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**SENSITIZATION OF CD8 T CELLS DURING ACUTE VIRAL INFECTIONS
IMPACTS BYSTANDER AND LATECOMER CD8 T CELL RESPONSES**

A Dissertation Presented

By

HEATHER D. MARSHALL

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

October 19, 2009

IMMUNOLOGY & VIROLOGY PROGRAM

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(October 19, 2009)

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Abstract

Many virus infections induce a transient state of immune suppression in the infected host. Virus-induced T cell suppression can be caused by T cell activation-induced cell death (AICD), dendritic cell (DC) apoptosis, DC dysfunction, and/or the enhanced expression of immune-suppressive cytokines. It has been previously demonstrated that naïve bystander CD8 T cells derived from hosts experiencing an acute virus-specific T cell response underwent AICD when polyclonally activated by anti-CD3 *in vitro* (Zarozinski et al., 2000). Susceptibility of naïve bystander T cells to AICD could prevent the development of a new T cell response during an ongoing immune response, and thus render infected hosts immune suppressed. Although immune suppression could result in an enhanced susceptibility to superinfections, virus-infected individuals are more commonly resistant to superinfecting pathogens. Because of these seemingly contradictory conditions, we sought to investigate how acute viral infections impact naïve bystander CD8 T cells *in vivo*. More specifically, we asked whether bystander CD8 T cells are susceptible to immune suppression or whether they can contribute to the resistance to superinfections. In order to address this, we examined the responses of bystander CD8 T cells activated with cognate antigen during acute viral infections *in vivo*. We generated several *in vivo* models using P14 (LCMV glycoprotein-specific), HY (male antigen-specific), and OT-I (ovalbumin-specific) transgenic CD8 T cells, which we defined as bystander during acute infections with lymphocytic

choriomeningitis virus (LCMV), Pichinde virus (PV), vaccinia virus (VV), and murine cytomegalovirus (MCMV).

Consistent with the enhanced susceptibility to cell death noted *in vitro*, we found that bystander CD8 T cells activated with cognate antigen *in vivo* during acute viral infections underwent markedly reduced proliferation. Virus-induced transient T cell suppression *in vivo* was not exclusively mediated by Fas-FasL- or TNF-induced AICD or due to an enhanced susceptibility to apoptosis. Instead, immune suppression *in vivo* was associated with a delayed onset of division, which we found not to be due to a defect in antigen presentation, but rather due to a T cell intrinsic defect.

Despite the suppressed proliferation of TCR-stimulated bystander CD8 T cells *in vivo*, we found an enhancement of the effector functions exerted by bystander CD8 T cells activated during acute viral infections. During acute viral infections or after stimulation with type 1 IFN (IFN- $\alpha\beta$) inducers, some bystander CD8 T cells were sensitized to immediately exert effector functions such as IFN- γ production and degranulation upon stimulation with high affinity cognate antigen. Sensitization of naïve CD8 T cells required self-MHC I and indirect effects of IFN- $\alpha\beta$, while IL-12, IL-18, and IFN- γ were not individually required. IL-15 was not required for the rapid expression of IFN- γ , but was required for up-regulation of granzyme B (GrzB). P14 and OT-I CD8 T cells, which are capable of homeostatic proliferation, could be sensitized by poly(I:C), but HY CD8 T cells, which are poor at homeostatic proliferation, could not, suggesting that the requirement for MHC I may be to present low affinity cryptically cross-reactive

self antigens. Sensitized naive CD8 T cells up-regulated the t-box transcription factor Eomesodermin (Eomes), which can regulate these rapid effector functions.

In conclusion, we demonstrate in this thesis that acute viral infections impact naïve bystander CD8 T cells such that their response to cognate antigen is altered. Prior to cognate antigen engagement, bystander CD8 T cells up-regulated Eomes, CD122, and GrzB. Following cognate antigen engagement, bystander CD8 T cells rapidly degranulated and expressed the effector cytokine IFN- γ . The ability of bystander CD8 T cells to rapidly exert effector functions may contribute to the resistance of virus-infected individuals to superinfections. Despite these rapid effector functions, the proliferation of TCR-stimulated bystander CD8 T cells was markedly inhibited. This reduced proliferation was found not to be a defect in antigen presentation, but was a T cell intrinsic defect in initiating division. Thus, bystander CD8 T cells were also susceptible to virus-induced immune suppression.

It is also likely that virus-specific CD8 T cells that are not activated until later in the response, so-called latecomer CD8 T cells, may also be susceptible to immune enhancement and suppression. Thus, latecomer CD8 T cells would be able to rapidly exert effector functions at the expense of proliferation. Taken together, we propose that during an immune response, due to spatial and temporal gradients of antigen and inflammation, it is likely that a combination of heterogeneous T cells with different signal strengths and sequences of exposure from cytokines and peptide-MHC constitute the total T cell response to pathogens.

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List of Abbreviations

7-AAD	7-aminoactinomycin D
ACAD	activated cell autonomous cell death
ADAR	adenosine deaminase
AICD	activation-induced cell death
APL	altered peptide ligand
ATF	activating transcription factor
Bad	Bcl-2/Bcl-xL associated death promoter
Bax	Bcl-2 associated X protein
BCG	Bacillus Calmette-Guerin
Bid	BH3 ineracting domain
Bik	Bcl-2 ineracting killer
Bim	Bcl-2-interacting molecule
Bmf	Bcl-2 modifying factor
Bok	Bcl-2-related ovarian killer
Cbl	Casitas B-lineage lymphoma
CBP	CREB-binding protein
CDKi	cyclin-dependent kinase inhibitor
CDR	complement-determining region
CFSE	caboxyfluorescein succinimidyl ester
CPE	cytopathic effects
CRAC	Ca ²⁺ release-activated Ca ²⁺

CREB	cyclic AMP-responsive-element-binding protein
CsA	cyclosporin A
Csk	C-src tyrosine kinase
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DHT	delayed-type hypersensitivity
DNA	deoxyribonucleic acid
Eomes	eomesodermin
FADD	fas-associated death domain
FLICE	caspase-8/FADD-like interleukin-1 β converting enzyme
FLIP	FLICE-like inhibitory protein
GAS	gamma-activated sequence
GITR	glucocorticoid-induced TNF receptor
GP	glycoprotein
GrzB	granzyme B
HBSS	Hank's balanced salt solution
HCMV	human cytomegalovirus
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immuno-deficiency virus
HPK1	hematopoietic progenitor kinase 1

HSV	herpes simplex virus
HVEM	herpesvirus entry mediator
IFN	interferon
i.p.	intraperitoneal
i.v.	intravenous
IP ₃	inositol 1,4,5-triphosphate
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
Jak	janus kinase
KO	knockout
KLRG1	killer cell lectin-like receptor 1
LAT	linker of activated T cells
Lck	leukocyte-specific protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
Mcl-1	myeloid cell leukemia 1
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation antigen 5

MFI	mean fluorescence intensity
MHC	minor histocompatibility
MIP-1 β	macrophage inflammatory protein 1 β
NFAT	nuclear factor of activated T cells
NF κ B	nuclear factor kappa B
NK	natural killer
NP	nucleoprotein
OAS	oligoadenylate synthetase
PCR	polymerase chain reaction
PEC	peritoneal exudates cells
PKC	protein kinase C
PKR	protein kinase R
PLC	phospholipase C
poly(I:C)	polyriboinosinic polyribocytidylic acid
pfu	plaque forming units
Puma	p53 up-regulated modulator of apoptosis
PV	Pichinde virus
RAR	retinoid acid receptor
RIGI	retinoic acid-inducible gene
RLR	RIG-I-like receptors
RNA	ribonucleic acid
RSV	respiratory syncytial virus

RUNX3	runt-related transcription factor 3
STAT	signal transducer and activator of transcription
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFRSF	TNF receptor super family
TRAIL	TNF-related apoptosis-inducing ligand
TSC-22	TGF- β -stimulated clone 22
TUNEL	terminal transferase dUTP nick end labeling
VSV	vesticular stomatis virus
VV	vaccinia virus

Chapter I: Introduction

A. Generation of TCR diversity and recognition of peptide-MHC

The diversity of the T cell repertoire in an individual is generated by somatic rearrangement of germline V, D, and J gene segments, deletion or insertion events at the junctions of these segments, and combinatorial diversity of protein chains (Davis and Bjorkman, 1988). The germline-encoded TCR segments have broad reactivity with self-MHC molecules (Germain, 1990; Jerne, 1971), while the junctions, or complement-determining regions (CDR) react with peptides bound by the MHC molecule. T cells develop in the thymus and are positively selected based on their ability to bind self-peptide-MHC with an intermediate affinity. Those T cells that recognize self-peptide with high affinity undergo negative selection and those T cells that do not recognize self-peptide-MHC undergo death by neglect. The process of T cell selection in the thymus imparts central tolerance, such that mature naïve T cells that exit the thymus will have some reactivity to MHC, but little reactivity to self-peptides that would result in autoimmunity. Reactivity to self-MHC is not only necessary for a proper T cell response to foreign antigen-loaded MHC, but also for peripheral T cell survival *in vivo* (Brockner, 1997; Kassiotis et al., 2002; Kirberg et al., 1997; Markiewicz et al., 1998; Polic et al., 2001; Tanchot et al., 1997).

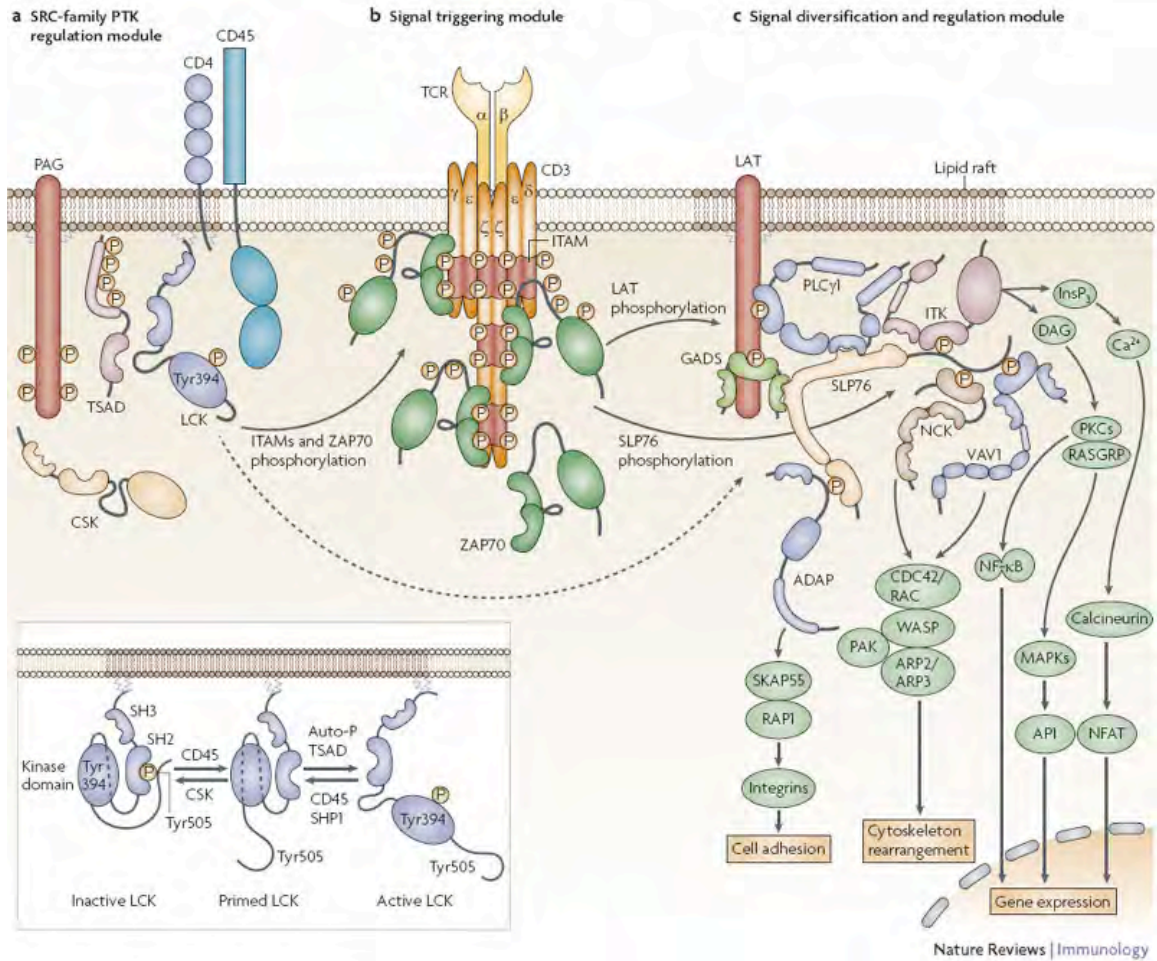
MHC molecules are polymorphic membrane-bound proteins with peptide binding segments that are the target of interaction with the TCR. Alleles of MHC proteins differ in their peptide-binding pockets, resulting in the ability of each allele to bind to only

certain peptide sequences or motifs (Garrett et al., 1989; Matsumura et al., 1992; Stern and Wiley, 1994). Most peptides that can stably bind MHC will have the proper motif for that MHC, but other peptides may interact with faster off-rates (Cerundolo et al., 1991). In general, MHC molecules cannot discriminate between self- or pathogen-encoded peptides. The result is that even on infected cells, the majority of MHC molecules will be presenting peptides derived from the abundant self-protein pool (Chicz et al., 1992; Hunt et al., 1992; Rudensky et al., 1991). Due to the diverse nature of TCRs and the diverse pool of peptide ligands, any given TCR will bind an array of peptide-MHC molecules with a variety of affinities that results in a continuum of agonist, partial agonist, antagonist, and non-reactive TCR-peptide pairs (Evavold et al., 1995; Williams et al., 1998). Consistent with this idea, it has been estimated that a single TCR may be able to recognize up to one million peptide-MHC complexes (Mason, 1998). The signaling events resulting from these associations will determine the response, or lack-there-of, of a given T cell.

B. T cell activation

Signaling from the TCR is a complex cascade of phosphorylation events that culminate in cytoskeleton rearrangement and gene expression (Figure 1.1). The cascade begins with the activation of src family kinases, most notably Lck (Samelson et al., 1986; Straus and Weiss, 1992). Active Lck phosphorylates the tyrosines in the TCR ζ chain and CD3 ϵ , γ , and δ chains in their immunoreceptor tyrosine-based activation motifs (ITAM) (Wange and Samelson, 1996). ZAP-70 is recruited and binds to the

Figure 1.1. T cell receptor signaling cascades. (Acuto et al., 2008)



phosphorylated ITAM in the TCR ζ chain, where it is subsequently phosphorylated by Lck (Chan et al., 1995; Isakov et al., 1995). Kinase-active ZAP-70 then phosphorylates an adapter protein called LAT (Zhang et al., 1998a), which associates with SLP-76 via the adapter molecule Gads (Liu et al., 1999). Itk is recruited to this complex and mediates the phosphorylation and activation of PLC- γ (Bunnell et al., 2000; Liu et al., 1998). Once activated, PLC- γ cleaves phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate (IP₃) and diacylglycerol. IP₃ is responsible for initiating the release of intracellular Ca²⁺ from the endoplasmic reticulum (ER) by binding receptors on the ER membrane and opening Ca²⁺ release-activated Ca²⁺ (CRAC) channels (Lewis and Cahalan, 1995). The rise in Ca²⁺ in combination with diacylglycerol activates protein kinase C (PKC) and is responsible for activating the phosphatase calcineurin, which dephosphorylates NFAT, resulting in its translocation to the nucleus where it is free to interact with members of the AP-1 family and initiate gene transcription (Jain et al., 1995; Karin et al., 1997; Ullman et al., 1990).

Knowledge of the signaling events emanating from the TCR after stimulation with agonist peptide has allowed researchers to assay a spectrum of peptide ligand-MHC pairs with known functional output (agonist, partial agonist, antagonist, and null) to determine if the TCR signaling events are differentially regulated by altered peptide ligands (APL). Two independent groups found that agonist peptide induced two forms of phosphorylated TCR ζ monomers (p23 and p21), while partial agonist peptides only induced p21 phospho- ζ and partially bound non-activated ZAP-70 (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). Formation of p21 phospho- ζ has also been reported

in response to antagonist peptides that show no agonist function at any concentration, providing evidence for TCR signaling without a typical activation response (Germain and Stefanova, 1999).

In addition to the signals provided through the TCR, other surface molecules have been shown to impact the response of the T cell. The most significant positive contributors to TCR signaling are the T cell co-receptors CD8 and CD4 as well as the costimulatory molecules CD28, CD27, 4-1BB (CD137), OX40 (CD134), CD30, herpesvirus entry mediator (HVEM), and glucocorticoid-induced TNF receptor (GITR) (Greenwald et al., 2005). It has been proposed that the co-receptors function to stabilize the interaction between the TCR and MHC, as they bind MHC I and II molecules, respectively, and positively regulate the downstream signaling events (Gao et al., 2002). CD28 has also been shown to positively regulate downstream signaling events from the TCR by enhancing IL-2 production and IL-2R expression, and accelerating entry into the cell cycle (Acuto and Michel, 2003). The costimulatory signals provided by CD28 are considered “signal 2” of TCR stimulation and are required for maximal activation of naïve T cells (Acuto and Michel, 2003). In addition, cytokines such as IL-2, IFN- $\alpha\beta$, IFN- γ , and IL-12 are considered “signal 3” contributors to T cell activation and may or may not be required for CD8 T cell activation and memory generation under some infection conditions (Curtsinger et al., 2003; Curtsinger et al., 2005; Kolumam et al., 2005; Thompson et al., 2006; Whitmire et al., 2007; Williams et al., 2006).

In addition to positive costimulation, there are a variety of negative feedback mechanisms in place to attenuate TCR signaling. These negative signaling molecules

include Csk, CD45, c-Cbl, and CTLA-4. Although these molecules have been shown to be of vital importance to the attenuation of T cell activation, none has been shown to play a role in the discrimination among peptide ligands that may result in different levels of activation. In contrast to the inhibitory molecules above, the tyrosine phosphatase SHP-1 has been linked to ligand discrimination. The presence of SHP-1 in TCR complexes has been shown to be rapid for antagonist peptides, intermediate for partial agonists, and slowest for agonist peptides (Germain and Stefanova, 1999). Thus, the engagement of antagonist or partial antagonist peptide-MHC is of sufficient duration and efficacy to yield the signals required for SHP-1 association with the TCR complex. It appears that the slow association of SHP-1 to the TCR complex in response to agonist ligands is the result of ERK-1 binding to Lck and outcompeting SHP-1 for binding (Stefanova et al., 2003).

C. Effector functions of virus-specific CD8 T cells

The signaling events emanating from the TCR (signal 1), costimulatory receptors (signal 2), and cytokine receptors (signal 3) culminate in the induction of a differentiation program to allow antigen-specific T cells to respond to the infectious pathogen. The effector functions of CD4 and CD8 T cells are quite diverse and although of importance to the control of some pathogens, the functions of the effector CD4 T cell subsets will not be discussed further here.

The CD8 T cell response to acute viral infections can be divided into three distinct phases: expansion, contraction, and memory. The expansion phase is so named because

the virus-specific CD8 T cells undergo rapid and robust division. Studies have estimated approximately one division every 6 hours and as many as 15 or more divisions per cell, resulting in a dramatic rise in CD8 T cell number (Masopust and Ahmed, 2004; Masopust et al., 2007; Murali-Krishna et al., 1998). Following the clearance of the pathogen, most of the virus-specific CD8 T cells undergo apoptosis during the contraction phase. Some virus-specific CD8 T cells survive contraction and develop into memory CD8 T cells to, along with CD4 T cell and B cell memory, provide the host with lifelong immunity to the infecting pathogen.

Naïve CD8 T cells start to divide approximately 20 hours after antigen contact (Zimmermann et al., 1999). Prior to the onset of division, newly activated T cells up-regulate the transcription of cyclin D and G1 genes (Teague et al., 1999). After the onset of division, T cells have also up-regulated transcription of cyclins A1, B, and E (Teague et al., 1999). Conversely, some proteins that are involved in suppressing cell division, such as the retinoid acid receptor (RAR) and the proliferation inhibitors D52, TGF- β -stimulated clone 22 (TSC-22), and cyclin-dependent kinase inhibitors (CDKi) p27 and p21 are down-regulated in activated T cells (Grayson et al., 2001; Teague et al., 1999). The expression of molecules involved in the cell cycle is tightly regulated, and the altered expression after TCR stimulation allows for the newly activated T cells to divide very rapidly.

Concomitant to the expansion phase and rapid proliferation, the virus-specific CD8 T cells differentiate into effector CD8 T cells (also known as cytotoxic T lymphocytes or CTL). This process does not require sustained antigen presentation,

indicating that the initial TCR priming from the peptide-MHC is sufficient to program the differentiation of the parental CD8 T cell, which instructs the daughter cells to continue dividing and differentiating (Kaech and Ahmed, 2001). The differentiation of naïve CD8 T cell to CTL is associated with large-scale changes in chromatin structure, gene expression, membrane structure, migratory potential, survival, self-renewal, and effector functions (Kaech et al., 2002).

Gene expression profiling studies have demonstrated enhanced transcription of adhesion molecules (galectin-3, P-selectin ligand, CD11c), trafficking molecules (CXCR4, CCR2, CCR5), and cytoskeletal proteins (filamin, talin) that ultimately lead to migration of virus-specific CTL to infected peripheral tissues (Kaech et al., 2002; Teague et al., 1999). In addition to expression of proteins required for migration, virus-specific CTL also up-regulate effector molecules that allow them to combat the virus and lyse virus-infected cells. Some of these molecules are cytokines and chemokines such as IFN- γ , TNF, MIP-1 β , and Rantes, while some are molecules involved in cytolytic activity such as granzymes, perforin, and Fas ligand (Carding et al., 1993; Grayson et al., 2001; Kaech et al., 2002; Teague et al., 1999). Cytokine production and cytolytic activity make up the two arms of CTL effector functions and play complementary roles in controlling virus infections (Slifka and Whitton, 2000).

Cytolytic ability of CTL can be acquired after one cell division, and efficiency of cytolysis has been shown to increase with subsequent divisions (Oehen and Brduscha-Riem, 1998). In another study, enhanced cytolytic ability correlated with the increased expression of perforin and granzymes in CTLs that had undergone 6-8 divisions

compared to those CTL that had undergone only 3 divisions (Jenkins et al., 2008). Effector CTL are able to rapidly lyse virus-infected cells; one study has shown that nearly 100% of peptide-pulsed target cells are eliminated within an hour after transfer into lymphocytic choriomeningitis virus (LCMV)-infected mice at the peak of the CTL response (Barber et al., 2003). Memory CD8 T cells retain the ability to quickly lyse infected cells within 4 hours, and if these experiments are normalized to the frequency of peptide-specific CTL, effector and memory CD8 T cells have similar (within 2-fold) lytic ability (Barber et al., 2003; Ganusov and De Boer, 2008; Regoes et al., 2007; Yates et al., 2007).

A lot of work has focused on the regulation of effector molecule expression by CTL, as its dysregulation could be detrimental to the host and result in immune-pathology. Naïve CD8 T cells require approximately 3 days to start transcribing IFN- γ , while effector and memory CTL retain the ability to turn on IFN- γ transcription very rapidly (Aune et al., 1997; Zimmermann et al., 1999). The rapidity with which virus-specific CTL can express IFN- γ increases with time as the cells differentiate into memory (Liu et al., 2004). Upon removal of specific peptide antigen, CTL rapidly turn off expression of IFN- γ (Slifka et al., 1999). Since cytokine mRNAs are notoriously unstable, the cessation of transcription results in a rapid decline in IFN- γ protein available to secrete (Sachs, 1993; Slifka et al., 1999). Restimulation with peptide results in rapid transcription of IFN- γ that can reach initial levels within 1 hour (Slifka et al., 1999). Importantly, *in vivo* virus-specific CTL are not continuously producing IFN- γ , but retain

the ability to turn on its expression when re-encountering antigen, potentially in a local environment of infected tissue (Liu and Whitton, 2005).

IFN- γ transcription can be regulated by the chromatin accessibility of the locus and the expression of transcription factors. In naïve CD8 T cells, the IFN- γ locus is highly methylated and in a closed, condensed conformation, which inhibits transcription of the gene (Kersh et al., 2006; Northrop et al., 2006; Zhou et al., 2004). After activation, the IFN- γ locus is unmethylated, acetylated, and the chromatin opens up to allow access to the transcriptional machinery (Fitzpatrick et al., 1998; Kersh et al., 2006; Northrop et al., 2006; Zhou et al., 2004). In memory CD8 T cells, the IFN- γ locus remains acetylated and in an open conformation, but it is methylated. Following restimulation, the IFN- γ locus is rapidly unmethylated to allow access to the transcriptional machinery and subsequent transcription (Kersh et al., 2006). Interestingly, forced histone acetylation of the IFN- γ locus by chemical inhibitors of deacetylases induces IFN- γ transcription under conditions in which the T cells produce very little IFN- γ (Northrop et al., 2008). Stable epigenetic changes at the IFN- γ locus play a key role in keeping memory CD8 T cells poised to rapidly respond to reencounter with antigen. It is likely that stable epigenetic modifications at multiple loci involved in survival, metabolism, proliferation, and effector functions occur in memory CD8 T cells (Pearce and Shen, 2006).

Another layer of regulation is the expression of transcription factors that drive the transcription of the effector molecules IFN- γ , granzymes, and perforin. The transcription of effector molecule genes are regulated by a variety of transcription factors including T-bet, Eomesodermin (Eomes), cyclic AMP-responsive-element-binding protein 1

(CREB1), activating transcription factor (ATF) 1 and 2, JUN, OCT1, signal transducer and activator of transcription 1 (STAT1), STAT4, runt-related transcription factor 3 (RUNX3), and REL.

The T box transcription factors T-bet and Eomes are the principle transcription factors in the regulation of IFN- γ transcription by virus-specific CD8 T cells (Intlekofer et al., 2008; Intlekofer et al., 2005; Mayer et al., 2008; Sullivan et al., 2003). The expression of these transcription factors is low in naïve CD8 T cells, and they are both up-regulated in effector CTL, with T-bet being the prominent transcription factor present (Cruz-Guilloty et al., 2009; Takemoto et al., 2006). Eomes, in contrast, is up-regulated further in memory CD8 T cells (Takemoto et al., 2006). Knockout and knockdown studies of both transcription factors have revealed partially overlapping and compensatory functions in CD8 T cells (Intlekofer et al., 2007; Intlekofer et al., 2005; Mayer et al., 2008; Pearce et al., 2003; Sullivan et al., 2003). Due to partially redundant functions, CD8 T cells lacking both transcription factors have the most pronounced defect in effector functional capabilities. LCMV-specific CD8 T cells lacking T-bet and Eomes express very little IFN- γ , perforin, and GrzB and instead express an aberrantly high amount of IL-17 (Intlekofer et al., 2008).

As a secreted factor, IFN- γ can induce a wide range of responses. IFN- γ signals through the IFN- γ receptor (IFN- γ R) that induces the activation of Janus kinase 1 and 2 (Jak1, 2) and subsequent phosphorylation of STAT1 (Bach et al., 1997; Schindler, 1999). Activated STAT dimers translocate to the nucleus and transactivate genes with gamma-activated sequences (GAS) and in some cases IFN-stimulated response elements (ISRE).

NF κ B and c-Jun have also been identified as transcription factors activated during IFN- γ signaling and some STAT1-independent genes have also been identified (Ramana et al., 2002). IFN- γ , like IFN- $\alpha\beta$, can induce the upregulation of MHC. However, IFN- γ is much more potent at upregulating MHC II expression (Boehm et al., 1997). IFN- γ is also a potent activator of macrophages by specifically inducing the production of nitric oxide synthase, whose product, nitric oxide, plays an important role in the host response against microbial and viral pathogens (Nathan, 1997).

D. Generation and maintenance of memory CD8 T cells

Following clearance of the virus, most of the virus-specific CD8 T cells undergo apoptosis during the contraction phase, but some virus-specific T cells survive contraction, differentiate into memory, and provide the host with immunity to the virus. There are certain surface receptors expressed on effector CD8 T cells during the acute phase of the immune response that have been associated with the ability to survive the contraction phase and to generate memory. These markers include IL-7R α (CD127) and killer cell lectin-like receptor 1 (KLRG1). Virus-specific CTL that retain high expression of IL-7R α and do not up-regulate KLRG1 have a longer lifespan and can proliferate well in response to homeostatic cytokines and antigen, thus have the highest capacity to develop into memory T cells (referred to as memory precursor effector cells (MPEC)). In contrast, those T cells that have down-regulated IL-7R α and up-regulated KLRG1 have a shorter lifespan and reduced proliferative capacity and are thus more

terminally differentiated (referred to as short-lived effector cells (SLEC)) (Huster et al., 2004; Joshi et al., 2007; Kaech et al., 2003; Sarkar et al., 2008).

Although it is not clear exactly how or when virus-specific effector CD8 T cells differentiate into memory cells, it is clear that specific signals during the initial priming of naïve T cells are required for the generation and maintenance of functional memory CD8 T cells. Some of these requirements include the signal 3 cytokines IFN- $\alpha\beta$ and/or IL-12 and IL-2 provided by CD4 T cells (CD4 help) (Curtsinger et al., 2005; Thompson et al., 2006; Williams et al., 2006). Inflammation can also impact memory development by inducing expression of T-bet and driving a SLEC fate (Joshi et al., 2007), and a lack of inflammation during T cell activation, via DC immunization, results in the rapid acquisition of memory characteristics (Badovinac et al., 2005).

Memory CD8 T cells can be divided into two subsets, central (T_{CM}) and effector (T_{EM}) based on anatomical location, proliferative capacity, and effector functions. T_{CM} express the lymph node homing molecules CD62L and CCR7 and have a higher proliferative capacity, while T_{EM} lack expression of these molecules, reside in nonlymphoid tissues, and have been shown to acquire effector functions such as cytolysis more rapidly (Masopust et al., 2001; Sallusto et al., 1999; Wherry et al., 2003). There is a linear relationship between these two memory subsets, as T_{EM} can convert to T_{CM} (Wherry et al., 2003). Thus T_{CM} are true memory T cells with the ability to self-renew and proliferate in response to secondary challenge.

E. Susceptibility of T cells to cell death during the acute immune response

During the acute immune response to LCMV, T cells are susceptible to cell death in three distinct phases, which serve to maintain the homeostasis of the T cell repertoire for further antigenic challenges. These phases are distinguished kinetically throughout the immune response and by mechanisms of induction. The first phase is a IFN- $\alpha\beta$ -dependent attrition of memory T cells that occurs 2-4 days post infection (McNally et al., 2001). The second phase is a susceptibility of both highly activated virus-specific CD8 T cells and bystander CD8 T cells to activation-induced cell death (AICD) mediated by repeated TCR stimulation and Fas-FasL interactions (Razvi and Welsh, 1993; Zarozinski et al., 2000). The final phase is activated cell autonomous cell death (ACAD) that is responsible for the contraction of the immune response mediated by a Bcl2-interacting molecule (Bim)- and p53 up-regulated modulator of apoptosis (Puma)-dependent cell death pathway initiated by cytokine withdrawal (Erlacher et al., 2006; Krammer et al., 2007; Razvi and Welsh, 1995).

Infection of mice with LCMV induces a profound IFN- $\alpha\beta$ response early after infection, and this IFN- $\alpha\beta$ has been shown to be required for maximal expansion of LCMV-specific T cells (Aichele et al., 2006; Kolumam et al., 2005; Quigley et al., 2008; Thompson et al., 2006). The IFN- $\alpha\beta$ that is induced early after infection is also required for the early deletion of memory phenotype (CD44^{hi}) T cells that precedes the expansion of virus-specific T cells (Jiang et al., 2005; McNally et al., 2001). Both virus-specific and bystander T cells are susceptible to this IFN- $\alpha\beta$ -induced attrition (Bahl et al., 2006). The loss of memory T cells may allow for a more diverse T cell response by diminishing

the frequency of potentially cross-reactive memory cells, which could otherwise dominate the response (Bahl et al., 2006) (Bahl et al., manuscript in preparation). The IFN- $\alpha\beta$ -dependent apoptosis of T cells requires the pro-apoptotic BH3-only Bcl-2 family member Bim and may also involve the TNF receptor superfamily member Fas (Bahl et al., manuscript in preparation). These two molecules are members of different apoptotic pathways, but have also been shown to converge during the contraction of the immune response to LCMV (Weant et al., 2008).

Naïve CD8 T cells are relatively resistant to apoptosis, but effector CTL are highly susceptible to AICD. During the acute phase of LCMV infection, virus-specific CTL downregulate Bcl-2 while simultaneously upregulating Bax and Bad, inducing a pro-apoptotic state (Grayson et al., 2001). Activated T cells also downregulate a negative regulator of Fas signaling, FLICE-like inhibitory protein (FLIP) allowing them to be more sensitive to Fas-induced apoptosis (Algeciras-Schimnich et al., 1999; Irmeler et al., 1997). Highly activated virus-specific CD8 T cells upregulate the death domain containing receptors Fas and TNFR p55 and p75 (Miyawaki et al., 1992; Ryffel and Mihatsch, 1993). Activated T cells can undergo AICD via ligation of FasL, TNF, or TRAIL (Hildeman et al., 2002b). In addition, in highly activated T cells, hematopoietic progenitor kinase 1 (HPK1) gets cleaved by active caspase 3 (Chen et al., 1999). The C terminal domain of HPK1 blocks NF κ B activation, thus inhibiting cell survival and promoting cell death (Arnold et al., 2001; Brenner et al., 2005).

In addition to highly activated virus-specific CTL, naïve bystander CD8 T cells have also been shown to be susceptible to AICD if they get stimulated through their TCR

in vitro (Zarozinski et al., 2000). During an acute immune response, virus-specific CTL up-regulate FasL in order to engage Fas on, and subsequently lyse, virus-infected cells. FasL expression on virus-specific CTL was necessary for the AICD of TCR-stimulated bystander CD8 T cells and suggests that the up-regulation of Fas on newly activated non-virus-specific CD8 T cells may direct them down a death pathway instead of a survival and proliferative pathway. The susceptibility of bystander CD8 T cells to AICD could contribute to virus-induced immune suppression by inhibiting the development of a new T cell response to a superinfecting pathogen during an ongoing immune response.

Following clearance of the viral infection, most virus-specific CTL will undergo ACAD during the contraction phase of the immune response. Apoptosis of virus-specific CTL during the contraction phase is only minimally due to AICD, as T cells deficient in Fas, FasL, or other TNFSF receptors that can induce apoptosis undergo relatively normal contraction (Lohman et al., 1996; Razvi et al., 1995; Reich et al., 2000; Weant et al., 2008). The mechanisms that control whether a virus-specific CD8 T cell survives or undergoes ACAD during the contraction phase are not well characterized, but there are several factors that have been shown to play a role in this cell fate decision.

Cytokine withdrawal during the acute immune response is thought to play a role in initiating ACAD. Cytokines of the common γ -chain family, including IL-2, IL-7, and IL-15 have been shown to modulate CD8 T cell survival and proliferation. Due to the expression of IL-2 and the high affinity IL-2R α chain (CD25) initiated by antigen, IL-2 signaling is thought to potentially augment T cell programming and play a role in memory potential. *In vivo*, IL-2 enhances but is not essential for CD8 T cell expansion.

Moreover, CD8 T cells deficient in CD25 expand and contract normally, but develop into poor memory cells (Williams et al., 2006). Enhancing and/or maintaining IL-2 signaling can prevent the contraction of virus-specific T cells, and removal of IL-2 results in rapid T cell apoptosis back down to homeostatic levels (Blattman et al., 2003). IL-7 is also thought to play a role in memory differentiation because virus-specific CD8 T cells that retain high expression of IL-7R α were shown to have an enhanced ability to generate memory cells (Kaech et al., 2003). Although expression of the IL-7R α chain is necessary for the survival of memory T cells, its expression is not sufficient to force the survival of effector CD8 T cells during contraction (Hand et al., 2007). IL-15 has also been shown to play an important role in the survival of memory T cells, but it appears to be more important for the homeostatic division and maintenance of memory cells rather than their establishment (Becker et al., 2002; Judge et al., 2002).

The withdrawal of cytokine signaling induces stress within activated T cells that results in the activation of pro-apoptotic molecules of the BH3-only Bcl-2 protein family including Bim and Puma. In the steady state, anti-apoptotic Bcl-2-like proteins (Bcl-2, Bcl-xL, Mcl-1) neutralize the pro-apoptotic BH3-only proteins (Bim, Puma, Noxa, Bad, Bmf, Bik, Bid) via heterodimeric interactions (Cheng et al., 2001). After activation, T cells downregulate Bcl-2 (Grayson et al., 2001), thus freeing the pro-apoptotic proteins. Free BH3-only proteins, such as Bim and Puma, bind Bax-like proteins (Bax, Bak, Bok) on the mitochondrial membrane and initiate cytochrome c release and downstream apoptosis (Martinou and Green, 2001).

Bim deficiency delays the contraction of LCMV-specific CD8 T cells and results in enhanced accumulation of memory CD8 T cells (Pellegrini et al., 2003; Weant et al., 2008; Wojciechowski et al., 2006). The accumulation of memory CD8 T cells can be enhanced further by blocking ACAD and AICD via combined deficiencies of Bim and Fas (Weant et al., 2008). Apoptosis requires the downregulation of Bcl-2 in activated T cells as overexpression of Bcl-2 can rescue cytokine withdrawal-mediated apoptosis of T cells *in vitro* (Erlacher et al., 2006; Strasser et al., 1991) and T cell contraction in response to superantigen *in vivo* (Hildeman et al., 2002a). Further, Bcl-2 overexpression or Bim deficiency rescues the development and function of T cells in IL-7R α knockout and common γ chain knockout mice (Akashi et al., 1997; Kondo et al., 1997; Maraskovsky et al., 1997; Pellegrini et al., 2004). The enhanced survival of T cells lacking Bim is abrogated in T cells lacking both Bim and Bcl-2, suggesting that the survival initiated by the loss of Bim depends on the presence of Bcl-2 (Wojciechowski et al., 2007). It should be noted that Bcl-2 overexpression does not always rescue the contraction of activated T cells, particularly following LCMV infection (Petschner et al., 1998; Razvi et al., 1995). This may be due to a role for Fas in the contraction of LCMV-specific T cells (Weant et al., 2008) and suggests that under some infection conditions, particularly ones that initiate multiple apoptotic pathways, Bcl-2 overexpression cannot inhibit contraction. This issue is further clouded by the recent retractions of a series of publications by Luke van Parijs and thus more work is needed to clarify the role of Bcl-2 during T cell contraction.

In addition to Bim, another BH3-only Bcl-2 family protein, Puma also plays a role in the contraction of T cell responses, as Puma deficient T cells accumulate after HSV infection, albeit not to the extent of Bim deficient T cells (Fischer et al., 2008). In support of the combined roles of Bim and Puma, double deficiency of both molecules results in enhanced survival of activated T cells after cytokine withdrawal *in vitro* (Erlacher et al., 2006). The enhanced survival of Bim and Puma double deficient T cells is less than Bcl-2 overexpressing transgenic T cells in this experimental system, suggesting that additional BH3-only proteins may also contribute to cytokine withdrawal-mediated apoptosis of T cells (Erlacher et al., 2006). Thus, CD8 T cell contraction following pathogen clearance is mediated by the pro-apoptotic activities of Bim, Puma, and perhaps other BH3-only proteins, but can be overridden, under some conditions, by the anti-apoptotic effects of Bcl-2.

F. *Arenaviridae*

The prototype virus in the *arenaviridae* family is LCMV, which has been used extensively in the laboratory for the study of anti-viral immune responses in mice and most of the research discussed in sections C & D was generated using this viral system. Studies using LCMV have contributed to many fundamental immunological concepts including immunological tolerance, virus-induced immune-pathology, MHC restriction, NK cell activation, immune deficiency, autoimmunity, immunological memory, and cellular immunity (Welsh, 2000). Two Nobel prizes have been awarded to investigators based on studies that utilized LCMV.

Members of the virus family *arenaviridae* are natural rodent viruses that cause asymptomatic persistent infections in their rodent hosts. There are currently 18 known species of arenaviruses, which are divided into two antigenic groups based on serology. Old World arenaviruses include LCMV, Lassa fever virus, and Mopeia virus. New World arenaviruses include Machupo virus, Junin virus, and Pichinde virus (PV). Despite their natural rodent hosts, some arenaviruses such as Junin and Lassa fever can cause severe hemorrhagic diseases in humans (Frame et al., 1970; Johnson et al., 1965; Salas et al., 1991), and it is reported that LCMV could account for about 8% of nonbacterial meningitis in North America (Adair et al., 1953; Meyer et al., 1960).

Arenaviruses are enveloped, pleomorphic spheres ranging in size from 50-300 nm in diameter and are granular in appearance due to the presence of ribosomes (Welsh, 2000). Virions contain a bi-segmented ambisense RNA genome consisting of small (S) and large (L) segments (Auperin et al., 1984b; Pedersen, 1973; Ramsingh et al., 1980). The S segment is approximately 3,500 nucleotides and encodes the nucleoprotein (NP) and the envelope glycoproteins (GP1 and GP2) (Auperin et al., 1984a; Clegg et al., 1991; Wilson and Clegg, 1991). The L segment is roughly 7,100 nucleotides and encodes the viral polymerase (L) and the Z protein (Iapalucci et al., 1989a; Iapalucci et al., 1989b; Salvato et al., 1989; Salvato et al., 1992; Singh et al., 1987). Due to the ambisense orientation of the genome, NP and L mRNAs are transcribed directly from the RNA genome, while GP and Z mRNAs are transcribed from negative sense anti-genome regions (Auperin et al., 1984a; Auperin et al., 1984b).

The NP is a 60-68 kd protein and is the major structural protein in the nucleocapsids of arenavirions (Bruns et al., 1986). The GP is transcribed as a polyprotein that is post-translationally cleaved into GP1 and GP2 (Buchmeier et al., 1987; Wright et al., 1990). GP1 is a 40-46 kd protein that is responsible for binding to the cellular receptor, while GP2 is a 35 kd protein that serves as the fusion protein. GP1 and GP2 oligomerize to form tetrameric spikes on the enveloped membrane to facilitate binding and fusion to target cells. The L protein is 180-250 kd and serves as an RNA-dependent RNA polymerase that is required for viral replication (Salvato et al., 1989; Sanchez and de la Torre, 2005). The Z protein is the smallest viral protein at 11 kd and has recently been reported to be required for viral budding (Perez et al., 2003; Strecker et al., 2003). The GP1 encoded by Old World arenaviruses and clade C of the New World viruses binds to the ubiquitous cellular glycoprotein alpha-dystroglycan, which allows for wide host range and tissue infectivity (Borrow and Oldstone, 1992, 1994; Cao et al., 1998; Spiropoulou et al., 2002). Despite wide tissue infectivity, LCMV Armstrong strain replicates predominantly within lymphoid tissues, while other strains such as WE demonstrate a wider target range. Macrophages have been identified as an early and prominent cell type infected, followed by infection of epithelial cells and marginal zone and follicular cells of secondary lymphoid tissues.

There are currently three predominant strains of LCMV used in the laboratory to study anti-viral immunity: Armstrong, Traub, and WE. The Armstrong strain was isolated from a monkey inoculated with a serial passage of cerebrospinal fluid from a suspected human case of St. Louis encephalitis virus (Armstrong C, 1934).

Subsequently, Eric Traub isolated a serologically identical virus from a persistently infected mouse colony (Traub, 1935), while WE virus was isolated from an employee (initials W.E.) exposed to Traub's mouse colony that presented with a fatal hemorrhagic disease (Scott, 1936).

Some isolates of LCMV such as Armstrong clone 13 and WE docile cause immune suppression in immuno-competent adult mice (Ahmed et al., 1984; Pfau et al., 1982). These virus strains have been used extensively to study the concept of clonal exhaustion, whereby high doses of viral antigen drive the loss of function and eventual deletion of virus-specific T cells, a phenomenon that may be shared among some chronic viral infections. The Armstrong clone 13 strain only differs from its parental strain by two amino acids, one in the L protein and one in the GP1 protein (Matloubian et al., 1990; Salvato et al., 1991). The GP1 mutation has been shown to confer broader cell tropism and enhanced affinity for the alpha-dystroglycan receptor, while the L mutation confers enhanced replication (Matloubian et al., 1993). Thus, these combined mutations result in increased infectivity and generation of more viral progeny, perpetuating the increased viral titers that lead to T cell exhaustion.

In addition to its use in T cell exhaustion studies, LCMV clone 13 is also the virus used in the congenitally infected LCMV carrier mouse model. These mice are infected *in utero* by using persistently infected female mice as breeders. Infection with LCMV *in utero* results in the development of an LCMV-tolerant immune system and persistent viral replication throughout the life of the mice (King et al., 1992). This model has been used to study the concepts of tolerance and bystander T cell activation (see section J).

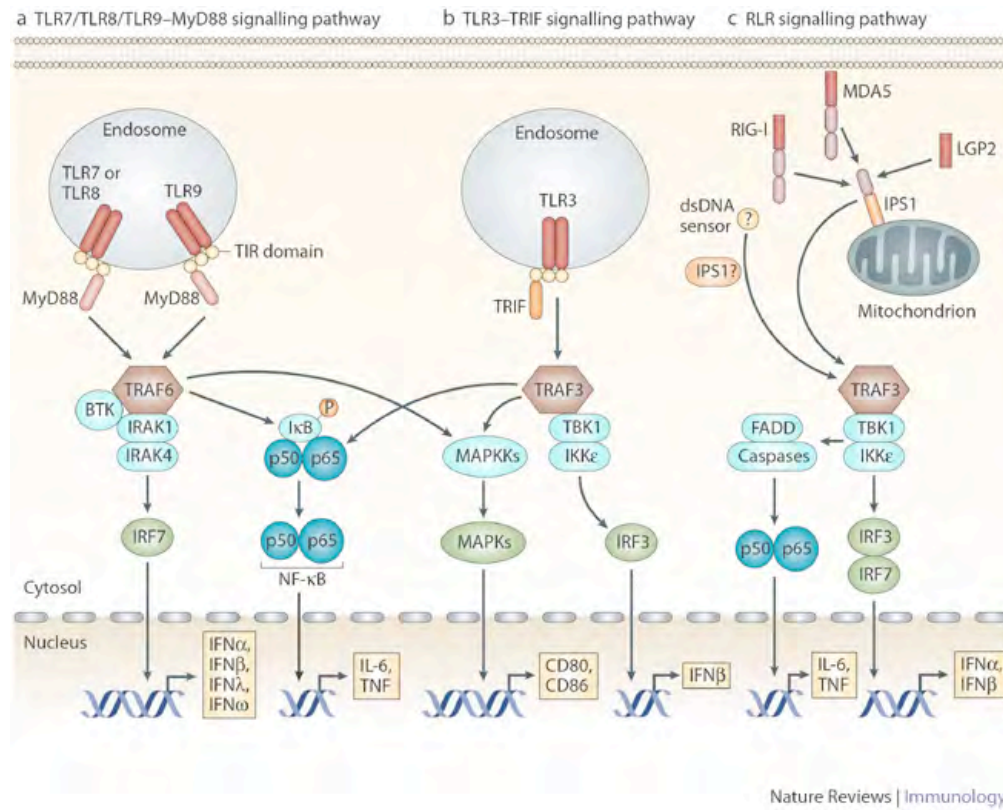
G. Innate type I IFN (IFN- $\alpha\beta$) response to arenavirus infections

Infection of wild type mice with LCMV Armstrong induces a rapid and prominent IFN- $\alpha\beta$ response (Merigan et al., 1977; Welsh, 1978) possibly due to the recognition of viral RNAs by toll-like receptors (TLRs) and/or retinoic acid-inducible gene (RIG)-I-like receptors (RLRs). TLR 3, 7, and 8, MDA5 and RIG-I are RNA sensors (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004; Yoneyama et al., 2005) that recognize various forms, lengths, structures, and sequences of RNA to distinguish among viral and cellular RNAs (Kato et al., 2008; Marques et al., 2006; Yoneyama et al., 2004). Depending on the RNA intermediates generated during replication, the subcellular location of viral RNA, and the expression of TLRs and RLRs in infected cells, different viruses can be recognized by different TLRs and RLRs (Kato et al., 2006; Kumar et al., 2006; Loo et al., 2008; Melchjorsen et al., 2005).

Signaling through TLRs and RLRs by viral RNA results in the activation of the IKK $\alpha/\beta/\gamma$ complex and mitogen activated protein kinase (MAPK) cascades, which lead to the activation of NF- κ B, AP-1, and TANK-binding kinase 1 (TBK1) (Figure 1.2). Active TBK1 phosphorylates the interferon regulatory factors (IRF) 3 and 7, which homo- and hetero-dimerize and translocate to the nucleus (Fitzgerald et al., 2003; Lin et al., 2000; Sharma et al., 2003). IRF3/7 homo- and hetero-dimers interact with the CREB-binding protein (CBP) and promote transcription of the type I IFNs: IFN- α and IFN- β (Sato et al., 1998b; Suhara et al., 2000; Yoneyama et al., 1998). In the steady state, IRF3 is constitutively expressed, while IRF7 is expressed at relatively low levels (Marie et al., 1998; Sato et al., 1998a). The result of this expression profile is that upon TLR or RLR

Figure 1.2. Intracellular nucleic acid sensors and signaling pathways.

(Gilliet et al., 2008)



stimulation, most of the phosphorylated IRF forms are IRF3/3 homodimers and few IRF3/7 heterodimers. IRF3/3 homodimers potently transactivate the IFN- β and IFN- $\alpha 4$ genes (Sato et al., 1998b). Signaling of IFN- β and IFN- $\alpha 4$ through the heterodimeric $\alpha\beta$ IFN-I receptor (IFN- $\alpha\beta$ R) activates the interferon-stimulated gene factor 3 (ISGF3) complex composed of IRF9, STAT1, and STAT2 that is responsible for the induction of IRF7 transcription (Marie et al., 1998; Sato et al., 1998a). The increased expression of IRF7 results in the production of phosphorylated IRF7/7 homodimers in response to further TLR or RLR stimulation and transactivation of the IFN- β and IFN- $\alpha 1,2,4,5,6$, and 8 genes, which potentiates further increased IFN- $\alpha\beta$ production in a positive feedback loop (Marie et al., 1998; Sato et al., 1998a; Sato et al., 2000).

Although many virus-infected cells are capable of producing IFN- $\alpha\beta$, plasmacytoid (pDC) and conventional dendritic cells (cDC) are the primary IFN- $\alpha\beta$ producers in response to viral infections (Cella et al., 1999; Diebold et al., 2003). DCs are also a prominent cell type infected by arenaviruses, providing a mechanism for the strong induction of IFN- $\alpha\beta$ by LCMV. It has not yet been determined which specific TLRs and/or RLRs recognize arenavirus RNAs, but it is clear that signaling through these receptors plays an important role in the induction of IFN- $\alpha\beta$, the generation of a strong CD8 T cell response, and the proper control of the virus, as shown by infection of mice lacking the TLR adapter protein MyD88 (Jung et al., 2008; Rahman et al., 2008; Zhou et al., 2005). TLR3 and 7/9 are probably not the sole LCMV RNA sensors, as mice deficient in TLR3 or TLR7 and 9 mount normal LCMV-specific T cell responses (Edelmann et al., 2004; Jung et al., 2008). Mice deficient in the RLR adaptor protein

MAVS also mount normal LCMV-specific CD8 T cell responses, indicating that RIG-I and MDA5 might not be responsible for LCMV RNA detection (Jung et al., 2008). It is also important to note that the induction of IFN- α and IFN- β may be differentially regulated by TLR and RLR pathways, as mice deficient in TLR7/9 can induce IFN- β but not IFN- α in response to LCMV (Jung et al., 2008). It is likely that multiple mechanisms and potentially unknown RNA sensors and/or pathways exist to detect arenaviral RNAs and that blockade or deficiency in any one pathway may not completely abrogate IFN- $\alpha\beta$ induction.

The synthetic dsRNA analog polyriboinosinic:polyribocytidylic acid (poly(I:C)) has been used extensively in the laboratory to mimic viral dsRNA induction of IFN- $\alpha\beta$ and will also be used in this study. Poly(I:C) is detected primarily by MDA5 as MDA5 deficiency, but not TLR3 or RIG-I deficiency, abrogates the induction of IFN- $\alpha\beta$ following stimulation with poly(I:C) (Gitlin et al., 2006; Kato et al., 2006).

As a secreted factor, IFN- $\alpha\beta$ plays a role in regulating a multitude of immune responses via signaling through the IFN- $\alpha\beta$ R and leading to the transcription of hundreds of IFN-stimulated genes (ISG), including amplification of more and different IRFs to drive further transcription of IFN- $\alpha\beta$ in the positive feedback loop described above. In mammals, there is one *IFN- β* gene and thirteen *IFN- α* genes whose transcription is regulated by the expression of various IRFs, of which there are nine members, that bind to ISRE sequences in ISG promoters. Some ISGs are antiviral genes such as protein kinase R (PKR), 2'-5' oligoadenylate synthetase (OAS), adenosine deaminase (ADAR), and the GPTase Mx. Others are involved in IFN signaling, such as STAT proteins and

IRFs, and there are also many ISGs with as yet unidentified functions (Samuel, 2001; Sen and Sarkar, 2007).

As its name implies, IFN- $\alpha\beta$ can directly interfere with viral replication, but it also has pleiotropic effects on a variety of cells in the immune system. IFN- $\alpha\beta$ has been shown to mature DCs, promote Th1 cytokine production from CD4 T cells, and activate NK cells (Krug et al., 2003; Way et al., 2007; Welsh, 1978). IFN- $\alpha\beta$ has also been implicated in driving the apoptosis of memory T cells early after viral infection. IFN- $\alpha\beta$ also has pleiotropic effects on T cells, as it supports effector the differentiation, proliferation, and survival of activated T cells, while inhibiting the proliferation of naïve T cells. Direct signaling of IFN- $\alpha\beta$ via STAT1 in CD8 T cells is required for the maximal expansion of virus-specific T cells during acute infection with LCMV (Aichele et al., 2006; Kolumam et al., 2005; Quigley et al., 2008; Thompson et al., 2006).

H. T cell response to arenavirus infections

The CD8 T cell response to LCMV infection in mice has been extensively characterized. Despite the multiple effector functions of CTLs and the dependence on virus-specific CTL for clearance of LCMV, viral clearance only requires a CTL response with functional cytolytic activity via perforin (Fung-Leung et al., 1991; Kagi et al., 1994). Infection of C57BL/6 mice with LCMV induces a reproducible and potent H2^b-restricted CD8 T cell response. The CD8 T cell response may be so large because LCMV does not inhibit MHC class I antigen presentation, and it induces a strong IFN- $\alpha\beta$ response, both of which enhance CD8 T cell activation and proliferation (Bukowski and Welsh, 1985;

Kolumam et al., 2005). CD8 T cells from C57BL/6 mice can recognize at least 20 LCMV-encoded peptides (Kotturi et al., 2007; van der Most et al., 1998). GP_{33/34}- and NP₃₉₆-specific CD8 T cells co-dominate the response, followed by sub-dominant responses of L₄₅₅, GP₄₄, GP₂₇₆, L₃₄₉, and others (Table 1.1). During the acute phase of the immune response, GP_{33/34}- and NP₃₉₆-specific CD8 T cells comprise as much as a third of the overall CD8 T cell response, while the subdominant epitope-specific CD8 T cell pool, of ~20 or more specificities, make up the rest. These responses can be detected by a number of assays, including the expression of IFN- γ and TNF after *in vitro* restimulation and direct staining with MHC H2^b-peptide multimeric complexes. Less well studied is the CD8 T cell response of C57BL/6 mice infected with PV. There are four described H-2^b CD8 T cell epitopes NP₃₈, NP₁₂₂, NP₁₆, and NP₂₀₅ (Brehm et al., 2002) (Table 1.2). The NP₃₈ response is dominant, followed by subdominant responses of the others, and there are as of yet no described CD4 T cell epitopes.

I. Transient virus-induced immune suppression

Many virus infections induce a transient state of immune suppression that can be measured by reduced T cell responses *in vitro* to mitogens or specific recall antigens and by diminished delayed-type hypersensitivity (DTH) responses *in vivo* to antigens such as tuberculin (Kantzler et al., 1974; Mims and Wainwright, 1968; Welsh et al., 1995). Although some viruses encode proteins to inhibit various phases of the immune response, many do not. The diverse natures of the many viruses that induce immune suppression

Table 1.1. H2^b (B6) CD8 T cell epitopes in LCMV¹

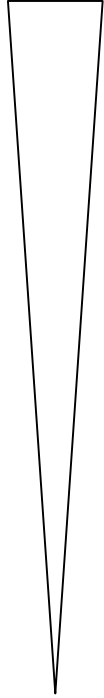
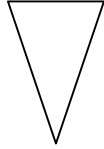
Epitope	aa sequence	MHC restriction	Immuno-dominance
NP ₃₉₆	FQPQNGQFI	D ^b	
GP ₃₃	KAVYNFATC	D ^b	
GP ₃₄	AVYNFATC	K ^b	
L ₄₅₅	FMKIGAHPI	D ^b	
GP ₄₄	FALISFLLL	D ^b	
GP ₂₇₆	SGVENPGGYCL	D ^b	
L ₃₄₉	SSLIKQSKF	K ^b	
L ₆₈₉	KFMLNVSYL	D ^b	
L ₂₀₆₂	RSIDFERV	K ^b	
NP ₂₀₅	YTVKYPNL	K ^b	
L ₁₃₆₉	FAAEFKSRF	K ^b	
L ₁₅₆	ANFKFRDL	K ^b	
GP ₁₁₈	ISHNFCNL	K ^b	
NP ₁₆₅	SLLNNQFGTM	D ^b	
NP ₂₃₈	SGYNFSLGAAV	K ^b	
GP ₂₂₁	SQTSYQYL	K ^b	
L ₁₈₇₈	GPRQSFVS	K ^b	

Table 1.2. H2^b (B6) CD8 T cell epitopes in PV¹

Epitope	aa sequence	MHC restriction	Immuno-dominance
NP ₃₈	SALDFHKV	K ^b	
NP ₁₂₂	VYEGNLTNTQL	D ^b	
NP ₁₆	RGLSNWTHPV	D ^b	
NP ₂₀₅	YTVKFPNM	K ^b	

¹ Epitope designation (gene and start site), amino acid sequences, and T cell MHC restriction are listed in order of relative immunodominance. Only epitopes generating ~2% or more of the CD8 T cell response are listed.

suggests broad and possibly host-regulated mechanisms. In fact, in several acute and persistent viral infections the failure of T cells to proliferate in response to these mitogens or antigens *in vitro* has been linked to the Fas-FasL AICD of T cell populations (Akbar et al., 1993; Fugier-Vivier et al., 1997; Zarozinski et al., 2000). Measles virus, vaccinia virus (VV), and herpes simplex virus (HSV) have also been shown to induce apoptosis of DC (Bosnjak et al., 2005; Engelmayer et al., 1999; Fugier-Vivier et al., 1997; Jones et al., 2003; Muller et al., 2004), presumably leading to reduced T cell responses. Infections of human or mouse DC with human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), measles virus, adenovirus, respiratory syncytial virus (RSV), VV, or LCMV clone 13 can interfere with DC maturation and inhibit MHC and costimulatory molecule expression (Andrews et al., 2001; Engelmayer et al., 1999; Moutaftsi et al., 2002; Munir et al., 2008; Newton et al., 2008; Servet-Delprat et al., 2000; Sevilla et al., 2004; Smith et al., 2005). Still others have reported that production of virus-induced immune-suppressive cytokines such as IL-10 or TGF- β (Alonso et al., 2003; Brockman et al., 2009; Diaz-San Segundo et al., 2009; Newton et al., 2008; Tinoco et al., 2009) or reduced production of inflammatory cytokines such as IL-12 (Moutaftsi et al., 2002; Servet-Delprat et al., 2000; Smith et al., 2005) may suppress T cell outgrowth.

Immune suppression can have detrimental outcomes that include increased risk of superinfections and poor vaccine efficacy. Some virus infections that are associated with increased incidence of superinfections include measles, influenza, RSV, hepatitis viruses, and HIV (Alonso et al., 2003; Avadhanula et al., 2006; Brogden et al., 2005; Huang et al., 2000; Kantzler et al., 1974; Slifka et al., 2003). Respiratory viruses can induce a

favorable niche for the colonization of bacteria by destroying respiratory epithelium and inducing expression of bacterial adhesion receptors (Avadhanula et al., 2006; Hament et al., 1999). In addition to creating a favorable environment for bacterial co-infection, immune suppression is also often associated with the enhanced risk of superinfections during respiratory virus infections (Alonso et al., 2003; Kantzler et al., 1974; Slifka et al., 2003). The most obvious example of how immune suppression can increase the risk of superinfections is the increased incidence of viral and bacterial infections in HIV-infected individuals (Antonucci et al., 2003; Girardi et al., 2001; Smith, 2004). Although HIV directly destroys an entire lymphocyte compartment, many viruses are capable of suppressing and/or subverting the immune system in more subtle ways.

Vaccination efficacy can be compromised by an ongoing viral infection, and this may be linked to this transient virus-induced immune suppression. A moderate-to-severe illness is a CDC-recommended precaution to vaccination; however, even a mild illness may reduce vaccine efficacy. This is not simply due to an interferon response restricting the replication of a live virus vaccine, as HCV-infected patients generated reduced antibody responses to a recombinant HBV protein vaccine (HB-Vax) compared to healthy controls (Wiedmann et al., 2000). Nematode and malaria infections have also been shown to reduce the protective efficacy of heterologous vaccines, whether they be live (e.g. BCG) or subunit (e.g. meningococcal polysaccharide) vaccines (Elias et al., 2005; Greenwood et al., 1980; Su et al., 2006).

J. Homeostasis of bystander T cells during acute viral infections

Because LCMV induces such a massive CD8 T cell response, many investigators have debated how much of the response is antigen-specific and how much might be bystander T cell activation. The issue is further complicated by the loose utilization of the term “bystander” in the literature. The strict definition is that bystander T cells are not stimulated through their TCR and, therefore, are not activated in an antigen-specific manner. The problem with this definition is that it may be difficult in some situations to determine whether a T cell has been stimulated through its TCR. This is apparent in the context of auto-antigens, where during a virus infection it is unclear whether self peptide-MHC can provide some TCR signaling in the presence of a virus-induced cytokine milieu. The degeneracy of T cell recognition of peptide-MHC further confounds the issue of bystander activation. It has been estimated that a single TCR may be able to recognize up to one million peptide-MHC complexes, leading to cross-reactivity against multiple epitopes (Mason, 1998). Due to the spectrum of affinities a given TCR may have for these many peptide antigens, cross-reactivities may be difficult to detect and may be misinterpreted as bystander activation. For the purpose of this thesis, we define bystander T cells as T cells not specific for the virus that is driving the T cell response, (i.e. they are not activated by viral antigens). In this context, activation is defined as induction of T cell proliferation (increase in cell number) and altered expression of a subset of activation markers whose regulation is dependent upon TCR stimulation.

The LCMV carrier mouse affords a unique model in which to investigate the contribution of bystander T cells to the LCMV-induced T cell response since the host T

cells are tolerant to the virus. Adoptive transfer of LCMV-specific memory T cells results in a T cell response in which the adoptively transferred T cells proliferate and reduce viral titers. It has been shown in this model that naïve host CD8 T cells do not participate in the response by proliferation, blastogenesis, or regulation of activation markers (Zarozinski and Welsh, 1997). In the same study, it was shown that TCR transgenic HY CD8 T cells with a fixed TCR also do not participate in the LCMV-induced T cell response, providing further evidence that LCMV induces little bystander T cell activation (Zarozinski and Welsh, 1997).

Early techniques to detect virus-specific CD8 T cells (limiting dilution analysis and single cell cytotoxicity assays) could only account for approximately 10% of the total CD8 T cell response as being LCMV-specific (Moskophidis et al., 1987). More sensitive techniques to define antigen-specific T cell responses (intracellular cytokine staining and staining with peptide-MHC multimer complexes) have minimized the expected contribution of bystander T cell activation, as 80-95% of the CD8 T cells present at the peak of an LCMV response can now be accounted for as LCMV-specific (Masopust et al., 2007; Murali-Krishna et al., 1998). Despite these studies, there are still a variety of publications that suggest bystander T cell activation during acute infections; most of these studies do not exclude the possibility of cross-reactivity.

In support of the theory that cytokines can induce CD8 T cell activation, it has been demonstrated that CD8 T cells from naïve mice can secrete IFN- γ in response to combinations of IL-2, IL-15, IL-12, and IL-18 *in vitro*, but only memory phenotype (CD44^{hi}) CD8 T cells are capable of IFN- γ production (Berg et al., 2002;

Lertmemongkolchai et al., 2001). Effector and memory CD8 T cells from *Listeria monocytogenes*- or LCMV-infected mice have the capacity to secrete IFN- γ in response to IL-12 and IL-18 stimulation in the absence of cognate antigen *in vitro* (Berg et al., 2003; Raue et al., 2004). The enhanced ability of activated CTL to produce IFN- γ in response to IL-12 and IL-18 requires initial TCR stimulation by antigens *in vivo*, which induces IL-12 responsiveness via upregulation of IL-12R, that induces IL-18R expression and downstream IL-18 responsiveness (Tomura et al., 1998). IL-12 signaling via STAT4 induces T-bet expression, which can initiate IFN- γ transcription as well as enhanced expression of IL-12R β (Afkarian et al., 2002; Takemoto et al., 2006; Yang et al., 2007). IL-18, IL-15, IL-21, and IFN- γ itself may also participate in this feedback loop by enhancing IFN- γ expression in T cells (Strengell et al., 2003). In this capacity, it is possible that bystander memory CD8 T cells can provide some IFN- γ -mediated cross-protection during subsequent heterologous infections. However, naïve bystander CD8 T cells are not activated in this manner (Berg et al., 2003).

It has also been demonstrated that IL-2 and IL-15 can induce cytolytic potential of naïve CD8 T cells in the absence of cognate antigen, but only if T cells are cultured *in vitro* for 3 days and after proliferation and conversion to memory phenotype (CD44^{hi}) (Tamang et al., 2006). This type of activation is similar to the transient “activation” of T cells after homeostatic proliferation (Goldrath et al., 2000). It has also been suggested that cytokines such as IFN- $\alpha\beta$ can induce the proliferation and activation of naïve T cells in the absence of antigen (Tough et al., 1996), but IFN- $\alpha\beta$ causes apoptosis of lymphocytes, creating immunological space that induces homeostatic proliferation and

subsequent “activation” (Goldrath et al., 2000; Kim et al., 2002; McNally et al., 2001). It is also important to note that homeostatic proliferation requires low affinity self-peptide-MHC and thus is not driven solely by cytokine signals in a purely bystander manner (Goldrath and Bevan, 1999; Kieper et al., 2004; Seddon et al., 2000).

Bystander T cells can also be affected by chemokines during acute infections. CCR5 signals have been shown to affect naïve bystander CD8 T cell trafficking in two separate experimental systems. In one study, it was demonstrated that naïve OT-I CD8 T cells could specifically traffic to a non-antigen-laden footpad if a CD4 epitope and CpG were administered into that footpad (Castellino et al., 2006). In another study, it was demonstrated that bystander polyclonal CD8 T cells slowed their movement in LNs and formed stable conjugates with DCs presenting peptide to TCR transgenic CD8 T cells (Hugues et al., 2007). In both systems, CCR5 signals in the bystander T cells were required (Castellino et al., 2006; Hugues et al., 2007).

In addition to the non-cognate T-DC conjugates described above, bystander OT-I CD8 T cells have also been shown to form contacts with DCs in VV-infected mice (Hickman et al., 2008). These T-DC contacts were longer than T-DC contacts in uninfected mice, but shorter than in VV-OVA-infected mice where DCs would be presenting cognate peptide (Hickman et al., 2008). Although bystander CD8 T cells make stable non-cognate T-DC conjugates, it is unclear what kind of signals bystander T cells may receive through the TCR from non-cognate peptide-MHC *in vivo*.

There are a couple of studies that suggest that naïve CD8 T cells can be sensitized or conditioned in a bystander manner to respond differently upon cognate antigen

stimulation. One such study has shown that IL-15 can induce cytolytic potential of naïve (CD44^{hi}-depleted) CD8 T cells within 24 hours *in vitro* (Tamang et al., 2008). IL-15 did not induce grzB expression and cytotoxicity was perforin-dependent in this *in vitro* system (Tamang et al., 2008). In a different study, *Mycobacterium avium* infection conditioned OT-I transgenic CD8 T cells to produce IFN- γ in response to a brief anti-CD3 stimulation *ex vivo* (Gilbertson et al., 2004). This group ruled out typical cross-reactivity by demonstrating that the OT-I cells did not proliferate during the infection, but did not otherwise determine a mechanism (Gilbertson et al., 2004).

Despite the debate of whether T cells can or cannot be activated in a TCR-independent bystander manner, it is clear that bystander T cells are affected by ongoing immune responses. The examples presented above demonstrate that bystander T cells can be affected by cytokines, chemokines, and mature APCs displaying non-cognate peptide-MHC. Additionally, virus-specific T cells recruited later in the immune response, or “latecomer” T cells, may also be sensitized by inflammatory signals prior to antigen stimulation, and it is likely that a combination of T cells with different signaling sequences from cytokine receptors and TCR constitute the total T cell response to pathogens. It is important to investigate how bystander and latecomer T cells may be sensitized or conditioned by ongoing immune responses, particularly in respect to how those T cells respond to cognate antigen stimulation; that will be the focus of this thesis.

Chapter II: Materials and Methods

A. Mice

C57BL/6J (Ly5.2⁺) male mice, B6.Smm.C3H-Tnfsf6^{gld} (*gld*), B6.129P2-B2m tm1Unc/J (β 2m KO), B6.129S7-IFNg<tm1Ts>/J (IFN- γ KO), and B6.129P2-*Il18*^{tm1Aki}/J (IL-18 KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-H-2Kb<tm1>H-2Db<tm1> (K^bD^b KO), C57BL/6-H-2Kb<tm1> (K^b KO), C57BL/6-H-2Db<tm1> (D^b KO), C57BL/6NTac-IL15<tm1>N5 (IL-15 KO) (Kennedy et al., 2000) and TCR-LCMV P14/Rag2 knockout mice were purchased from Taconic Farms (Germantown, NY). B6.SJL (Ly5.1⁺) male and female mice were purchased from Taconic Farms or bred within the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). TCR transgenic mice (P14 (Pircher et al., 1989), HY (Kisielow et al., 1988), and OT-I (Barnden et al., 1994)), IFN- $\alpha\beta$ receptor KO mice (IFN- $\alpha\beta$ R KO) (Muller et al., 1994), B6.MRL-*Tnfrsf6*^{lpr} (*lpr*) mice (Andrews et al., 1978), and B6.129-IL-12 β (IL-12 p40 KO) mice were bred at UMMS. TCR transgenic HY mice were crossed to *lpr* mice to generate HY transgenic T cells expressing a mutated Fas protein. B6.Tg(HY)-*Tnfrsf6*^{lpr}F2 mice (referred to as HY/*lpr*) were screened via surface expressing of the HY TCR and Fas after *in vitro* anti-CD3 stimulation. TCR transgenic P14 mice were crossed to IFN- $\alpha\beta$ R KO mice to generate P14 transgenic T cells that do not express IFN- $\alpha\beta$ R. P14 IFN- $\alpha\beta$ R KO mice were screened via surface expression of V α 2⁺ TCR and genomic PCR for the knockout IFN- $\alpha\beta$ R locus. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of UMMS.

B. Virus stocks and inoculations

LCMV, strain Armstrong, and Pichinde virus, strain AN3739, were propagated in baby hamster kidney cells (BHK21) as previously described (Welsh and Seedhom, 2008; Yang et al., 1989). VV, strain WR, was propagated in NCTC 929 cells and purified over a sucrose gradient (Selin et al., 1998). VV recombinants expressing LCMV NP or GP were kindly provided by Dr. J. Lindsay Whitton (Scripps Research Foundation, La Jolla, CA) (Whitton et al., 1988). MCMV, strain Smith, was propagated *in vivo* in salivary glands of BALB/c mice (Selin et al., 1998). Mice were injected intraperitoneally (i.p.) with 4×10^5 plaque forming units (pfu) of LCMV, 1.5×10^7 pfu of PV, 1×10^6 pfu of VV, 6×10^5 pfu of MCMV, 5×10^6 pfu of VV-NP, or 5×10^6 pfu of VV-GP. The higher than wild type dose for VV recombinants is used because the recombinants are attenuated, due to the inactivation of the *thymidine kinase* gene.

To induce IFN- $\alpha\beta$ *in vivo*, mice were inoculated with 200 μg poly(I:C) (InvivoGen, San Diego, CA) i.p. unless otherwise described. To activate P14 TCR transgenic CD8 T cells *in vivo*, mice were inoculated with 5 μg GP₃₃₋₄₅ 13mer peptide or GP₃₃₋₄₁-labeled DC2.4 cells intravenously (i.v.). DC2.4 cells were incubated with 1 μM GP₃₃₋₄₁ for 45 minutes at 37 °C and washed 4 times in HBSS to label. To block TNF, mice were inoculated with 100 μg Enbrel® (etanercept) i.p. (Moreland, 1998). To block TCR signaling, mice were inoculated i.p. with cyclosporin A (CsA; Sigma-Aldrich, St. Louis, MO) at a dose of 40 mg/kg diluted in olive oil (Sigma-Aldrich). To deplete NK cells, mice were inoculated i.v. with 25 μg anti-NK1.1 (PK136) or IgG2a (Cl.18.4) isotype control (BioXcell, West Lebanon, NH). To deplete CD4 T cells, mice were

inoculated i.v. with 100 μg anti-CD4 (GK1.5) antibody or IgG2b (LTF-2) isotype control (BioXcell).

C. Adoptive transfers

Spleens were harvested from TCR transgenic mice (P14, HY, or OT-I) and single cell suspensions were prepared. Red blood cells were lysed with a 0.84% NH_4Cl solution and lymphocytes were washed with HBSS. Where described, cells were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) by incubation in 2 μM CFSE in HBSS (Invitrogen, Carlsbad, CA) at 37°C for 15 minutes. For the experiments in Chapter III, 5-20 $\times 10^5$ total splenocytes or 0.5-5 $\times 10^5$ T cell enriched splenocytes from TCR transgenic mice were injected into congenic recipient mice i.v. For the experiments in Chapter IV, 3-10 $\times 10^6$ total splenocytes or 0.5-5 $\times 10^6$ T cell enriched splenocytes from TCR transgenic mice were injected into congenic recipient mice i.v.

To enrich for T cells, total splenocytes were stained with biotinylated anti-NK1.1 (PK136; BD Pharmingen), anti-I-A^b (AF6-120.1; BD Pharmingen), and anti-CD19 (1D3; BD Pharmingen) antibodies followed by staining with streptavidin microbeads (Miltenyi Biotec, Auburn, CA). The cells were then passed through a magnetic LS column and the flow through was collected. Enrichment of transgenic T cells was assayed via flow cytometry.

D. Synthetic peptides

Synthetic peptides were used to stimulate T cell responses *in vivo* and *ex vivo*. All peptides were purchased from 21st Century Biochemicals (Marlboro, MA) and were purified with reverse phase-HPLC to 90% purity. For *ex vivo* stimulations, P14 transgenic T cells were stimulated with the LCMV epitope GP₃₃₋₄₁ (KAVYNFATC) (Pircher et al., 1989), HY transgenic T cells were stimulated with the Y-chromosome encoded Smcy epitope (KCSRNRQYL) (Rocha and von Boehmer, 1991), and OT-I transgenic T cells were stimulated with OVA₂₅₇₋₂₆₄ (SIINFELK) (Barnden et al., 1994). For *in vivo* stimulations, P14 transgenic T cells were stimulated with the elongated GP₃₃₋₄₅ (KAVYNFATCGIFA) 13mer epitope.

Virus-infected mice were tested for host CD8 T cell responses with the following peptides: LCMV and VV-GP infections - GP₃₃₋₄₁ (KAVYNFATC); LCMV and VV-NP infections - NP₃₉₆₋₄₀₄ (FQPQNGQFI) (Lewicki et al., 1995); PV infections - PV₃₈₋₄₅ (SALDFHKV) (Brehm et al., 2002); VV, VV-GP, and VV-NP infections - B8R (TSYKFESV) (Moutaftsi et al., 2002); and MCMV - M45₉₈₅₋₉₉₃ (HGIRNASFI) (Gold et al., 2002).

E. Intracellular cytokine and effector molecule staining

Cytokine production was evaluated after stimulation with peptides using the Cytofix/Cytoperm Kit Plus (with GolgiPlug; BD Pharmingen). Splenocytes ($2-4 \times 10^6$) were plated in replicates (as many as 10 wells/spleen for some experiments) in 96-well plates with 1-5 μ M synthetic peptide, 10 U/ml human rIL-2, and 0.2 μ l GolgiPlug (BD

Pharmingen) for 5 hours at 37°C. For positive controls, splenocytes were stimulated with 1 µg purified anti-mouse CD3ε mAb (145-2c11; BD Pharmingen). Following stimulations, splenocytes were washed in flow cytometry buffer (2% FCS in HBSS) and blocked with α-Fc (2.4G2; BD Pharmingen) for 15 minutes at 4°C. Splenocytes were then stained with a combination of fluorescently-labeled monoclonal antibodies (mAb) specific for CD8 (53-6.7; BD Pharmingen), Ly5.2/CD45.2 (104; BD Pharmingen), Ly5.1/CD45.1 (A20; eBioscience (San Diego, CA) or BioLegend (San Diego, CA)), Thy1.2/CD90.2 (53-2.1; BD Pharmingen), Thy1.1 /CD90.1 (H1S51; eBioscience), Vα2 TCR (B20.1; eBioscience), HY TCR (T3.70; eBioscience), CD44 (IM7; BD Pharmingen), CD122 (TM-β1; BD Pharmingen), CD62L (MEL-14, BD Pharmingen), CD127 (A7R34; eBioscience), and CD43 (1B11; BioLegend) for 20 minutes at 4°C. Subsequent fixation and permeabilization of the cells was performed via Cytotfix/Cytoperm for 20 minutes at 4°C. Following permeabilization, cells were stained with fluorescently-labeled mAbs specific for IFN-γ (XMG1.2; BD Pharmingen or eBioscience), TNF (MP6-XT22; BD Pharmingen), and/or GrzB (Invitrogen). Eomes protein was stained with anti-mouse/human Eomes (TBR2; eBioscience) after fixation and permeabilization with the FoxP3 staining buffer kit (eBioscience) as per manufacturer's instruction.

F. *In vivo* cytokine production assay

To assess *in vivo* IFN- γ production, mice were inoculated directly with 250 μ g brefeldin A (Sigma-Aldrich) and GP₃₃₋₄₅ 13mer peptide i.v. Six hours later, spleens were harvested, single cell suspensions prepared, and stained as described above without the need for *ex vivo* restimulation. This is a modified protocol that has been previously described (Liu and Whitton, 2005).

G. Degranulation assay

To assay the ability of CD8 T cells to undergo antigen-driven degranulation, splenocytes were stimulated with synthetic peptides as stated above with the addition of 0.5 μ l/well anti-CD107a (1D4B; BD Pharmingen) and anti-CD107b (ABL-93; BD Pharmingen) FITC-labeled antibodies and 0.2 μ l/well of GolgiStop (BD Pharmingen).

H. Apoptosis assays

Apoptosis was evaluated via flow cytometry by phosphatidyl serine exposure (annexin-V staining), terminal transferase dUTP nick end labeling (TUNEL), and/or caspase activation. For Annexin-V staining, splenocytes were first surface stained with the antibodies listed above, followed by washes and staining in Annexin-V binding buffer with fluorescently labeled Annexin-V (BD Pharmingen) at a 1:20 dilution for 15 minutes at room temperature. The cells were then washed in Annexin-V binding buffer and analyzed immediately via flow cytometry. For TUNEL analysis, splenocytes were first plated in multiple replicates at 2×10^6 cells/well in 48-well plates and cultured *ex vivo* at

4°C or 37°C for 5 hours. Cells were then washed and stained with fluorescently-labeled mAb in HBSS followed by fixation with Cytotfix (BD Pharmingen). Nuclear membrane permeabilization was achieved by incubation in 70% ethanol at -20°C for up to 2 days, followed by staining with the Apo-BrdU-Red™ *In Situ* DNA Fragmentation Assay Kit (BioVision, Mountain View, CA). To detect caspase activation, splenocytes were first surface stained, followed by staining with CaspGLOW™ fluorescein active stains specific for caspase 3 (FITC-DEVD-FMK), caspase 8 (FITC-IETD-FMK), or total caspases (FITC-Z-VAD-FMK). All active caspase stains were purchased from BioVision.

For some experiments, membrane integrity was analyzed via staining with 7-aminoactinomycin D (7-AAD; BD Pharmingen) or LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen).

I. Cell cycle analysis

In addition to the analysis of CFSE dilution to measure cell division, DNA content and Ki-67 expression were measured. DNA content was measured with Vybrant® DyeCycle™ (Invitrogen), a cell membrane permeant, DNA-selective, fluorescently-labeled dye. Following surface stains as described above, splenocytes were incubated with 100µl of a 1:1000 dilution of Vybrant® DyeCycle™ violet stain for 30 minutes at 37°C and immediately analyzed by flow cytometry. Ki-67 expression was also measured as a marker for dividing cells by staining cells intracellularly with anti-human Ki-67 (B56; BD Pharmingen).

J. Flow Cytometry

Freshly stained and previously fixed samples were acquired using a BD Biosciences LSRII with FACS Diva software and analyzed with FlowJo software (Treestar Inc, Ashland, OR). In order to analyze enough bystander TCR transgenic CD8 T cells, the threshold for acquisition was set to CD8⁺ events only, and the storage and stoppage gates were set on CD8⁺ events only. By setting these parameters, FACS Diva ignored all other (CD8⁻) events that ran through the cytometer and allowed for the acquisition of up to approximately 3-4 x 10⁶ CD8 T cell events/sample (approximately an entire naïve mouse spleen worth of CD8 T cells). It should be noted that for most experiments in this study 3-6 x 10⁵ CD8⁺ T cell events were collected/sample.

K. Functional IFN quantification bioassay

Serum and human rIFN- α (PBL Interferon Source, Piscataway, NJ) were serially diluted (2-fold) across a 96-well flat bottom plate and NCTC L929 cells were added at 2 x 10⁴ cells/well. The following day, 7.5 x 10⁵ pfu vesicular stomatitis virus (VSV) was added/well. Cell morphology and cytopathic effects (CPE) were monitored 2 days post infection and the amount of total functional IFN was measured as the last dilution of serum or control rIFN- α to provide approximately 50% protection from VSV-mediated CPE.

L. RNA isolation and quantitative real-time PCR

P14 transgenic CD8 T cells (7-AAD⁻, CD8⁺, V α 2⁺, and congenic marker⁺) were sorted to 93-99% purity on a Mo-Flo sorter (Cytomation, Fort Collins, CO). RNA was isolated from sorted P14 CD8 T cells with an RNeasy kit (Qiagen, Valencia, CA) and evaluated spectrophotometrically at 260nm to determine concentration. cDNA was generated using SuperScript III first-strand synthesis system (Invitrogen) on a PTC-200 Thermo Cycler (MJ Research, Waltham, MA) at 25°C for 10 minutes followed by 50°C for 50 minutes. Relative mRNA concentrations were determined by quantitative real-time PCR using SYBR Green PCR core reagent kit (Applied Biosystems, Foster City, CA) on an iCycler iQ (Bio-Rad, Hercules, CA). The following primers were used: *18SrRNA* sense 5'-TGGTGGAGGGATTTGTCTGG-3' and anti-sense 5'-TCAATCTCGGGTGGCTGAAC-3', *eomesodermin* sense 5'-TGAATGAACCTTCCAA GACTCAGA-3' and anti-sense 5'-GGCTTGAGGCAAAGTGTTGACA-3', *T-bet* sense 5'-TTCCCATTCCTGTCCTTCACC-3' and anti-sense 5'TGCCTTCTGCCTTTCCACA C-3'. For the generation of standard curves, cDNA clones of 18SrRNA, *eomesodermin*, and *t-bet* were used. All primers and cDNA plasmid clones were generously provided by Dr. Leslie J. Berg.

M. Statistical Analyses

Where appropriate, Student's *t* tests were calculated using GraphPad InStat software. Significance was set at $p < 0.05$ and denoted as * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$. All results are expressed as the mean \pm standard deviation.

Chapter III: Virus-induced transient immune suppression inhibits antigen-induced bystander CD8 T cell proliferation *in vivo*

Many virus infections induce a transient state of immune suppression that can result in superinfections and poor vaccine efficacy. There is evidence that immune suppression can be caused by T cell AICD, DC apoptosis, DC dysfunction, and/or be cytokine-mediated. It has been previously demonstrated that naïve bystander CD8 T cells derived from hosts experiencing an acute virus-specific T cell response underwent AICD when polyclonally triggered through their TCR *in vitro* (Zarozinski et al., 2000). Susceptibility to AICD could cause immune suppression by inhibiting the development of a new T cell response during an ongoing immune response. In this chapter we sought to investigate whether naïve bystander CD8 T cells would be susceptible to immune suppression if activated by cognate antigen during an acute viral infection *in vivo*. Consistent with the enhanced susceptibility to cell death noted *in vitro*, we found that bystander CD8 T cells that are activated with cognate antigen *in vivo* during acute viral infections undergo markedly reduced proliferation. This virus-induced transient immune suppression *in vivo* was associated with a delayed onset of division, which we found not to be a defect in antigen presentation, but rather due to a T cell intrinsic defect. The inhibition of TCR-stimulated bystander CD8 T cell proliferation during acute viral infections could lead to a superinfection or poor vaccine efficacy, as co-infecting pathogen- or vaccine-specific T cells may be unable to mount a sufficient immune response.

A. *In vivo* bystander CD8 T cell models

In order to study how bystander and latecomer CD8 T cells are affected by acute viral infections, we developed *in vivo* models to track and specifically activate bystander CD8 T cells. We tested three TCR transgenic CD8 T cells for this purpose and defined bystander as a transgenic CD8 T cell population that did not divide or proliferate (lose CFSE or increase in cell number), or alter the expression of activation markers (CD44, CD43, CD62L) during the viral infection.

We found that P14 CD8 T cells, specific for the LCMV epitope GP₃₃₋₄₁, did not divide, as measured by a lack of dilution of CFSE throughout infection of mice with PV (Figure 3.1A). Additionally, P14 CD8 T cells did not expand in terms of frequency or cell number throughout the PV infection and instead were reduced in frequency and number during the PV infection, as they may have been susceptible to IFN- $\alpha\beta$ -induced attrition and got diluted out by the expansion of PV-specific T cells (Figure 3.1B). P14 CD8 T cells also remained phenotypically naïve during the PV infection as demonstrated by the expression patterns of CD44, CD43, and CD62L (Figure 3.2). Naïve CD8 T cells are CD44^{lo}, CD43^{lo}, and CD62L^{hi} as shown by the host and P14 CD8 T cells in naïve mice (left, grey and black histograms respectively). Activated PV-specific CD8 T cells are CD44^{hi}, CD43^{hi}, CD62L^{lo}, CD127^{lo} and CD122^{hi} as shown by the host CD8 T cells at day 5 of PV (right, grey histograms), while the P14 CD8 T cells remained CD44^{lo}, CD43^{lo}, and CD62L^{hi} (right, black histograms). There was a moderate down-regulation of CD127 and up-regulation of CD122 on a portion of the P14 CD8 T cells in the PV-

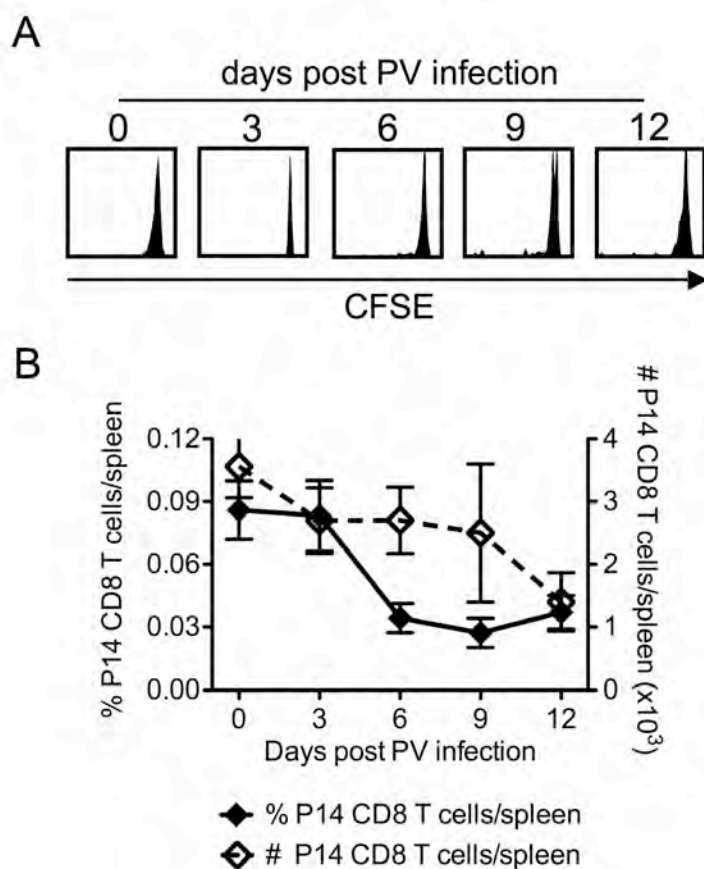


Figure 3.1. P14 CD8 T cells do not divide or expand during PV infection. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. (A) CFSE profiles of donor P14 CD8 T cells at 0, 3, 6, 9, and 12 days post infection. (B) Frequency (left axis, closed diamonds) and number (right axis, open diamonds) of P14 CD8 T cells during PV infection.

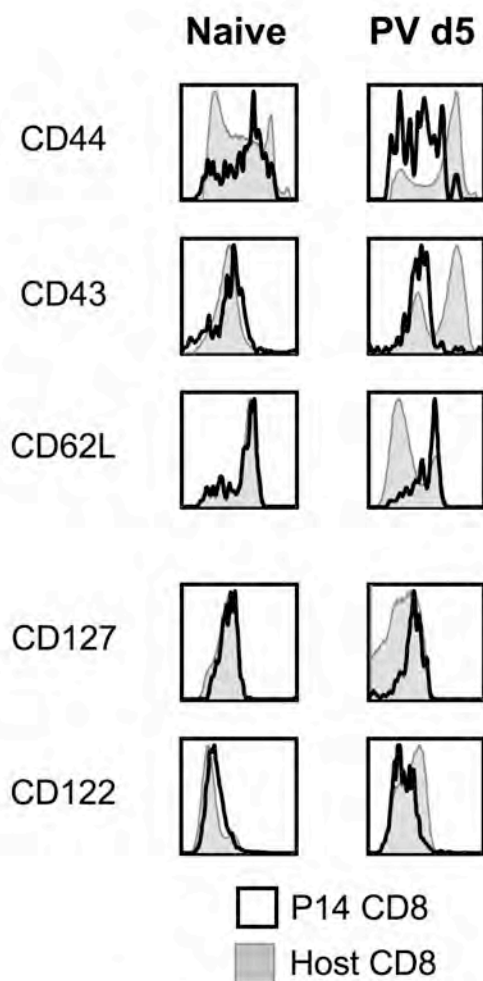


Figure 3.2. P14 CD8 T cells remain phenotypically naïve during PV infection. P14 CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5, splenocytes were harvested and stained for the surface markers shown. Representative examples of host CD8 T cells (shaded) and P14 CD8 T cells (thick black) are overlaid from the same host.

infected mice, but the expression of these activation markers was not altered to the extent of that of the host CD8 T cells, and a majority of the P14 CD8 T cells retained the naïve phenotype. The regulation of CD127 and CD122 activation markers will be discussed further in chapter IV.

HY transgenic CD8 T cells are specific for the male (Y chromosome-encoded) *Smcy* epitope and remained phenotypically naïve during LCMV infection of female mice. HY CD8 T cells did not divide (CFSE^{hi}) and remained CD44^{lo}, CD43^{lo}, CD62L^{hi}, and CD127^{hi} (Figure 3.3). OT-I CD8 T cells are specific for the SIINFEKL epitope of ovalbumin (OVA₂₅₇₋₂₆₄) and, like the HY CD8 T cells, remained phenotypically naïve during LCMV infection (Figure 3.4).

We have used these three TCR transgenic CD8 T cell models (P14 + PV, HY or OT-I + LCMV) extensively throughout this thesis to study the impact of viral infections on bystander CD8 T cells. We have also used these TCR transgenic CD8 T cells in other viral infection models (VV, VV-GP, VV-NP, and MCMV) and have ensured that they are bystander by the lack of division, proliferation, and upregulation of CD44 (data not shown).

B. Transient inhibition of TCR-stimulated bystander CD8 T cell proliferation during acute viral infections

To determine if bystander CD8 T cells were susceptible to immune suppression when activated by cognate antigen during an acute viral infection *in vivo*, we first

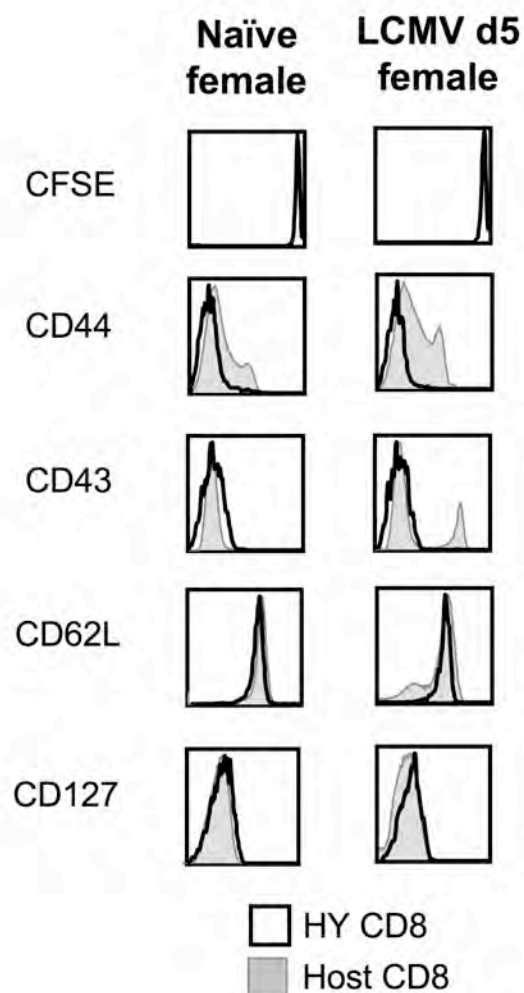


Figure 3.3. HY CD8 T cells remain phenotypically naïve during LCMV infection of female mice. HY CD8 T cells were adoptively transferred into female congenic recipients followed by infection with 4×10^5 pfu LCMV Armstrong i.p. At day 5, spleens were harvested and stained for the surface markers shown. Representative examples of host CD8 T cells (shaded) and HY CD8 T cells (thick black) are overlaid from the same host except for CFSE profiles, which are donor HY CD8 T cells only.

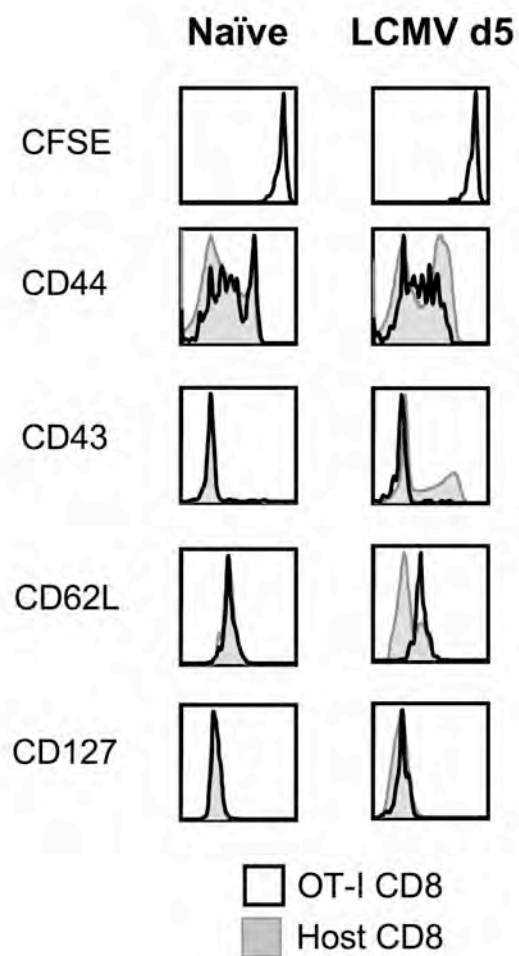


Figure 3.4. OT-I CD8 T cells remain phenotypically naive during LCMV infection. OT-I CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 5×10^4 pfu LCMV i.p. At day 5, spleens were harvested and stained for the surface markers shown. Representative examples of host CD8 T cells (shaded) and OT-I CD8 T cells (thick black) are overlaid from the same host except for CFSE profiles, which are donor OT-I CD8 T cells only.

adoptively transferred HY transgenic CD8 T cells into naïve or day 5 LCMV-infected female or male congenic recipients. Because of their male antigen specificity, HY CD8 T cells become activated in male but not female mice (Rocha and von Boehmer, 1991). Two days later, proliferation was measured by calculating the number of HY CD8 T cells in the spleen of each group of mice. We found a slight reduction in the number of HY CD8 T cells recovered from the LCMV-infected female mice compared to the naïve female mice, and the HY CD8 T cells that remained had a naïve phenotype. However, the HY CD8 T cells became activated and proliferated in the naïve male mice, resulting in an increase in their overall cell number, but this proliferation was greatly inhibited in the LCMV-infected male mice (Figure 3.5A).

Since the HY CD8 T cells were directly transferred into virus-infected mice, we also employed a second model in which P14 CD8 T cells were adoptively transferred into naïve congenic recipients, followed by infection with PV. In this model, the bystander CD8 T cells were present *in vivo* during the entire virus infection. At day 5 of PV infection, mice were inoculated with the highly immunogenic GP₃₃₋₄₅ 13mer peptide i.v. to specifically activate the P14 CD8 T cells. Two days later, the same time after activation as the HY experiment, the number of P14 CD8 T cells in the spleen of each group was determined. Without antigen there was a slight decrease in the number of P14 CD8 T cells recovered from the PV-infected mice, possibly due to IFN- $\alpha\beta$ -induced T cell attrition (McNally et al., 2001). After infusion of GP₃₃₋₄₅ antigen the P14 CD8 T cells in the naïve mice proliferated, but this proliferation was inhibited in the PV-infected mice,

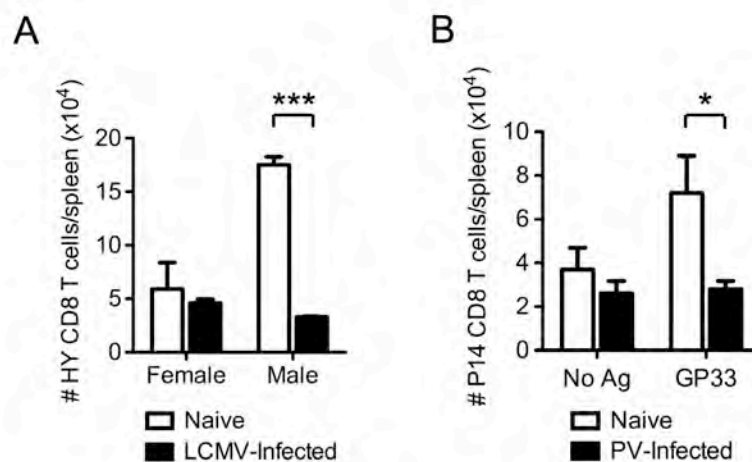


Figure 3.5. Reduced proliferation of TCR-stimulated bystander CD8 T cells during acute viral infections. (A) HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV day 5-infected female and male congenic mice. Two days post transfer, splenocytes were isolated and stained for CD8⁺ HY TCR⁺ cells. The number of HY CD8 T cells/spleen was graphed. (B) P14 transgenic CD8 T cells were adoptively transferred into congenic mice. One day post transfer, mice were infected with 1.5×10^7 pfu PV and at day 5 of PV, inoculated with 5 μ g GP₃₃₋₄₅ 13mer peptide i.v. Two days post peptide treatment, splenocytes were isolated and stained for CD8⁺ Va2 TCR⁺ cells. The number of P14 CD8 T cells/spleen was graphed.

much like the inhibition of proliferation of HY cells in LCMV-infected male mice (Figure 3.5B). These data demonstrate, in two independent experimental models, that acute viral infections inhibited the proliferation of TCR-stimulated bystander CD8 T cells.

We next addressed the kinetics of virus-induced immune suppression by transferring HY CD8 T cells into naïve or LCMV-infected male mice at days 3, 6, 9, and 12 post infection and examining the proliferation of HY CD8 T cells 3 days later. The proliferation of HY CD8 T cells activated on day 3 and day 6 of LCMV infection was inhibited compared to the proliferation in naïve mice (Figure 3.6). HY CD8 T cells activated on day 3 of LCMV infection had the most pronounced defect in proliferation, followed by HY CD8 T cells activated on day 6, day 9, and proliferation was back to normal naïve levels at day 12 post infection (Figure 3.6). In contrast, activation of CD8 T cells at the onset of virus infection, either involving HY cell transfer immediately prior to the time of LCMV infection or GP₃₃₋₄₅ inoculation immediately prior to the time of PV infection, markedly enhanced the proliferation of the bystander CD8 T cells (Figure 3.7). These data indicated that viral infections acted as a stimulatory adjuvant when bystander T cells were exposed to the inflammatory milieu and antigens at the beginning of infections but were immunosuppressive when bystander T cells engaged antigen as the infection progressed.

Due to the lack of expansion of TCR-stimulated bystander CD8 T cells in Figure 3.5, we questioned whether there was a specific deletion of cells following TCR stimulation *in vivo*, which we might expect if the cells were susceptible to AICD. To do

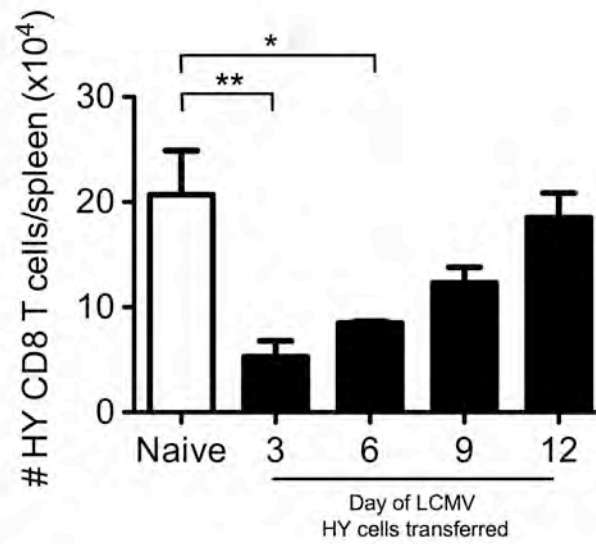


Figure 3.6. Transient susceptibility to reduced proliferation during acute viral infection. HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV-infected congenic male mice at days 3, 6, 9, or 12 post infection. Three days post transfer, spleens were harvested and the number of HY CD8 T cells/spleen was assessed.

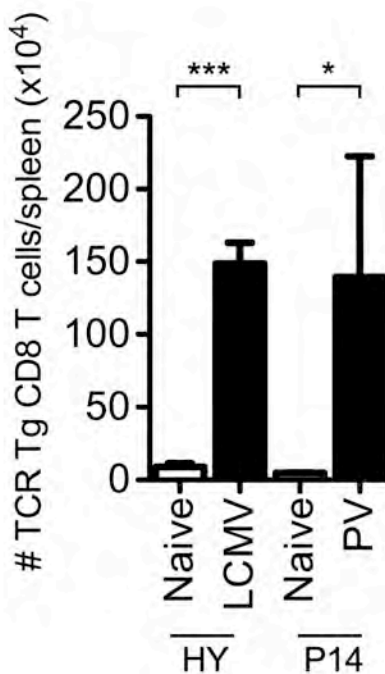


Figure 3.7. Enhanced proliferation of bystander CD8 T cells activated at the onset of virus infection. HY transgenic CD8 T cells were adoptively transferred into naïve congenic male mice and one group was infected with 5×10^4 pfu LCMV a few hours later. At day 5 post infection, spleens were harvested and the number of HY CD8 T cells/spleen was assessed. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic mice. The following day, mice were inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. and one group was infected with 1.5×10^7 pfu PV i.p. At day 4 post infection, spleens were harvested and the number of P14 CD8 T cells/spleen was assessed.

this, we first investigated the overall kinetics of proliferation by calculating the number of P14 CD8 T cells at 0.5, 1, 1.5, 2, 4, and 8 days post peptide inoculation on day 5 of PV infection. First, we found that the number of P14 CD8 T cells activated in the naïve mice remained relatively stable until day 1.5, when they began an expansion phase that peaked 4 days post peptide treatment (Figure 3.8, open diamonds). In contrast, the P14 CD8 T cells activated in PV-infected mice underwent a slight attrition within the first day post peptide treatment, but then also proliferated with kinetics similar to that of the naïve mice (Figure 3.8, black circles). At the peak of the proliferative response, day 4 post peptide treatment, the total cell number for the P14 CD8 T cells activated in the PV-infected mice was greatly reduced compared to the P14 CD8 T cells activated in naïve mice. These data indicated that there was only a moderate attrition of TCR-stimulated P14 CD8 T cells following activation, and this cell loss probably could not account for the reduced expansion of bystander CD8 T cells activated during acute viral infections.

We also utilized the HY LCMV model to ask whether the reduced expansion was due to a rapid deletion of TCR-stimulated CD8 T cells by calculating the number of HY CD8 T cells just 2 hours post adoptive transfer into naïve and LCMV-infected female and male mice. In this model, we did find that the HY CD8 T cells transferred into the male LCMV-infected mice underwent a rapid depletion, prior to division of the cells, and supportive of the hypothesis that bystander CD8 T cells may be susceptible to AICD (Figure 3.9).

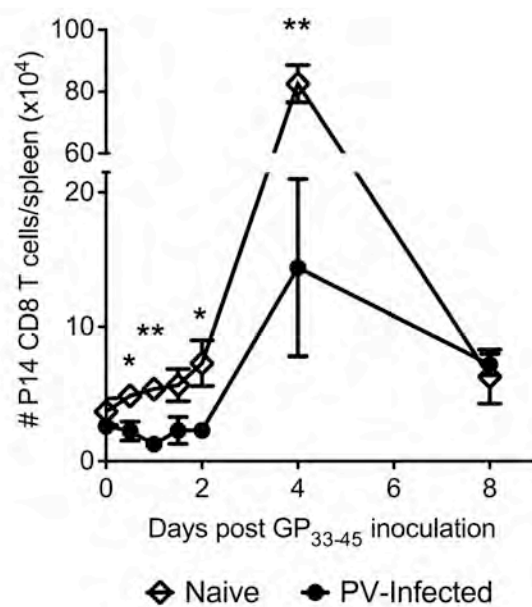


Figure 3.8. Kinetics of proliferation of TCR-stimulated bystander P14 CD8 T cells during acute PV infection. P14 transgenic CD8 T cells were adoptively transferred into congenic mice. One day post transfer, mice were infected with 1.5×10^7 pfu PV and at d5 of PV, inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. Spleens were harvested at 0, 0.5, 1, 1.5, 2, 4, and 8 days post peptide inoculation and the number of P14 cells/spleen was assessed.

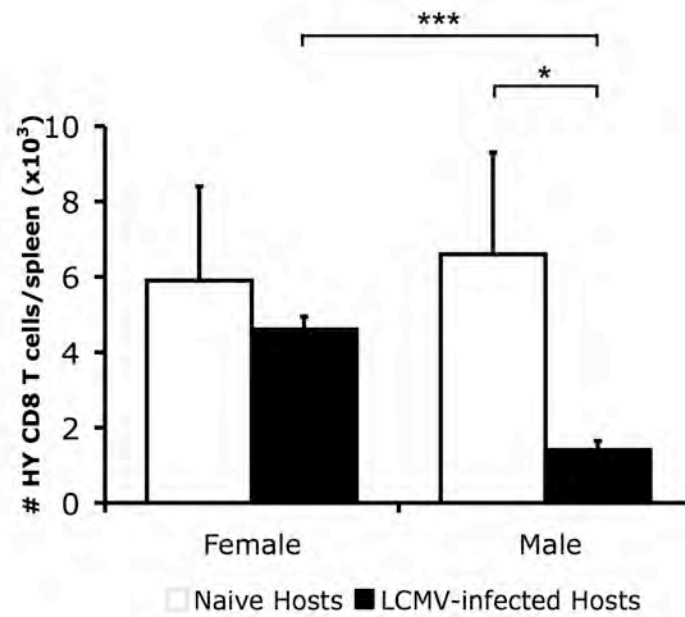


Figure 3.9. Rapid depletion of TCR-stimulated bystander HY CD8 T cells in LCMV-infected male mice. Female HY transgenic splenocytes were adoptively transferred into female and male naïve or LCMV-infected d5 congenic recipients. Two hours post transfer, spleens were harvested and the number of HY transgenic CD8 T cells/spleen was assessed.

C. Reduced proliferation and rapid depletion of TCR-stimulated bystander CD8 T cells is not due to sequestration in peripheral tissues

Due to the rapid depletion and reduced expansion of HY CD8 T cells in the spleen of LCMV-infected male mice, we asked whether the transgenic cells were being sequestered in other tissues. Trafficking of T cells can be altered during viral infection (Castellino et al., 2006; Hugues et al., 2007) and cells injected into the tail vein traffic through the lungs and liver before reaching the spleen, so it was possible that the HY CD8 T cells got trapped in the lung or liver on their way to the spleen following i.v. injection, or else got recruited to lymph nodes. We first tested whether the rapid depletion of HY CD8 T cells was due to getting trapped in the lung or the liver following i.v. injection. Two hours post transfer, we found a similarly low number of HY CD8 T cells in the liver and the lungs of LCMV-infected mice compared to the naïve mice (Figure 3.10). There were slightly more HY CD8 T cells in the lungs and livers of the virus-infected animals. However, this increase could not account for the loss of cells in the spleen, as there were approximately 10-fold fewer HY T cells recovered from the lungs and liver than in the spleen. We also enumerated the number of HY CD8 T cells in the spleen, inguinal lymph nodes, bone marrow, peritoneum, lung, and liver two days post transfer into male mice and found that HY CD8 T cells could not be recovered in any significant number in these tissues, indicating that they were not being specifically recruited to any of these other compartments (Figure 3.11). These data indicated that the rapid depletion and reduced proliferation of HY CD8 T cells in the spleen was not due to specific trafficking or sequestration in peripheral tissues.

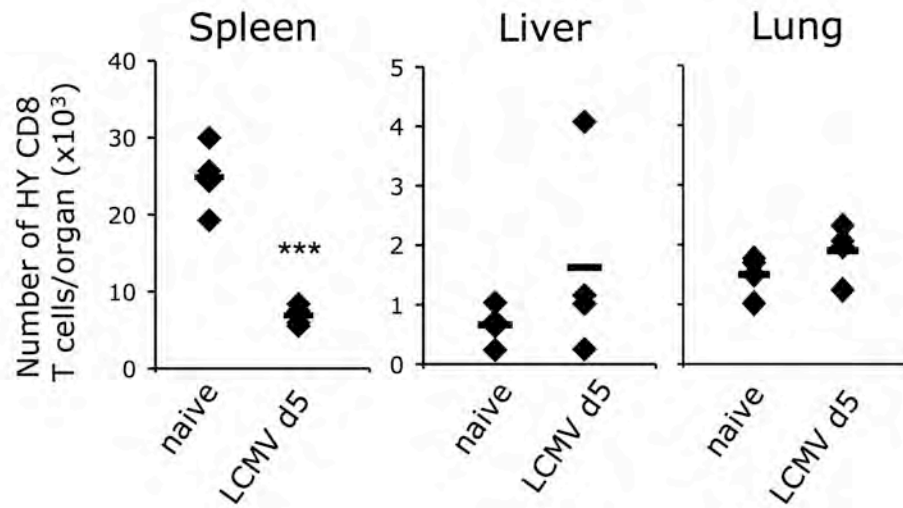


Figure 3.10. Rapid depletion of TCR-stimulated bystander HY CD8 T cells is not due to sequestration in peripheral tissues. HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV day 5 congenic male recipients. Two hours post transfer, indicated organs were harvested and the number of HY CD8 T cells/organ was assessed.

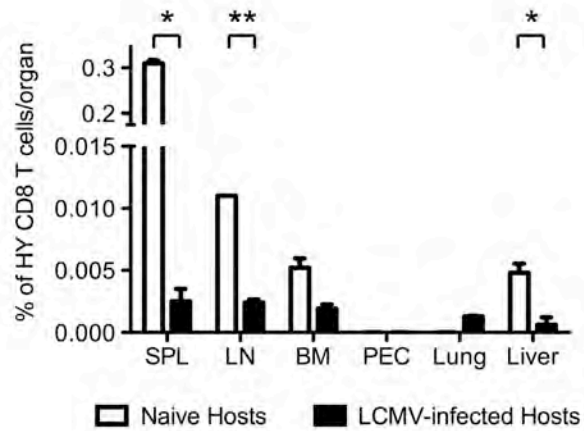


Figure 3.11. Reduced proliferation of TCR-stimulated bystander HY CD8 T cells is not due to sequestration in peripheral tissues. HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV day 5 congenic male recipients. Two days post transfer, indicated organs were harvested and the frequency of HY CD8 T cells/organ out of total lymphocytes was assessed.

D. Reduced proliferation of TCR-stimulated bystander CD8 T cells *in vivo* is not exclusively mediated by Fas-FasL- or TNF-induced AICD

The reduced proliferation of TCR-stimulated bystander CD8 T cells during acute viral infections would have been predicted by *in vitro* studies showing susceptibility of virus-induced T cell populations to AICD (Zarozinski et al., 2000). We therefore tested whether the reduced proliferation of defined bystander T cell populations was a consequence of AICD *in vivo*, by testing the influence of Fas-FasL and TNF in this process. To investigate the role of Fas-FasL, we studied the fate of HY transgenic T cells bred onto the B6.MRL-*Tnfrsf6^{lpr}* (*lpr*, Fas mutant) background and the fate of HY or P14 CD8 T cells in B6.Smm.C3H-*Tnfrsf6^{gld}* (*gld*, FasL-deficient) mice. HY/*lpr* CD8 T cells lack a functional Fas surface receptor and cannot, therefore, undergo apoptosis initiated by FasL (Nagata and Suda, 1995). *Gld* mice lack a functional FasL and cannot, therefore initiate apoptosis in Fas-expressing T cells (Nagata and Suda, 1995). To investigate the role of TNF, we treated mice with the human TNF receptor fusion protein Enbrel® to neutralize serum TNF (Moreland, 1998). Using these systems we found that HY and P14 CD8 T cell proliferation was inhibited in virus-infected mice whether Fas-FasL, TNF, or both pathways were blocked (Table 3.1), indicating that Fas-FasL and TNF were not required for the reduced proliferation of TCR-stimulated bystander CD8 T cells.

Table 3.1. Fas-FasL and TNF are not required for the inhibition of TCR-stimulated bystander CD8 T cell proliferation²

# Exps	TCR Tg	Host mice	Treatment	Functional Fas-FasL/TNF	Fold inhibition
7	HY	WT		+/+	4.2 ± 1.4
8	P14	WT		+/+	4.4 ± 1.9
2	HY	<i>gld</i>		-/+	4.9 ± 1.9
4	HY/lpr	WT		-/+	4.4 ± 1.1
4	P14	<i>gld</i>		-/+	4.5 ± 3.7
3	HY	WT	Enbrel®	+/-	4.0 ± 1.6
3	P14	WT	Enbrel®	+/-	5.1 ± 4.5
1	HY	<i>gld</i>	Enbrel®	-/-	4.3 ± 0.0
1	HY/lpr	WT	Enbrel®	-/-	4.1 ± 0.0
2	P14	<i>gld</i>	Enbrel®	-/-	3.8 ± 0.9

² HY or HY/*lpr* transgenic CD8 T cells were adoptively transferred into naïve or day 5 LCMV-infected WT or *gld* recipient male mice. Two days post adoptive transfer, the number of HY CD8 T cells in the spleen of individual mice was determined. P14 transgenic CD8 T cells were adoptively transferred into naïve WT or *gld* recipient mice followed by infection with 1.5×10^7 pfu PV i.p. At day 5 of PV infection, mice were inoculated with GP₃₃₋₄₅ i.v. Two days post peptide treatment, the number of P14 CD8 T cells in the spleen of individual mice was determined. Enbrel®-treated mice were inoculated i.v. with 100 µg Enbrel® i.p. at day 4 of virus infection (one day prior to transgenic T cell activation). Fold inhibition was calculated as the number of transgenic CD8 T cells activated in naïve mice over the number of transgenic CD8 T cells activated in virus-infected mice. The numbers depict the average fold inhibition for all experiments performed ± SD.

E. Reduced proliferation of TCR-stimulated bystander CD8 T cells may only minimally be due to enhanced susceptibility to apoptosis

Despite not finding a role for Fas-FasL or TNF in mediating AICD, we further explored the possibility that TCR-stimulated bystander CD8 T cells underwent cell death through another pathway, particularly due to the rapid depletion of HY CD8 T cells after activation in LCMV-infected male mice (Figure 3.9). We assessed apoptosis by annexin-V reactivity, TUNEL, and active caspase staining in TCR-stimulated HY and P14 CD8 T cells. Annexin-V reactivity is a measure of phosphatidyl serine exposure on the cell membrane and marks cells in the early phase of apoptosis. The TUNEL assay measures DNA fragmentation, which is an outcome of apoptosis, and thus TUNEL staining measures late stage apoptosis. Caspases are a family of cysteine proteases, which play an essential role in apoptosis and can be stained with fluorescent inhibitors that will only bind the active form. We used multiple assays to detect apoptosis because it is sometimes difficult to assess apoptosis of lymphocytes during acute viral infections directly *ex vivo* because of the rapid clearance system of phagocytes.

We found that TCR-stimulated HY CD8 T cells in LCMV-infected male mice were only slightly more apoptotic as measured by annexin-v reactivity and TUNEL staining than TCR-stimulated HY CD8 T cells in naïve male mice (Figure 3.12 and 3.13) (HY CD8 T cells averaged $9.3 \pm 3.1\%$ annexin-v⁺ and $16.5 \pm 7\%$ TUNEL⁺ in LCMV-infected male mice, while HY CD8 T cells averaged $10.7 \pm 2.1\%$ annexin-v⁺ and $12.6 \pm 2.1\%$ TUNEL⁺ in naïve male mice). Activated HY CD8 T cells were also slightly more apoptotic in naïve hosts than unstimulated HY CD8 T cells (HY CD8 T cells averaged

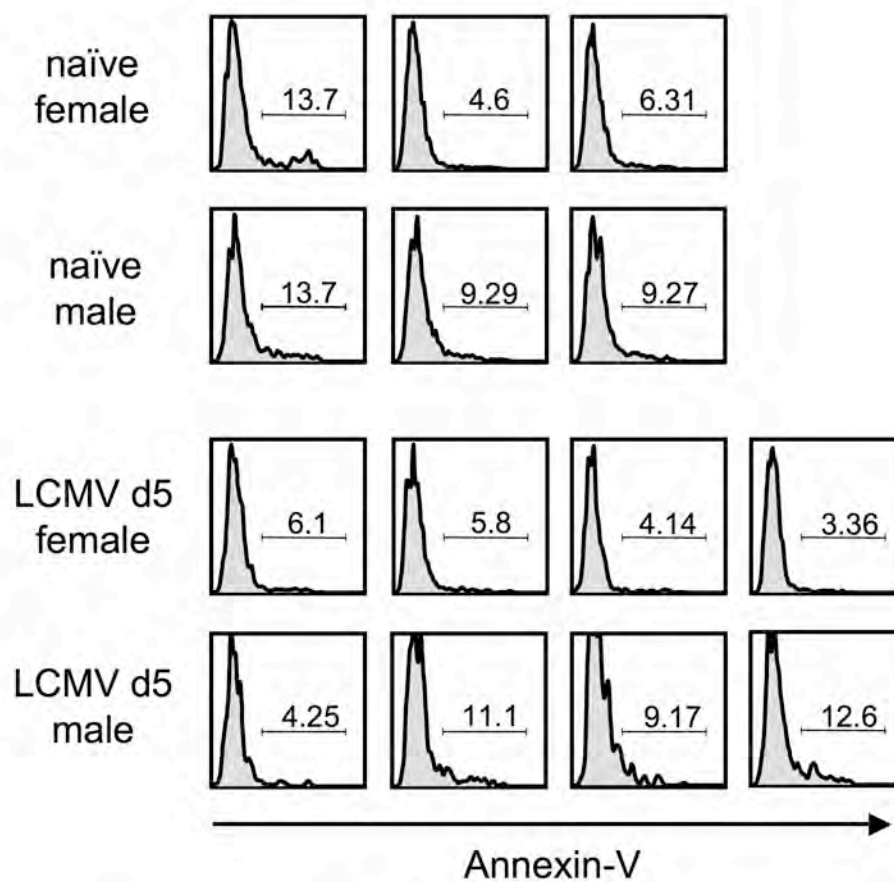


Figure 3.12. HY CD8 T cells undergo minimal apoptosis within 2 hours of activation in LCMV-infected male mice. HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV-infected day 5 female and male congenic recipients. Two hours post transfer, spleens were harvested and splenocytes were stained with Annexin-V. Annexin-V profiles of HY CD8 T cells are shown.

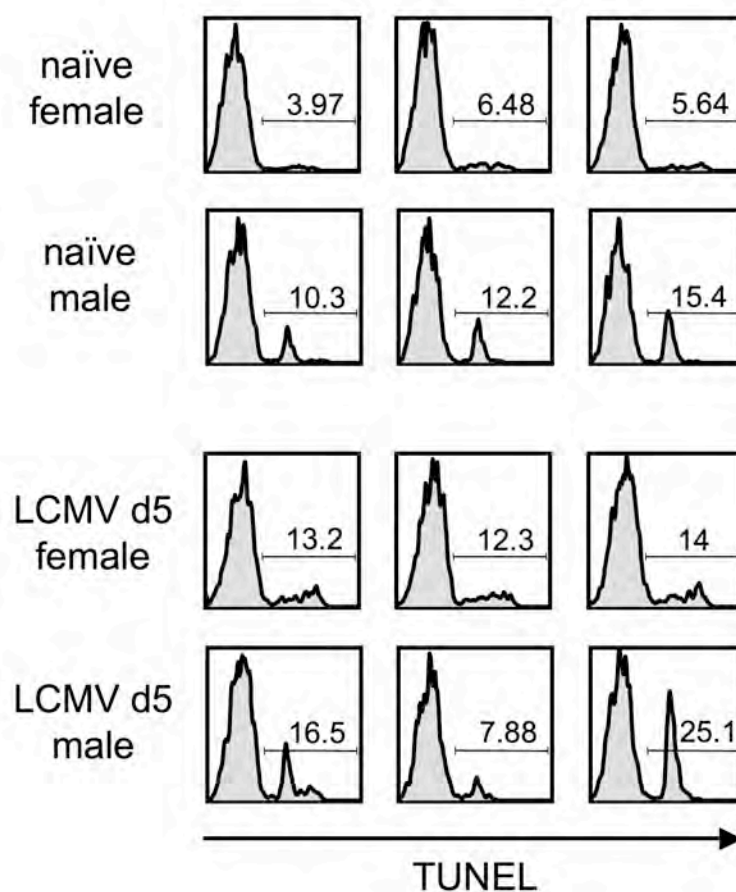


Figure 3.13. HY CD8 T cells undergo minimal apoptosis within 2 hours of activation in LCMV-infected male mice. HY transgenic CD8 T cells were adoptively transferred into naïve or LCMV-infected day 5 female and male congenic recipients. Two hours post transfer, spleens were harvested and DNA fragmentation was assessed via TUNEL after a brief *in vitro* culture of splenocytes for 5 hours at 37°C. TUNEL staining profiles of HY CD8 T cells are shown.

10.7 ± 2.1% annexin-v⁺ and 12.6 ± 2.1% TUNEL⁺ in naïve male mice, while HY CD8 T cells averaged 8.2 ± 3.9% annexin-v⁺ and 5.4 ± 1% TUNEL⁺ in naïve female mice), but only the TUNEL data was statistically significant (p = 0.01). These data indicated that activation of HY CD8 T cells induced some apoptosis, but that activation of bystander HY CD8 T cells during acute LCMV infection did not significantly drive the cells into apoptosis.

We also found that P14 CD8 T cells stimulated with GP₃₃₋₄₅ peptide in PV-infected mice were also only slightly more apoptotic as measured by annexin-v reactivity than GP₃₃₋₄₅-stimulated P14 T cells in naïve mice (P14 CD8 T cells averaged 16.5 ± 5.6% annexin-v⁺ in peptide-treated PV-infected mice, while P14 CD8 T cells averaged 10 ± 2.8% annexin-v⁺ in peptide-treated naïve mice) (Figure 3.14). The frequency of P14 CD8 T cells harboring active caspase 3, caspase 8, or total active caspases increased with stimulation, regardless of whether the P14 CD8 T cells were activated during an acute PV infection or in naïve mice (Figure 3.15). It was also apparent that the levels of active caspase 3 and caspase 8 were higher in P14 CD8 T cells stimulated with cognate peptide in naïve mice compared to PV-infected mice, although only caspase 8 activation reached statistical significance (caspase 3 activation in GP₃₃-stimulated P14 CD8 T cells from naïve mice versus PV-infected mice: p = 0.06, caspase 8: p = 0.005). Only total active caspases were higher on P14 CD8 T cells activated with GP₃₃₋₄₅ peptide in PV-infected mice compared to naïve mice, although this difference was not statistically significant (p = 0.56).

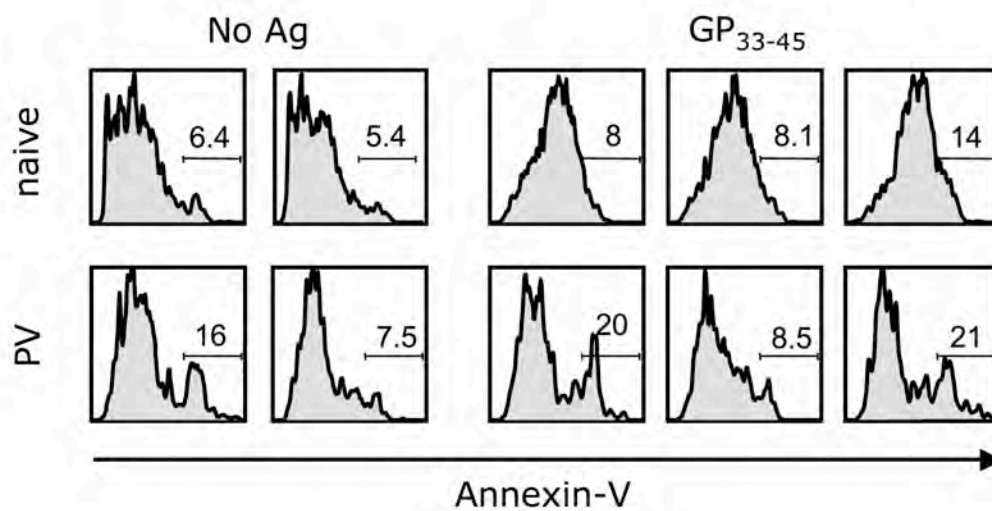


Figure 3.14. P14 CD8 T cells undergo minimal apoptosis 2 days after activation in PV-infected mice. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 of PV, mice were inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. Two days post peptide inoculation, spleens were harvested and stained for Annexin-V. Annexin-V profiles of P14 CD8 T cells are shown.

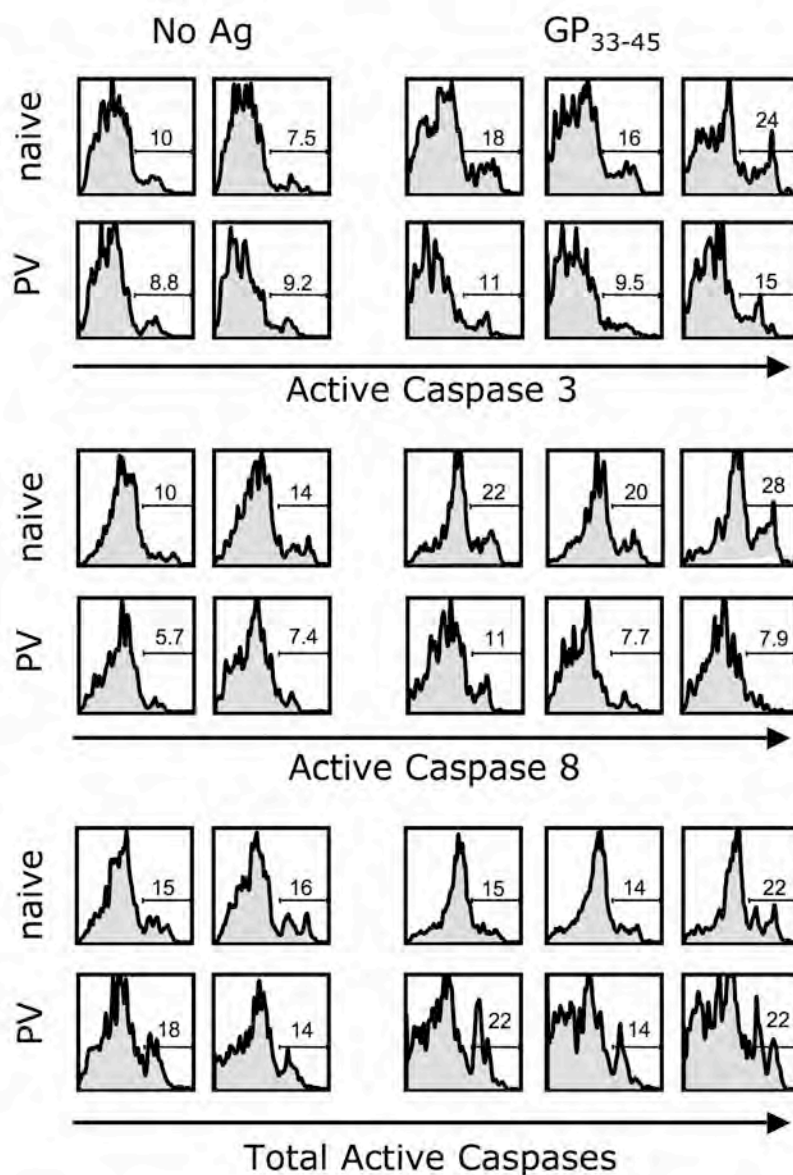


Figure 3.15. P14 CD8 T cells only minimally increase total active caspases within 2 days of activation in PV-infected mice. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 of PV, mice were inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. Two days post peptide inoculation, spleens were harvested and stained for active caspases. Active caspase profiles of P14 CD8 T cells are shown.

Similar results were obtained at various time points between 1 hour and 8 days post T cell activation and in different tissues (spleen, inguinal lymph nodes, bone marrow, peritoneum, lung, liver, and blood) for both TCR transgenic models (data not shown). These data indicate that the reduced expansion of TCR-stimulated bystander CD8 T cells may only minimally be due to enhanced susceptibility to cell death and that the mechanisms behind the rapid depletion of HY CD8 T cells in LCMV-infected male mice remain unknown.

F. Reduced proliferation of TCR-stimulated bystander CD8 T cells *in vivo* is associated with delayed division

Because the reduced proliferation of TCR-stimulated bystander CD8 T cells could not be explained by their death, we questioned whether a defect in division accounted for the reduced proliferation. Thus, CFSE-labeled P14 CD8 T cells were assessed for dilution of CFSE at various time points between 0 and 5 days after GP₃₃₋₄₅ peptide inoculation. As shown in Figure 3.16A, P14 CD8 T cells activated by GP₃₃₋₄₅ in naïve mice started to divide approximately 48 hours post peptide treatment and by 120 hours many of the P14 CD8 T cells in the naïve mice had fully diluted the CFSE. In contrast, only $22.9 \pm 8\%$ ($n = 12$) of the P14 CD8 T cells activated during the acute PV infection had divided at least once by 72 hours post peptide treatment compared to $87 \pm 6.4\%$ ($n = 10$) of P14 CD8 T cells activated in naïve mice at the same 72 hour time point. It was only at 96 hours and later that a larger proportion of P14 cells activated in the PV-infected mice had divided (Figure 3.16A).

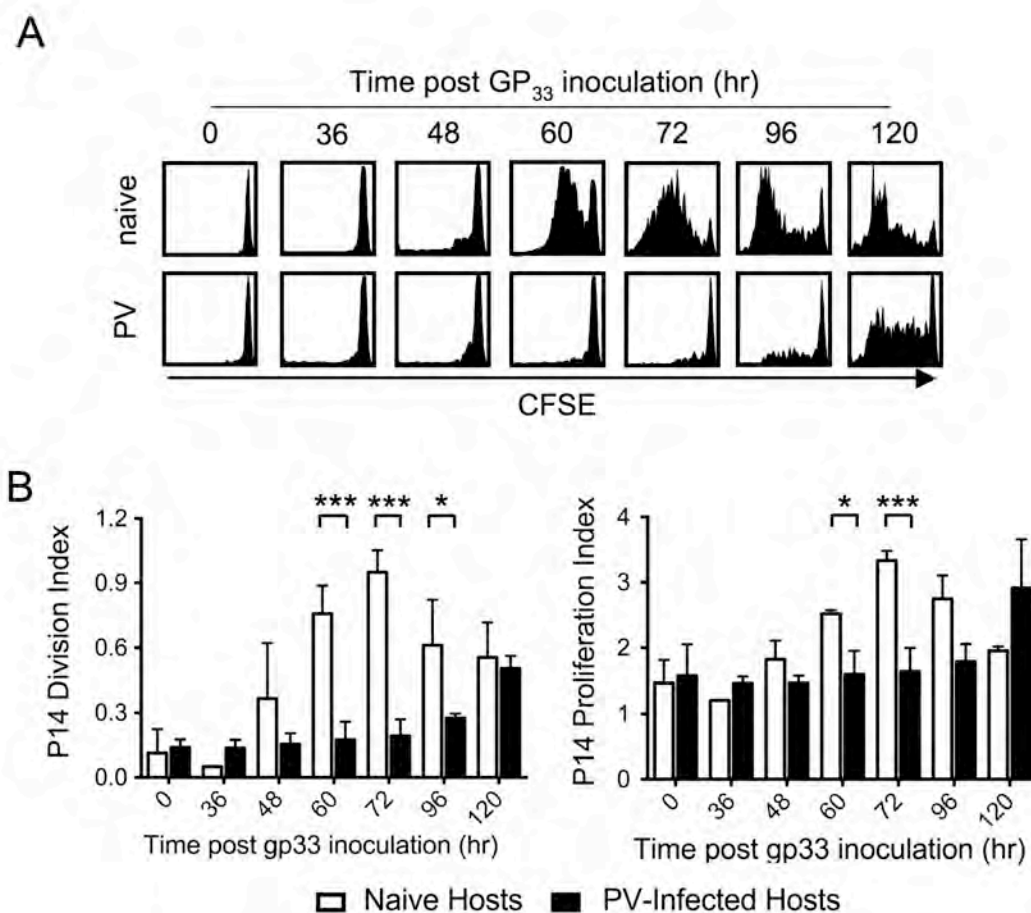


Figure 3.16. P14 CD8 T cells undergo delayed division as measured by CFSE dilution upon activation in PV-infected mice. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. Day 5 of PV, mice were inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. At indicated time points post peptide inoculation, spleens were harvested and division was monitored. (A) CFSE profiles of P14 transgenic CD8 T cells. (B) Division and proliferation indexes were calculated and graphed.

The kinetics of CFSE dilution can also be analyzed by calculating the division and proliferation indexes. The division index is a measure of the average number of divisions that any cell in the starting population underwent, while the proliferation index is the average number of divisions that any divided cell underwent (ignores undivided cells). The division and proliferation indexes for P14 CD8 T cells activated by GP₃₃₋₄₅ peptide in naïve mice peaked at 72 hours post peptide treatment, while both indexes were still increasing at 120 hours for the P14 cells activated in PV-infected mice (Figure 3.16B).

In addition to assessing CFSE dilution, we also measured the frequency of P14 CD8 T cells in different phases of the cell cycle at 0, 3, 4, and 5 days post GP₃₃₋₄₅ peptide treatment. This was achieved with the use of a DNA-specific fluorescent dye, and cells in G₀-G₁, S, and G₂-M phases of the cell cycle were separated by the intensity of fluorescence (a measure of the amount of DNA). Although this is a less precise measurement of division, because any divided CFSE^{lo} cell could be in G₀-G₁ at any given point, it is still a useful measure of division of a population as a whole. We found that 3 days post peptide treatment, fewer P14 CD8 T cells in naïve mice were in G₀-G₁ and more cells were in G₂-M phases of the cell cycle compared to day 0. In contrast, there was not an increase in the frequency of P14 CD8 T cells in G₂-M at day 3 post peptide treatment in the PV-infected mice, although the difference between the naïve mice and PV-infected mice at this time point were not quite statistically different ($p = 0.09$). There was not a significant increase in the frequency of P14 cells in G₂-M phases

of the cell cycle until 5 days post peptide treatment for the P14 CD8 T cells activated during the acute PV infection (Figure 3.17A). Additionally, the expression of Ki-67 is often used as a marker of cell division (Gerdes et al., 1983), and P14 CD8 T cells activated with GP₃₃₋₄₅ in naïve mice up-regulated Ki-67 within 3 days post peptide treatment. This up-regulation, however, was delayed in the P14 CD8 T cells activated in the PV-infected mice (Figure 3.17B). The kinetics of CFSE dilution, cell cycle progression, and Ki-67 expression suggested that there was a delay in the division of TCR-stimulated bystander P14 CD8 T cells during acute viral infection.

G. Reduced proliferation of TCR-stimulated bystander CD8 T cells is not due to a defect in antigen presentation during acute viral infection

The delayed onset of TCR-stimulated bystander CD8 T cell division could be a T cell intrinsic defect or could be a consequence of aberrant antigen presentation during the acute viral infections. Antigen presentation could be inhibited because of DC apoptosis or the reduced expression of MHC or costimulatory molecules during acute viral infections. Since our experiments rely on host APCs to present the endogenous male *Smcy* epitope to HY CD8 T cells or the inoculated GP₃₃₋₄₅ peptide to P14 CD8 T cells, we asked if the proliferation of bystander CD8 T cells could be rescued if exogenous GP₃₃₋₄₁-pulsed DCs were administered to PV-infected mice instead of GP₃₃₋₄₅ peptide. We inoculated naïve and PV-infected mice with 10^7 DC (unpulsed) or DC-GP₃₃ (peptide-pulsed) cells i.v. and measured the division and proliferation of the P14 CD8 T cells two

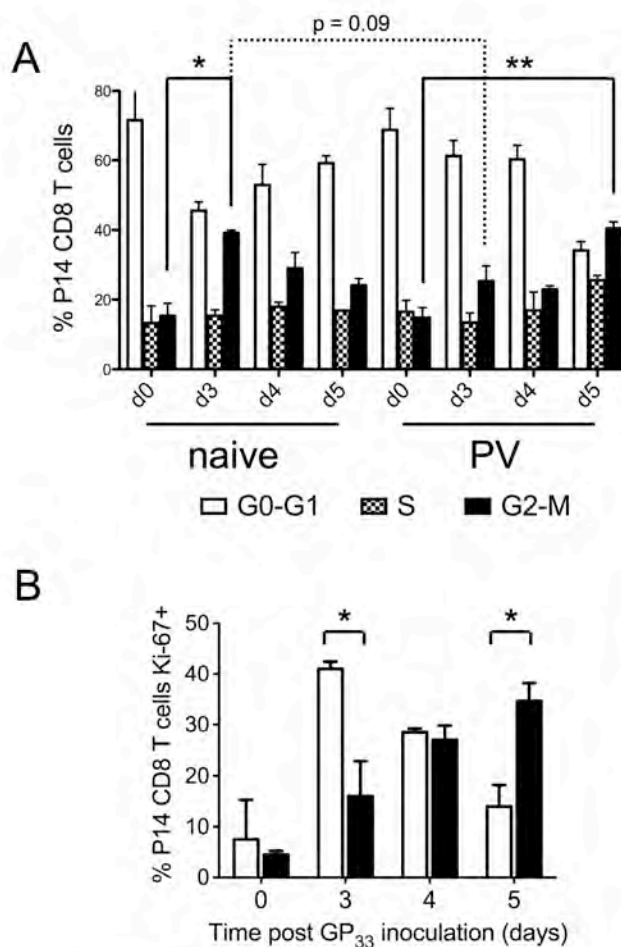


Figure 3.17. P14 CD8 T cells undergo delayed division as measured by cell cycle progression and Ki-67 expression upon activation in PV-infected mice. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 of PV, mice were inoculated with $5 \mu\text{g}$ GP₃₃₋₄₅ 13mer peptide i.v. At indicated time points post peptide inoculation, spleens were harvested, cell cycle progression and Ki-67 antigen was assessed. (A) Cells were stained with DyeCycle DNA-specific dye and the frequency of P14 cells in G0-G1, S, or G2-M phases of the cell cycle were assessed. (B) The frequency of P14 cells that were Ki-67⁺ was plotted.

days later. The administration of DC-GP₃₃ activated the P14 CD8 T cells in naïve mice to divide (Figure 3.18A) and proliferate (Figure 3.18B). The division and proliferation of P14 CD8 T cells in response to DC-GP₃₃ were inhibited in the PV-infected mice, much like they were in response to peptide infusion, suggesting that the reduced proliferation of P14 CD8 T cells in PV-infected mice was not due to a defect in the processing or presentation of the GP₃₃₋₄₅ peptide, but was a T cell intrinsic defect. Additionally, we found that the frequency of total CD11c⁺ DC in the spleen at day 5 of PV ($1.7 \pm 0.17\%$) was not different than that found in naïve mice ($1.5 \pm 0.08\%$), indicating that DC apoptosis was probably not the cause of reduced T cell proliferation.

As an alternative approach, we asked whether bystander HY CD8 T cells from LCMV-infected female mice could proliferate normally if transferred into uninfected male mice displaying the cognate ligand. If the reduced proliferation of HY CD8 T cells in LCMV-infected male mice was due to a defect in antigen presentation during the virus infection, then we would predict that HY CD8 T cells from LCMV-infected female mice would be able to proliferate normally when transferred into naïve male mice. To do this, we directly infected HY transgenic female mice with LCMV, harvested splenocytes from naïve and day 5 LCMV-infected HY transgenic female mice, enriched for T cells using magnetic beads, and transferred equal numbers of naïve donor HY or bystander donor HY CD8 T cells into naïve male recipient mice. The bystander donor HY CD8 T cells were not able to divide or proliferate to the extent of naïve HY CD8 T cells in the naïve male recipient mice (Figure 3.19). These data indicate that the defect in proliferation of HY CD8 T cells in LCMV-infected male mice was not due to a defect in antigen

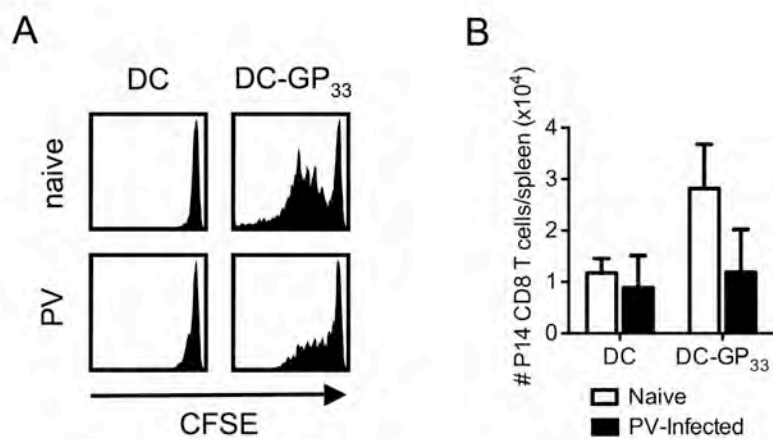


Figure 3.18. Delayed division of bystander P14 CD8 T cells after activation with DC-GP₃₃ in PV-infected mice. WT P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 of PV, unlabelled or GP₃₃₋₄₁-labelled DC2.4 cells (DC or DC-GP₃₃ respectively) were injected i.v. Three days post DC transfer, spleens were harvested and (A) CFSE profiles and (B) the number of P14 CD8 T cells were assessed.

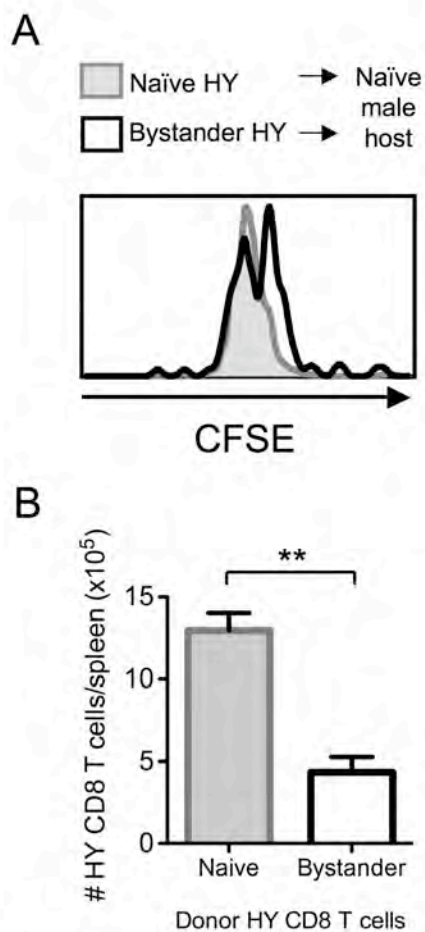


Figure 3.19. Bystander-sensitized HY CD8 T cells undergo reduced proliferation upon activation in naïve male mice. Female HY transgenic were infected with 5×10^4 pfu LCMV i.p. At day 5, splenocytes were harvested, depleted of I-A⁺, NK1.1⁺, and CD19⁺ cells, and the same number of HY CD8 T cells were adoptively transferred into naïve congenic male recipients. Three days post transfer, spleens were harvested and (A) the dilution of CFSE and (B) the number of HY cells in the spleen were assessed.

presentation, but was a T cell intrinsic inhibition. It was possible that this experimental setup resulted in the transfer of virus from the LCMV-infected HY mice to the naïve male mice, but we noted that the host CD8 T cells in the naïve male mice receiving LCMV-infected HY T cells remained CD44^{lo} and CD62L^{hi}, suggesting that little to no virus was transferred (data not shown). Taken together, these data demonstrate that the inhibition of TCR-stimulated bystander CD8 T cell proliferation was not due to a defect in antigen presentation during the acute viral infections, but was probably a T cell intrinsic defect.

H. Conclusions

Many viruses induce a transient state of immune suppression, and there is *in vitro* evidence linking immune suppression to T cell AICD, aberrant co-stimulation, and DC dysfunction. Due to the lack of consensus from *in vitro* systems, we sought to investigate the transient immune suppression induced by acute infections with the arenaviruses LCMV and PV using adoptive transfer models to track and specifically activate TCR transgenic bystander CD8 T cells *in vivo* (Figure 3.1-3.3). In this chapter we demonstrate that the proliferation of bystander CD8 T cells activated by cognate antigen during the early acute phase of virus infection *in vivo* was inhibited (Figure 3.5). Susceptibility to immune suppression was transient, as by day 12 post infection, TCR-stimulated bystander CD8 T cells underwent normal proliferation (Figure 3.6), yet the proliferation

of TCR-stimulated bystander CD8 T cells was enhanced if the T cells were activated immediately prior to the viral infection (Figure 3.7). Thus, while there was a strong adjuvant effect of viral infections on T cell proliferation at the beginning of infection, there was a strong suppression of T cell proliferation as early as three days into the infection. Our studies focused on T cells with specificities for ligands other than those encoded by the infecting virus, but we presume that these findings would be relevant to latecomer virus-specific T cells whose TCR do not encounter antigen until later in infection. Despite the susceptibility of bystander CD8 T cells to AICD *in vitro* (Zarozinski et al., 2000), we found that the reduced proliferation of bystander CD8 T cells *in vivo* was not exclusively mediated by Fas-FasL- or TNF-induced AICD (Table 3.1). In addition, we could not convincingly show an enhanced susceptibility to apoptosis *in vivo* (Figures 3.12-3.15), though it should be noted that apoptotic cells can sometimes be cleared *in vivo* before they are readily detectable. Nevertheless, most of the inhibition in antigen-induced proliferation could be explained by a delay in the onset of division (Figures 3.16 and 3.17), that was a T cell intrinsic defect (Figures 3.18 and 3.19).

Chapter IV: IFN- $\alpha\beta$ and self-MHC induce Eomes and sensitize naïve bystander

CD8 T cells to rapid effector functions during acute viral infections

Due to the delayed proliferation of TCR-stimulated bystander CD8 T cells, we sought to determine if the effector functions of such T cells were altered by ongoing acute viral infections. In order to do this, we utilized the TCR transgenic bystander T cell models described in chapter III, except that for most experiments described in this chapter, bystander CD8 T cells were isolated and activated with cognate peptide *in vitro*. Despite the suppression of TCR-stimulated bystander CD8 T cell proliferation *in vivo*, we found an enhancement of the effector functions exerted by bystander CD8 T cells activated during acute viral infections. During acute viral infections or after stimulation with type 1 IFN (IFN- $\alpha\beta$) inducers, some bystander CD8 T cells were sensitized to immediately exert effector functions such as IFN- γ production and degranulation upon stimulation with high affinity cognate antigen. Sensitization of naïve CD8 T cells required self-MHC I and indirect effects of IFN- $\alpha\beta$, while IL-12, IL-18, and IFN- γ were not individually required. IL-15 was not required for the rapid expression of IFN- γ , but was required for up-regulation of GrzB. P14 and OT-I CD8 T cells, which are capable of homeostatic proliferation, could be sensitized by poly(I:C), but HY CD8 T cells, which are poor at homeostatic proliferation, could not, suggesting that the requirement for MHC I may be to present low affinity cryptically cross-reactive self antigens. Sensitized naïve CD8 T cells up-regulated the t-box transcription factor Eomes, which was dependent on IFN- $\alpha\beta$ and MHC I, and can regulate these rapid effector functions.

A. Naïve bystander CD8 T cells are transiently sensitized to rapidly express IFN- γ upon cognate antigen stimulation during acute viral infection and poly(I:C) treatment

In order to determine if virus infections altered the effector functions of TCR-stimulated bystander CD8 T cells, we first asked how naïve P14 CD8 T cells would respond if activated with cognate peptide GP₃₃₋₄₁ at day 5, during the early acute phase of PV infection. This would simulate the activation of bystander CD8 T cells during a superinfection or the activation of virus-specific latecomer CD8 T cells. To address this, we adoptively transferred P14 transgenic CD8 T cells into congenic recipients and infected mice with PV the following day. At day 5 post infection we harvested spleens and stimulated the spleen leukocytes with cognate peptide GP₃₃₋₄₁ specific to the P14 cells for 5 hours *ex vivo*. As predicted, naïve P14 CD8 T cells isolated from naïve mice produced very little IFN- γ in response to GP₃₃₋₄₁ stimulation. However, a substantial number of the P14 CD8 T cells from the PV-infected mice were able to rapidly express IFN- γ after, and only after, GP₃₃₋₄₁ stimulation *in vitro* (Figure 4.1A). IFN- γ expression was induced between 2 and 4 hours post peptide stimulation (Figure 4.1B), with kinetics similar to effector and memory CD8 T cells. Additionally, if mice were inoculated directly with brefeldin A, to block golgi-mediated cytokine secretion, and the highly immunogenic GP₃₃₋₄₅ 13mer peptide simultaneously i.v, the P14 CD8 T cells residing in PV-infected mice began expressing IFN- γ *in vivo* within 6 hours of peptide inoculation (Figure 4.2). The PV-induced sensitization to rapid IFN- γ expression was transient, as the ability to rapidly express IFN- γ in response to cognate antigen was decreased at day

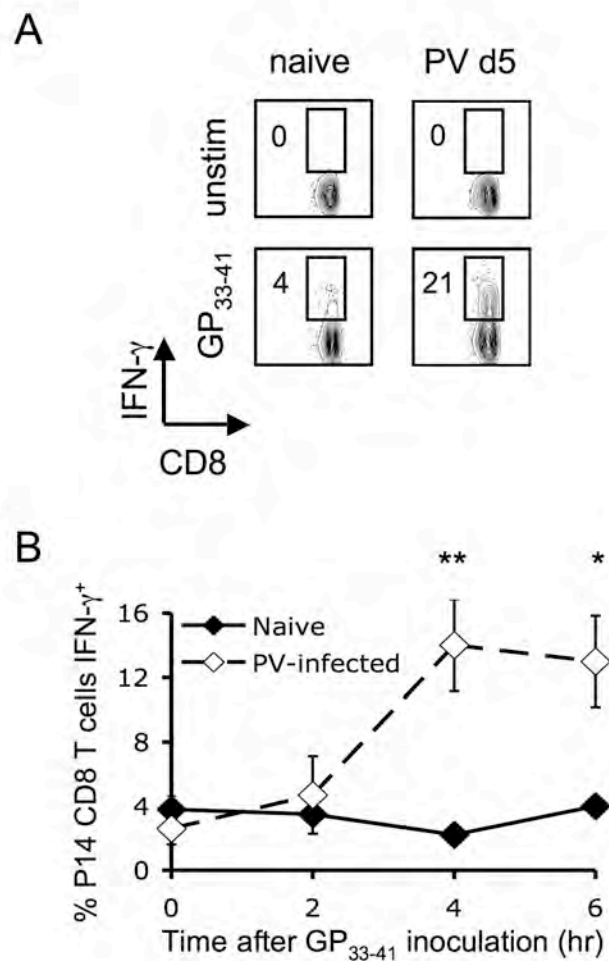


Figure 4.1. PV infection sensitizes bystander P14 CD8 T cells to rapid IFN- γ production upon cognate antigen stimulation. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 post infection, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) P14 transgenic CD8 T cells were gated and IFN- γ production was assessed. (B) P14 transgenic CD8 T cells were stimulated with GP₃₃₋₄₁ peptide for 2, 4, or 6 hours *ex vivo* and IFN- γ production was assessed.

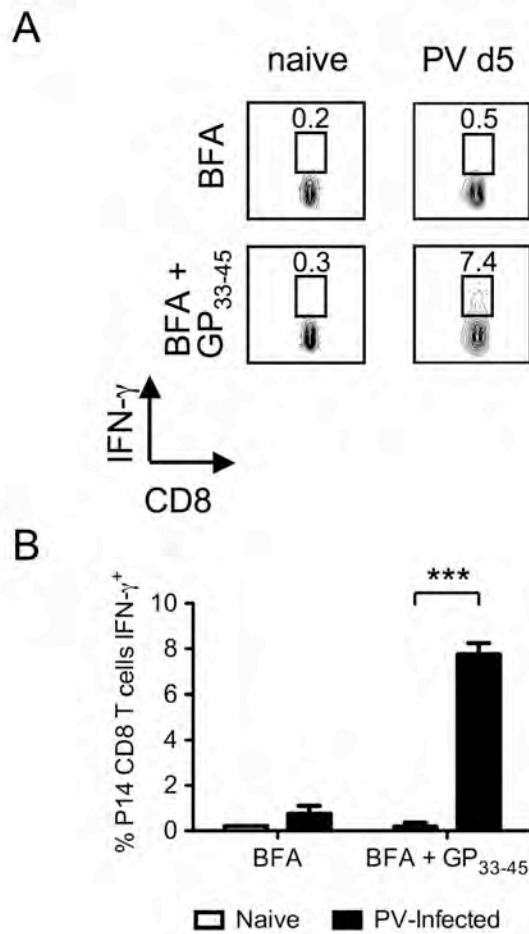


Figure 4.2 PV infection sensitizes bystander P14 CD8 T cells to rapid *in vivo* IFN- γ production upon cognate antigen stimulation. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At day 5 post infection, mice were inoculated with 250 μ g brefeldin A (BFA) i.v. or BFA + 5 μ g GP₃₃₋₄₅ 13mer peptide i.v. Six hours later, spleens were harvested and stained directly for cytokine production. (A) P14 CD8 T cells were gated and the ability of the transgenic T cell to produce IFN- γ was assessed. (B) The frequency of P14 CD8 T cells that were able to produce IFN- γ was graphed.

15 and was down to background levels by day 20 post PV infection (Figure 4.3). Together, these data show that an acute viral infection transiently sensitized naïve bystander CD8 T cells to rapidly initiate IFN- γ synthesis in response to cognate peptide stimulation with kinetics similar to effector and memory CD8 T cells.

We next questioned whether a pro-inflammatory state, in the absence of a virus infection, could sensitize bystander CD8 T cells to the rapid induction of IFN- γ in response to cognate peptide. To do this, we adoptively transferred P14 transgenic CD8 T cells followed by inoculation with the potent IFN- $\alpha\beta$ -inducer poly(I:C). We found that poly(I:C), like PV, could sensitize P14 CD8 T cells to rapid IFN- γ expression upon GP₃₃₋₄₁ stimulation and, although the kinetics were different than the PV infection, the sensitization of P14 CD8 T cells by poly(I:C) was also transient (Figure 4.4).

We also questioned whether poly(I:C) treatment could sensitize polyclonal naïve CD8 T cells to rapidly turn on expression of IFN- γ in response to an anti-CD3 stimulation. We found that, like P14 T cells, polyclonal CD8 T cells in untreated mice were poor producers of IFN- γ , and of all the IFN- γ -producing cells, most of them were of an effector or memory phenotype (CD44^{hi}) as the average ratio of CD44^{hi}:CD44^{lo} IFN- γ ⁺ CD8 T cells was 8.2 (3 independent experiments, n=12) (Figure 4.5). After poly(I:C) treatment, many CD44^{hi} CD8 T cells undergo IFN- $\alpha\beta$ -induced apoptosis (Bahl et al., 2006; McNally et al., 2001), as reflected by a loss of IFN- γ -producing CD44^{hi} CD8 T cells in Figure 4.5. Concomitant with the loss of IFN- γ ⁺ CD44^{hi} CD8 T cells, there was an increase in the frequency of IFN- γ ⁺ naïve CD44^{lo} CD8 T cells, as the average ratio of

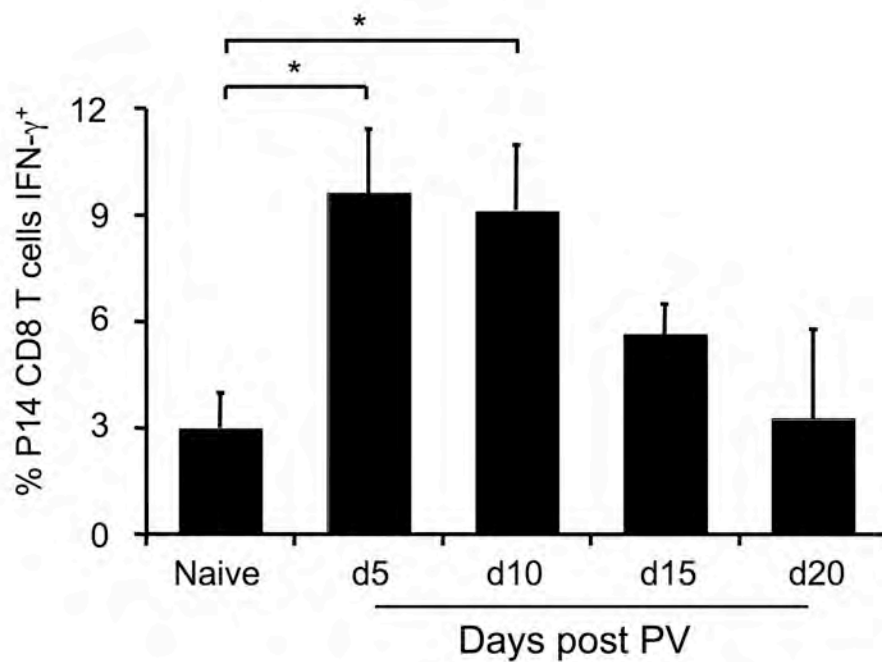


Figure 4.3. PV infection transiently sensitizes bystander P14 CD8 T cells to rapid IFN- γ production upon cognate antigen stimulation. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 1.5×10^7 pfu PV i.p. At days 0 (naïve), 5, 10, 15, and 20 post infection, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo* and the frequency of P14 CD8 T cells producing IFN- γ was assessed.

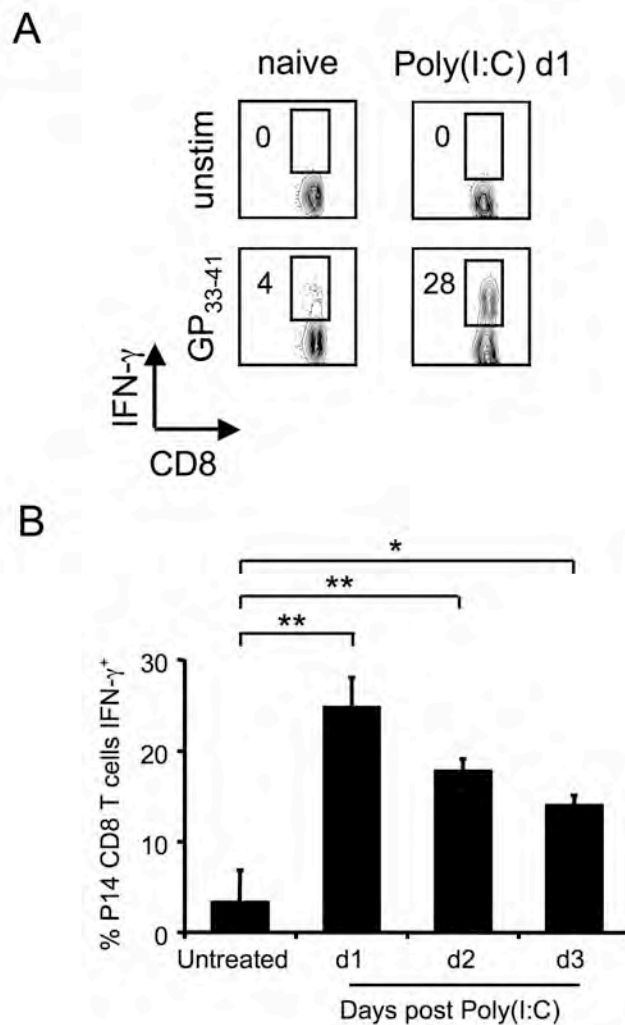


Figure 4.4. Poly(I:C) transiently sensitizes P14 transgenic CD8 T cells to rapid IFN- γ production upon cognate antigen stimulation. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by inoculation with 200 μ g poly(I:C). One day post inoculation, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) P14 CD8 T cells were gated and the ability of the transgenic T cell to produce IFN- γ was assessed. (B) The frequency of P14 CD8 T cells that were able to produce IFN- γ on days 1, 2, and 3 post poly(I:C) inoculation was graphed.

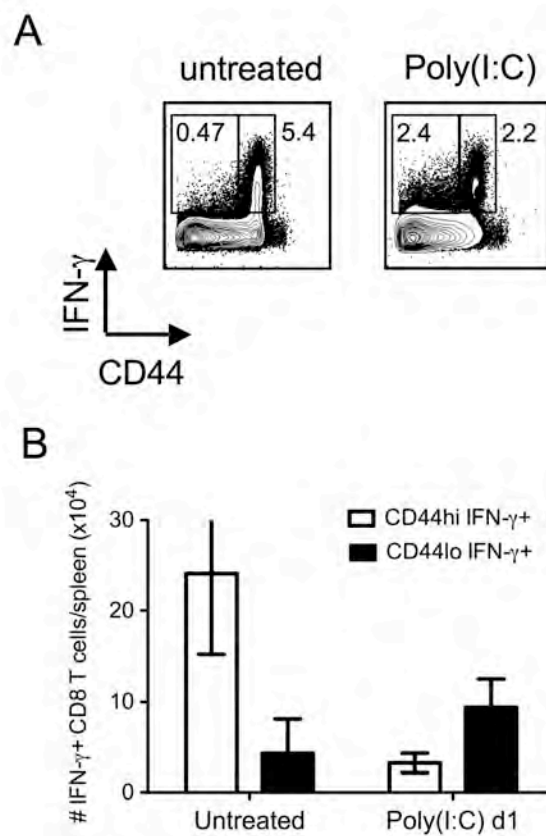


Figure 4.5. Sensitization of naive polyclonal CD8 T cells after poly(I:C) treatment. C57BL/6J mice were inoculated with 200 μ g poly(I:C) i.p. At days 0 (untreated) and 1 post poly(I:C), splenocytes were stimulated with 1 μ g anti-CD3 for 5 hours *ex vivo*, and then stained for intracellular accumulation of IFN- γ . (A) CD8 T cells were gated and IFN- γ versus CD44 was plotted. (B) The number of IFN- γ -producing CD44^{hi} and CD44^{lo} CD8 T cells was graphed.

CD44^{hi}:CD44^{lo} IFN- γ ⁺ CD8 T cells was reduced to 0.94 (3 independent experiments, n=14). This change in ratio was partly due to the apoptotic loss of CD44^{hi} CD8 T cells, but it also reflected an increase in the absolute number of IFN- γ -producing CD44^{lo} naïve CD8 T cells (Figure 4.5B). The ability of naïve phenotype CD44^{lo} CD8 T cells to rapidly produce IFN- γ in response to anti-CD3 was also enhanced during PV infection (frequency of IFN- γ ⁺ CD44^{lo} CD8 T cells after anti-CD3 stimulation from naïve mice = 0.33% \pm 0.1, n=10; frequency of IFN- γ ⁺ CD44^{lo} CD8 T cells after anti-CD3 stimulation at day 5 of PV infection = 0.98% \pm 0.6, n=15). The differences in the frequencies of IFN- γ -producing polyclonal CD8 T cells and transgenic P14 CD8 T cells could be partially due to the stimulation conditions, as we found that P14 CD8 T cells activated by cognate peptide were better able to rapidly express IFN- γ than P14 cells activated with anti-CD3 (frequency of IFN- γ ⁺ P14 CD8 T cells after GP₃₃₋₄₁ stimulation = 25.3% \pm 9; frequency of IFN- γ ⁺ P14 CD8 T cells after anti-CD3-stimulation = 12.5% \pm 5, from the same 4 experiments, n=25). This may indicate that a larger proportion of the naïve polyclonal population is sensitized than the anti-CD3 experiment would predict and that anti-CD3 is not as good a stimulus as a cognate peptide.

B. Induction of granzyme B and enhanced antigen-driven degranulation of naïve bystander CD8 T cells during acute viral infection and poly(I:C) treatment

Due to the ability of naïve bystander CD8 T cells in virus-infected or poly(I:C)-treated mice to rapidly express the effector cytokine IFN- γ upon cognate antigen stimulation, we questioned whether these naïve bystander CD8 T cells would also have

the ability to be cytolytic. We measured their expression of GrzB immediately *ex vivo* and their ability to undergo antigen-driven degranulation *in vitro* by staining for the surface expression of LAMP-1 and LAMP-2 (CD107a and CD107b) in response to GP₃₃₋₄₁ stimulation. In the absence of exposure to cognate GP₃₃₋₄₁ ligand, GrzB was induced in P14 CD8 T cells after poly(I:C) treatment in terms of the frequency of P14 cells expressing GrzB (%) and the relative amount of GrzB per cell (MFI) (Figure 4.6A). GrzB induction was even greater in naïve P14 CD8 T cells during PV infection, especially in terms of GrzB expression per cell. The ability of P14 CD8 T cells to undergo antigen-driven degranulation, as measured by surface expression of CD107, was also significantly enhanced for P14 CD8 T cells after poly(I:C) treatment and PV infection (Figure 4.6B). It is also noteworthy that after GP₃₃₋₄₁ stimulation, bystander-sensitized P14 CD8 T cells lose some GrzB expression (average ratio of GrzB MFI in P14 CD8 T cells after peptide stimulation over no stimulation = 0.87 ± 0.009 from 3 independent experiments, n = 15), suggesting that the enhanced degranulation may be of some GrzB-containing particles. PV infection and poly(I:C) treatment can thus sensitize naïve P14 CD8 T cells such that they will upregulate GrzB prior to cognate antigen stimulation *in vivo* and prime cells for IFN- γ production and degranulation upon ligand exposure *in vitro*.

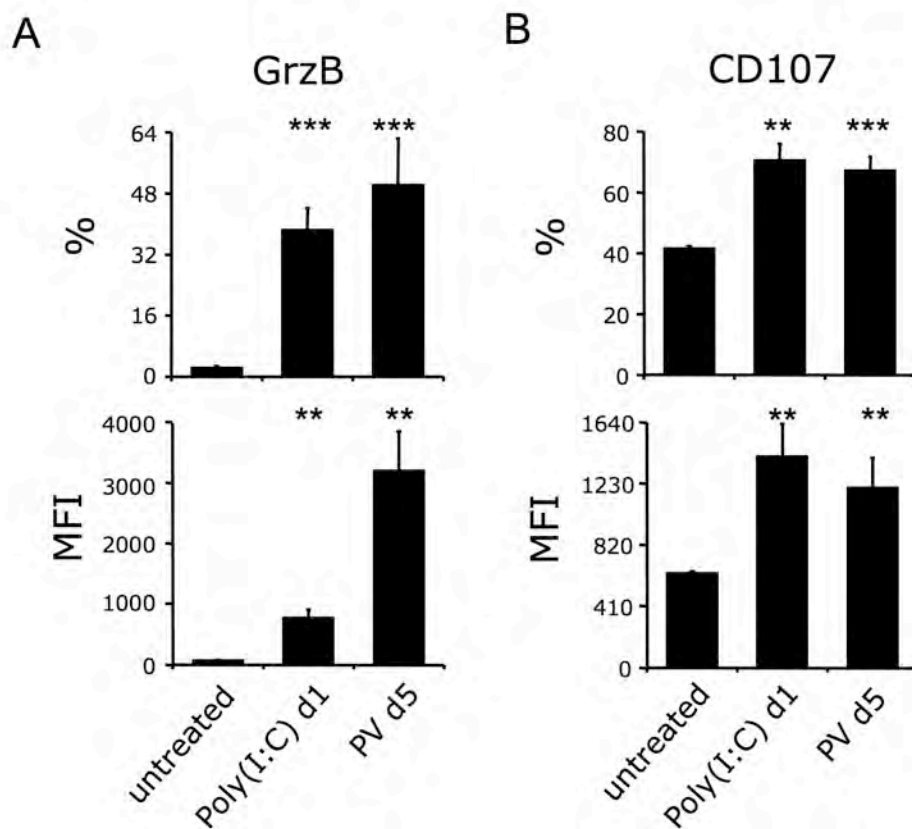


Figure 4.6. Induction of GrzB and enhanced antigen-driven degranulation of bystander P14 CD8 T cells during acute PV infection and poly(I:C) treatment. P14 transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by inoculation with 200 μ g poly(I:C) or 1.5×10^7 pfu PV i.p. At day 0 (untreated), day 1 post poly(I:C), or day 5 post PV, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) Granzyme B expression was assessed by intracellular staining directly *ex vivo* and (B) degranulation (CD107 surface expression) was assessed after GP₃₃₋₄₁ peptide stimulation. The frequency of P14 CD8 T cells staining positive and MFI for both molecules were graphed.

C. Not all viral infections sensitize all bystander CD8 T cells to exert rapid effector functions: roles for IFN- $\alpha\beta$ and cryptic cross-reactivity

To determine whether other viral infections could sensitize P14 CD8 T cells and whether TCR transgenic CD8 T cells of other specificities could be sensitized, we examined the sensitization of P14, HY, and OT-I transgenic CD8 T cells by LCMV, PV, VV, and MCMV. Examples from 8 independent experiments showing the IFN- γ production of cognate peptide-stimulated TCR transgenic CD8 T cells from all of these models are shown in Figure 4.7. The cumulative data generated from >30 experiments using all of these models are listed in Table 4.1, which depicts the average ratio of cognate peptide-induced IFN- γ production for TCR transgenic CD8 T cells in infected/treated mice over the IFN- γ production for transgenic CD8 T cells from control mice. P14 CD8 T cells were sensitized to rapidly express IFN- γ in response to cognate peptide stimulation by PV and poly(I:C), but not by VV or MCMV. HY transgenic CD8 T cells were not sensitized by any of the stimuli, and OT-I CD8 T cells were sensitized well by LCMV and moderately by PV and poly(I:C) (Figure 4.7 and Table 4.1).

D. Indirect effects of IFN- $\alpha\beta$ are required for the sensitization of naïve bystander CD8 T cells

Due to the ability of LCMV, PV, and poly(I:C) to sensitize naïve CD8 T cells and the inability of VV, a poor inducer of IFN- $\alpha\beta$, to do so, we questioned whether IFN- $\alpha\beta$ levels correlated with the sensitization process. Mice were thus inoculated with 10-fold

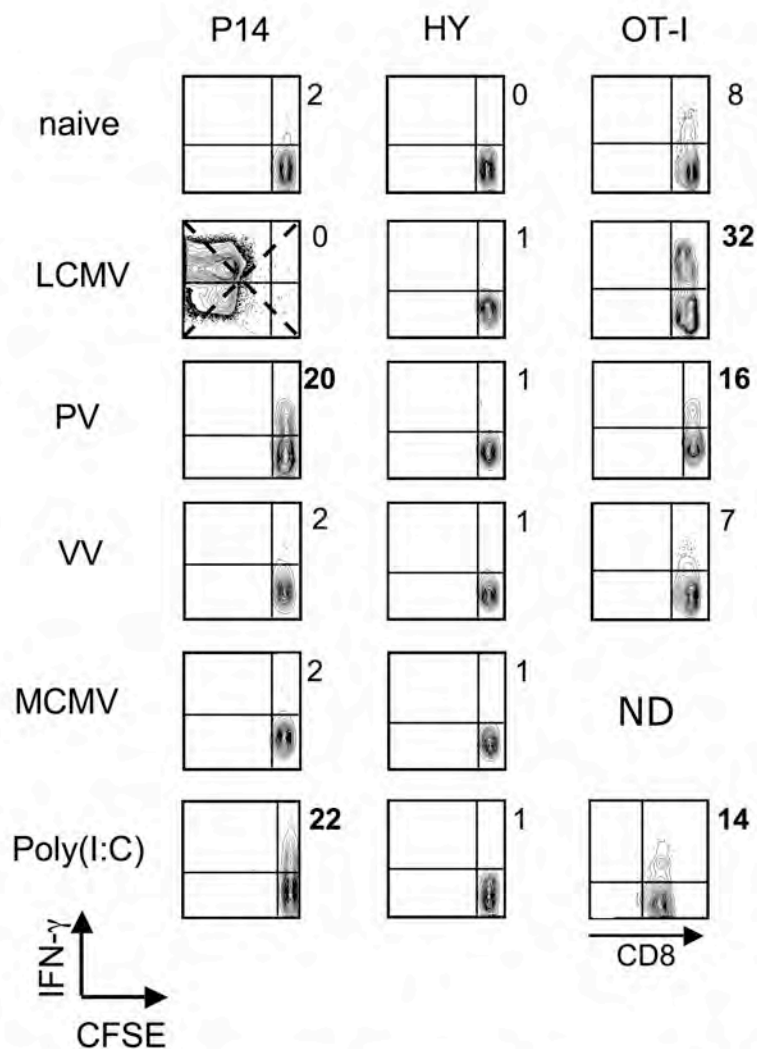


Figure 4.7. Not all virus infections or pro-inflammatory stimuli sensitize all bystander CD8 T cells. P14, HY, or OT-I transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by inoculation with 5×10^4 pfu LCMV, 1.5×10^7 pfu PV, 1×10^6 pfu VV, or 200 μ g poly(I:C) i.p. At days 0 (naive) and 5 post infection or day 1 post poly(I:C), splenocytes were stimulated with GP₃₃₋₄₁, Smcy, or SIINFEKL peptides *ex vivo*. Each transgenic population was gated and the ability of the transgenic CD8 T cells to produce IFN- γ in response to their cognate peptide was assessed. IFN- γ vs. CFSE is shown from representative mice from 8 independent experiments and the numbers depict the frequency of CFSE^{hi} IFN- γ ⁺ events, except for OT-I + poly(I:C) which shows IFN- γ vs CD8.

Table 4.1. Not all virus infections or pro-inflammatory stimuli sensitize bystander CD8 T cells³

TCR T _g	Virus infection or pro-inflammatory stimulus				
	LCMV	PV	VV	MCMV	Poly(I:C)
P14	N/A	5.8±4	1.1±0.09	1.1±0.1	5.8±5
HY	0.93±0.13	0.96±0.04	0.5±0.5	1.4±0	0.66±0.3
OT-I	2.9±2	1.7±0.6	1.2±0.4	ND	2.1±0.2

³ P14, HY, or OT-I transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by infection with 5×10^4 pfu LCMV, 1.5×10^7 pfu PV, 1×10^6 pfu VV, 6×10^5 pfu MCMV, or 200 µg poly(I:C) i.p. At day 5 post infection (or day 1 post poly(I:C) inoculation), splenocytes were stimulated with GP₃₃, Smcy, or SIINFEKL peptides *ex vivo*. Each transgenic population was gated and the ability of the transgenic T cells to produce IFN-γ in response to its cognate peptide was assessed. Numbers depict the average ratio of IFN-γ production from infected/treated mice over IFN-γ production from naïve/untreated mice stimulated with cognate peptide ± standard deviation from >30 total experiments. N/A – not applicable and ND – not done.

dilutions of poly(I:C) from 0.01-100 $\mu\text{g}/\text{mouse}$. Decreasing the dose of poly(I:C) resulted in lower induction of total functional IFN, as assessed by a VSV-mediated CPE inhibition bioassay (Figure 4.8A). There was a linear correlation between the amount of functional IFN induced and the ability of P14 CD8 T cells to rapidly express IFN- γ in response to GP₃₃₋₄₁ stimulation ($r^2 = 0.560$, $p < 0.005$, $n = 32$) (Figure 4.8B). Likewise, we also found correlations between the induction of functional IFN and the up-regulation of GrzB ($r^2 = 0.563$, $p < 0.005$, $n = 18$) and the ability of T cells to undergo enhanced antigen-driven degranulation ($r^2 = 0.389$, $p < 0.05$, $n = 18$) (Figure 4.8C,D).

We next asked if IFN- $\alpha\beta$ was required for the sensitization of naïve P14 CD8 T cells by using IFN- $\alpha\beta\text{R}$ KO mice, which induce less total functional IFN than the WT mice (Figure 4.9B), likely due to the inhibition of a positive feedback loop initiated by IFN- $\alpha 4$ and IFN- β signaling (Marie et al., 1998; Sato et al., 1998a). We found that P14 CD8 T cells were less efficiently sensitized to rapidly express IFN- γ in response to cognate peptide stimulation in the poly(I:C)-treated IFN- $\alpha\beta\text{R}$ KO mice compared to WT mice (Figure 4.9A). These results are consistent with the correlation of total functional IFN and the ability of P14-CD8 T cells to rapidly express IFN- γ (Figure 4.8B) and suggest that IFN- $\alpha\beta$ is required for the sensitization of naïve bystander CD8 T cells during acute viral infections and poly(I:C) treatment.

Knowing that IFN- $\alpha\beta$ was involved in the sensitization of bystander CD8 T cells, we next asked whether direct or indirect IFN- $\alpha\beta$ signals were required. In order to address whether direct IFN- $\alpha\beta$ signals were required, P14 transgenic mice were crossed to IFN- $\alpha\beta\text{R}$ KO mice to generate P14 CD8 T cells that did not express the IFN- $\alpha\beta\text{R}$ and

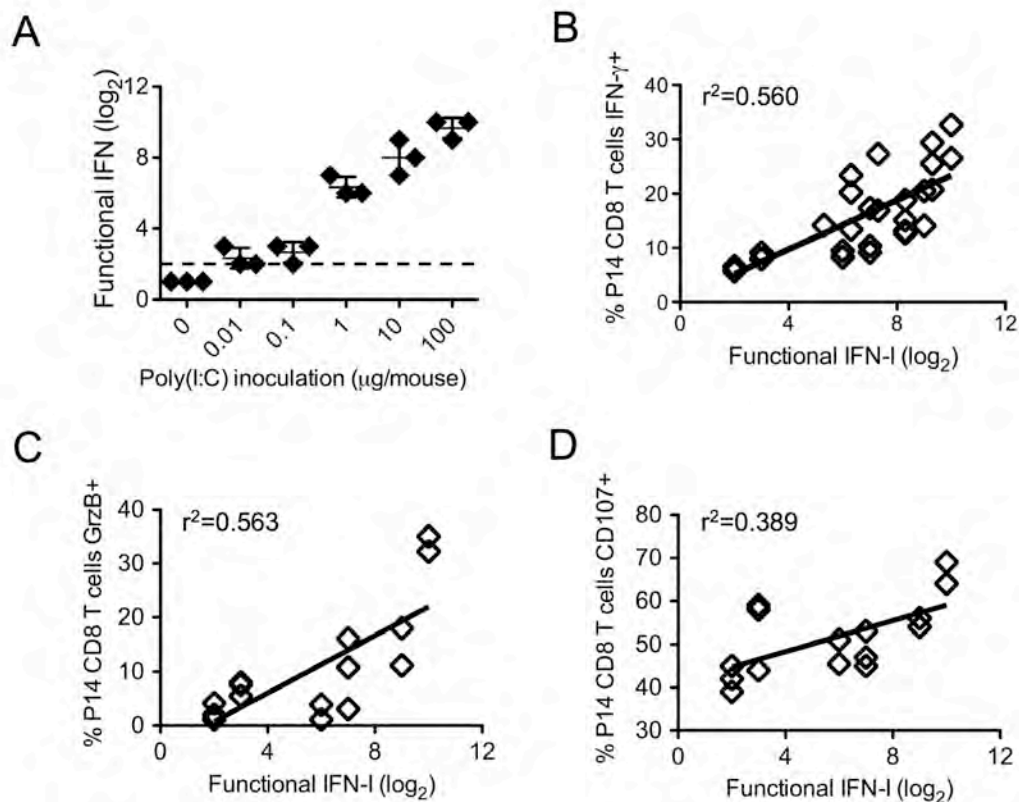


Figure 4.8. Level of functional IFN correlates with sensitization to rapid effector functions. P14 CD8 T cells were adoptively transferred into congenic recipients followed by inoculation with 0-100 μg poly(I:C) i.p. One day post poly(I:C) inoculation, serum was collected for bioassay and splenocytes were collected for stimulation. (A) Log₂ functional IFN for each individual mouse was plotted. Dashed line depicts the level of detection for the assay. (B) The level of functional IFN was plotted against the ability of P14 T cells in each individual mouse to express IFN-γ in response to GP₃₃₋₄₁ peptide stimulation from 2 independent experiments. (C) The level of functional IFN was plotted against the expression of granzyme B in P14 T cells in each individual mouse. (D) The level of functional IFN was plotted against the ability of P14 T cells in each individual mouse to degranulate in response to GP₃₃₋₄₁ peptide stimulation.

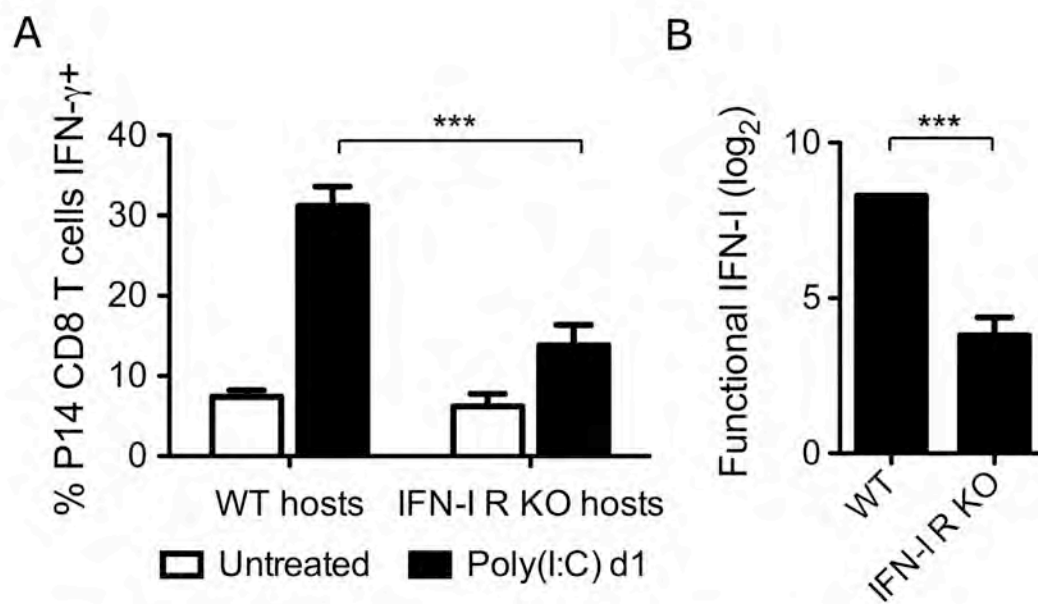


Figure 4.9. IFN- $\alpha\beta$ is required for sensitization to rapid IFN- γ production upon cognate antigen stimulation. P14 CD8 T cells were adoptively transferred into WT or IFN-IR KO congenic recipients, followed by inoculation with 200 μ g poly(I:C) i.p. One day post poly(I:C) inoculation, serum was collected for bioassay and splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) The ability of P14 CD8 T cells to rapidly express IFN- γ was assessed. (B) The \log_2 of functional IFN in both poly(I:C)-treated groups was assessed.

thus could not respond directly to IFN- $\alpha\beta$ signals. WT or IFN- $\alpha\beta$ R KO P14 CD8 T cells were adoptively transferred into congenic WT or IFN- $\alpha\beta$ R KO recipients, which were then inoculated with poly(I:C). In the IFN- $\alpha\beta$ R KO host mice, WT or IFN- $\alpha\beta$ R KO P14 CD8 T cells (WT \rightarrow KO and KO \rightarrow KO) were much less efficiently sensitized by poly(I:C) than either type of P14 cells in WT host mice (WT \rightarrow WT and KO \rightarrow WT) (Figure 4.10A). The most pronounced defect in IFN- γ production was the KO \rightarrow KO group, but the ratio of IFN- γ ⁺ P14 CD8 T cells for the poly(I:C)-treated mice over the untreated mice in this group was similar to the WT \rightarrow KO group. These data indicated that IFN- $\alpha\beta$ was required for sensitization, but direct IFN- $\alpha\beta$ signals on the P14 CD8 T cells were not required for their sensitization.

E. Influence of other cytokines and cytokine-producing cells on sensitization

Due to the indirect requirement for IFN- $\alpha\beta$ in the sensitization of naïve P14 CD8 T cells, we questioned whether IFN- $\alpha\beta$ was inducing sensitization by way of another mediator. NK cells are activated by IFN- $\alpha\beta$ (Welsh, 1978), and CD4 T cells can be Th1-skewed by IFN- $\alpha\beta$ (Way et al., 2007), so it was possible that NK cells or CD4 T cells, perhaps by secreting IFN- γ , were mediating the sensitization of the P14 CD8 T cells. To address this, we depleted mice of NK cells or CD4 T cells prior to poly(I:C) inoculation. Depletion of NK cells or CD4 T cells did not affect the sensitization of P14 CD8 T cells after poly(I:C) treatment (Figure 4.11). Further, P14 CD8 T cells were sensitized by poly(I:C) in IFN- γ KO mice (Figure 4.12A) and administration of a dose of neutralizing

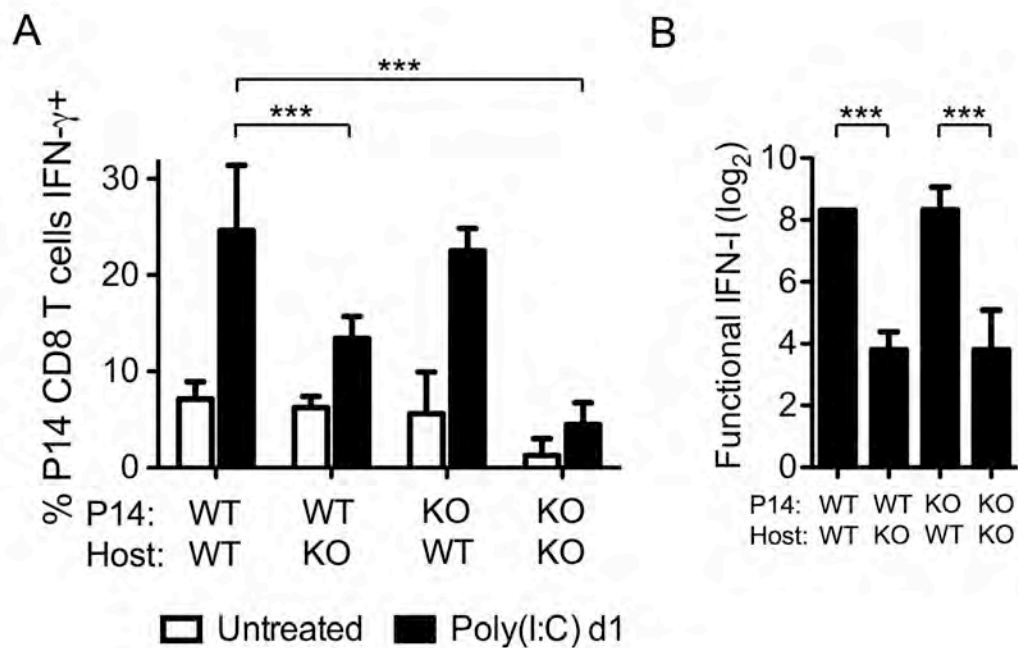


Figure 4.10. Indirect requirement for IFN- $\alpha\beta$ in the sensitization of bystander CD8 T cells. WT or IFN-IR KO P14 CD8 T cells were adoptively transferred into WT or IFN-IR KO congenic recipients, followed by inoculation with 200 μ g poly(I:C) i.p. One day post poly(I:C) inoculation, serum was collected for bioassay and splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) The ability of P14 CD8 T cells to rapidly express IFN- γ was assessed. (B) The log₂ of functional IFN in all poly(I:C)-treated groups was assessed.

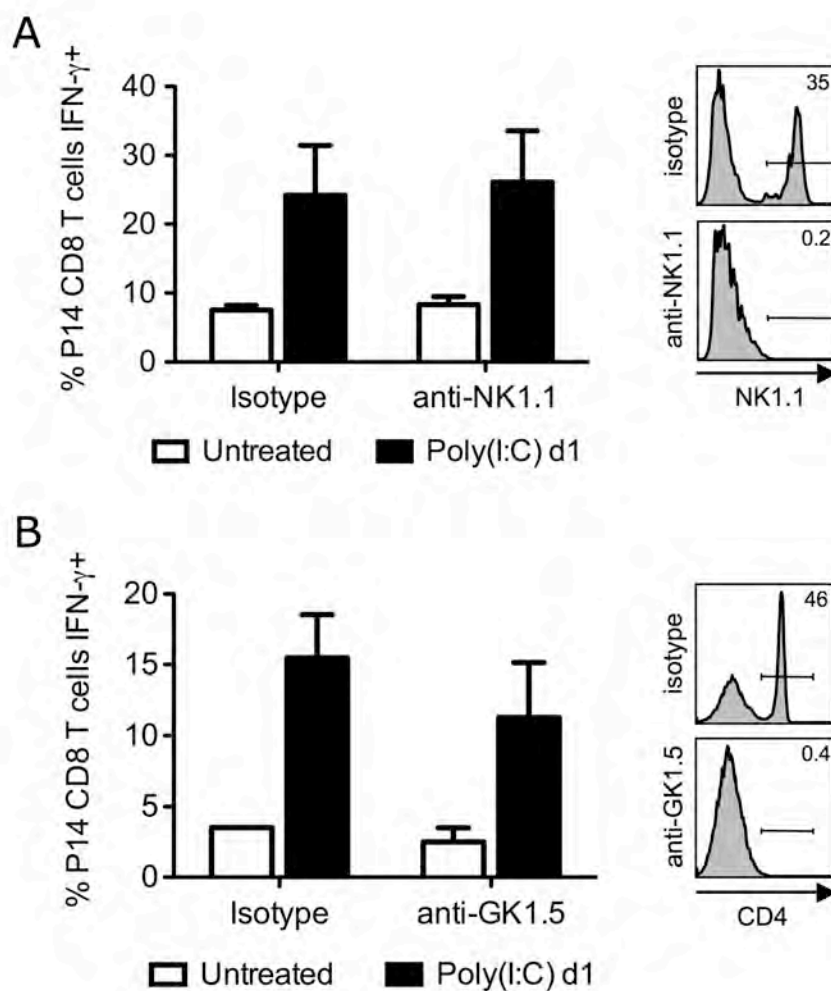


Figure 4.11. NK cells & CD4 T cells are not required for sensitization of bystander CD8 T cells to rapid IFN- γ production upon cognate antigen stimulation. P14 CD8 T cells were adoptively transferred into congenic recipients, followed by i.v. inoculation with (A) anti-NK1.1 or IgG2a or (B) anti-GK1.5 or IgG2b to deplete NK cells or CD4 T cells respectively. One day post antibody treatment, mice were inoculated with 200 μ g poly(I:C) i.p. One day post poly(I:C) inoculation, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo* and the ability of P14 CD8 T cells to rapidly express IFN- γ was assessed. Splenocytes were also stained with anti-NK1.1 and anti-CD4 of different clones than the depletion antibodies to assess depletion.

antibody to IFN- γ , known to be effective in other studies in our laboratory (Chen et al., 2001), also did not inhibit the sensitization of P14 CD8 T cells (data not shown). We questioned whether another cytokine induced by viral infections and poly(I:C) could be mediating sensitization. Since IL-12, IL-15, and IL-18 have been shown to induce IFN- γ expression by effector and memory CD8 T cells (Berg et al., 2002; Berg et al., 2003; Lertmemongkolchai et al., 2001; Raue et al., 2004), we tested the requirements for these cytokines by using cytokine knockout mice. We found that none of these cytokines, at least individually, were required for the poly(I:C)-induced sensitization of naive P14 CD8 T cells to rapid IFN- γ production (Figures 4.12B,C and Figure 4.13A). Each knockout mouse group induced a slightly lower frequency of IFN- γ -producing P14 CD8 T cells than did their WT counterparts, but these differences were not statistically significant (poly(I:C)-treated WT versus IFN- γ KO: $p = 0.89$, poly(I:C)-treated WT versus IL-12 KO: $p = 0.06$, poly(I:C)-treated WT versus IL-18 KO: $p = 0.35$, and poly(I:C)-treated WT versus IL-15 KO: $p = 0.71$). We did notice, however, that IL-15 was required for up-regulation of GrzB (Figure 4.13C) and may also play a role in the enhanced degranulation in response to cognate peptide stimulation (Figure 4.13B). Antigen-driven degranulation of P14 CD8 T cells was reduced in the poly(I:C)-treated IL-15 KO mice in 2 independent experiments, but these results did not reach statistical significance ($p = 0.11$, $n = 7$) (Figure 4.13B and data not shown).

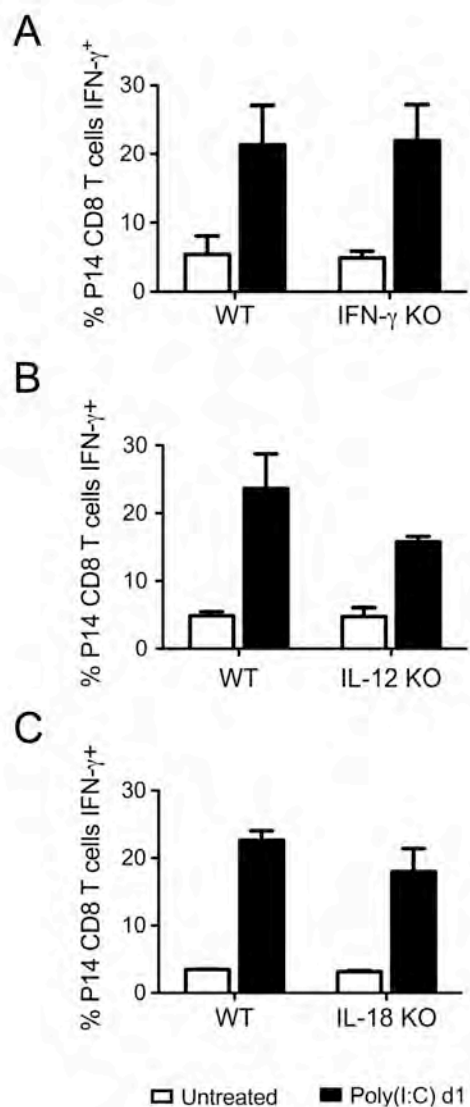


Figure 4.12. IFN- γ , IL-12, & IL-18 are not required for the sensitization to rapid IFN- γ production of P14 CD8 T cells after poly(I:C) treatment. P14 CD8 T cells were adoptively transferred into WT, (A) IFN- γ KO, (B) IL-12 KO, or (C) IL-18 KO congenic recipients followed by inoculation with 200 μ g poly(I:C) i.p. At days 0 (untreated) and 1 post poly(I:C), splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo* and intracellular accumulation of IFN- γ was assessed.

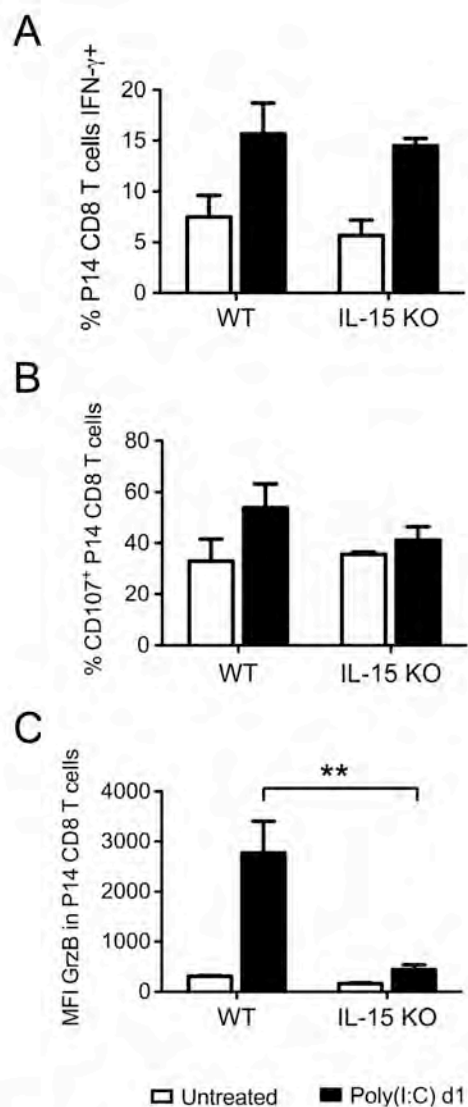


Figure 4.13. IL-15 is not required for sensitization of bystander CD8 T cells to rapid IFN- γ production, but is required for up-regulation of GrzB. P14 transgenic CD8 T cells were adoptively transferred into naïve or IL-15 KO congenic recipients. One day later, mice were inoculated with 200 μ g poly(I:C) i.p. At days 0 (untreated) or 1 post poly(I:C), splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo* and (A) intracellular accumulation of IFN- γ , (B) CD107, or (C) intracellular granzyme B were assessed.

F. MHC I is required for the sensitization of naïve bystander CD8 T cells

The fact that CD8 T cells of different specificities would be sensitized differently by poly(I:C) suggested that their TCR may play a role in sensitization. T cells are selected in the thymus for their low avidity to self antigens, and naïve T cells require MHC-presented self antigens to undergo homeostatic proliferation (Goldrath and Bevan, 1999; Kieper et al., 2004; Seddon et al., 2000). Notably, female HY transgenic CD8 T cells are known to be poor at homeostatic proliferation in female mice (Kieper et al., 2004; Lin et al., 2007) and could not be sensitized in our systems. This suggested that low affinity cryptically cross-reactive self-antigen in the presence of pro-inflammatory signals may sensitize P14 and OT-I CD8 T cells, which readily undergo homeostatic proliferation. If this were the case, CD8 T cell sensitization would require TCR signaling by class I MHC.

To test the requirement for pMHC-TCR, we first sought to determine if P14 CD8 T cells could be sensitized by poly(I:C) in $\beta 2m$ knockout mice, which poorly express class I MHC (Koller et al., 1990; Lehmann-Grube et al., 1994). We compensated for the lack of MHC I antigen presentation *ex vivo* by providing congenic naïve splenocytes to efficiently present the GP₃₃₋₄₁ peptide to the P14 CD8 T cells. Naïve T cells make TNF but not IFN- γ on exposure to their MHC-displayed ligand (Brehm et al., 2005), and this set up allowed us to control for TCR stimulation *in vitro*, as under these conditions of stimulation P14 cells taken from $\beta 2m$ KO mice produced TNF in response to GP₃₃₋₄₁ (Figure 4.14A). However, P14 CD8 T cells from poly(I:C)-treated $\beta 2m$ KO mice were unable to rapidly express IFN- γ in response to peptide stimulation (Figure 4.14A,B),

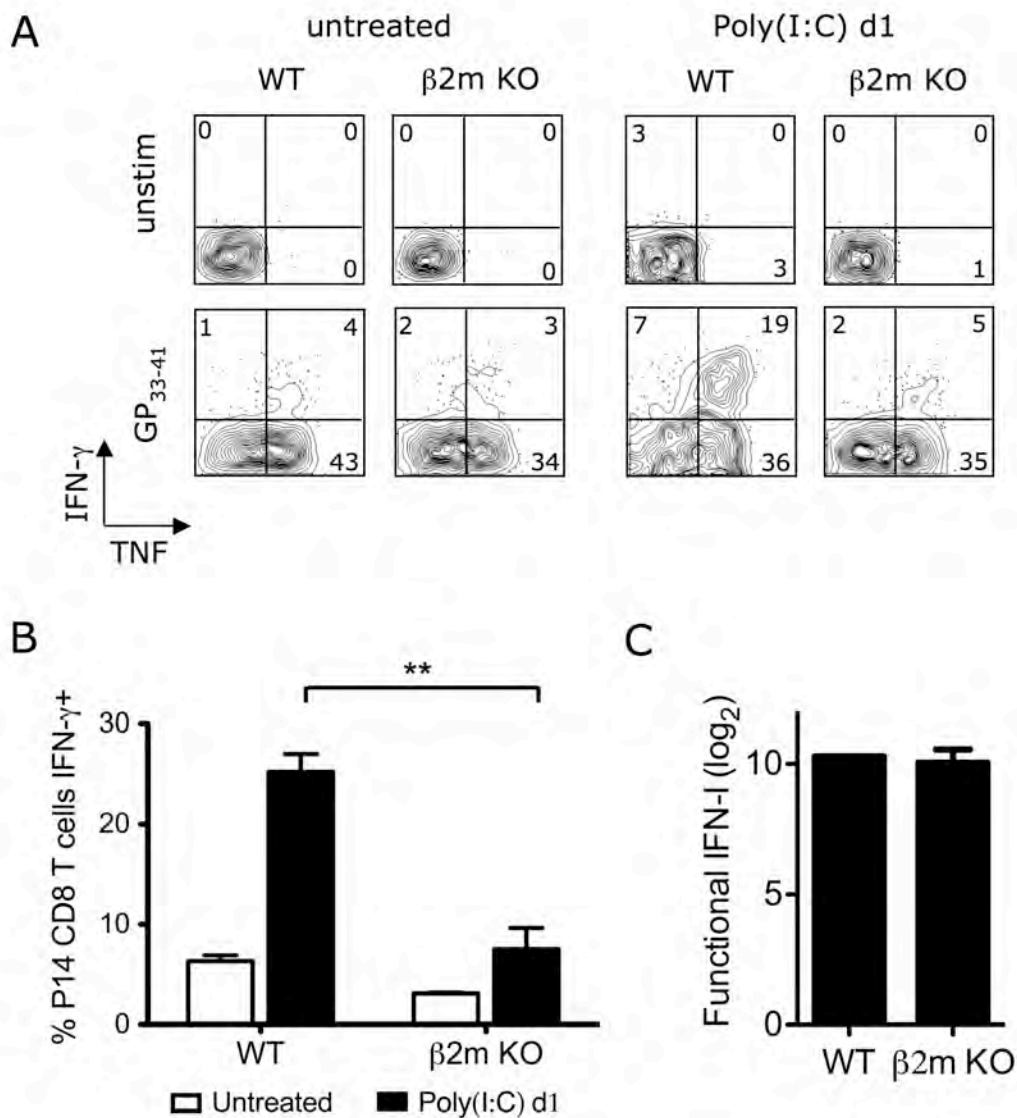


Figure 4.14. Requirement for class I antigen presentation for sensitization. P14 transgenic CD8 T cells were adoptively transferred into naïve WT or β 2m KO congenic recipients followed by inoculation with 200 μ g poly(I:C) i.p. At day 1 post poly(I:C), splenocytes were stimulated with GP₃₃₋₄₁ peptide + exogenous WT splenocytes *ex vivo*. (A) P14 CD8 T cells were gated and IFN- γ and TNF production was assessed. (B) The frequency of IFN- γ producing P14 cells was graphed. (C) Functional IFN production was assessed via VSV-induced CPE bioassay and the log₂ of the reciprocal of the serum dilution that provided 50% protection was graphed.

suggesting that they required class I antigen presentation *in vivo* for sensitization. This difference between $\beta 2m$ KO and WT mice was not due to a defect in IFN- $\alpha\beta$ induction, because similar levels of functional IFN were induced in both strains of mice at 1 day post poly(I:C) inoculation (Figure 4.14C).

In addition to the requirement for class I antigen presentation, we also asked whether TCR stimulation was required for the sensitization of naïve P14 CD8 T cells in poly(I:C)-treated mice also treated with cyclosporin A (CsA). CsA is an inhibitor of the Ca^{2+} -dependent phosphatase calcineurin and blocks T cell activation (O'Keefe et al., 1992; Trenn et al., 1989). This was a technically challenging experiment to design because of the need to optimize doses and kinetics which would prevent TCR stimulation *in vivo* yet allow for TCR stimulation with cognate peptide *ex vivo*. We found that, consistent with the requirement for class I antigen presentation, TCR stimulation may also be required for the priming of naïve P14 CD8 T cells, as CsA treatment reduced the frequency of P14 CD8 T cells that could rapidly express IFN- γ , but not TNF (Figure 4.15A,B). The difference was not quite statistically significant ($p = 0.16$), but if the P14 cells were capable of activation by cognate peptide *ex vivo*, there is a possibility that they received some TCR signaling by self-MHC *in vivo* prior to the time of sacrifice.

We also questioned whether cognate MHC (H2D^b for P14) was required for sensitization or if any MHC I could sensitize the P14 CD8 T cells. To address this, we adoptively transferred P14 CD8 T cells into congenic WT, K^bD^b KO, K^b KO, and D^b KO mice, followed by inoculation with poly(I:C). As shown in Figure 4.16, P14 CD8 T cells were only sensitized in mice expressing H2D^b (WT and K^b KO). Their ability to rapidly

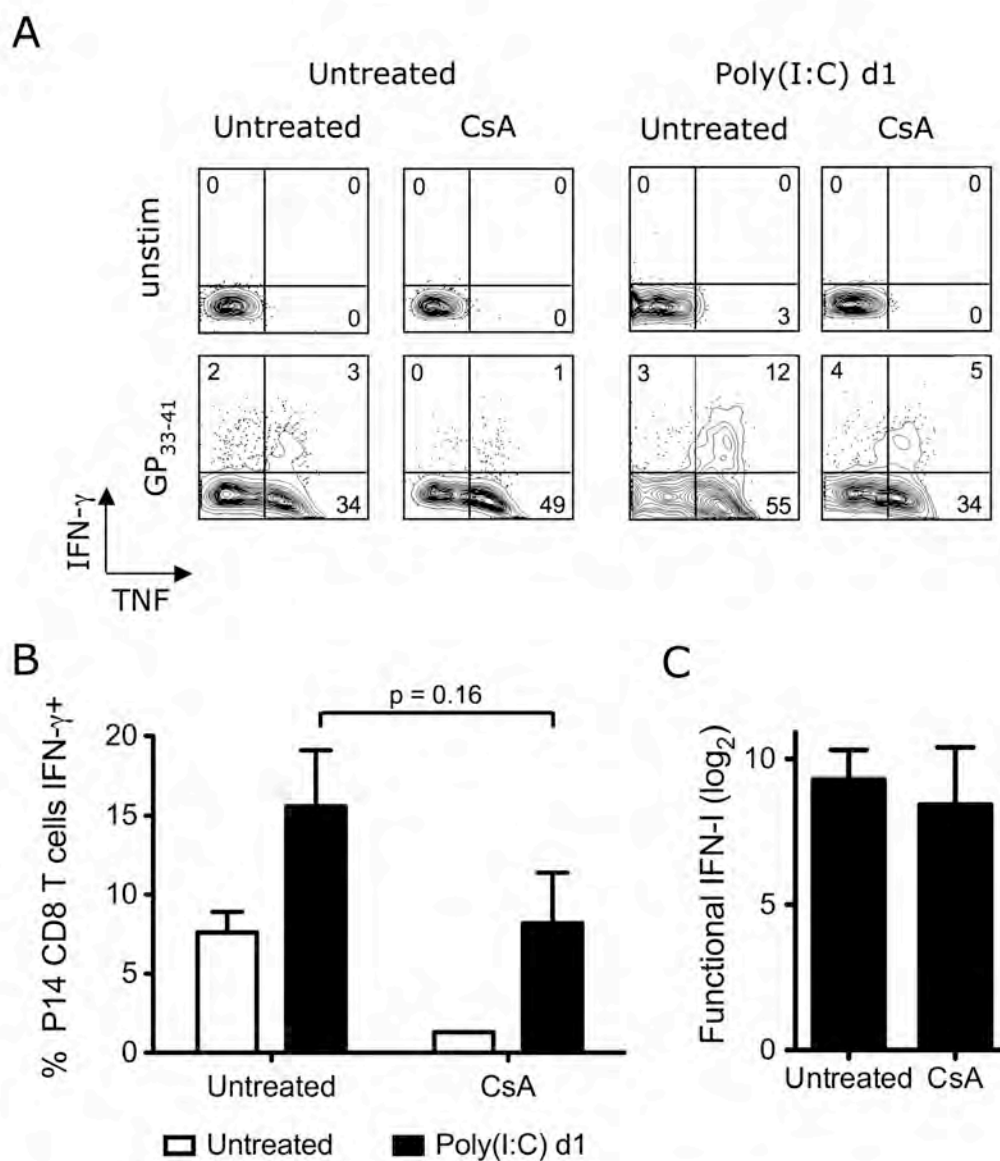


Figure 4.15. Requirement for TCR stimulation for sensitization. P14 transgenic CD8 T cells were adoptively transferred into naïve WT congenic recipients followed by inoculation with 40 mg/kg cyclosporin A i.p at t=0. At t=16hr, mice were inoculated with 200 μ g poly(I:C) i.p. At t=40hr, splenocytes were stimulated with GP₃₃₋₄₁ peptide *ex vivo*. (A) P14 CD8 T cells were gated and IFN- γ and TNF production was assessed. (B) The frequency of IFN- γ producing P14 cells was graphed. (C) Functional IFN production was assessed via VSV-induced CPE bioassay and the log₂ of the reciprocal of the serum dilution that provided 50% protection was graphed.

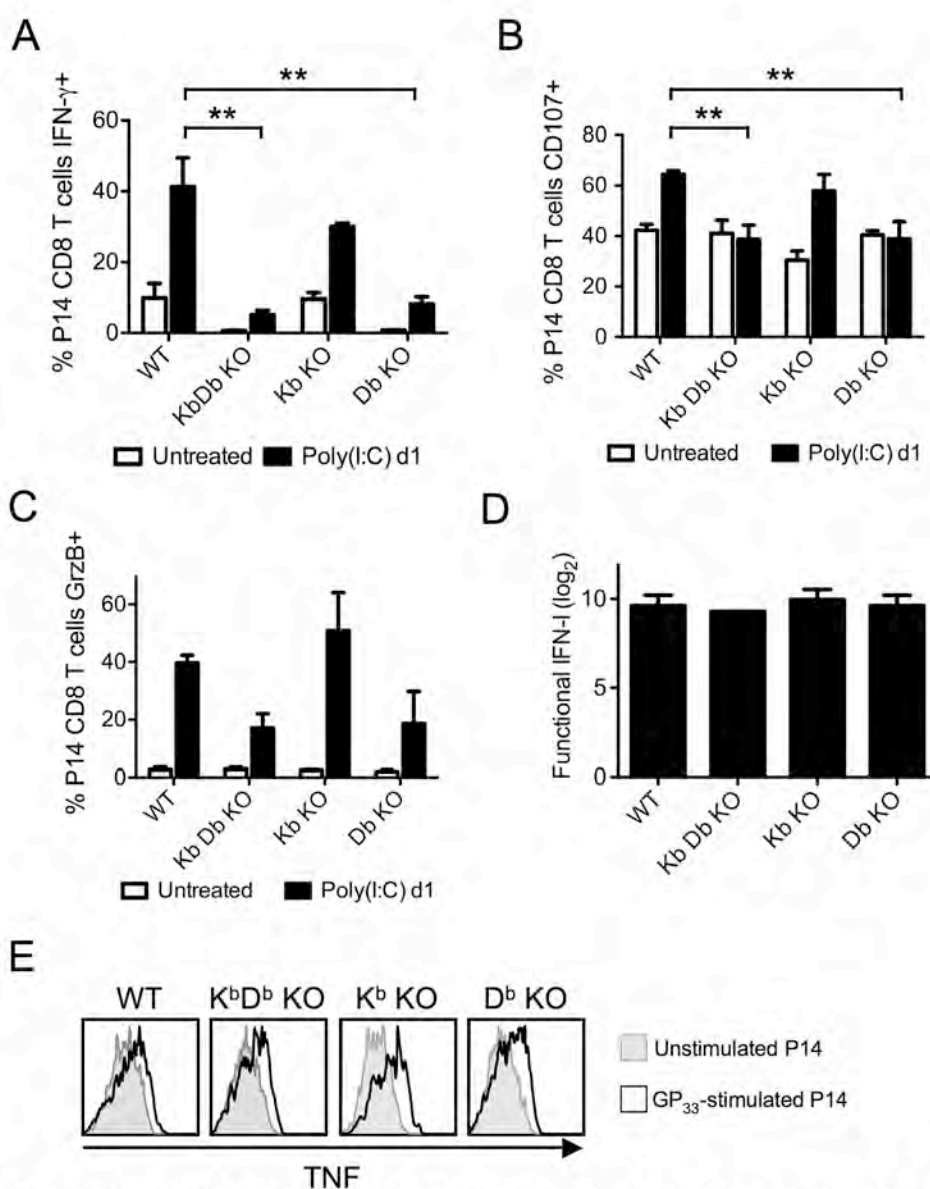


Figure 4.16. H2D^b is required for the sensitization of P14 CD8 T cells after poly(I:C) treatment. P14 transgenic CD8 T cells were adoptively transferred into naive WT, K^bD^b KO, K^b KO, or D^b KO congenic recipients followed by inoculation with 200 μg poly(I:C) i.p. At days 0 (untreated) and 1 post poly(I:C), splenocytes were stimulated with GP₃₃₋₄₁ peptide + exogenous WT splenocytes *ex vivo*. The frequency of (A) IFN-γ-producing, (B) CD107⁺, and (C) GrzB⁺ P14 CD8 T cells were graphed. (D) Functional IFN production was assessed via VSV-induced CPE bioassay and the log₂ of the reciprocal of the serum dilution that provided 50% protection was graphed. (E) TNF production by unstimulated (grey histograms) and GP₃₃₋₄₁-stimulated (black lined histograms) P14 CD8 T cells was assessed.

express IFN- γ and undergo enhanced degranulation in response to cognate peptide stimulation was abrogated in mice lacking H2D^b but still expressing H2K^b (Figure 4.16A,B). The immediate *ex vivo* expression of granzyme B was up-regulated in the P14 CD8 T cells after poly(I:C) inoculation in all of the mice, but this up-regulation was diminished in those mice lacking H2D^b (Figure 4.16C), suggesting that there is some MHC I-independent regulation of GrzB expression. Importantly, the inability of P14 CD8 T cells to be sensitized in mice lacking H2D^b was not due to a defect in IFN induction (Figure 4.16D) or due to an inability to be activated *in vitro*, as the P14 CD8 T cells from all recipient mice could induce TNF expression in response to GP₃₃₋₄₁ stimulation (Figure 4.16E). P14 CD8 T cells also could not be sensitized by H2^d MHC alleles, as P14 CD8 T cells were refractory to sensitization by poly(I:C) in BALB/c mice (ratio of IFN- γ ⁺ P14 cells in poly(I:C)-treated Balb/c mice over untreated mice = 1.2 from 2 independent experiments, n = 10). These data suggest that cognate MHC (H2D^b) displaying cryptic (i.e. unidentified) self peptides were required for the sensitization of naïve P14 CD8 T cells after poly(I:C) treatment.

Although we have defined the P14 CD8 T cells as bystander, it was apparent that they were receiving some signal through their TCR during acute viral infections and after poly(I:C) treatment. We questioned whether this cryptic TCR signal induced any other phenotypic changes. Despite remaining CD44^{lo}, CD43^{lo}, and CD62L^{hi}, sensitized P14 CD8 T cells down-regulated IL-7R α (CD127) after poly(I:C) treatment (Figure 3.2 and 4.17), but HY CD8 T cells, which could not be sensitized for IFN- γ production, only minimally down-regulated IL-7R α (Figure 4.17A). Other signals in addition to TCR

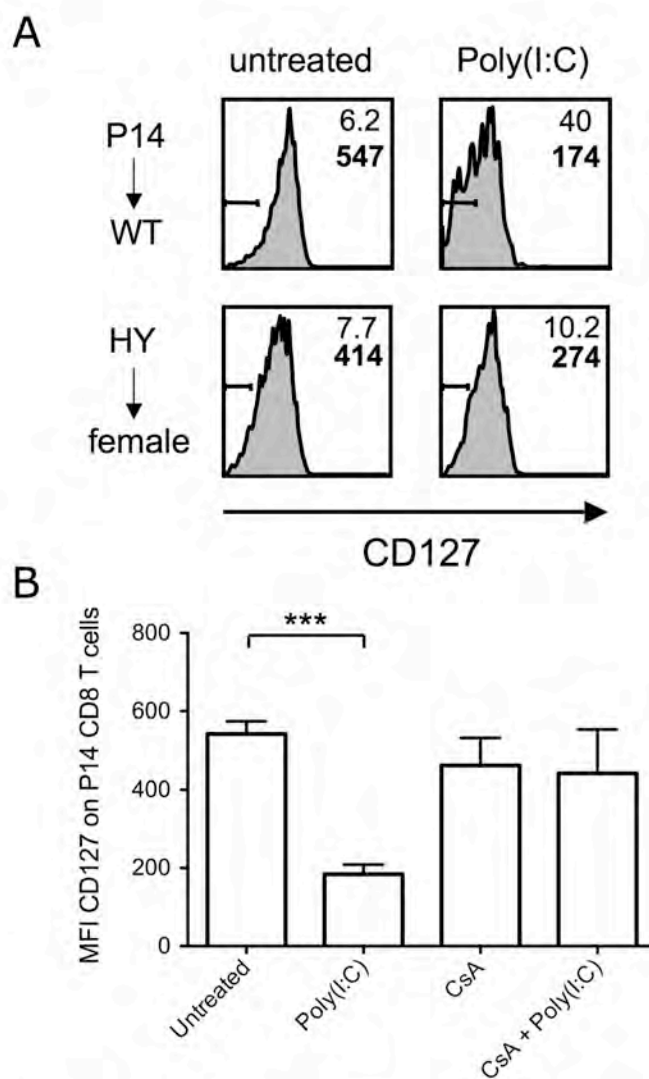


Figure 4.17. Downregulation of IL-7R α on P14 CD8 T cells after poly(I:C) treatment mediated by TCR stimulation. (A) P14 or HY CD8 T cells were adoptively transferred into WT male or female congenic recipients respectively. The following day, mice were inoculated with 200 μ g poly(I:C) i.p. One day post poly(I:C), splenocytes were stained with anti-CD127 and representative examples are shown. Top numbers in upper right are the frequency of CD127^{lo} transgenic T cells and the bottom bold numbers are MFI of CD127. (B) P14 CD8 T cells were adoptively transferred into congenic recipients, which were then inoculated with Cyclosporin A and/or poly(I:C) (described in figure 4.15). Splenocytes were stained with anti-CD127 one day post poly(I:C) inoculation and the MFI of CD127 was plotted.

stimulation have been shown to down-regulate IL-7R α , including IL-7 itself (Vranjkovic et al., 2007), but we found that blocking TCR signaling *in vivo* with CsA prevented the down-regulation of IL-7R α (Figure 4.17B) and the down-regulation of IL-7R α was reduced in mice lacking H2-D^b (data not shown). However, some down-regulation of IL-7R α did occur after poly(I:C) inoculation of K^bD^b KO mice and D^b KO mice, suggesting that other signals that can potentially be inhibited by CsA, in addition to TCR stimulation, may contribute the down-regulation of IL-7R α .

Because OT-I CD8 T cells were sensitized by LCMV and not by VV, we asked whether VV-recombinant viruses expressing the LCMV NP or GP (VV-NP or VV-GP respectively) could sensitize the OT-I CD8 T cells. We found that VV-NP could marginally sensitize OT-I cells, but this sensitization was not much greater than WT VV (Figure 4.18). Since IFN- $\alpha\beta$ was required for sensitization and VV is a poor IFN- $\alpha\beta$ inducer, we questioned whether VV-NP could sensitize OT-I cells if IFN- $\alpha\beta$ was also induced. To do this, we adoptively transferred OT-I transgenic CD8 T cells, infected mice with VV or VV-NP, and on day 2 some mice in each infection group were also inoculated with poly(I:C) to induce IFN- $\alpha\beta$. On day 5, we assayed the ability of the OT-I CD8 T cells to produce IFN- γ in response to SIINFEKL stimulation *ex vivo*. As shown in figure 4.18, poly(I:C) increased the sensitization by VV-NP but not by WT VV, suggesting that both IFN- $\alpha\beta$ and a potential low affinity LCMV NP epitope, that does not activate the T cells in a typical fashion, may drive the sensitization of OT-I CD8 T cells. The increase in IFN- γ production by OT-I CD8 T cells in VV-NP + poly(I:C)-treated mice was not quite statistically significant over VV ($p = 0.08$), VV + poly(I:C) ($p = 0.37$),

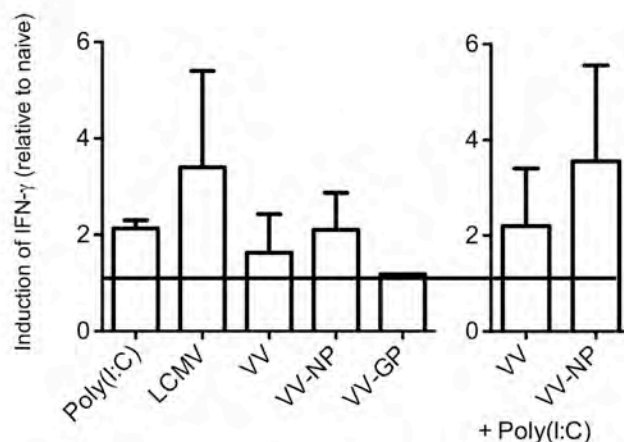


Figure 4.18. LCMV NP epitope and poly(I:C) sensitize OT-I CD8 T cells to rapid IFN- γ production upon cognate antigen stimulation. OT-I transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by inoculation with 200 μ g poly(I:C), 5×10^4 pfu LCMV, 1×10^6 pfu VV, 5×10^6 pfu VV-NP, 5×10^6 pfu VV-GP i.p or infection with virus followed by inoculation with 200 μ g poly(I:C) at day 2. At day 5 post infection (or day 1 post poly(I:C) for poly(I:C) only group), splenocytes were stimulated with SIINFEKL peptide *ex vivo*. OT-I transgenic T cells were gated and their ability to produce IFN- γ was assessed. The frequency of OT-I IFN- γ^+ from naïve mice was set to 1 and the induction of IFN- γ for all the other groups was graphed. Cumulative data from 1-6 experiments/group is depicted.

or VV-NP ($p = 0.19$), but these data suggest that pathogen-encoded peptides may also be able to sensitize bystander CD8 T cells and further work is needed to substantiate these findings.

G. IFN- $\alpha\beta$ - and MHC I-dependent induction of Eomes in sensitized P14 CD8 T cells during PV infection and poly(I:C) treatment

IFN- γ gene transcription is regulated by chromatin accessibility and by the expression of appropriate transcription factors. The t-box transcription factors T-bet and Eomes are important transcription factors in the regulation of CTL effector genes (Cruz-Guilloty et al., 2009; Intlekofer et al., 2008; Intlekofer et al., 2005; Mayer et al., 2008; Pearce et al., 2003; Sullivan et al., 2003). We questioned whether T-bet or Eomes were induced in bystander-sensitized CD8 T cells, thus enabling those cells to rapidly exert effector functions. P14 CD8 T cells from naïve, LCMV day 6 (effector), LCMV day 40 (memory), poly(I:C) day 1, PV day 5, and PV day 20 infected mice were purified by cell sorting immediately *ex vivo* and without any exposure to cognate ligand. Their RNA was then extracted and used for quantitative real-time PCR to quantify mRNA levels of the transcription factors. As shown in Figure 4.19A and consistent with published reports, effector CD8 T cells up-regulated mRNA for T-bet (6-fold) and Eomes (4-fold), and memory CD8 T cells further up-regulated Eomes (8-fold) but not T-bet mRNA (Cruz-Guilloty et al., 2009; Takemoto et al., 2006). The sensitized bystander P14 CD8 T cells up-regulated Eomes, but not T-bet mRNA, after poly(I:C) inoculation and PV infection. Poly(I:C) induced about a 2-fold induction of Eomes, and PV infection induced about 4-

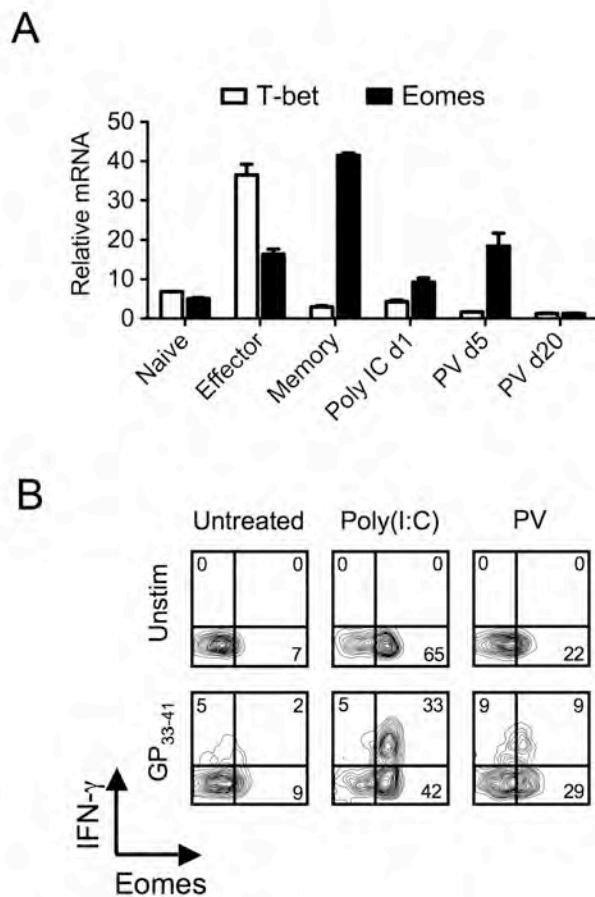


Figure 4.19. Transient induction of Eomes, but not T-bet, in bystander P14 CD8 T cells during acute viral infection and poly(I:C) treatment. (A) P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by inoculation with 5×10^4 pfu LCMV, 1.5×10^7 pfu PV, or $200 \mu\text{g}$ poly(I:C) i.p. P14 cells were sorted from naïve P14 mice (Naïve), d6 post LCMV (Effector), d42 post LCMV (Memory), d1 post Poly(I:C), d5 post PV, or d20 post PV. RNA was extracted and converted to cDNA, followed by real-time PCR using primers for T-bet and Eomes. Representative experiment that depicts the relative mean from multiple experiments, sorts, and real-time PCR reactions. (B) P14 transgenic CD8 T cells were adoptively transferred into naïve congenic recipients followed by inoculation with 1.5×10^7 pfu PV or $200 \mu\text{g}$ poly(I:C) i.p. Day 0 (untreated), day 1 post poly(I:C), or day 5 post PV infection, splenocytes were stimulated *ex vivo* with GP₃₃₋₄₁ and intracellular IFN- γ and Eomes was assessed.

fold induction over naïve levels (Figure 4.19A). Importantly, the high expression of Eomes mRNA in bystander CD8 T cells was transient, and by day 20 after PV infection, at a time in which the P14 CD8 T cells could no longer rapidly express IFN- γ (Figure 4.3), the level of Eomes mRNA was below that detected in naïve CD8 T cells.

Eomes protein was also induced in sensitized P14 CD8 T cells after PV infection and poly(I:C) treatment and was expressed directly *ex vivo* without a requirement for exposure to GP₃₃₋₄₁ (Figure 4.19B). Strikingly, most of the P14 CD8 T cells up-regulated Eomes protein after poly(I:C) treatment ($71\% \pm 7.7$, $n = 8$), a frequency nearly twice that ($38\% \pm 9$, $n = 8$) of the cells that rapidly synthesized IFN- γ in response to GP₃₃₋₄₁ stimulation *ex vivo* (Figure 4.19B). Importantly, about half of the polyclonal naïve CD8 T cells in WT B6 mice also up-regulated Eomes expression after poly(I:C) treatment (Figure 4.20B), arguing that this up-regulation is a common event and not restricted to only a rare transgenic T cell population.

Since MHC I and indirect effects of IFN- $\alpha\beta$ were required for the sensitization of P14 CD8 T cells to rapidly express IFN- γ in response to cognate antigen, we also tested whether these signals were required for the induction of Eomes. To address this, we measured the induction of Eomes protein in P14 and naïve (CD44^{lo}) host CD8 T cells in H2D^b KO and IFN- $\alpha\beta$ KO mice. As shown in Figure 4.20A, P14 CD8 T cells did not up-regulate Eomes protein in response to poly(I:C) treatment in H2D^b KO KO mice, supporting the concept that recognition of MHC was needed for sensitization. In contrast, induction of Eomes protein in naïve polyclonal CD8 T cells in the H2D^b KO mice was like that of wild-type (Figure 4.20B), indicating that these host CD8 T cells,

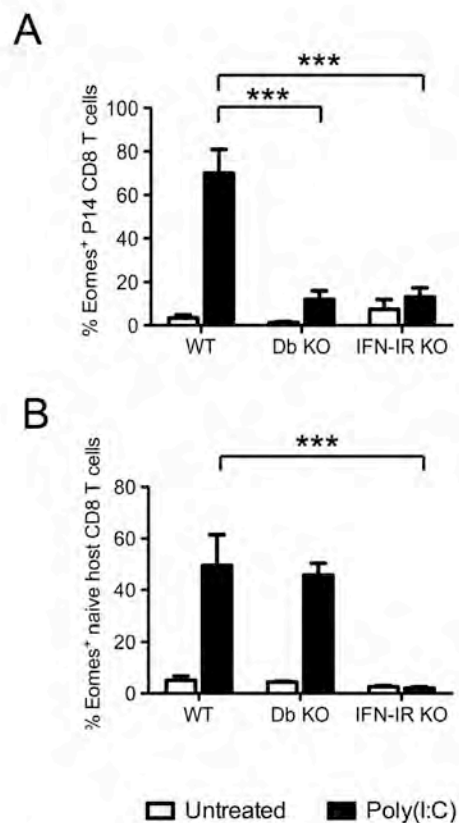


Figure 4.20. Up-regulation of Eomes in bystander-sensitized CD8 T cells requires MHC I and IFN- $\alpha\beta$. P14 transgenic CD8 T cells were adoptively transferred into naïve congenic WT, H2D^b KO, or IFN-IR KO recipients followed by inoculation with 200 μg poly(I:C) i.p. One day later, splenocytes were stained immediately *ex vivo* for intracellular Eomes. (A) The frequency of Eomes⁺ P14 CD8 T cells was graphed. (B) The frequency of Eomes⁺ CD44^{lo} naïve host CD8 T cells was graphed.

which had been selected in a MHC H2K^b environment, received sufficient stimulation for sensitization. Neither donor P14 cells nor polyclonal host naïve cells were sensitized in mice lacking receptors for IFN- $\alpha\beta$ (Figure 4.20A,B), probably due to the diminished induction of IFN- $\alpha\beta$ (Figure 4.9B). Taken together, these data show that IFN- $\alpha\beta$ and cognate MHC I are required for the up-regulation of Eomes in bystander-sensitized CD8 T cells, and we suggest that the expression of this transcription factor allows for the rapid synthesis of IFN- γ in response to cognate antigen stimulation.

Additionally, another known target of Eomes is the β chain of the IL-2R and IL-15R, CD122 (Intlekofer et al., 2005), which was not up-regulated to the level on virus-specific CD8 T cells, but was moderately induced on bystander P14 and naïve polyclonal CD8 T cells during acute viral infection and after poly(I:C) treatment (Figure 3.2) as the average ratio of CD122 MFI on CD44^{lo} naïve CD8 T cells from PV-infected mice over untreated mice was 1.6 ± 0.4 (3 independent experiments, $n = 12$), suggesting that Eomes expression in bystander-sensitized CD8 T cells may also induce IL-15 responsiveness. This is interesting given the result that IL-15 was required for the up-regulation of GrzB and possibly for enhanced degranulation of bystander-sensitized P14 CD8 T cells after poly(I:C) treatment (Figure 4.13B,C).

H. Conclusions

We demonstrate in this chapter that IFN- $\alpha\beta$ -inducing acute viral infections and TLR agonists sensitize naïve phenotype bystander CD8 T cells to gain a transient ability to rapidly express IFN- γ in response to high affinity cognate antigen (Figures 4.1-4.5).

Bystander-sensitized CD8 T cells also up-regulated GrzB prior to high affinity cognate antigen engagement and underwent enhanced antigen-driven degranulation after cognate antigen stimulation (Figure 4.6), suggesting that they may also be cytolytic. Associated with this acquisition of effector functions was the up-regulation of mRNA for the t-box transcription factor Eomes, known to regulate CTL effector functions (Figure 4.19). Hence, these naïve bystander T cells were conditioned to behave like memory cells on exposure to high affinity cognate ligand.

Taken together, we propose the model shown in Figure 4.21, whereby the arenaviruses LCMV and PV and the TLR agonist poly(I:C) induce IFN- $\alpha\beta$ (Merigan et al., 1977; Welsh, 1978). IFN- $\alpha\beta$ has pleotropic effects on many cell types, including the up-regulation of MHC I and the induction of cytokines, including IL-15 (Samuel, 2001; Zhang et al., 1998b). We propose that the enhanced expression of MHC I during these inflammatory conditions results in TCR signaling by low affinity cryptically cross-reactive self peptide-MHC. These signals up-regulate the t-box transcription factor Eomes, which allows for the rapid expression of IFN- γ upon cognate antigen stimulation. Eomes-induced up-regulation of CD122 and concomitant IL-15 responsiveness also regulates the expression of GrzB and perhaps the ability of bystander or latecomer CD8 T cells to undergo enhanced antigen-driven degranulation. Therefore, inflammatory signals during acute viral infections initiate the sensitization of naive bystander and latecomer CD8 T cells such that they will rapidly exert effector functions upon cognate antigen stimulation.

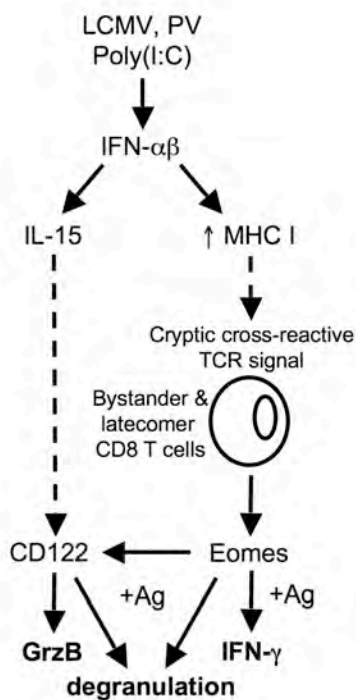


Figure 4.21. Model of mechanisms that sensitize bystander CD8 T cells during acute viral infections. IFN- $\alpha\beta$ induced by viral infection or TLR agonist poly(I:C) induces expression of IL-15 and up-regulation of MHC I. Up-regulation of MHC I enhances presentation of self and virus-encoded peptides, which signal through the TCR of bystander CD8 T cells, inducing Eomes expression. Eomes induces expression of CD122, which confers IL-15 responsiveness and induces GrzB expression. In response to cognate antigen, the expression of Eomes allows for the rapid synthesis of IFN- γ . IL-15 expression is also required for the enhanced cognate antigen-driven degranulation. Dashed lines depict signals through receptors, while full lines depict the induction of molecules from those signals.

Chapter V: Discussion

We demonstrate in this thesis that naïve bystander and latecomer CD8 T cells are affected by inflammatory signals during acute viral infections such that their response to cognate antigen stimulation is altered. Prior to cognate antigen engagement, bystander CD8 T cells up-regulated Eomes, CD122, and GrzB. Following cognate antigen engagement, bystander CD8 T cells rapidly degranulated and expressed the effector cytokine IFN- γ . The ability of bystander CD8 T cells to rapidly exert effector functions may contribute to the resistance of virus-infected individuals to superinfections. Despite these rapid effector functions, the proliferation of TCR-stimulated bystander CD8 T cells was markedly inhibited. This reduced proliferation was found not to be a defect in antigen presentation, but was a T cell intrinsic defect in initiating division. Thus, bystander CD8 T cells were also susceptible to virus-induced immune suppression.

It is also likely that virus-specific CD8 T cells that are not activated until later in the response, so-called latecomer CD8 T cells, may also be susceptible to immune enhancement and suppression. Thus, latecomer CD8 T cells would be able to rapidly exert effector functions at the expense of proliferation. Taken together, we propose the model shown in Figure 5.1, whereby virus-specific CD8 T cells activated early during infection have the highest proliferative potential, but do not have the ability to rapidly exert effector functions. In contrast, sensitized bystander and latecomer CD8 T cells that are activated during the infection are able to rapidly exert effector functions at the expense of proliferation. Suppression in the form of reduced proliferation was not

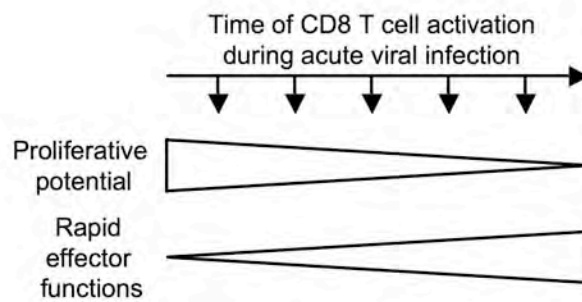


Figure 5.1. Model of the inverse correlation between the proliferative potential and rapid effector functions driven by the time of CD8 T cell activation during acute viral infections. We propose that virus-specific CD8 T cells that get recruited into the immune response early will have the highest proliferative potential but will be poor at exerting rapid effector functions. In contrast, CD8 T cells that get recruited into the response later, so called late-comer T cells, will be able to exert rapid effector functions at the expense of proliferation.

caused by the ability to exert rapid effector functions, as demonstrated by HY CD8 T cells. The HY cells are not sensitized to rapidly exert effector functions in female mice due to the lack of self-antigen signals, yet still undergo poor proliferation when activated during an acute viral infection. It is conceivable that sensitized bystander CD8 T cells would be immediately able to respond and limit the early replication of a superinfecting pathogen without having to first undergo multiple rounds of proliferation. Furthermore, sensitized latecomer CD8 T cells could immediately respond to the ongoing infection while early-activated virus-specific CD8 T cells have already commenced their proliferative phase. Taken together, we propose that during an immune response, due to spatial and temporal gradients of antigen and inflammation, it is likely that a combination of heterogeneous T cells with different signal strengths and sequences of exposure from cytokines and peptide-MHC constitute the total T cell response to pathogens.

In chapter IV, we demonstrate that IFN- $\alpha\beta$ -inducing acute viral infections and TLR agonists sensitize naïve phenotype bystander CD8 T cells to gain an ability to rapidly express IFN- γ in response to high affinity cognate antigen (Figures 4.1-4.5). Bystander-sensitized CD8 T cells also up-regulated GrzB prior to high affinity cognate antigen engagement and underwent enhanced antigen-driven degranulation after cognate antigen stimulation (Figure 4.6), suggesting that they may also be cytolytic. Associated with this acquisition of effector functions was the up-regulation of the t-box transcription factor Eomes (Figure 4.19), known to regulate CTL effector functions. Hence, these naïve bystander T cells were conditioned to behave like memory cells on exposure to high affinity cognate ligand.

The sensitization likely required low affinity MHC-TCR interactions that did not fully activate the T cells, because if MHC I was reduced, absent, or of the wrong allotype or if TCR signaling was inhibited by CsA treatment, sensitization was reduced (Figures 4.14-4.16). The specificity of the TCR also likely played a role, as P14 and OT-I CD8 T cells could be sensitized by poly(I:C), but HY CD8 T cells could not (Figure 4.7 and Table 4.1). We questioned whether this sensitization event was a general occurrence among bystander cells and found that during PV infection or after poly(I:C) treatment about 1-3% of the naïve non-transgenic CD8 T cells gained the ability to rapidly express IFN- γ in response to anti-CD3 stimulation (Figure 4.5). This small percent may, however, be a substantial underestimate of the sensitized cell population, as anti-CD3 was inefficient at measuring the sensitization of P14 cells, in comparison to the cognate peptide. Further, about half of naïve host polyclonal CD8 T cells synthesized Eomes protein after poly(I:C) (Figure 4.20B), arguing that a substantial proportion of the T cells may become sensitized by virus-induced cytokines and that once sensitized, their response to cognate ligand would be altered.

The rules for the T cell differentiation events determined by studying naïve T cells from unstimulated mice would be different for T cells first exposed to virus-induced cytokines. It could thus be predicted that latecomer T cells recruited at later stages of an immune response would behave differently than those stimulated at the beginning of a response. Further, because different responses were seen in transgenic T cells of different specificities, it could be argued that this bystander sensitization may provide a spectrum of T cells receiving a variety of strength of signals and perhaps ultimately

inducing different fates. It is likely, therefore, that some cells would become highly sensitized, while others would not, and some may be in an intermediate state. Cells that do not become sensitized may be more like the HY transgenic T cells, because of the absence of sufficient quantities of self male ligand in female mice that would enable them to undergo homeostatic proliferation. Although sensitization probably required TCR stimulation through pMHC, bystander CD8 T cells were not activated in a typical fashion, as they remained phenotypically naïve and did not divide or proliferate during the virus infection (Figure 3.1-3.4). Nevertheless, they received some signal that induced expression of mRNA and protein for Eomes, which are known to be induced by TCR stimulation (Cruz-Guilloty et al., 2009). The signals emanating from the TCR of bystander-sensitized T cells *in vivo* are currently unknown. Antagonist and partial agonist APLs can induce p21 phosphorylated TCR- ζ and partially bound non-phosphorylated ZAP-70 (Madrenas et al., 1995; Sloan-Lancaster et al., 1994), so it will be of interest to determine the status of the phosphorylation events in bystander-sensitized T cells *in vivo*. It will also be of interest to examine phosphorylation after cognate antigen stimulation *ex vivo* to determine if the TCR signaling cascade is altered when sensitized CD8 T cells are activated by cognate antigen compared to naïve CD8 T cells from untreated mice.

There are still a number of questions regarding the MHC-TCR interactions involved in CD8 T cell sensitization during acute viral infections. For instance, we find that P14 CD8 T cells can only be sensitized by H2D^b, which is the MHC responsible for presenting the cognate epitope GP₃₃₋₄₁. However, it is unknown if P14 CD8 T cells are

selected only on H2D^b in the thymus. Further, it is unknown whether naïve CD8 T cells can only be sensitized by the selecting-MHC I. Additionally, we found that MHC II-restricted LCMV GP₆₁-specific TCR transgenic CD4 T cells (SMARTA) could not be sensitized by PV (data not shown). However, it is currently unknown whether CD4 T cells of other specificities and/or other virus infections or inflammatory responses could sensitize CD4 T cells. Since CD4 T cells do not express Eomes, it is also of interest to determine whether T-bet could function like Eomes in the sensitization of CD4 T cells.

Sensitization also required IFN- $\alpha\beta$ but not direct IFN- $\alpha\beta$ signaling on the CD8 T cells (Figures 4.8-4.10). IFN- $\alpha\beta$ did not sensitize CD8 T cells by way of NK cells or CD4 T cells, nor were IFN- γ , IL-12, IL-15, or IL-18 required, at least individually, for the sensitization to rapid IFN- γ production (Figures 4.11-4.13). Nevertheless, it is possible that combinations of IFN- γ , IL-12, IL-15, and IL-18 signals could sensitize P14 CD8 T cells, because combinations of these cytokines can promote IFN- γ production by effector and memory CD8 T cells better than any of them individually (Berg et al., 2002; Raue et al., 2004). Due to the requirement for MHC I (Figures 4.14-4.16), it is probable that one indirect role of IFN- $\alpha\beta$ during PV infection or poly(I:C) treatment is to up-regulate expression of MHC I, which may then signal the T cells. T cells are positively and then negatively selected in the thymus under conditions when IFN is not up-regulating MHC, and it has been a mystery why T cells selected at one threshold of MHC do not become auto-aggressive during viral infections which induce high levels of IFN, which in turn enhances MHC expression throughout the host (Bukowski and Welsh, 1985). Here we show that these T cells may become sensitized but not fully activated by the enhanced

expression of self-MHC during acute viral infections. Full activation only occurs on exposure to their high affinity ligand and not to cryptically cross-reactive self-ligands present in the host. The simplest explanation of our results would be that the IFN $\alpha\beta$ -induced upregulation of class I MHC was all that was needed to sensitize the T cells. We cannot, however, rule out that other indirect IFN-induced events modulate this process.

Many viral and bacterial infections induce IFN- $\alpha\beta$ via signaling through TLR and RLR pathways so it is likely that the sensitization of T cells demonstrated in this study is not restricted to arenavirus infections of mice. Interestingly, MCMV infection induces IFN- $\alpha\beta$ (Zuniga et al., 2008) but was not able to sensitize P14 CD8 T cells (Table 4.1). This result is interesting given the fact that MCMV encodes a few immune evasion genes known to inhibit class I antigen presentation (Kavanagh and Hill, 2001). During MCMV infection, the up-regulation of MHC I, and thus the enhanced presentation of low affinity self-antigens, may be inhibited by a class of MHC I retention genes (including m152 and m6) (Kavanagh and Hill, 2001; Wagner et al., 2002). Although it is unknown whether the up-regulation of MHC I on infected cells mediates sensitization, it will be of interest to determine if MCMV mutant viruses specifically knocked-out in MHC I retention genes (Δ m152 (Krmptic et al., 1999) and/or Δ m6 (Wagner et al., 2002)) are able to sensitize P14 CD8 T cells.

IFN- γ transcription is a tightly controlled process, regulated by chromatin accessibility and the expression of transcription factors. The t-box transcription factors T-bet and Eomes play an essential role in the induction of IFN- γ transcription in virus-specific CD8 T cells (Intlekofer et al., 2008; Intlekofer et al., 2005; Mayer et al., 2008;

Sullivan et al., 2003). We found that Eomes was transiently induced in bystander-sensitized CD8 T cells during PV infection and poly(I:C) treatment and all IFN- γ -producing P14 CD8 T cells were Eomes^{mid-hi} (Figure 4.19). This probably imparts the ability to rapidly express IFN- γ in response to high affinity cognate antigen. Additionally, another known target of Eomes, the β chain of the IL-2R and IL-15R, CD122 (Intlekofer et al., 2005), was moderately induced on bystander P14 and naïve polyclonal CD8 T cells during acute viral infection and after poly(I:C) treatment (Figure 3.2), suggesting that Eomes expression in bystander-sensitized CD8 T cells may also induce IL-15 responsiveness. This is interesting given the result that IL-15 was required for the up-regulation of GrzB and possibly for enhanced degranulation of bystander-sensitized P14 CD8 T cells after poly(I:C) treatment (Figure 4.13B,C). IFN- $\alpha\beta$ has been shown to induce expression of IL-15 (Zhang et al., 1998b), among other cytokines and IL-15 can induce GrzB and perforin expression and concomitant cytolytic ability of CD8 T cells (Liu et al., 2002; Tamang et al., 2008; Tamang et al., 2006). It is unknown whether Eomes-induced expression of CD122 is required for this process, although we did find some up-regulation of GrzB in the absence of cognate MHC I (Figure 4.16C), suggesting that full up-regulation of GrzB may require Eomes-induced IL-15 responsiveness.

A very limited number of publications have demonstrated effector functions mediated by naïve CD8 T cells. In one report, *Mycobacterium avium* infection conditioned OT-I transgenic CD8 T cells to produce IFN- γ in response to a brief anti-CD3 stimulation *ex vivo* (Gilbertson et al., 2004). This group ruled out typical cross-

reactivity by demonstrating that the OT-I cells did not proliferate during the infection, but the expression of activation markers such as CD44 and the role of self MHC were not assessed. (Gilbertson et al., 2004). *Mycobacterium* infections induce the expression of an array of pro-inflammatory cytokines including IL-1, IL-6, IL-12, IL-15, IL-18, and IFN- γ (van Crevel et al., 2002), which may have the capacity to mediate the sensitization of OT-I CD8 T cells in addition to cryptic cross-reactive TCR stimulation. Another study has demonstrated that IL-15 could induce cytolytic potential of naïve (CD44^{hi}-depleted) polyclonal CD8 T cells *in vitro* (Tamang et al., 2008). In that system IL-15 did not induce GrzB expression and cytotoxicity was perforin-dependent, but it further supports the hypothesis that IL-15 may regulate the cytolytic ability of naïve CD8 T cells.

Virus infections have been implicated as a risk factor for transplant rejection and autoimmunity. Virus-induced IFN- $\alpha\beta$ has been shown to break transplantation tolerance induced by costimulation blockade (Miller et al., 2008; Thornley et al., 2007). Costimulation blockade induces apoptosis of allo-reactive T cells, and it was demonstrated that TLR agonists and recombinant IFN- β could prevent the deletion of allo-reactive T cells (Thornley et al., 2007), but it is also possible that sensitization of allo-reactive T cells may also function in this process. Thus, Eomes-expressing allo-reactive T cells induced by a virus infection could facilitate rapid graft rejection, but the role of Eomes in breaking transplantation tolerance has yet to be assessed.

Viral infections have also had a longstanding association with certain autoimmune diseases. There are several mechanisms described as to how viral infections could induce or exacerbate autoimmune diseases, including the induction of general inflammatory

responses, cross-reactivity, and bystander lymphocyte activation (Munz et al., 2009). Mouse models have uncovered roles for T cell cross-reactivity between pathogen-encoded and self-antigens, but pathogen-derived triggers of human autoimmune diseases have been difficult to identify (Munz et al., 2009). If auto-reactive T cells can be sensitized during acute viral infections, the precise self- or pathogen-encoded epitopes would be difficult to identify, as we have shown that sensitization does not alone induce a typical T cell response (proliferation, regulation of activation markers, etc.). It is likely that sensitization would be driven by non-cognate self- or pathogen-derived epitopes and that sensitized auto-reactive T cells may have to encounter cognate self-antigen for auto-immunity to ensue. Because sensitized T cells remain phenotypically naïve during the viral infection, their contribution to auto-immunity has yet to be uncovered.

During an ongoing immune response the ability of a small proportion of naïve bystander CD8 T cells to rapidly exert effector functions in response to a superinfecting pathogen may prevent an invasion by such a pathogen. Acute viral infections often render hosts resistant to superinfecting viruses, despite transient states of immune suppression where proliferative T cell responses to recall antigens are inhibited, as shown in chapter III. Resistance to superinfection may well be due to the IFN- $\alpha\beta$ levels generated during infection, but we suggest that bystander-sensitized T cells may also function in this process. It is also possible that virus-specific CD8 T cells, particularly those that are not activated early in infection, or so-called latecomer T cells, may also be sensitized like bystander CD8 T cells. In this regard, latecomer T cells would be

immediately able to produce effector cytokines and to lyse virus-infected cells without having to proliferate and may immediately assist in clearing the pathogen.

Many viruses induce a transient state of immune suppression, and there is *in vitro* evidence linking immune suppression to T cell AICD, aberrant co-stimulation, and DC dysfunction. Due to the lack of consensus from *in vitro* systems, we sought to investigate the transient immune suppression induced by acute infections with the arenaviruses LCMV and PV using adoptive transfer models to track and specifically activate TCR transgenic bystander CD8 T cells *in vivo* (Figures 3.1-3.3). In Chapter III, we demonstrated that the proliferation of bystander CD8 T cells activated by cognate antigen during the early acute phase of virus infection *in vivo* was inhibited (Figure 3.5). Susceptibility to immune suppression was transient, as by day 12 post infection, TCR-stimulated bystander CD8 T cells underwent normal proliferation (Figure 3.6), yet the proliferation of TCR-stimulated bystander CD8 T cells was enhanced if the T cells were activated immediately prior to the viral infection (Figure 3.7). Thus, while there was a strong adjuvant effect of viral infections on T cell proliferation at the beginning of infection, there was a strong suppression of T cell proliferation as early as three days into the infection. Our studies focused on T cells with specificities for ligands other than those encoded by the infecting virus, but we presume that these findings would be relevant to latecomer virus-specific T cells whose TCR do not encounter antigen until later in infection. Despite the susceptibility of bystander CD8 T cells to AICD *in vitro* (Zarozinski et al., 2000), we found that the reduced proliferation of bystander CD8 T cells *in vivo* was not exclusively mediated by Fas-FasL- or TNF-induced AICD (Table

3.1). In addition, we could not convincingly show an enhanced susceptibility to apoptosis *in vivo*, though it should be noted that apoptotic cells can sometimes be cleared *in vivo* before they are readily detectable. Nevertheless, most of the inhibition in antigen-induced proliferation could be explained by a delay in the onset of division (Figure 3.12-3.15).

Infections of human or mouse DC with HCMV, MCMV, measles virus, adenovirus, RSV, VV, or LCMV clone 13 can interfere with DC maturation and inhibit MHC and costimulatory molecule expression (Andrews et al., 2001; Engelmayer et al., 1999; Moutaftsi et al., 2002; Munir et al., 2008; Newton et al., 2008; Servet-Delprat et al., 2000; Sevilla et al., 2004; Smith et al., 2005), and measles virus, VV, and HSV have been shown to induce apoptosis of DC (Bosnjak et al., 2005; Engelmayer et al., 1999; Fugier-Vivier et al., 1997; Jones et al., 2003; Muller et al., 2004), all of which can presumably lead to reduced T cell responses. However, we found that administration of peptide-labeled DC could not rescue the inhibition of bystander CD8 T cell proliferation (Figure 3.18), suggesting that DC dysfunction *in vivo* was not the cause of immune suppression during acute infections of mice with PV. Additionally, the reduced expression of the homeostatic chemokines CCL21 and CXCL13 in lymphoid organs during acute viral infections has been postulated to inhibit the development of new T cell responses by hindering the localization of naïve T cells with DCs during ongoing anti-viral immune responses (Mueller et al., 2007). This does not appear to be the cause of immune suppression in our models because the bystander donor HY CD8 T cells sensitized by virus infection in female mice could not proliferate to the extent of naïve

HY CD8 T cells when transferred into uninfected male mice (Figure 3.19), where there should be normal APC and normal gradients of chemokines expressed.

Although we could demonstrate reduced proliferation and delayed division with both P14 and HY CD8 T cell models, it remains unknown as to the mechanisms behind the rapid depletion of the HY CD8 T cells in LCMV-infected male mice (Figure 3.9). We could not convincingly show that the HY CD8 T cells underwent apoptosis when transferred directly into the LCMV-infected male mice (Figures 3.12 and 3.13), nor could we recover the cells in other tissues (Figures 3.10 and 3.11). One could speculate that the up-regulation of adhesion receptors on blood vessel walls during viral infection (Thomsen et al., 2003) could bind the T cells soon after they were injected into the tail vein, and thus prevent them from reaching any specific tissue. However, if this were the mechanism of depletion, it would also have to require antigen, as there were significantly more HY T cells recovered from LCMV-infected female mice. Whole mouse fluorescent microscopy (Reinhardt et al., 2001) may be a useful tool to locate the HY CD8 T cells in this model.

Inhibition of TCR-stimulated bystander CD8 T cell proliferation could result in reduced vaccine efficacy if vaccines are administered during an acute viral infection. The long-term fate of the bystander CD8 T cells in our models is unknown, as these models are not suitable for studying the differentiation and maintenance of memory CD8 T cells. The P14 system relies on peptide treatment to activate the CD8 T cells without engaging CD4 T cell help. Thus the generation and maintenance of functional memory P14 CD8 T cells may be compromised in the control naïve mice because of this lack of help. The

HY system relies on the high and constant expression of antigen in male mice, which has been shown to eventually anergize adoptively transferred female HY CD8 T cells rather than driving them into memory pools (Rocha and von Boehmer, 1991). Other model systems have investigated the role of inflammation in the development of T cell memory. Lack of inflammation during T cell activation, via DC immunization, has been shown to enhance the rate at which T cells acquire memory characteristics (Badovinac et al., 2005; Joshi et al., 2007). And simultaneous exposure of T cells to bacteria- or CpG-induced inflammation inhibited this rapid transition to memory (Joshi et al., 2007; Pham et al., 2009), but prior exposure of T cells to CpG-induced inflammation 2 days before DC immunization did not inhibit the rapid transition to memory (Pham et al., 2009). Although we cannot study the long term fate of TCR-stimulated bystander CD8 T cells using our models, we know that these naïve T cells are capable of immediate effector functions, including IFN- γ production and degranulation when exposed to cognate ligand (Chapter IV). The ability to rapidly exert effector functions was associated with the up-regulation of the t-box transcription factor Eomes, which required IFN- $\alpha\beta$ and self peptide-presenting MHC I. Thus, bystander CD8 T cells had acquired a characteristic of memory prior to any TCR stimulation. We propose that CD8 T cells that receive inflammatory signals prior to cognate peptide-induced TCR stimulation are diverted down a differentiation pathway that is represented by the rapid acquisition of effector functions at the expense of proliferation. Thus, bystander-sensitized CD8 T cells behaved more like short-lived effector cells (SLEC) than memory precursor cells (MPEC), despite the expression of Eomes. Inflammation in the form of IL-12 can tip the

balance toward increased SLEC development, but T-bet, not Eomes, is the primary CTL transcription factor that controls that cell fate decision (Joshi et al., 2007). Thus, the kind and duration of inflammatory stimuli before, during, or after T cell activation may have diverse implications in the development and maintenance of memory. Models utilizing different inflammatory stimuli and immunization schemes will have to be established to investigate the long-term fate of TCR-stimulated bystander and latecomer CD8 T cells, particularly in regards to how vaccine-induced immunity may be compromised during acute viral infections.

In conclusion, we have shown in this thesis that acute viral infections impact naïve bystander and latecomer CD8 T cells such that their response to cognate antigen is altered. Prior to cognate antigen engagement, bystander CD8 T cells up-regulate Eomes, CD122, and GrzB. Following cognate antigen engagement, bystander CD8 T cells rapidly degranulate GrzB-containing particles and express the effector cytokine IFN- γ . Despite these rapid effector functions, the proliferation of TCR-stimulated bystander CD8 T cells was markedly inhibited. This reduced proliferation was found not to be a defect in antigen presentation, but was a T cell intrinsic defect in initiating division. Finally, we propose that the virus-specific CD8 T cell response is composed of a heterogeneous population of T cells that have received different strengths and sequences of TCR and cytokine receptor stimulation that will impact their ability to exert effector functions, proliferate, and potentially develop into memory. Dissecting these signals will help define how T cells respond to infectious pathogens and aid in the development of more efficacious vaccination strategies.

Chapter VI: References

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