfer. Donor CD8 T cells were visualized via an IFN- γ assay. **A**, percentages of both LCMV and PV donor (Ly5.2) CD8 T cells as well as recipient (Ly5.1) CD8 T cells present post-treatment. **B**, percentages of LCMV donor (Ly5.2) CD8 T cells as well as recipient (Ly5.1) CD8 T cells present in the inguinal lymph nodes 16 hours post-transfer. **C**, absolute numbers of donor LCMV-specific and PV-specific memory CD8 T cells present post-treatment. Numbers were calculated based on percentages obtained the flow cytometry plots in **A**. Representative data with $n=3$ per group. FACS plots represent 1 of 3 mice. The data is representative of 2 independent experiments.

Chapter V: Early CD8 T Cell Attrition Contributes to the Development of a Diverse Immune Response Following Viral Infection

As indicated in the previous chapter, CD8 T cells, regardless of specificity, are susceptible to the type 1 IFN-induced attrition associated with viral infections. Using computer models, we show how this concept is important in the context of heterologous immunity. These models indicate that the attrition of cross-reactive memory CD8 T cells reduces their immunodominance and allows for a more diverse immune response, since memory CD8 T cells are more susceptible to the type 1 IFN-induced attrition than naïve CD8 T cells.

As mentioned previously, others have shown that old mice develop less initial lymphopenia relative to young mice (Jiang, Anaraki et al. 2003). Therefore, using the LCMV/PV cross-reactive model, I also question whether the reduced apoptosis of the cross-reactive memory CD8 population (NP205), in old LCMV-immune mice, allow it to dominate the immune response following heterologous virus challenge (PV). LCMV and Pichinde virus (PV) encode epitopes in the nucleoprotein, NP205-212, sharing 6 of 8 amino acids. In a naïve host, NP205 is normally a subdominant epitope for both viruses, but due to a selective expansion of NP205-specific cross-reactive memory CD8 T cells, the NP205-specific T cell response became dominant when LCMV-immune mice were infected with PV, demonstrating how cross-reactive expansion can alter the hierarchy of T cell responses (Brehm, Pinto et al. 2002). This model shows that the early attrition of T cells allows for the generation of a more diverse T cell

response to infection by reducing the immunodomination caused by cross-reactive T cells.

A. Computer modeling predicts that early attrition of memory CD8 T cells allows for more diversity in newly arising T cell responses

Computer simulations were used to determine the impact of virus-induced lymphopenia on the T cell response to a virus infection in virtual mice that had a partially cross-reactive pool of memory T cells. The simulations first showed that, in the absence of a memory cell lymphopenia, cross-reactive T cells would dominate a new T cell response and inhibit the emergence of a complex naïve T cell response specific to a new pathogen (Figure 22A). The simulation tests that, after a successful interaction with an APC, the T cell is protected from type 1 IFN-mediated death for a designated number of time steps, decided at the beginning of the simulation. Thus, during the heterologous virus infection, only cross-reactive memory T cells are protected from depletion during the early lymphopenia. This is contrasted with the model in which even cross-reactive clones are susceptible to the early lymphopenia.

A virtual mouse was challenged with 70 particles of virus, which were cleared before time step 500, thanks to an effective immune response. The CD8 memory T cells were transferred into different virtual recipients, and a second challenge with 240 particles of a partially cross-reactive virus was administered. The larger amount of challenge virus was necessary to elicit a valid response, because of the cross-reactive CD8 T cell pool. The two simulations modelled

were (1) specific protection from active attrition conferred to cross-reactive clones (Figure 22A) and (2) absence of any kind of protection from attrition (Figure 22B). Here a low affinity cross-reactive clone is depicted in red. In the absence of attrition, this low affinity clone can dominate the immune response against high and medium affinity clones (green and blue) originating at low frequency from a naive memory pool (Figure 22A and 22B). Figure 22B shows that if this low affinity cross-reactive clone undergoes apoptosis, an immunodominant response can be generated by higher affinity clones (i.e. green) from the naïve repertoire.

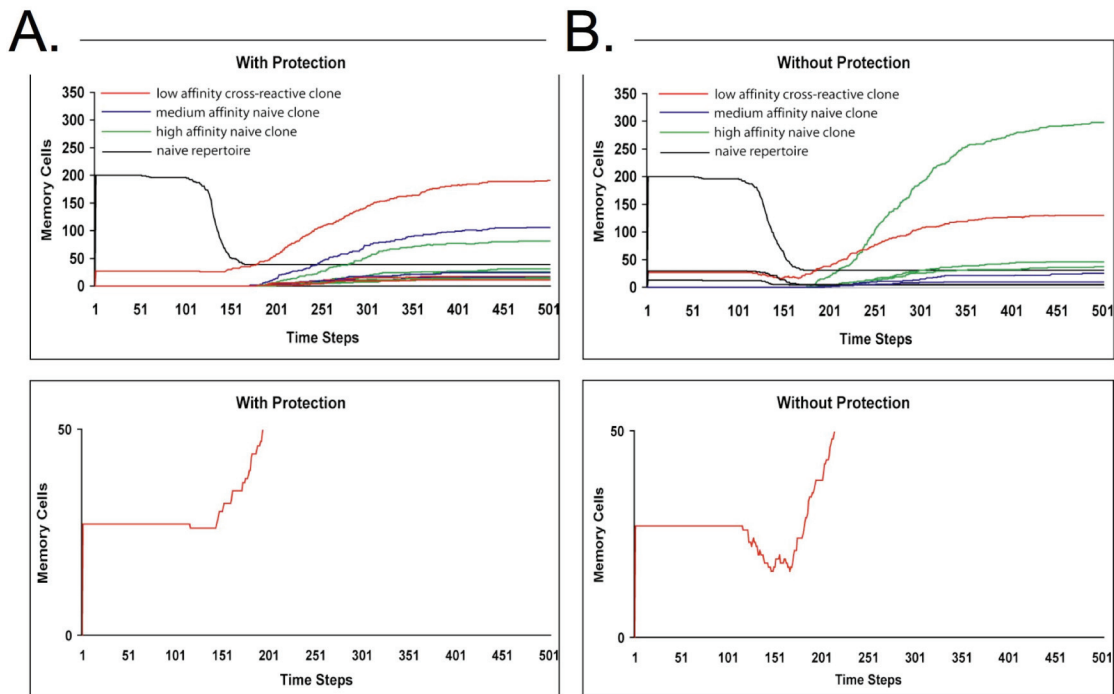


Figure 22 - Computer modelling indicates that the early apoptosis of memory cells allows for more diversity in arising T cell responses. A virtual mouse was challenged with 70 particles of virus. CD8 memory T cells with different affinities (low affinity cross-reactive clone = red, medium affinity naive clones = blue, high affinity naive clones = green, naïve repertoire = black) were transferred into virtual recipients followed by a secondary challenge with 240 particles of a partially cross-reactive virus. *A*, simulation depicting protection from active attrition, where a low cross-reactive affinity clone (red) dominates the immune response. *B*, simulation depicting an absence of protection from active attrition, where a high affinity naïve clone (green) generates an immunodominant response. Plots below *A* and *B* enlarge the otherwise compressed plots for the low affinity cross-reactive clone (red) in order to demonstrate the attrition without ligand induced protection. The plots are representative of 30 repeated runs. IMMSIM computer simulations were conducted by Claudio Calcagno, Dario Ghersi, Roberto Puzone, and Franco Celada at the University of Genoa, Genoa, Italy.

B. Reduced attrition of cross-reactive memory cells in old LCMV-immune mice limits the diversity of the ensuing immune response to heterologous Pichinde infection

The computer simulation supports a model in which the apoptosis of cross-reactive memory CD8 T cells allows for the generation of a diverse immune response. I pre-bled both old (18-22 month old) and young (6-8 month old) LCMV-immune mice to determine the frequency of both dominant (GP33) and subdominant (NP205) epitope-specific memory CD8 T cells via intracellular IFN γ assay. Old mice had GP33 frequencies ranging from 5-15% of the CD8 population, while the young mice had frequencies ranging from 2-7% of the CD8 population (Figure 23A). Old mice had NP205 frequencies ranging from 2-3% of the CD8 population, while young mice had similar frequencies, ranging from 1-2% of the CD8 population (Figure 23A). Young and old LCMV-immune mice were infected with PV, which, like LCMV, induces a potent type 1 IFN response. At day 8 post-infection, the frequency of the cross-reactive memory (NP205) and non-cross-reactive PV-specific (NP38) CD8 T cells was determined via intracellular IFN γ assay. Young and old naïve mice, age matched to their LCMV-immune counterparts, were also infected with PV, to ensure that both old and young naïve mice mount a similar immune response to PV. Both had a comparable NP38-specific response at day 8 post-infection (15.9% and 17.6% of the CD8 population, respectively) (Figure 23B). At day 8 post-infection, 2 out of 3 young LCMV-immune mice had an NP205 to NP38 ratio under 1, suggesting that the loss of the cross-reactive, NP205-specific memory CD8 population allowed

for the successful expansion of the newly arising NP38-specific response (Figure 23B). One young LCMV-immune mouse, however, had an NP205 to NP38 ratio over 1 (5.5) (Figure 22B); this may be due to private specificity of this particular mouse's TCR repertoire (Kim, Cornberg et al. 2005; Cornberg, Chen et al. 2006). Private specificity suggests that the differences in TCR repertoire between mice might dictate the nature of the cross-reactive response. All three old mice had NP205 to NP38 ratios over 1, suggesting that the lack of cross-reactive, NP205-specific memory CD8 cell attrition allowed this population to dominate, thereby, hindering the expansion of the newly arising NP38- response (Figure 23B).

There was a substantial loss of GP33-specific memory CD8 cells at day 8 post-PV infection, relative to day 0, in young LCMV-immune mice (Figure 23A and 23B). This was not surprising, considering that non-cross-reactive memory cells are lost upon heterologous virus challenge (Selin, Vergilis et al. 1996; Selin, Lin et al. 1999; Liu, Andreansky et al. 2003). The GP33-specific memory CD8 response, however, remained quite high in old LCMV-immune mice, at day 8 post-PV, compared to day 0 (Figure 23A and 23B); this is most likely due the reduced lymphopenia in old mice relative to young mice following PV-infection. The NP205 to NP38 ratio was compared between additional young and old LCMV-immune, at day 8 post-PV infection, and this revealed young mice to have a significantly lower ratio relative to old mice (1.5 vs. 2.5, respectively) (Figure 23C). Collectively, these results suggest that the early virus-induced lymphopenia limits the size of the cross-reactive memory pool (NP205), thus

allowing for the successful expansion of non-cross-reactive clones from the naïve pool.

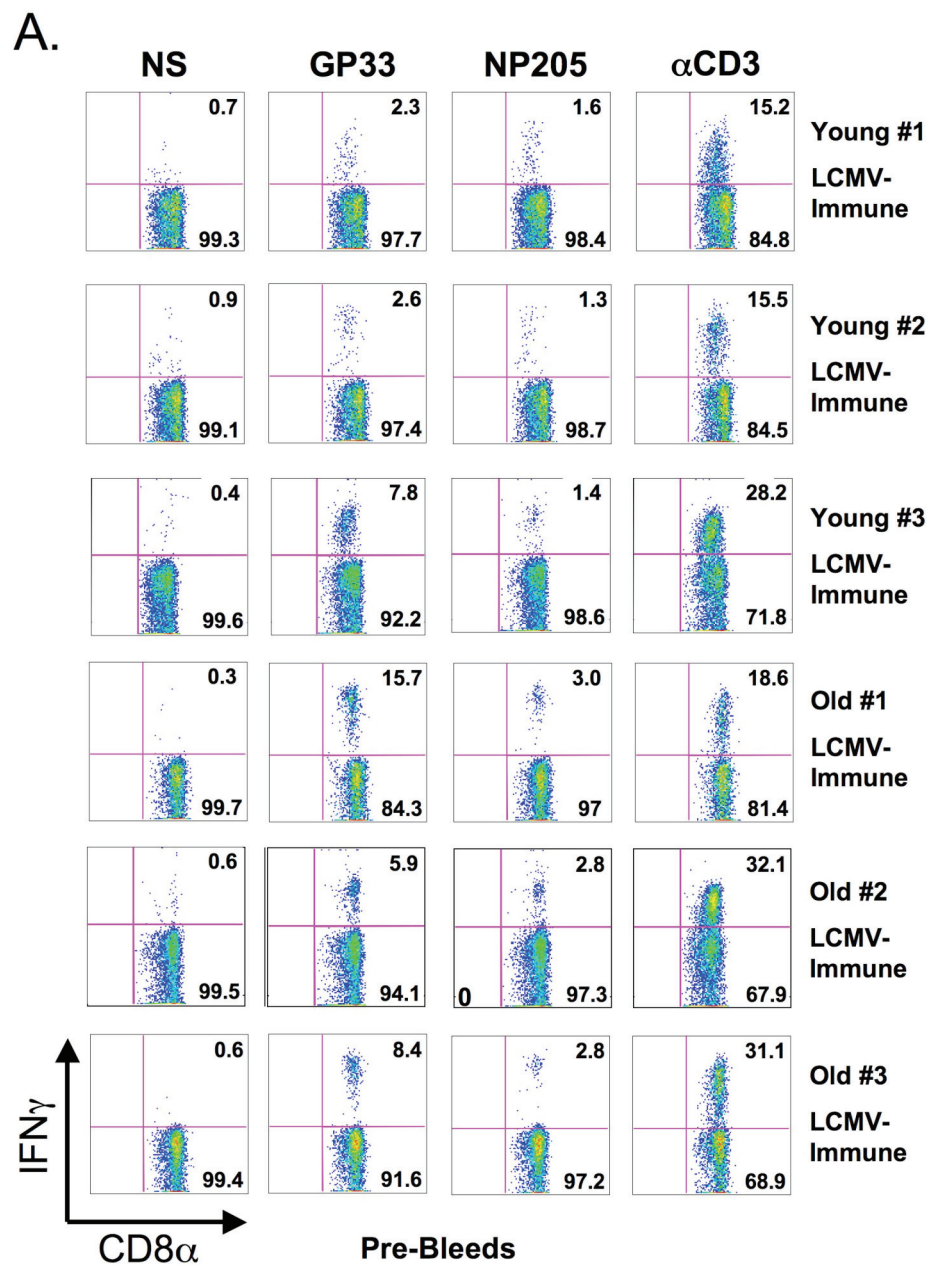


Figure 23

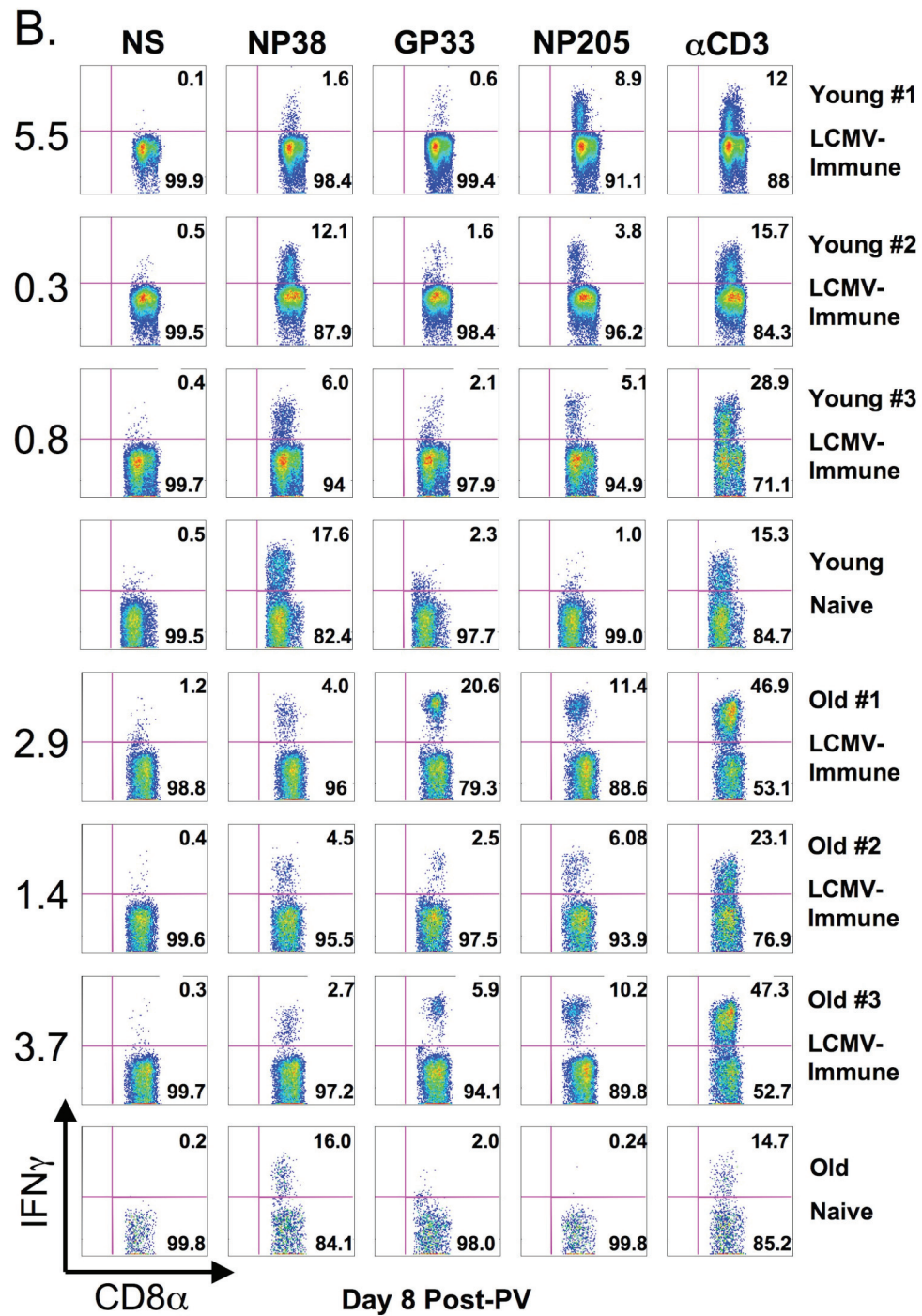


Figure 23

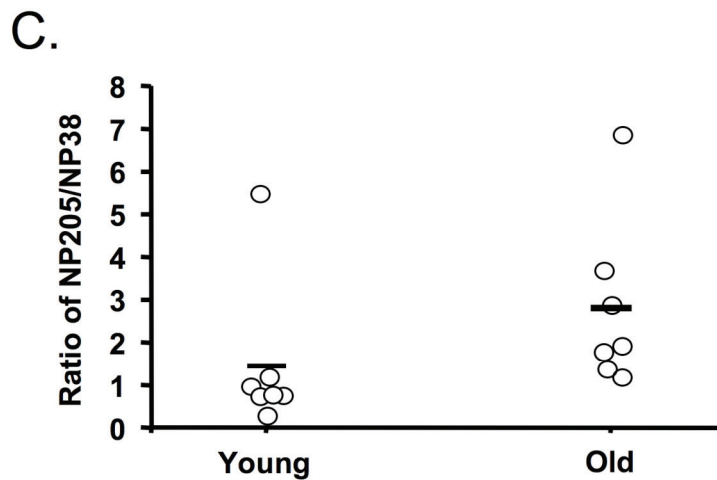


Figure 23 – Reduced attrition in old LCMV-immune mice limits the diversity in the arising T cell response following heterologous Pichinde viral challenge. Young (6-8 months) and old (18-22 months) LCMV-immune mice were pre-bled to determine GP33 and NP205 frequencies prior to infection with PV, via intracellular cytokine assay. Mice were re-bled on day 8 post-PV and the ratios of NP205 to NP38 response were determined via intracellular cytokine assay. *A*, frequencies of GP33- and NP205-specific CD8⁺ T cells prior to PV infection, as revealed by intracellular IFN γ assay. Negative and positive stimulations were included as well (unstimulated, NS, and anti-CD3 stimulation, respectively) *B*, frequencies of NP38-, GP33-, and NP205-specific CD8⁺ T cells, at day 8 post-PV infection, as revealed by intracellular IFN γ assay. Young and old naïve mice were infected with PV to ensure they mount comparable NP38-responses at day 8 post-infection. Numbers on the far left indicates the ratio of the frequencies of NP205- to NP38-specific CD8⁺ T cells. *C*, the ratio of the NP205 to NP38 response for multiple young and old LCMV-immune mice (day 8 post-PV infection). Ratios were based upon intracellular IFN γ assay data. FACS plots represent 1 of 7 mice. The data are representative of 2 independent experiments.

Chapter IV: Discussion

In this thesis, I re-evaluated the early type 1 IFN-dependent loss of T cells associated with viral infections. I showed that the attrition of T cells is due to apoptosis, as revealed by an increase in DNA fragmentation following LCMV infection. I provided evidence that the apoptosis might occur through a mitochondrial-induced pathway involving the pro-apoptotic molecule Bim. I also showed that a population of “lymphoid” CD8 α^+ DCs, which accounted for the previously observed increase in Annexin V reactivity, may aid in the rapid clearance of apoptotic cells during the early type 1 IFN-induced lymphopenic phase. Moreover, I provide evidence that the type 1 IFN-dependent attrition of CD8 T cells is non-selective, as both antigen-specific and non-specific CD8 T cells are equally susceptible. This non-selective depletion of memory CD8 T cells may allow for the generation of a more diverse T cells response to infection by reducing the immunodomination caused by cross reactive T cells as indicated by the computer generated models. I also show that the reduced attrition in LCMV-immune aged mice results in the immunodomination of cross-reactive T cells (NP205) following heterologous PV infection. A more in depth discussion of these results follows.

A. A re-assessment of the type 1 IFN-induced apoptosis of T cells

Previously, our lab had shown that the type 1 IFN-induced attrition of $CD8\alpha^+CD44^{hi}$ cells correlated with an increase in Annexin V reactivity, suggesting that this loss occurred through an apoptotic mechanism (Figure 1) (McNally, Zarozinski et al. 2001). It was believed that this $CD8\alpha^+CD44^{hi}$ population consisted of primarily T cells, but I found a population of “lymphoid” $CD8\alpha^+$ DCs, within the $CD8\alpha^+CD44^{hi}$ gate, that increased upon poly(I:C) treatment (Figure 6). This population accounted for the previously observed increase in Annexin V reactivity at 12 hours following poly(I:C) treatment (Figures 1 and 2).

This observation required a re-evaluation of the type 1 IFN-induced attrition of $CD8^+CD44^{hi}$ T cells with an anti- $CD8\beta$ antibody, which is a more exclusive stain for $CD8^+$ T cells than an anti- $CD8\alpha$ antibody. The attrition of both $CD8\alpha^+CD44^{lo}$ and $CD8\beta^+CD44^{lo}$ populations was similar, since the $CD8\alpha^+$ DC population was only in the $CD44^{hi}$ gate (Figure 4C). There was, however, a significant loss of splenic $CD8\beta^+CD44^{hi}$ T cells at 12 hours following poly(I:C) treatment (Figure 9). A type 1 IFN-dependent loss in splenic $CD8\beta^+CD44^{hi}$ T cells was also observed at 3 days post-LCMV-Armstrong infection (Figure 12).

Three potential mechanisms that could account for this loss were migration, increased adherence, and death. Although Inflammatory cytokines can alter the trafficking patterns of T cells, I observed a similar loss of $CD8\beta^+CD44^{hi}$ T cells in spleen, PEC, lungs, iLNs, and peripheral blood following poly(I:C) treatment (Figure 10). Although it is difficult to rule out trafficking into

compartments that I have not yet examined, these data indicate that the loss of CD8 β ⁺CD44^{hi} T cells is not due to redistribution into other organs.

Inflammatory cytokines can also affect the adherence properties of T cells. Others have reported a similar transient decline of antigen-specific memory CD8 T cells, assessed by flow cytometry, in the spleens of mice early after *Listeria monocytogenes* infection. This decline, however, was a consequence of the T cells undergoing an APC-dependent conditioning phase that renders them undetectable by flow cytometry, but readily detectable by histological techniques (Jabbari, Legge et al. 2006). This conditioning phase, termed T cell conditioning, is a process whereby APCs laden with antigen form aggregates with antigen-specific T cells, thereby preventing them from being properly extracted or processed for analysis by flow cytometry (Maxwell, Rossi et al. 2004). A disruption of this interaction through protease treatment can enhance the recovery of antigen-specific memory CD8 T cells, but it cannot account for the loss of a significant number of cells (Jabbari, Legge et al. 2006), suggesting that T cell conditioning alone cannot account for the attrition of antigen-specific memory CD8 T cells during infection.

LCMV infection causes substantial reductions in the frequencies of CD8 T cells that are specific to a heterologous virus as early as 2 days post-infection, and these frequencies do not recover as the infection resolves, a finding that neither migration or T cell conditioning can account for (Kim and Welsh 2004). This suggests that the long-term loss in memory might, in large part, be a consequence of the type 1 IFN-dependent apoptotic loss in memory CD8 T cells.

Others have shown that type 1 IFN can induce the activation of multiple caspases in certain tumor cell lines, including caspases 3 and 9, which are both associated with mitochondrial-induced apoptosis (Thyrell, Erickson et al. 2002). Caspase 8, which is typically associated with death receptor-mediated apoptosis, was also activated in certain cells lines, but to a lesser extent (Thyrell, Erickson et al. 2002; Panaretakis, Pokrovskaja et al. 2003). The attrition of wildtype CD8 β^+ CD44^{hi} and CD4⁺CD44^{hi} T cells at 3 days post-LCMV infection, correlated with a significant increase in the activation of caspases 8 and 3 (Figure 12). No significant increase in the activation of caspase 3 was observed within the IFN1-R KO CD8 β^+ CD44^{hi} or CD4⁺CD44^{hi} T cell population and this was consistent with the reduced attrition of both populations (Figure 12). This finding confirms a previous study that associates the activation of caspase 3 with the type 1 IFN-dependent depletion of CD8 T cells (Jiang, Gross et al. 2005). Caspase 8 activation was significantly increased in the IFN1-R KO CD8 β^+ CD44^{hi} T cell population at 3 days post-LCMV infection (Figure 12), suggesting that it may not contribute to type 1 IFN-induced attrition of CD8 β^+ CD44^{hi} T cells. Caspase 8 activation is also associated with proliferation (Chun, Zheng et al. 2002; Salmena, Lemmers et al. 2003; Kang, Ben-Moshe et al. 2004; Su, Bidere et al. 2005), but our lab has previously shown that there is no proliferation of CD8 T cells during the early type 1 IFN-induced lymphopenia, but that they will proliferate upon recovery (McNally, Zarozinski et al. 2001). I have attempted to block the poly(I:C)-induced attrition of CD8 T cells using a pan-caspase inhibitor *in vivo*, but this has proven futile. There was still a significant cell loss, potentially

through a caspase-independent, programmed necrosis pathway, as suggested by others (Nussbaum and Whitton 2004). Nevertheless, these findings support a role for caspases in the type 1 IFN-induced attrition of T cells.

Other groups, however, have reported caspase activation without significant apoptosis prior to T cell expansion (Alam, Cohen et al. 1999; Kennedy, Kataoka et al. 1999). I found a significant increase in DNA fragmentation, as revealed by TUNEL, within the CD8 β ⁺CD44^{hi} and CD4⁺CD44^{hi} T cell population at 3 days post-LCMV infection (Figure 14). This suggests that the attrition of both CD8 β ⁺CD44^{hi} and CD4⁺CD44^{hi} T cells occurs through an apoptotic mechanism, as DNA fragmentation is one of the hallmark characteristics of the late stage of apoptosis.

Bim, a pro-apoptotic molecule involved in mitochondrial-induced cell death, has previously been shown to be involved in apoptotic events following either an acute or persistent LCMV infection. It is critical for eliminating most activated effector T cells at the resolution of an acute LCMV infection and as a result, functional memory cells are significantly increased in Bim KO mice following the contraction of the immune response (Wojciechowski, Jordan et al. 2006). Bim has also been demonstrated to have a critical role during chronic LCMV-Clone 13 by downregulating CD8 T cell responses. While NP396-specific CD8 T cells were rapidly deleted in wildtype mice, no decrease was observed in Bim KO mice (Grayson, Weant et al. 2006).

Here I report the first evidence of Bim's involvement in the early apoptosis of T cells following LCMV infection. The attrition of CD8 β ⁺CD44^{hi} and

CD4⁺CD44^{hi} T cells was reduced in Bim KO mice at 3 days post-LCMV infection, relative to wildtype mice (Figure 15A-B). The reduced attrition corresponded with a decrease in DNA fragmentation, as revealed by TUNEL, in Bim KO T cells, relative to T cells (Figure 15C). Nevertheless, Bim KO mice infected with LCMV were not completely resistant to the early lymphopenia (Figure 15A), suggesting that additional pro-apoptotic family members might be involved. T cell homeostasis is more severely disturbed in Bax/Bak double-deficient mice than in Bim KO mice, suggesting that other molecules that signal upstream of Bax/Bak may compensate for the loss of Bim (Wojciechowski, Tripathi et al. 2007). Moreover, physical interactions between MCL-1 and Bak prevent Bak homodimerization, mitochondrial pore formation, and spontaneous induction of apoptosis that may bypass Bim (Willis, Chen et al. 2005).

Bim-dependent apoptosis can be dependent on the covalent modification of Bim by phosphorylation at multiple sites by members of the MAPK family members, which play a crucial role in type 1 IFN-mediated responses. Bim's pro-apoptotic function may be increased or decreased depending on the site of phosphorylation (Ley, Ewings et al. 2005). I examined the attrition of CD8 β ⁺CD44^{hi} T cells at 3 days post-LCMV infection, in mice deficient in JNK1, a kinase that can phosphorylate Bim at multiple sites, as well as mice in which certain phosphorylation sites on Bim have been mutated (Bim3A, BimTA, and BimL). Mice deficient in Bmf, a closely related homolog of Bim, as well as mice in which a phosphorylation site on Bmf has been mutated, were also used (a brief description of each knockout and mutant mice used is provide in Table 2). All

mice showed a similar decrease in overall CD8 β ⁺CD44^{hi} T cell number coupled with a corresponding increase in TUNEL staining, relative to the wildtype control at 3 days post-LCMV infection (data not shown).

Table 2 - Description of Bim-Related Mutant and Knockout Mice^a

Mutant	Description
JNK1KO	Knockout of JNK1; member of the MAP kinase family
BmfKO	Knockout of Bmf, a close homolog of Bim; believed to have a similar function to Bim
BmfSA	Site of JNK phosphorylation is removed; phosphorylation at this site increases Bmf activity
Bim3A	Sites of MAP kinase phosphorylation have been removed; these are required of Ub-mediated degradation of Bim
BimTA	Site of JNK phosphorylation is removed; phosphorylation at this site increases Bim activity
BimL	The exon that encodes the major isoform of Bim (BimEL) is deleted; mice only express the short isoform (BimS)

^aMice were generated by Dr. Roger J. Davis (University of Massachusetts Medical School)

I have not yet established whether type 1 IFN induction leads to an increase in Bim activity and/or expression, resulting in the apoptosis of T cell. Type 1 IFN has been shown to cause the attrition of certain cancer cell lines through a mitochondrial-induced pathway involving Bim (Otsuki, Yamada et al. 1998; Chen, Gong et al. 2001). As mentioned previously, serum type 1 IFN serum levels are similar between both wildtype and Bim KO mice, at 3 days post-LCMV infection. Therefore, the reduced attrition in Bim KO mice, infected with LCMV, is not due to lower levels of type 1 IFN induction.

I also investigated whether TNFRSF members might be involved in the type 1 IFN-induced attrition of CD8⁺ T cells. TRAIL expression is induced by type 1 IFN on multiple cells types and has been shown to kill certain normal and cancer cells (Wiley, Schooley et al. 1995; Pan, O'Rourke et al. 1997; Martin-Villalba, Herr et al. 1999; Lamhamedi-Cherradi, Zheng et al. 2003; Mundt, Kuhnel et al. 2003). Although it has been reported that the depletion of T cells during the early response to *listeria monocytogenes* infection was partially dependent on TRAIL (Zheng, Jiang et al. 2004), I found that the poly(I:C)-induced attrition of CD8 α ⁺CD44^{hi} cells in TRAIL KO mice was similar to that of wildtype mice (Table 1). Poly(I:C) also induces TNF (Zhang, Sun et al. 1998), which can be cytotoxic to cells, but both TNFR1 and TNFR2 KO CD8 α ⁺CD44^{hi} cells mice were equally susceptible to the early type 1 IFN-induced attrition (Table 1). Mice that over-express viral FLICE (caspase-8)-like inhibitor proteins (v-FLIPs) are resistant to death receptor-induced apoptosis and programmed necrosis (Woelfel, Bixby et al. 2006). CD8 α ⁺CD44^{hi} cells from these mice, however, were also susceptible

to the type 1 IFN-induced attrition (Table 1). Although these experiments were conducted prior to the discovery of the sub-population of $CD8\alpha^+$ DCs within the $CD8\alpha^+CD44^{hi}$ gate, the substantial depletion exhibited by all KO mice following poly(I:C) treatment, suggests that these members of the TNFRSF may not play a role in the type 1 IFN-induced attrition of CD8 T cells, although this requires further confirmation.

Regardless of the additional mechanisms involved, it is still unclear whether the loss of T cells results from either the direct or indirect effects of type 1 IFN. I have, however, preliminary evidence that supports an indirect role for type 1 IFN using an adoptive transfer model. I adoptively transferred bona fide IFN1-R KO memory $CD8^+CD44^{hi}$ T cells into wildtype recipient mice followed by inoculation with poly(I:C). These memory cells were generated using a less virulent vaccinia virus (VV) variant, expressing the entire glycoprotein of LCMV (VV-GP), since IFN1-R KO mice have difficulty clearing most other viruses. Since the donor IFN1-R KO memory population lacks IFN1-R expression, any donor cell loss, following poly(I:C) treatment, could be attributed to the indirect effects of type 1 IFN, whereas no loss would suggest a direct effect. There was a significant loss in both VV-specific cells (B8R) and LCMV-specific cells (GP33), at 12 hours post-poly(I:C) treatment (Figure 24), suggesting that attrition of bona fide IFN1-R KO $CD8^+CD44^{hi}$ T cells might occur through the indirect effects of type 1 IFN.

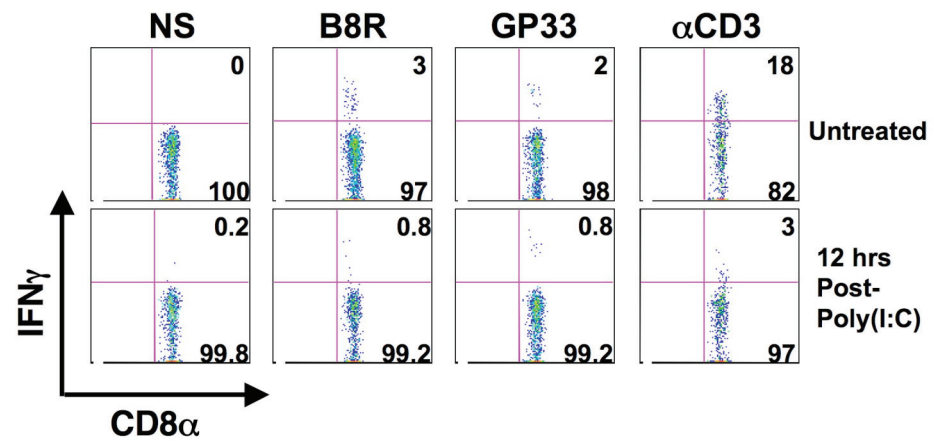


Figure 24 – Donor IFN1-R KO memory CD8 T cells undergo attrition in wildtype host following poly(I:C) treatment. Two $\times 10^7$ CFSE-labeled VV-GP, IFN1-R KO memory CD8 T cells were adoptively transferred into 129SVE (wildtype) hosts i.v. Wildtype hosts were inoculated with poly(I:C) 1 day post-transfer. Splenocytes were harvested 12 hours post-poly(I:C). Gates were set on the CFSE⁺ donor cells and visualized via an IFN- γ assay. Plots show the percentage of VV-GP-specific IFN1-R KO memory CD8 T cells. FACS plots represent 1 of 3 mice. The data is representative of 1 independent experiments.

B. A role for “lymphoid” CD8 α ⁺ DCs during the type 1 IFN-induced early lymphopenia

I document a type 1 IFN-dependent increase in CD8 α ⁺ DCs that was greater following poly(I:C) treatment, relative to LCMV infection. This is not surprising since CD8 α ⁺ DCs can be activated through TLR3, a receptor for poly(I:C) (Schulz, Diebold et al. 2005). Interestingly, this increase in CD8 α ⁺ DCs correlated with the decrease in the CD8 β ⁺CD44^{hi} T cells (Figure 6 and 9). Therefore, I questioned whether CD8 α ⁺ DCs might aid in the rapid clearance of apoptotic cells, following poly(I:C) treatment, *in vivo*. There was a slight increase in the uptake of CFSE-labeled congenic donor cells, by host CD8 α ⁺ DCs, at 9 and 12 hours post-poly(I:C) treatment (Figure 17). Others have shown that the engulfment of apoptotic cells results in an increase in class II expression, a result consistent with my finding of increased class II expression at 12 hours post-poly(I:C) treatment (Figure 5B and 17) (Clayton, Savage, 2003). T cells and DCs are in close contact during antigen presentation, but this would not explain the increased uptake of CFSE⁺ donor cell label by host DCs, since doublets were gated out using pulse-width, prior to analysis. Nevertheless, there are very few CFSE⁺ donor cells taken up by the host CD8 α ⁺ DCs. This was to be expected since there was a higher frequency of endogenous, unlabeled apoptotic cells also taken up. Another caveat is that we were unable to determine the phenotype of the donor cells taken up by host DCs. I have tried to detect T cell surface proteins (i.e. Thy1.2) within the host DCs, but the results were inconclusive.

Monocytes have been shown to phagocytose apoptotic bodies that are shed from the neighboring apoptotic cell and, in the process, can become “false positive” by the Annexin V assay (Marguet, Luciani et al. 1999). This suggests that $CD8\alpha^+$ DCs might become Annexin V⁺ upon engulfing apoptotic lymphocytes. The apoptotic phenotype, as indicated by an increase Annexin V reactivity, suggests that the plasma membrane of the $CD8\alpha^+$ DC might be fusing with the plasma membrane of the apoptotic body it engulfs. Phagocytic cells expressing T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4) have been shown to associate with exosomes carrying exposed PS, which may also account for the observed increase in Annexin V reactivity (Miyanishi, Tada et al. 2007). Nevertheless, increased Annexin V reactivity also suggests that the DCs could actually be dying. The typical lifespan of mature DCs is estimated at 3 days *in vivo* (Ingulli, Mondino et al. 1997; Kamath, Pooley et al. 2000). Also, DC populations begin to drop in number within 24 hours of infection of mice with LCMV and decline even further by day 3 post-infection. It is not known whether this is due to migration, phenotypic alterations, and/or cell death, but it is known to be type 1 IFN-dependent (Montoya, Edwards et al. 2005). Although contrary to the current dogma, it has been shown that the surface expression of phosphatidylserine on macrophages is required for phagocytosis of apoptotic lymphocytes. Pre-treating these macrophages with Annexin V was found to inhibit phagocytosis of apoptotic thymocytes (Callahan, Williamson et al. 2000). Interestingly, the apoptosis PCR array also revealed an increase in IL-10 RNA in $CD8\alpha^+CD44^{hi}$ population, containing $CD8\alpha^+$ DCs, at 6 hours post-

poly(I:C). Recently, it has been shown that exposure of DCs to IL-10 can suppress the induction of anti-apoptotic genes, which coincided with the spontaneous apoptosis of the DC (Chang, Baumgarth et al. 2007). In some cases, an impairment of apoptotic cell phagocytosis can lead to autoantibody production and may result in the breakdown of self tolerance (Asano, Miwa et al. 2004). We are currently setting up a system to determine what effect depleting DCs prior to poly(I:C) treatment has on the early attrition of T cells, utilizing CD11c-DTR mice. These mice can be depleted of DCs because the diphtheria toxin receptor is on the CD11c promoter (Jung, Unutmaz et al. 2002).

C. Antigen engagement does not protect CD8 T cells from the type 1 IFN-induced attrition associated with viral infections

I show here that antigen engagement by antigen-specific naïve (P14) or memory (LCMV-immune) CD8 T cells, with either peptide or viral antigen, does not protect these cells from depletion resulting from poly(I:C) inoculation or viral infection (LCMV). This is in contrast to an earlier report that concluded that depletion was selective for non-specific CD8 T cells and that antigen-specific CD8 T cells resisted deletion and instead underwent extensive proliferation (Jiang, Lau et al. 2003). We suggest that this paper may have missed an early depletion preceding the proliferation phase. Our study also seems at odds with the concept that antigen-engagement can up-regulate anti-apoptotic proteins such as Bcl-X_L and protect cells from apoptosis (Petschner, Zimmerman et al. 1998). Our results indicate that protection from apoptosis does not occur at

these very early stages of the type 1 IFN response. Poly(I:C) treatment up-regulated CD69 on both donor naïve P14 and OT-1 transgenic T cell populations (Figure 18A), consistent with the ability of type 1 IFN to nonspecifically up-regulate CD69 on naïve T cells (Sun, Zhang et al. 1998; Sun and Sprent 2000). In contrast, GP33-45 peptide up-regulated CD69 only on the GP33-specific P14 T cells, but not on the OVA-specific OT-1 cells, consistent with reports indicating that TCR engagement upregulates CD69 expression (Figure 18A). In addition, GP33-45 peptide inoculation down-regulated CD62L expression on P14 cells, but not on OT-1 cells (data not shown). These experiments show the nonspecific effects of the TLR agonist poly(I:C) and the very specific effects of peptide on the transgenic T cell populations. This peptide-dependent engagement of the T cells did not, however, selectively protect them from the apoptosis and attrition mediated by poly(I:C) (Figure 18). Therefore, the increased activation status of naïve antigen-specific cells via peptide inoculation did not confer resistance to type 1 IFN-induced depletion.

Memory CD8 T cells are more susceptible than naïve CD8 T cells to type 1 IFN-induced depletion (McNally, Zarozinski et al. 2001) (Figure 20A). Because the experiments with transgenic P14 T cells utilized immunologically naïve T cells, I also investigated whether the GP33-45 peptide would protect GP33-specific memory CD8 T cells from poly(I:C)-induced depletion. The GP33-specific memory population was susceptible to the poly(I:C)-induced attrition regardless of whether the GP33-45 peptide was administered beforehand (Figure 20B). Treatment with GP33-45 peptide alone resulted in a slight loss of GP33-specific

memory CD8 T cells, suggesting that memory CD8 T cells may be more susceptible to AICD than naïve CD8 T cells (Figure 20B). Another explanation is that the loss of GP33-specific memory cells may be due to T cell conditioning, which was described earlier.

Similar results showing a failure of antigen to protect T cells from deletion were found in virus-infected mice. When naïve transgenic P14 cells or LCMV-specific memory CD8 T cells were transferred into day 2 LCMV Clone 13-infected mice, where substantial levels of antigen should be present, the donor P14 cells were significantly depleted (Figure 21). Serum from day 2 LCMV Clone 13-infected mice and poly(I:C)-treated mice, 16 hours post-infection, had comparable type 1 IFN endpoint dilution titers of 1/6400 in a standard type 1 IFN bioassay using microtiter plates of L-929 cells challenged with vesicular stomatitis virus, suggesting that type 1 IFN induction was similar between both models used in this study. LCMV-specific and PV-specific memory CD8 T cells also underwent a significant depletion when transferred into a day 2 post-Clone 13-infected mouse, (Figure 21A) and this decrease occurred in all epitope-specific memory CD8 T cells tested (Figure 21C). Thus, antigen load has no protective effect on this early T cell attrition and, if anything, may enhance the attrition. The loss of memory CD8 T cells could not be attributed to trafficking into other organs, such as the peripheral blood, bone marrow, peritoneal cavity, lungs, or the lymph nodes (Figure 21B and data not shown). Collectively, these results show that antigen-specific naïve and memory CD8 T cells are not

protected from depletion during the early stages of the immune response to a viral infection.

D. Early attrition of CD8 T cells allows for greater diversity during challenge with a heterologous virus

CD8 T cell memory to virus is stable in the absence of infection, but apoptotic events that occur during the early immune response to infection can leave the host with a reduced population of memory CD8 T cells to previously encountered viruses (Selin, Vergilis et al. 1996; Selin, Lin et al. 1999; Liu, Andreansky et al. 2003). Although this early depletion may appear to be detrimental to the host, studies in which a lymphopenic state was induced via cytotoxic drugs or irradiation showed that such a lymphopenic state can lead to enhanced immune responses, by making room in lymphoid organs for development of T cell responses (Pfizenmaier, Jung et al. 1977; Peacock, Kim et al. 2003).

Our computer modeling predicts that if cross-reactive antigen prevents the type 1 IFN-induced deletion of a memory T cell population, those cross-reactive memory T cells, even those of low affinity, would compete against a more diverse and higher affinity new T cell response to the newly encountered pathogen (Figure 22). Therefore, an early depletion of these cells, even in the presence of TCR engagement with antigen, would allow for a more diverse response to occur. Thus, this early T cell attrition phenomenon that occurs under conditions of virus-induced lymphopenia may not only allow for room in the immune system

for a more vigorous T cell expansion but may also allow for a more diverse T cell response originating from naïve T cells.

Creating a biological model to study the consequence of the lack of type 1 IFN-induced attrition of memory CD8 T cells during the early immune response has, thus far, proven problematic, in part, because it is difficult to separate the T cell apoptosis-inducing properties of the type 1 IFN response from the myriad of other type 1 IFN-induced events that regulate antigen presentation and the subsequent immune response. Nevertheless, I took advantage of the finding that older mice develop less initial lymphopenia relative to younger mice (Jiang, Anaraki et al. 2003; Jiang, Gross et al. 2005) and questioned whether the reduced apoptosis of the cross-reactive memory CD8 population (NP205) in older LCMV-immune mice, following heterologous virus challenge (PV), would allow them to dominate the immune response. I show that the cross-reactive memory CD8 T cell response (NP205) was more immunodominating in older LCMV-immune mice relative to younger LCMV-immune mice, at day 8 post-PV challenge (Figure 23B). The diminished PV-response in older LCMV-immune mice relative to younger LCMV-immune mice could be attributed to older mice having a smaller immunologically naïve repertoire. Both young and old naïve mice, however, mounted a similar magnitude of a PV-specific response, at day 8 post-infection (Figure 23B). Since non-cross-reactive memory cells are lost upon heterologous virus challenge, there was a significant loss of GP33-specific cells at day 8 post-PV infection, relative to day 0, in young LCMV-immune mice (Figure 23A-B). The high GP33-specific response, in old LCMV-immune mice

(day 8 post-PV) could be attributed to the decreased amount of apoptosis following PV-infection, presumably due to lower type 1 IFN levels relative to young LCMV-immune mice (Jiang, Gross et al. 2005). The one young LCMV-immune mouse that had an NP205 to NP38 ratio over 1 (5.5) could be due to private specificity (Figure 22b). Nevertheless, the immunodominance of the cross-reactive population (NP205), at day 8 post-PV, was consistent between several old LCMV-immune mice, relative to young LCMV-immune mice (Figure 22c).

Bim KO mice undergo less T cell attrition than wildtype mice following LCMV infection. Therefore, the PV/LCMV cross-reactive model could be applied to Bim KO mice, which would remove the age variable. Interestingly, LCMV-immune Bim KO mice have a higher frequency of LCMV-specific memory CD8 T cells, since an increased number of effector cells survive the contraction phase (Wojciechowski, Jordan et al. 2006). Therefore, Bim KO LCMV-immune mice will have an increased frequency of NP205-specific memory cells, relative to wildtype LCMV-immune mice. When I challenge BIM KO LCMV-immune mice with PV, the reduced attrition might allow the cross-reactive NP205 response to dominate, thereby limiting the expansion of the NP38 non-cross-reactive response.

As previously mentioned, CD8 T cell memory to virus is stable in the absence of infection, but apoptotic events that occur during the early immune response to infection can leave the host with a reduced population of memory CD8 T cells to previously encountered viruses (Selin, Vergilis et al. 1996; Selin, Lin et al. 1999; Liu, Andreansky et al. 2003). The CD4 memory pool, however,

was found to be remarkably stable under these conditions (Varga, Selin et al. 2001). Active (cytokine secretion at the beginning of the response) and passive (competition for resources and space) are two mechanisms of T cell attrition (Selin, Cornberg et al. 2004). My findings suggest both memory CD4 and CD8 T cells are equally susceptible to active attrition, as both populations were susceptible to type 1 IFN-induced attrition (Figure 12 and 13). Therefore, the increased stability of CD4 memory, relative to CD8 memory, might be due to survival subsequent to passive attrition. Because the contraction of the CD8 response is much greater than the CD4 response, there would be more competition for space for CD8 T cells than CD4 T cells. This would be more detrimental for pre-existing memory CD8 T cells than CD4 T cells. Mathematical modeling of memory CD8 T clones during the primary and secondary response to cross-reactive viruses, suggest that either active attrition alone or both passive and active attrition could account for the observed memory loss, but passive attrition alone, could not (Selin, Cornberg et al. 2004). Collectively, these data suggest that the relative stability of memory CD4 T cells might be due to the lack of passive attrition during the contraction of the immune response.

Chapter VII: References

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