THE ROLE OF HETEROLOGOUS IMMUNITY IN VIRAL CO-INFECTIONS AND NEONATAL IMMUNITY

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LAURIE LEA KENNEY

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LAURIE LEA KENNEY

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Liisa K Selin, M.D., Ph.D., Thesis Advisor

Raymond Welsh, Ph.D., Member of Committee

Michael Brehm, Ph.D., Member of Committee

John Harris, Ph.D., Member of Committee

Dennis W. Metzger, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Francis Ka-Ming Chan, Ph.D., Chair of Committee

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program in Immunology and Virology
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* indicate co-first authorship

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Abstract

The dynamics of T cell responses have been extensively studied during single virus infection of naïve mice. During a viral infection, viral antigen is presented in the context of MHC class I molecules on the surface of infected cells. Activated CD8 T cells that recognized viral antigens mediate clearance of virus through lysis of these infected cells. We hypothesize that the balance between the replicating speed of the virus and the efficiency at which the T cell response clears the virus is key in determining the disease outcome of the host. Lower T cell efficiency and delayed viral clearance can lead to extensive T cell-mediated immunopathology and death in some circumstances. To examine how the efficiency of the immune response would impact immunopathology we studied several viral infection models where T cell responses were predicted to be less than optimal: 1. a model of co-infection with two viruses that contain a crossreactive epitope, 2. a viral infection model where a high dose infection is known to induce clonal exhaustion of the CD8 T cell response, 3. a neonatal virus infection model where the immune system is immature and 4. A model of beneficial heterologous immunity and T cell crossreactivity where mice are immunized as neonates when the T cell pool is still developing.

Model 1. Simultaneous co-infections are common and can occur from mosquito bites, contaminated needle sticks, combination vaccines and the simultaneous administration of multiple vaccines. Using two distantly related arenaviruses, lymphocytic choriomeningitis virus (LCMV) and Pichinde virus

(PICV), we questioned if immunological T cell memory and subsequent protection would be altered following a simultaneous co-infection, where two immune responses are generated within the same host at the same time. Co-infection with these two viruses, which require CD8 T cell responses to clear, resulted in decreased immune protection and enhanced immunopathology after challenge with either virus. After primary co-infection, each virus-specific immune response impacted the other as they competed within the same host and resulted in several significant differences in the CD8 T cell responses compared to mice infected with a single virus. Co-infected mice had a dramatic decrease in the overall size of the LCMV-specific CD8 T cell response and variability in which virus-specific response dominated, along with skewing in the immunodominance hierarchies from the normal responses found in single virus infected mice. The reduction in the number of LCMV-specific CD8 memory T cells, specifically cells with an effector memory-like phenotype, was associated with higher viral loads and increased liver pathology in co-infected mice upon LCMV challenge. The variability in the immunodominance hierarchies of co-infected mice resulted in an enhanced cross-reactive response in some mice that mediated enhanced immune-mediated fat pad pathology during PICV challenge. In both viral challenge models, an ineffective memory T cell response in co-infected mice facilitated increased viral replication, possibly leading to enhanced and prolonged accumulation of secondary effector T cells in the tissues, thereby leading to increased immune pathology. Thus, the magnitude and character of memory

CD8 T cell responses in simultaneous co-infections differed substantially from those induced by single immunization. This has implications for the design of combination vaccines and scheduling of simultaneous immunizations.

Model 2. The balance between protective immunity and immunopathology often determines the fate of the virus-infected host. Several human viruses have been shown to induce a wide range of severity of disease. Patients with hepatitis B virus (HBV), for example, show disease progression ranging from acute resolving infection to a persistent infection and fulminant hepatitis. Certain rapidly replicating viruses have the ability to clonally exhaust the T cell response, such as HBV and hepatitis C virus (HCV) in humans and the clone 13 strain of LCMV in mice. How rapidly virus is cleared is a function of initial viral load, viral replication rate, and efficiency of antigen-specific T cells. By infecting mice with three different inocula of LCMV clone 13, we questioned how the race between virus replication and T cell responses could result in different disease outcomes. A low dose of LCMV generated efficient CD8 T effector cells, which cleared the virus with minimal lung and liver pathology. A high dose of LCMV resulted in clonal exhaustion of T cell responses, viral persistence and little immunopathology. An intermediate dose only partially exhausted the CD8 T cell responses and was associated with significant mortality, and the surviving mice developed viral persistence and massive immunopathology, including necrosis of the lungs and liver. This was a T cell-mediated disease as T cell-deficient mice had no pathology and became persistently infected like mice infected with a high

dose of LCMV clone 13. This suggests that for non-cytopathic viruses like LCMV, HCV and HBV, clonal exhaustion may be a protective mechanism preventing severe immunopathology and death.

Model 3. Newborns are more susceptible to infections due to their lack of immunological memory and under-developed immune systems. Passive maternal immunity helps protect neonates until their immune systems have matured. We questioned if a noncytolytic virus that produces strong T cell responses in adult mice would also induce an equally effective response in neonatal mice. Neonates were infected with very low doses of LCMV Armstrong and surprisingly the majority succumbed to infection between days 7-11, which is the peak of the T cell response in adult mice infected with LCMV. Death was caused by T cell-dependent pathology and not viral load as 100% of T cell deficient neonates survived with minimal lung and liver pathology. This is similar to the adult model of medium dose LCMV clone 13, but T cell responses in neonates were not partially clonal exhausted. Furthermore, surviving neonates were not persistently infected, clearing virus by day 14 post infection. In adult mice direct intracranial infection leads to LCMV replication and CD8 T cell infiltration in the central nervous system (CNS), causing CD8 T cell-mediated death. However, this does not occur in adults during LCMV intraperitoneal (ip) infections. We questioned if unlike adults LCMV could be gaining access to the CNS in neonates following ip infection. Replicating LCMV was found in the brain of neonates after day 5 post infection along with virus-specific CD8 T cells

producing IFNγ at day 9 post infection. Neonates lacking perforin had complete survival when followed until day 14 post infection, suggesting perforin-mediated T cell-dependent immunopathology within the CNS of neonates was causing death after LCMV infection. Passive immunity from LCMV-immune mothers also protected 100% of pups from death by helping control viral load early in infection. We believe that the maternal antibody compensates for the immature innate immune response of neonates and controls viral replication early so the neonatal T cell response induced less immunopathology. Neonates are commonly thought to have less functional immune systems, but these results show that neonates are capable of producing strong T cell responses that contribute to increased mortality.

Model 4. Due to their enhanced susceptibility to infection neonatal and infant humans receive multiple vaccines. Several non-specific effects from immunizations have been observed, for example, measles or Bacillus Calmette-Guerin (BCG) vaccines have been linked to decreased death of children from infections other than measles virus or tuberculosis. These studies mirror the concepts of beneficial heterologous immunity, where previous immunization with an unrelated pathogen can result in faster viral clearance. LCMV-immune mice challenged with vaccinia virus (VV) have lower viral loads then naïve mice and survive lethal infections, but some mice do develop fat pad immunopathology in the form of panniculitis or acute fatty necrosis (AFN). We questioned how immunological T cell memory formed during the immature neonatal period would

compare to memory generated in fully mature adults during a heterologous viral challenge. Mice immunized as neonates had comparable reduction in VV load and induction of AFN, indicating that heterologous immunity is established during viral infections early in life. Interestingly, the LCMV-specific memory populations that expanded in mice immunized as neonates differed from that of mice immunized as adults. In adult mice 50% of the mice have an expansion of LCMV-NP205-specific CD8 T cells while the majority of neonates expanded the LCMV-GP34-specific CD8 T cell pool. This alteration in dominant crossreactivities may be due to the limited T cell receptor repertoire of neonatal mice. In naïve neonatal mice we found altered Vβ repertoires within the whole CD8 T cell pool. Furthermore, there was altered Vβ usage within virus-specific responses compared to adult mice and a wide degree of variability between individual neonates, suggesting enhanced private specificity of the TCR repertoire. Beneficial heterologous immunity is maintained in neonates, but there was altered usage of crossreactive responses.

As neonatal mice were found to be so sensitive to LCMV infection we questioned if neonates could control another arenavirus that did not replicate as efficiently in mice, PICV. Unlike LCMV infection, neonatal mice survived infection with PICV even with adult-like doses. However, viral clearance was protracted in neonates compared to adults, but was cleared from fat pad and kidney by day 11 post infection. The peak of the CD8 T cell response was similarly delayed. PICV-infected neonates showed dose-dependent PICV-specific CD8 T cell responses,

which were similar to adult responses by frequency, but not total number. As with LCMV infection there were changes in immunodominance hierarchies in neonates. Examination of the immunodominance hierarchies of PICV-infected neonates showed that there were adult-like responses to the dominant NP38-specific response, but a loss of the NP122-specific response. Six weeks post neonatal infection mice were challenged with LCMV Armstrong and there was a strong skewing of the PICV immunodominance hierarchy to the crossreactive NP205-specific response. These data further support the hypothesis that heterologous immunity and crossreactivity develop following neonatal immunization, much as occurs in adults, although TCR repertoire and crossreactive patterns may differ.

Changing the balance between T cell efficiency and viral load was found to altered the severity of the developing immunopathology after viral infection.

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List of Symbols, Abbreviations or Nomenclature

AFN	acute fatty necrosis
AIM	acute infectious mononucleosis
ALT	alanine aminotransferase
APC	antigen presenting cell
BALT	bronchus-associated lymphoid tissue
BCG	Bacillus Calmette-Guerin
CNS	central nervous system
CTLA4	cytotoxic T lymphocyte-associated protein 4
CDR3	complementarity-determining region
D	diversity
DC	dendritic cell
DTP	diphtheria tetanus pertussis
EBV	Epstein-Barr virus
Eomes	eomesodermin
ER	endoplasmic reticulum
ERAP	endoplasmic reticulum aminopeptidase
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HBV	hepatitis B virus
HCV	hepatitis C virus

HIV	human immunodeficiency virus
HSV	herpes simplex virus
IAV	influenza A virus
IFNαR	interferon α receptor
IL-7R	IL-7 receptor
INT	iodonitrotetrazolium chloride
IP	intraperitoneal
IV	intravenous
J	junctional
KLRG1	killer cell lectin-like receptor subfamily G member 1
LAG3	lymphocyte activation gene-3
LCMV	lymphocytic choriomeningitis virus
MCMV	murine cytomegalovirus
MPEC	memory precursor effector cell
NFkB	nuclear factor kappa B
PD1	programmed death 1
PDL	programmed death ligand
PEC	peritoneal exudate cells
PFU	plaque forming units
PI3K	phosphatyidylinositol 3 kinase
PICV	Pichinde virus
Poly IC	polyinosinic polycytidine acid

RTE	recent thymic emigrants
SLEC	short-lived effector cell
SOCS1	suppressor of cytokine signaling 1
TAP	transporter associated with antigen processing
T_{cm}	central memory T cell
TCR	T cell receptor
T_{em}	effector memory T cell
Treg	T regulatory cell
V	variable
VV	vaccinia virus

Chapter 1: Introduction

The dynamics of the CD8 T cell response during viral infection has been extensively studied during single virus infections in mouse models. Specifically, the lymphocytic choriomeningitis virus (LCMV) model of mouse infection has been a cornerstone in our current understanding of the activation, effector function and memory formation of CD8 T cells. During a viral infection the immune system generates CD8 T cell responses specific to viral antigens. T cells require specific signals to become properly activated and undergo clonal expansion and differentiate into effector cells (Kaech and Cui, 2012; Russ et al., 2012). Activated CD8 T cells recognize peptide presented by MHC class I complexes on infected cells and lyse these cells to clear virus, after which virus-specific T cells undergo contraction and form a heterogeneous memory population that protects individuals against a reinfection (Kaech and Cui, 2012; Russ et al., 2012). Under normal circumstances, reinfection results in the reactivation of memory CD8 T cells. Memory cells are at a higher frequency and require only reexposure to antigen to become reactivated causing faster viral clearance and less pathology compared to a primary infection (Arens and Schoenberger, 2010; Pihlgren et al., 1996). Understanding how to generate a protective memory response that rapidly clears virus without causing pathology is important, specifically in terms of vaccine development.

Memory CD8 T cells can also recognize and be reactivated against antigens from unrelated pathogens and significantly alter disease outcome. This is known as heterologous immunity and may more closely mimic what occurs in humans as they are sequentially infected with unrelated pathogens. Compared to single virus infections, heterologous infections change the dynamics of viral load and the CD8 T cell responses depending on the sequence of infections and characteristics of the crossreactive responses involved. Heterologous immunity can be beneficial or detrimental depending on the circumstances. For example, LCMV-immune mice infected with VV clear virus faster than naïve mice, but not as fast as VV-immune mice (Chen et al., 2003; Cornberg et al., 2010; 2007; Selin et al., 1998). In this circumstance even though virus is cleared faster some mice developed immunopathology in the form of acute fatty necrosis (AFN) (Nie et al., 2010; Selin et al., 1998; Yang et al., 1985). However, these mice are protected from lethal vaccinia virus (VV) infection (Chen et al., 2001). This is beneficial heterologous immunity. However, influenza A virus (IAV)-immune mice given LCMV clear virus more slowly than naïve mice and develop severe lung pathology (Chen et al., 2003; Wlodarczyk et al., 2013). The severity of lung pathology directly correlates with the size of the memory crossreactive CD8 T cell responses. Ablation of these crossreactive memory responses, by use of mutant viruses lacking these epitopes or peptide tolerization, leads to reduced pathology (Wlodarczyk et al., 2013). This is an example of detrimental heterologous immunity.

The goal of this thesis is to build on these findings and further examine how the context of viral infection, such as co-infection, antigen load or the maturity of the immune system could effect the dynamics of the CD8 T cell response, viral clearance and the induction of immunopathology. These are all common major variables that may play a role in the outcome to any human viral infection. It is important that we understand how these variables may modify outcome in order to appropriately design vaccines or therapies that prevent viral immunopathology. These studies will focus on the well-developed LCMV infection model in mice, along with virus infections such as Pichinde virus (PICV) and VV, which have been well defined in single virus and heterologous virus models.

The subsequent introduction is divided into separate sections that give more detail on topics directly related to this thesis.

Antigen processing and presentation is required for CD8 T cell priming

CD8 T cells sample their environment by interacting with peptides loaded in MHC class I molecules on the surfaces of cells. Under homeostatic situations the normal turnover of proteins results in degradation through the proteasome and trimming by peptidases (Rock et al., 1994b). The proteasome is vital for peptide presentation as blocking the major peptidase subunits of the proteasome with inhibitors blocked expression of MHC-peptide complexes on the surface of

cells (Rock et al., 1994b). Degraded peptides are shuttled from the cytoplasm into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) 1 and 2 where they undergo further trimming by aminopeptidases to 8-10 amino acids in length and are loaded into MHC class I molecules and transported to the surface (Neefjes and Sadaka, 2012). During infection, IFNγ signaling induces the immunoproteasome, which leads to increased presentation of viral antigens on the surface of cells (Groettrup et al., 1996; Hisamatsu et al., 1996; Nandi et al., 1996).

Antigen can be loaded into MHC class I molecules through direct presentation, which occurs in all cells when peptides are transported into the ER and loaded into MHC molecules. Dendritic cells (DC) can also present antigen through cross presentation or cross dressing (Joffre et al., 2012; Wakim and Bevan, 2011). During cross presentation peptides from necrotic cells are taken up by endocytosis, pinocytosis or phagocytosis into the cytoplasm (Rock and Shen, 2005). Peptides can then enter the direct presentation pathway by transport into the ER or be degraded by enodosomal proteases, such as cathepsins, and loaded in the MHC class I molecules in the endosome (Rock and Shen, 2005). Cross presentation has been found to occur only in CD8+ DC (Jung et al., 2002). DC can also present antigen by cross dressing through the transfer of membrane between cells, or trogocytosis (Wakim and Bevan, 2011). Peptide pulsed DC were found to share or pass on peptide-MHC class I complexes to a

population of unpulsed DC, which were able to activate CD8 T cells in culture (Wakim and Bevan, 2011).

CD8 T cell activation

Naïve CD8 T cells require three signals to become properly activated. Signal 1 is the interaction with cognate antigen in the context of MHC class I molecules on the surface of mature antigen presenting cells (APC). APC that encounter antigen through uptake of foreign antigen or infection must undergo maturation and migration to the draining lymph node to interact with naïve CD8 T cells. Maturation of APC occurs through innate receptor signaling, such as toll-like receptors (TLR) or intracellular receptors, such as RIGI or MDA5 (Russ et al., 2012). Upon activation, APC upregulate MHC molecules and costimulatory molecules, such as CD80 and CD86 (Acuto and Michel, 2003). Interaction of the co-receptor, CD8, on the surface of the T cell with the MHC class I molecule on the APC stabilizes this interaction and allows for proper signaling (Laugel et al., 2011). CD8 T cells also require costimulation, or signal 2, from the APC to become properly activated. CD80 and CD86 interact with CD28 on the surface of the CD8 T cells and induce signaling cascades that synergize with T cell receptor (TCR) signaling and lead to calcium flux (Acuto and Michel, 2003; Belz et al., 2007). Most notably, CD28 signaling in T cells causes activation of the transcription factor, nuclear factor kappa B (NFkB), and induction of IL-2 (Acuto and Michel, 2003; Narayan et al., 2006; Qiao et al., 2008). Proinflammatory

cytokine signaling is the third signal required for proper CD8 T cell activation (Curtsinger and Mescher, 2010). Numerous papers have been published on type I IFN and IL-12 signaling providing the 3rd signal for CD8 T cells during activation (Curtsinger and Mescher, 2010; Curtsinger et al., 2005; Keppler and Aichele, 2011; Keppler et al., 2012; Pham et al., 2011; Xiao et al., 2009). For instance, type I IFN receptor deficient CD8 T cells have decreased survival and limited effector functions after LCMV infection (Aichele et al., 2006; Kolumam et al., 2005). CD8 T cell responses to other intracellular pathogens, such as Listeria, require both of these proinflammatory cytokines, IL-12 and type I IFN, for their 3rd signal in activation (Xiao et al., 2009). It is the integration of signals 1, 2 and 3 that drives the differentiation of a naïve cell into an effector cell (Kaech and Cui, 2012; Russ et al., 2012).

Functional phenotypes of activated CD8 T cells

After receiving the three signals required for activation CD8 T cells undergo rapid division, which is correlated with differentiation and the acquisition of effector functions (Jenkins et al., 2008). Effector CD8 T cells produce proinflammatory cytokines, such as IFNγ and TNFα (La Gruta et al., 2004), and can induce apoptosis of infected cells through release of perforin (Kägi et al., 1994) and granzymes (Jenkins et al., 2007; Moffat et al., 2009; Peixoto et al., 2007) and Fas-FasL interactions (Kägi et al., 1994; Suda et al., 1995). The

effector CD8 T cell population is heterogeneous forming a continuum between terminally differentiated effectors, that make up the majority of the population and die during contraction, and a smaller pool of memory precursors that will survive into memory (**Figure 1.1**) (Kaech and Cui, 2012). Several studies have characterized the differences between a cell with high potential to become a memory cell versus one that has a greater probability of dying during contraction (Joshi et al., 2007; Kaech et al., 2003; Sarkar et al., 2008; Schluns et al., 2000). Memory-precursor effector cells (MPEC) have high expression of the IL-7 receptor (IL-7R), which provides survival signals and low expression of killer cell lectin-like receptor G1 (KLRG1), a marker of terminal differentiation (Joshi et al., 2007; Kaech et al., 2003; Sarkar et al., 2008; Schluns et al., 2000). MPEC also have increased proliferative potential in response to secondary antigen exposure, and have the ability to produce IL-2 and self-renew (Kaech et al., 2003). These characteristics allow for MPEC to be reactivated upon reinfection and produce a secondary round of effector cells to quickly clear infection. IL-7R high cells also have a greater potential to produce central memory cells (T_{cm}), which will be discussed below (Kaech and Cui, 2012). Terminally differentiated effectors, or short-lived effector cells (SLEC), express low levels of the IL-7R and high levels of KLRG1 (Hand et al., 2007). These cells have been shown to have decreased longevity (Kaech et al., 2003). After a simultaneous transfer of equal numbers of IL7R high or low CD8 T cells from mice at day 15 of LCMV infection into the same host reveals that IL7R low cells are not maintained over time

Figure 1.1

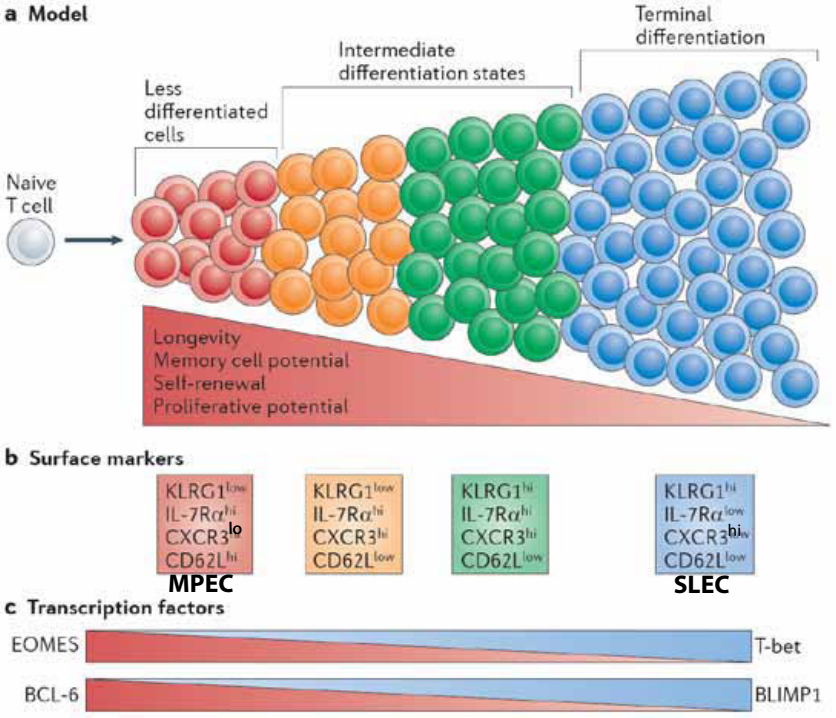


Figure 1.1. Effector CD8 T cell differentiation forms a continuum. A. Naïve CD8 T cells respond to the integration of TCR stimulation, costimulation and cytokine signaling and differentiate to form a heterogeneous effector CD8 T cell pool. Cells range from less differentiated, with increased memory cell potential, longevity, self-renewal and proliferation potential, to terminally differentiated cells that have a reduction in these properties. **B.** Less differentiated cells have increased expression of IL7R and CD62L, with decreased expression of KLRG1 and CXCR3, these cells are known as memory-precursor effector cells (MPEC). As cells differentiate these markers change and terminally differentiated cells have high expression of KLRG1 and CXCR3 with low expression of IL7R and CD62L and are called short-lived effector cells (SLEC). **C.** Differentiation is also linked with graded expression of transcription factor pairs, such as Eomes and Tbet and BCL-6 and BLIMP1. Less differentiated cells have high expression of Eomes and BCL-6, while terminally differentiated cells express high Tbet and BLIMP1. Modified from Kaech SM and W Cui. Transcriptional control of effector and memory CD8+ T cell differentiation. Nature Reviews Immunology 12: 749-761 (2012). Copyright license number 3196031411599.

(Kaech et al., 2003). More recently, the chemokine receptor CXCR3 has been associated with terminally differentiated effector CD8 T cells (Hu et al., 2011; Kohlmeier et al., 2011; Kurachi et al., 2011). CXCR3 is responsible for recruiting CD8 T cells to the site of infection. It is thought that CXCR3-expressing cells are more likely to undergo terminal differentiation due to their enhanced exposure to proinflammatory cytokines and antigen at the site of infection (Hu et al., 2011; Kohlmeier et al., 2011; Kurachi et al., 2011).

The fate of an individual cell is not preprogramed, but dependent on the integration of several factors including strength and duration of TCR stimulation, inflammatory cytokine environment, transcriptional regulation, metabolic switches and the segregation of factors during cell division (Kaech and Cui, 2012). For some pathogens, larger doses of antigen cause greater CD8 T cell expansion and lead to a bigger memory population (Badovinac et al., 2002; Kaech and Ahmed, 2001). One study controlled the length of time T cells could interact with antigen by transferring transgenic CD8 T cells and peptide-pulsed DC, which carried the transgene for diphtheria toxin, while at the same time infecting with Listeria (Prlic et al., 2006). The injection of diphtheria toxin would induce apoptosis of antigen presenting DC and stop T cell exposure to antigen. If DC were only allowed to interact with T cells for shorter periods of time mice developed smaller CD8 T cell populations indicating a role for length of exposure to antigen in influencing the size of the CD8 T cell response (Prlic et al., 2006). Inflammatory cytokines, such as IL-12 and type I IFN, also play a major role in

the differentiation of CD8 T cells during infection (Curtsinger and Mescher, 2010). Direct effects of type I IFN are required for CD8 T cell expansion during LCMV infection as IFNα receptor deficient CD8 T cells had decreased survival and limited effector functions (Curtsinger et al., 2005). Timing of signal 3 plays a major role on effector CD8 T cell proliferation and development of effector functions. Exposure of naive CD8 T cells to type I IFN prior to interaction with cognate peptide caused a transient inhibition of proliferation and unresponsiveness to inflammatory cytokines due to decreased IFNα receptor expression and upregulation of the protein known as suppressor of cytokine signaling (SOCS) 1 (Marshall et al., 2011); Stina Urban, unpublished data). SOCS1 is a cytokine-inducible protein that acts in a negative feedback loop to attenuate cytokine signaling (Yoshimura et al., 2007). It is the integration of several signals that ultimately shapes the CD8 T cell response through the expression of different transcription factors.

Multiple transcription factors are induced in response to CD8 T cell activation (**Figure 1.1**). Two T-box transcription factors, Tbet and Eomesodermin (Eomes) are coupled in CD8 T cell differentiation (Kaech and Cui, 2012). Tbet is induced by high levels of IL-12 signaling and promotes terminal differentiation (Joshi et al., 2007). Tbet induces the expression of IFNγ (Szabo et al., 2000) and granzyme B (Cruz-Guilloty et al., 2009). Eomes is induced in CD8 T cells subsequent to Tbet and stimulates MPEC development (Banerjee et al., 2010; Intlekofer et al., 2005). Eomes mediates memory formation by the upregulation of

CD122, which is required for IL-2 and IL-15 signaling, and memory formation and maintenance (Intlekofer et al., 2005). Blimp1 and Bcl-6 are two other transcription factors that are coupled and determinants in T cell differentiation (Kaech and Cui, 2012). Blimp1 is induced by IL-2, IL-12 and IL-21 signaling and is a transcriptional repressor of Bcl-6 and IL7R (Pipkin et al., 2010). Blimp1 expression in CD8 T cells declines with transition into the memory phase (Rutishauser et al., 2009), while Bcl-6 progressively accumulates in the memory population (Cui et al., 2011). These pairs of transcription factors are co-expressed in activated CD8 T cells and the balance between them determines where an individual cell will appear in the continuum between terminally differentiated cells and memory precursor cells (Kaech and Cui, 2012).

CD8 T cell memory: friend or foe?

After viral clearance, the effector T cell response contracts to form a memory pool. Memory cells provide an advantage to the host during secondary infection with the same pathogen. Compared to naïve T cells, antigen-specific memory cells are at a **higher frequency** due to the clonal expansion that occurred during the primary infection. Memory cells also have a **lower activation threshold** (Pihlgren et al., 1996) and do not require signal 2 or 3 to become reactivated (Arens and Schoenberger, 2010). Naïve cells are restricted to the blood and secondary lymphoid organs, while memory cells can enter **peripheral sites** allowing them to detect reinfection earlier (Arens and Schoenberger, 2010).

Collectively these attributes make memory cells superior to naïve cells during a reinfection. These are also important features for heterologous immunity, for viral clearance, but also make them a lethal weapon when not used appropriately. Similar to the effector phase, the memory pool is also a heterogeneous population. Two major subsets that make up the memory pool are central and effector memory cells (Sallusto et al., 2004).

Effector memory (T_{em}) cells are characterized by their low levels of CD62L and CCR7, receptors that allow trafficking through high endothelial venules into secondary lymphoid organs (Kohlmeier et al., 2011; Sallusto et al., 2004; Wakim et al., 2008). T_{em} cells express CCR5, which allows them to patrol the peripheral tissues for reinfection (Kohlmeier et al., 2011; Wakim et al., 2008). Upon reinfection T_{em} cells can rapidly exert effector functions, such as cytokine production and cytolytic activity, but the proliferation potential of this population is low, making this population unsustainable during infection (Masopust et al., 2001; Sallusto et al., 2004). Tissue resident T_{em} cells are more recently identified cells that are retained in the original site of infection (Gebhardt et al., 2009; Mackay et al., 2012). On the other hand, T_{cm}, which express high levels of CD62L and CCR7, play a supportive role during reinfection. T_{cm} cells reside within the secondary lymphoid tissues and during reinfection quickly divide to produce a large population of effectors cells (Masopust et al., 2001).

CD8 T cell immunodominance hierarchies

During a viral infection all viral peptides are processed, but only a small percentage are presented to T cells. Epitope-specific T cell populations can be arranged into a hierarchy based upon the size of each epitope-specific response, also known as an immunodominance hierarchy (Yewdell, 2006). The immunodominance of an epitope-specific population is influenced by multiple factors, including processing and presentation of viral antigen and the characteristics of the TCR repertoire. Altering antigen processing by studying mice that lack aminopeptidases (York et al., 2006) or components of the immunoproteasome (Kincaid et al., 2012) results in altered immunodominance hierarchies after LCMV infection. Mice lacking the endoplasmic reticulum aminopeptidase (ERAP) 1 develop reduced CD8 T cell responses to several LCMV epitopes, including NP396, GP33, NP205 and GP118 (York et al., 2006). Interestingly, there is an increase in the CD8 T cell response to the longer 11mer epitope, GP276 (York et al., 2006). This same increase in the GP276-specific CD8 T cell response was also found in mice that lacked the three main components of the immunoproteasome (Kincaid et al., 2012), indicating the major role that antigen processing and presentation plays in determining the immunodominance of an epitope. The kinetics of antigen presentation also alters immunodominance, as the LCMV nucleoprotein is processed and presented earlier during infection then the glycoprotein and results in earlier T cell responses to nucleoprotein derived epitopes (Probst et al., 2003). The TCR

repertoire (Brehm et al., 2002; Chen et al., 2001; Haeryfar et al., 2008; Kim et al., 2002; La Gruta et al., 2006), recruitment of T cells to the site of infection (Khanna and Lefrançois, 2008), and affinity of the TCR to the peptide-MHC complex (Khanna and Lefrançois, 2008) also affect how dominant an epitope-specific T cell population will be. Crossreactive memory responses that mediate heterologous immunity have been found to skew immunodominance hierarchies during sequential infections (Brehm et al., 2002; Chen et al., 2001; Kim et al., 2002). For example, infection with either LCMV or PICV alone induced a subdominant NP205-specific response, but a subsequent heterologous challenge with the other virus (either LCMV+PICV or PICV+LCMV) caused the NP205-specific response to increase and become dominant in most mice (Brehm et al., 2002; Chen et al., 2012). During a LCMV+PICV infection the memory LCMV-NP205-specific CD8 T cells were at a higher precursor frequency and had a lower activation threshold, which allowed them to outcompete the naïve PICV-specific CD8 T cell response after PICV challenge.

T cell clonal exhaustion

T cell clonal exhaustion is the stepwise loss of effector function that occurs during chronic infections (**Figure 1.2**). In the early stages of clonal exhaustion cells lose **cytotoxicity** and the ability to **proliferate and produce IL-2**

Figure 1.2

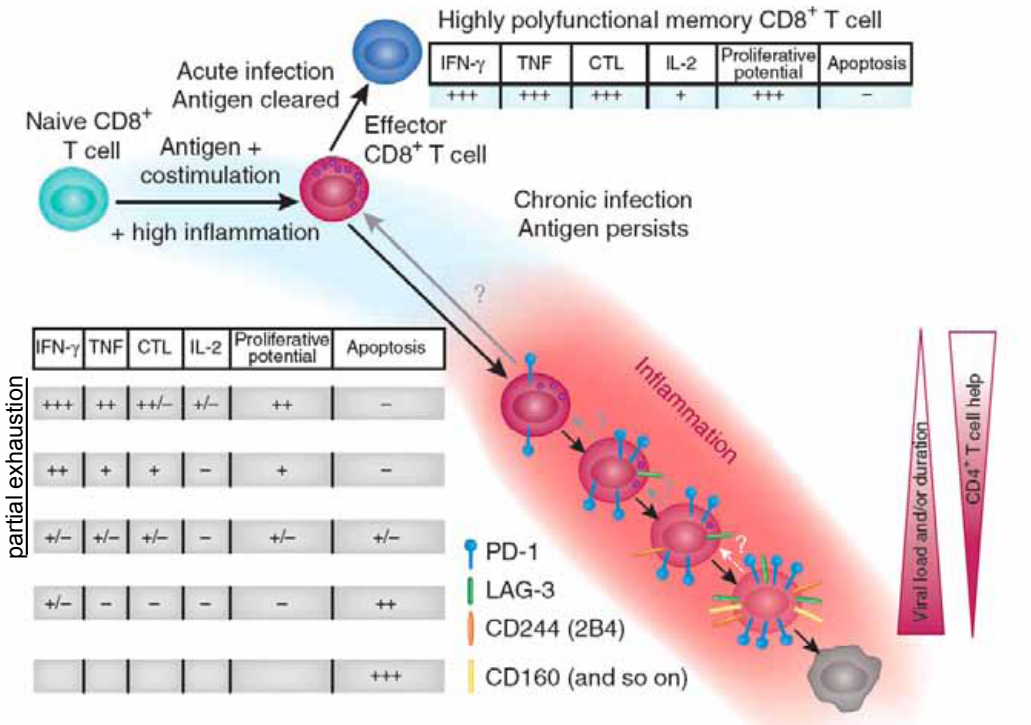


Figure 1.2. T cell exhaustion is a stepwise loss of function that occurs during persistent high viral load or loss of CD4 T cell help. Naive CD8 T cells are activated by antigen, costimulation and inflammatory cytokines. **Top.** During an acute infection CD8 T cells are fully functional, wherein they have high proliferation potential, are cytotoxic and can produce several cytokines. **Bottom.** However, during a chronic infection, where cells are exposed to persistent antigen, there is a stepwise loss of function as cells become exhausted. Cells first lose proliferation potential and cytotoxicity, followed by the loss of production of IL-2, TNFα and finally IFNγ. Cells that have only lost part of their function are considered partially exhausted. Exhausted cells express several markers, including PD-1, LAG-3, CD244(2B4) and CD160. In additional to high viral load, exhaustion is also induced by the loss of CD4 T cell help. Modified from Wherry EJ. T cell Exhaustion. Nature Immunology 12: 492-499 (2011). Copyright license number 3196040142324.

(Freeman et al., 2006; Wherry, 2011). As clonal exhaustion progresses cells lose the ability to produce **TNFα then IFNγ** (Freeman et al., 2006; Wherry, 2011). The final stage of clonal exhaustion is deletion of virus-specific cells through **apoptosis** (Freeman et al., 2006; Wherry, 2011). The extent of clonal exhaustion has been linked to high viral load, high epitope presentation and the lack of CD4 help (Battegay et al., 1994; Matloubian et al., 1994). More recently clonal exhaustion has been correlated with NK cell killing of activated CD4 T cells (Waggoner et al., 2012).

During an acute viral infection the CD8 T cell activation phase is followed by a negative regulation phase where activation signals are dampened to prevent over activation. Cytotoxic T lymphocyte associated protein 4 (CTLA-4) is upregulated on the surface of the activated T cells and binds to the costimulatory molecules CD80 and CD86 with higher affinity then CD28 (Chambers, 2001). This blocks the costimulation through CD28 and dampens downstream signaling through blocking AKT activation, the sernine threonine kinase downstream of phosphatidylinositol 3 kinase (PI3K), which is activated during costimulation (Keir et al., 2008). Another member of the CD28 family, programmed death (PD) 1 is also expressed on activated T cells and has been extensively studied within the LCMV system (Riley, 2009; Wherry, 2011). PD1 interacts with programmed death ligand (PDL) 1 or 2 and decreases CD28 costimulation through inhibiting PI3K activity, thereby preventing downstream signaling (Keir et al., 2008). Soluble inhibitory factors, such as IL-10, are expressed after the activation phase

and suppress the over activation of the T cell response in LCMV infection (Brooks et al., 2010; Ng and Oldstone, 2012). After an acute LCMV infection there is a burst of IL-10 at day 1 post infection, but during a persistent LCMV clone 13 infection, which induces clonal exhaustion, this transient burst is following by persistent IL-10 production (Brooks et al., 2010). Similarly, PD-1 expression of virus-specific CD8 T cells peaks at day 6 of an acute LCMV infection, but remains high on these cells during a persistent LCMV clone 13 infection (Barber et al., 2006). Therefore, during clonal exhaustion negative regulatory cell surface markers are used to identify cells that are at some stage of clonal exhaustion prior to apoptosis (**Figure 1.2**) (Wherry, 2011). Blocking one or more of these negative regulators after exhaustion has been shown to lead to recovery of function (Blackburn et al., 2009; Brooks et al., 2008a; 2008b; Nakamoto et al., 2009). For example one study blocked PD-1 signaling by administering a blocking antibody for PD-L1 into mice persistently infected with LCMV clone 13 and found an increase in both the cytokine production and proliferation of exhausted virus-specific CD8 T cells leading decreased viral load in several organs (Barber et al., 2006). Interestingly, when mice are deficient in PD-L1 they have a more functional CD8 T cell response after high dose LCMV clone 13 infection, as measured by IFNγ production on a per cell basis, but die between day 6-8 post infection (Barber et al., 2006). These studies suggest that clonal exhaustion is a mechanism of survival. This reversal of the clonal exhaustion phenotype could be further enhanced if anti-PD-L1 treatment was

given concurrently with a blocking antibody for lymphocyte activation gene-3 (LAG3), another inhibitory maker associated with clonal exhaustion (Blackburn et al., 2009). Recently two studies found that the blockade of type I IFN would also result in viral clearance of mice persistently infected with LCMV clone 13 (Teijaro et al., 2013; Wilson et al., 2013). Type I IFN signaling caused the negative regulatory environment as blocking type I IFN receptor signaling reduced IL-10 production and the expression of PD-L1 (Teijaro et al., 2013; Wilson et al., 2013).

In addition to signaling molecules, depletion of some cell types has also been found to induce clonal exhaustion or restore function to CD8 T cells (Battegay et al., 1994; Matloubian et al., 1994; Waggoner et al., 2012). Transient depletion of CD4 T cells at the time of infection with LCMV clone 13 resulted in life long infection, while non-depleted mice cleared virus after three months (Matloubian et al., 1994). Furthermore, the rapidly replicating WE strain of LCMV induced persistent infection at a 100-fold lower infecting dose in mice deficient of CD4 T cells compared to wildtype controls (Battegay et al., 1994), indicating a clear role for CD4 help in virus-specific CD8 T cell response during infection with viruses that can induce clonal exhaustion. More recently the depletion of NK cells was found to prevent clonal exhaustion of CD8 T cells during high dose LCMV clone 13 infection (Lang et al., 2012; Waggoner et al., 2012). This discovery led to the conclusion that NK cells can control the developing T cell response, both through the lysis of antigen presenting cells and the killing of activated CD4 T cells that are required to provide help to CD8 T cells (Waggoner et al., 2012).

T cell receptor repertoire and crossreactivity

One of the hallmarks of the immune system as a whole, but also the T cell pool, is an ability to respond to a wide range of pathogens. For T cells this ability to recognize a range of pathogens is dependent on the diversity of the TCR repertoire. The TCR interacts with short peptides, both pathogen and self-derived, presented on the cell surface in the context of MHC molecules. In the thymus imprecise somatic recombination of germline variable (V), diversity (D) and junctional (J) segments of the tcrβ locus and VJ recombination of tcrα locus, along with random pairing of the α and β subunits of the TCR significantly enhances diversity (**Figure 1.3**) (Nikolich-Zugich et al., 2004). The complementarity-determining regions (CDR) of the TCR account for the majority of the diversity, as these regions interact with antigen (Rock et al., 1994a). CDR 1 and 2 are located in the V segments of the TCR α and β subunits (Pannetier et al., 1993). The CDR3 is the most diverse as it overlaps the N terminal of the V segment and the C terminal of the J segment. In addition to the germline VDJ segments there is lack of precise joining between segments and non-template-encoded nucleotides (N) that are inserted in between these segments, highly diversifying the TCR and the CDR3 region (**Figure 1.3**) (Cabaniols et al., 2001).

Figure 1.3

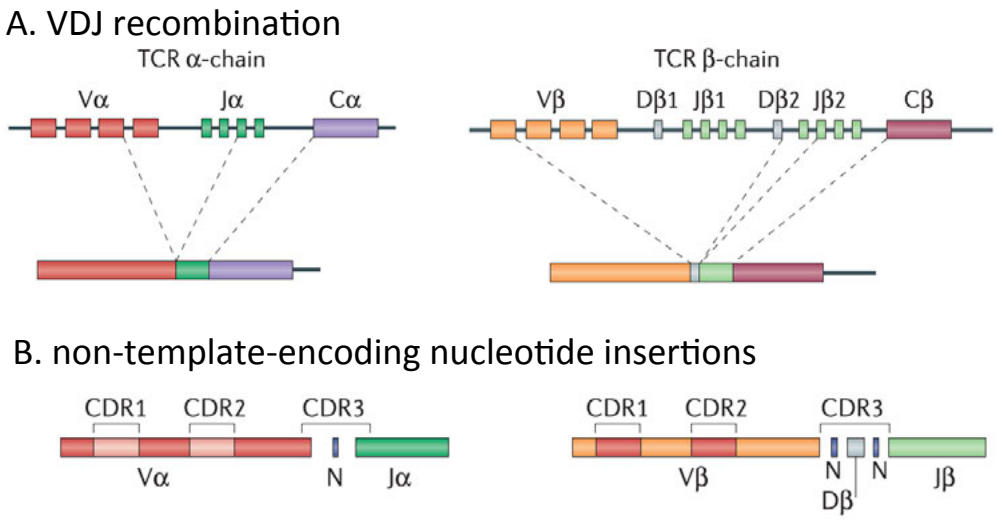


Figure 1.3. Generation of T cell receptor (TCR) diversity during VDJ rearrangement and non-template-encoding nucleotide insertions. A. During T cell development the TCR α- and β-chains undergo somatic recombination of the VJ segments in the α-chain and VDJ segments of the β-chain. **B.** There are three complementarity-determining regions (CDRs) or hypervariable regions in both the TCR α- and β-chains. The CDR1 and CDR2 regions are encoded within the V regions. The CDR3 regions is composed of the juxtaposition of the VJ segments in the α-chain and VDJ segments of the β-chain. WIthin the CDR3, insertion of non-template-encoding nucleotides between these segments further increases TCR diversity. Figure modified from Turner SJ et al. 2006. Structural determinants of T cell receptor bias in immunity. Nature Reviews Immunology. 6: 883-895 (2006). Copyright license number 3196040771397.

The number of antigens that the TCR repertoire of a single individual can recognize is further increased by crossreactivity of the TCR, or the ability to recognize multiple antigens. The TCR can interact with different peptides presented in MHC molecules. The number of antigens a single TCR can recognize has been estimated up to 10^6 (Mason, 1998). TCR-peptide interactions are usually dependent on a few key amino acids (**Figure 1.4**). A TCR may be able to recognize multiple antigens containing these same key amino acids. This is known as molecular mimicry and is dependent on sequence similarity (**Figure 1.4**) (Wilson et al., 2004). Crossreactivity is not always dependent on homologous peptide sequences and can be due to the 3-dimensional structure of the peptide this is known as alternative recognition (**Figure 1.4**). One major limitation to the diversity of the TCR repertoire is the spatial capacity of individual humans and mice. Even after thymic selection the theoretical estimate of possible TCR rearrangements results in diversity being 10^{13} different TCR, but the realistic estimates are 10^6-10^7 in mice and 10^7-10^8 in humans (Arstila et al., 1999; Casrouge et al., 2000; Nikolich-Zugich et al., 2004).

T cell responses and Heterologous immunity

Memory T cells can be crossreactive and recognize peptides derived from unrelated pathogens or cancers (Nilges et al., 2003; Urbani et al., 2005). Crossreactive immune responses can alter heterologous infection a phenomenon

Figure 1.4

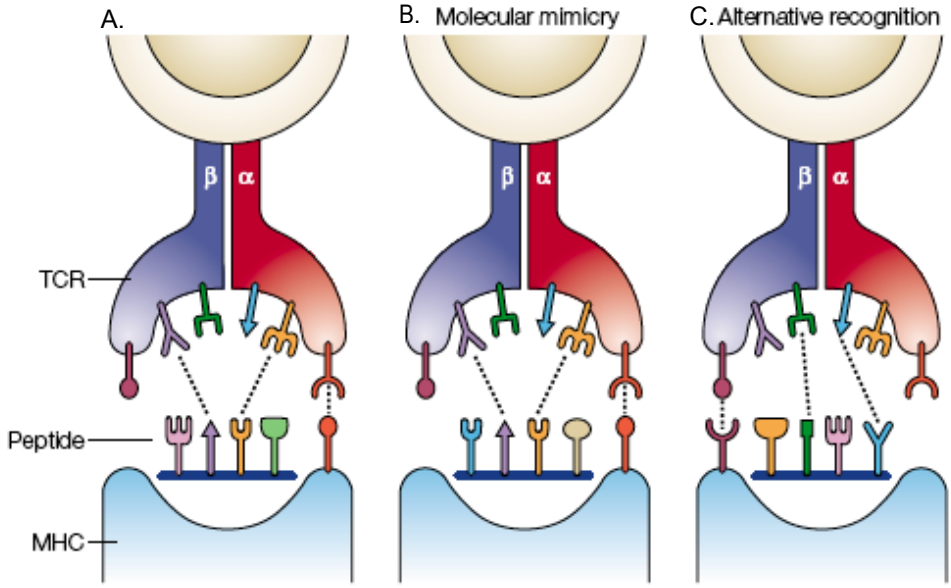


Figure 1.4. Possible mechanisms for T cell receptor crossreactivity. A. TCR interacts with peptide in the context of MHC class I. **B.** Crossreactivity occurs when peptide epitopes that share amino acids make contacts with the same TCR. **C.** However, alternative recognition is when other parts of the TCR interact with a different epitope and are not dependent of sequence similarity. Image taken from Welsh RM and Selin LK. No one is naïve: Significance of heterologous T cell immunity. 2002. Nature Reviews Immunology. 6:417-426 (2002). Copyright license number 3196040973332.

termed heterologous immunity (Welsh and Selin, 2002; Welsh et al., 2010). Heterologous immunity can be either beneficial or detrimental to the host (Chen et al., 2012; 2001; 2003; Cornberg et al., 2010; Nie et al., 2010; Selin et al., 1998; Wlodarczyk et al., 2013). For example, LCMV-immune mice have lower VV loads after challenge than naïve mice, although not as low as VV-immune mice. Unlike naïve mice, some LCMV-immune mice develop fat pad pathology or AFN during VV challenge (Selin et al., 1998), but since they are protected from a lethal VV challenge (Chen et al., 2001) this is considered beneficial heterologous immunity. On the other hand, detrimental heterologous immunity can occur if the crossreactive T cell response is not efficient at clearing the unrelated pathogen infection (Chen et al., 2003; Wlodarczyk et al., 2013). IAV-immune mice had higher viral loads than naïve mice after challenge with either LCMV or murine cytomegalovirus (MCMV) and developed more severe lung pathology (Chen et al., 2003; Wlodarczyk et al., 2013). In this case the virus lingered in the peripheral tissues allowing for increased infiltration of activated crossreactive T cells, thus causing immunopathology. This enhanced severity of pathology and protracted viral clearance is collectively known as detrimental heterologous immunity (Chen et al., 2003; Wlodarczyk et al., 2013).

Both beneficial and detrimental forms of heterologous immunity have been found between pathogens in both humans and mice. For example, IAV-specific CD8 T cells were found to recognize and be reactivated in response to HCV and Epstein-Barr virus (EBV) infections (Clute et al., 2005; Urbani et al., 2005). HLA-

A2 patients with EBV-induced acute infectious mononucleosis (AIM) have enhanced crossreactive IAV-M1-specific CD8 T cell responses compared to healthy donors (Clute et al., 2005). This suggests that IAV-specific memory cells can be reactivated upon EBV infection and induce an inappropriate CD8 T cell response that mediated severe pathology. Indeed the severity of disease in AIM patients correlated with the frequency of cells that were crossreactive between IAV-M1 and EBV-BMLF1 (Nuray Aslan, unpublished data). Interestingly, this same epitope crossreactivity between IAV and EBV was also found to be protective against EBV-infection in EBV-seronegative middle-aged adults (Levi Watkin, unpublished data). IAV M1-specific CD8 T cells isolated from EBV-seronegative donors expanded and produced cytokine in response to EBV peptides. These crossreactive IAV M1-specific cells could also kill EBV-infected targets suggesting that they could protect from EBV infection. These crossreactive IAV-M1-specific memory cells in EBV-seronegative donors differed from similar cells in EBV-seropositive individuals in the characteristics of their TCR repertoire. CDR3 region sequences from the crossreactive cells isolated from EBV-seronegative donors were highly oligoclonal, with a limited number of unique clones compared to EBV-seropositive donors (Levi Watkin, unpublished data). Crossreactive responses have also been found between different serotypes of Dengue virus (Beaumier and Rothman, 2009; Friberg et al., 2011a; 2011b; Kurane et al., 1989) and may be a factor in the increased severity of

disease, including dengue hemorrhagic fever, upon reinfection with a different serotype.

In mice cross-reactive responses have been identified between several pathogens including, LCMV and PICV, LCMV and VV, PICV and VV, IAV and LCMV, West Nile virus and Japanese Encephalitis virus and Bacillus Calmette-Guerin (BCG) and VV (Brehm et al., 2002; Chen et al., 2012; 2003; Cornberg et al., 2007; 2006; Kim et al., 2002; Mathurin et al., 2009; Trobaugh et al., 2010). The crossreactive epitopes and sequences of LCMV, PICV, VV and IAV are summarized in **Figure 1.5**. The LCMV and PICV and LCMV and VV models will be explained in more detail below, but both are models of beneficial heterologous immunity and are utilized in this thesis.

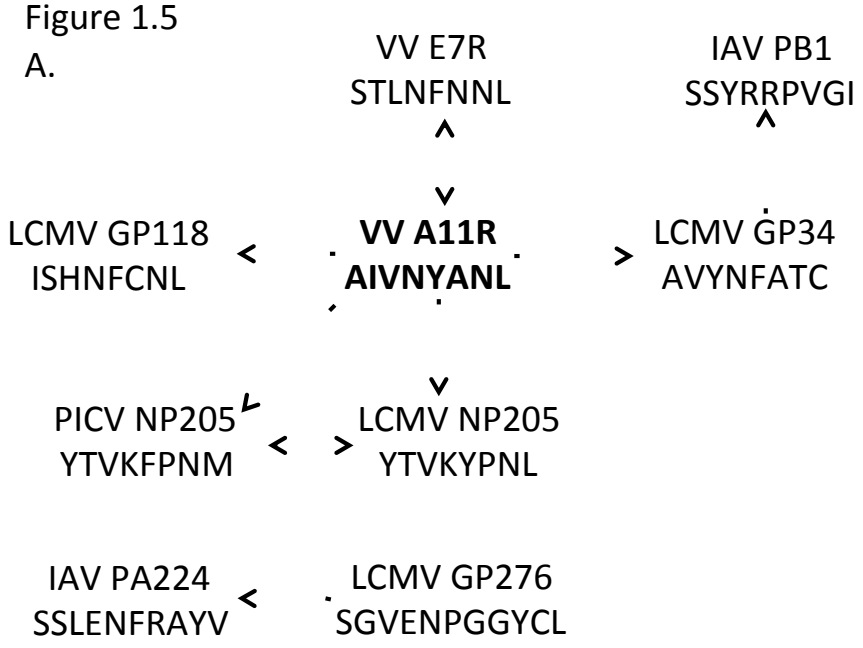
Private Specificity and heterologous immunity

Genetically identical naïve mice will all have the same, highly predictable virus-specific T cell immunodominance hierarchy to the different virus epitopes, a phenomenon known as **public specificity** (Cibotti et al., 1994). LCMV-immune mice all develop the same CD8 T cell immunodominance hierarchies, but within these polyclonal epitope-specific populations of individual mice there are different TCR, the Vβ repertoire and the CDR3 sequences of the epitope-specific responses are unique to individual hosts, which is known as **private specificity** (Cibotti et al., 1994). These differences in the TCR repertoire between genetically

identical mice arise during T cell development due to VDJ recombination, TCR α and β chain pairing and the insertion of noncoding nucleotides into the junctions between the VDJ segments (Nikolich-Zugich et al., 2004). This TCR variability, along with the stochastic events that occur during positive thymic selection and randomness of which T cell interacts with antigen first during an infection, control the private specificity of the TCR repertoire. The private specificity of an individual's TCR repertoire mediates crossreactive expansion during heterologous challenge (**Figure 1.5**) (Kim et al., 2005). In LCMV-immune mice challenged with VV there are multiple LCMV-specific memory responses that can be crossreactive and expand to skew the LCMV-specific immunodominance hierarchy (Kim et al., 2005). To elucidate the role of TCR repertoire in the unique expansion of different crossreactive responses in individual LCMV-immune mice challenged with VV one study transferred LCMV-immune splenocytes from a single donor into three naïve recipients, which were all subsequently challenged with VV (Kim et al., 2005). The donor populations in all three recipients showed expansion of the same crossreactive LCMV memory response, but differed depending on the donor, indicating that the private specificity of an individual's TCR repertoire controls crossreactivity (Kim et al., 2005). Private specificity was also found to control immunopathology in this model of heterologous immunity (Nie et al., 2010). Using a similar transfer model, this study examined the development of AFN, or fat pad immunopathology, after VV challenge

Figure 1.5

A.



B.

EPITOPE	SEQUENCE								% SIMILARITY
VV A11R	**A**	**I**	**V**	**N**	**Y**	**A**	**N**	**L**	
LCMV GP118	I	S	H	N	F	C	N	L	37.50%
LCMV GP34	A	V	Y	N	F	A	T	C	37.50%
LCMV NP205	Y	T	V	K	Y	P	N	L	50%
PICV NP205	Y	T	V	K	F	P	N	M	25%
VV E7R	S	T	L	N	F	N	N	L	37.50%
LCMV NP205	**Y**	**T**	**V**	**K**	**Y**	**P**	**N**	**L**	
PICV NP205	Y	T	V	K	F	P	N	M	75%

Figure 1.5. Crossreactivity patterns show a dynamic network of crossreactive responses in mice. A. A diagram of crossreactive CD8 T cell responses in LCMV, PICV, VV and IAV infection. Arrows indicate which memory epitope-specific CD8 T cells can be activated by which crossreactive epitopes. PICV NP205-, LCMV GP118-, GP34- and NP205-specific CD8 T cells can be activated by VV A11R. VV A11R can also crossreact with VV E7R in a reciprocal manner. LCMV NP205 can activate PICV NP205-specific CD8 T cells and vice versa. LCMV GP34 can be activated by IAV PB1 and LCMV GP276 can be activated by IAV PA224. B. Alignment of amino acid sequences of VVA11R and LCMV NP205 with their known crossreactive epitopes and the percentage of their sequence similarity.

(Nie et al., 2010). The level of severity of AFN between two recipients who received adoptive transfer of LCMV memory cells from one donor was found to be similar and controlled by the private specificity of the donor LCMV-immune splenocytes (Nie et al., 2010).

Neonatal Immune System

Neonates are highly susceptible to death from infection. Neonates lack protective memory and are still developing and undergoing a process of colonization of their skin and gut with microbial flora. Disparities in the innate, cellular and humoral arms of the immune system all play a role in the reduced immune responses of neonates to infection. To combat the susceptibility of neonates, three vaccines have been administered world-wide to newborns, including the HBV vaccine, BCG and the oral polio vaccines, in an effort to further protect this highly susceptible group (Sanchez-Schmitz and Levy, 2011). The immune system of a neonate is significantly different than that of an adult (**Figure 1.6**) making understanding how the neonatal immune system responds to vaccination or infection necessary to improve vaccines and treatment.

As their exposure to antigen in utero is limited neonates lack immunological memory and are more reliant on their innate immune responses (Krishnan et al., 2003). However, the innate immune system of the neonate is functionally distinct from that of an adult. For example, blood derived monocytes

Figure 1.6

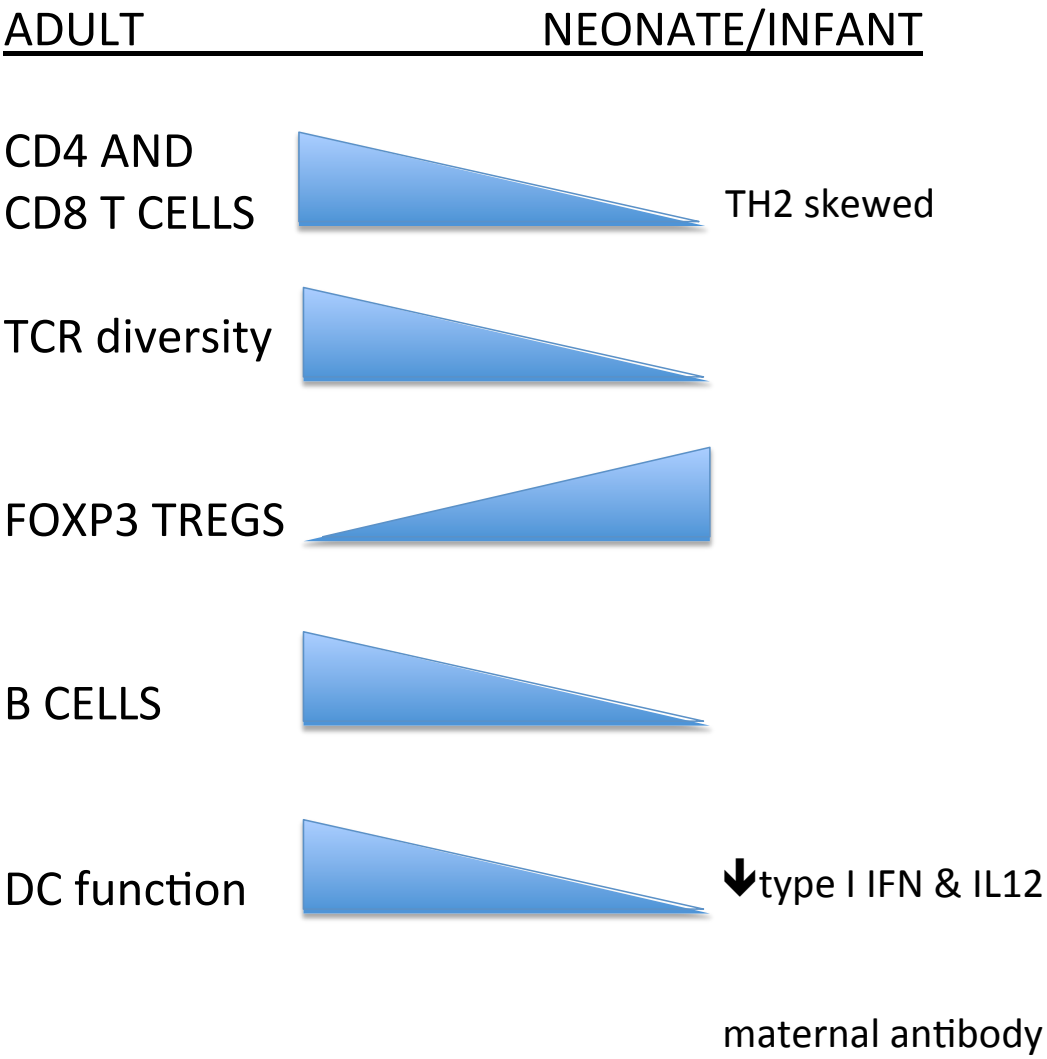


Figure 1.6. The immune system of neonates is immature. Neonates have decreased frequencies and total numbers of CD4 and CD8 T cells that are more Th2 prone compared with adults. The T cell receptor (TCR) diversity of neonates is limited in neonates and grows with age. Neonates have increased proportions of FoxP3+ T regulatory (Treg) cells and decreased B cell responses. Neonates have a less mature innate immune response, for example dendritic cells (DC) produce less type I IFN and IL-12 than adult DC. Neonates also have the unique feature of the presence of maternal antibody.

from human infants have reduced IFNα, IFNγ and IL-12 production upon TLR stimulation, but have an increase in IL-10 and Th17 inducing cytokines IL-6 and IL-23 (Kollmann et al., 2009). Compared to adults neonates were also found to have altered adaptive responses. Antibody responses are reduced, of shorter persistence with decreased affinity maturation and altered IgG isotype skewing (Siegrist and Aspinall, 2009). The T cell responses in neonates differ from that of adults by decreased number of T cells in the spleen (Ridge et al., 1996) frequencies of T cell subsets (Tang et al., 2008), and responsiveness to stimulation and TCR diversity (Rudd et al., 2011) resulting in altered T cell responses after infection. Additionally, neonatal T cells proliferate less, produce altered cytokine responses and have decreased cytotoxic activity under certain situations (Garcia et al., 2000). Neonatal T cells were thought to be unresponsive until 1996, when three seminal papers showed that neonatal T cells were more prone to Th2-skewing (Forsthuber et al., 1996; Ridge et al., 1996; Sarzotti et al., 1996). However, it is still not completely understood why this happens during some infections, but not others. For example, high doses of murine leukemia virus induced a Th2 response in neonatal mice, while low doses allowed for the induction of adult-like cytotoxic CD8 T cell and Th1-skewed CD4 T cell responses (Sarzotti et al., 1996). This may be partially due to the defectiveness of recent thymic emigrants (RTE) to develop Th1 responses (Haines et al., 2009; Opiela et al., 2009) and as RTE make up the majority of the peripheral T cells in neonates this may explain this Th2 bias. In utero FoxP3+ regulatory T cells

(Treg) are a dominating cell type suppressing immune responses to non-inherited maternal antigens (Tang et al., 2008). After birth this large proportion of Tregs may play a role suppressing a Th1 immune response.

Neonatal T cells also differ in the diversity of their TCR. During T cell development diversity is accrued in the TCR repertoire by VDJ recombination, αβ pairing of the TCR and noncoding nucleotide insertions and deletions in the CDR3 region of the TCR. These CDR3 insertions and deletions are mediated by the enzyme terminal deoxynucleotyl transferase (TdT), which is responsible for up to 90% of the TCR diversity (Cabaniols et al., 2001). In humans this enzyme is expressed in the thymus during the third trimester, but in mice it turned on between days 4-5 of age with increased CDR3 lengths not detected in single positive thymocytes until day 8 of age (Bogue et al., 1991). As mice age the CDR3 length of their TCR increases (Rudd et al., 2011). Shorter CDR3 regions have been associated with increased crossreactivity (Gavin and Bevan, 1995). T cell clones specific to IAV-NP366 were isolated from wildtype and TdT ko adult mice and assessed for their ability to lyse targets coated with a library of peptides. TdT ko clones were able to recognize a greater number of peptides, indicating that they are more crossreactive (Gavin and Bevan, 1995). This goes against what is found in adult studies with IAV M1-specific responses in HLA-A2 individuals, where increased alanine/glycine representation within the CDR3 loop is associated with increased flexibility and increased crossreactivity (Naumov et al., 2008). TdT ko T cells, with shorter CDR3 regions, can respond to more

antigens and this may be attributed to a greater interaction with the alpha chain of MHC class I instead of the peptide (Gavin and Bevan, 1995). The increased crossreactivity of the neonatal T cell repertoire may allow for smaller populations of T cells to protect against a wide range of pathogens (Gavin and Bevan, 1995). The limited TCR repertoire in mice lacking TdT also alters immunodominance hierarchies. TdT ko mice infected with the P8R strain of IAV or VV showed a shift in the immunodominance hierarchies (Haeryfar et al., 2008). Wildtype Balb/c mice infected with IAV have a dominant response to the epitope NP147, but in TdT ko mice NP147-specific cells are a subdominant response and HA518 becomes the dominant epitope (Haeryfar et al., 2008).

In the LCMV model the age of infection has been shown to alter disease outcome. Neonatal mice infected at day 1 of age become persistently infected for life due to the deletion of LCMV-specific CD8 T cells (Hotchin and Cinits, 1958; Volkert and Larsen, 1964). Only one LCMV study has examined the development of T cell responses in more mature infant mice (day 14 of age) (Belnoue et al., 2007). This study found that infant Balb/c mice cleared LCMV WE by 3 weeks post infection in the spleen and 4 weeks in the kidney and brain. Interestingly, viral clearance was found to be B cell-dependent as μMT ko mice, which lack B cells were persistently infected (Belnoue et al., 2007)

LCMV and Immune response to LCMV

LCMV is an old world arenavirus. All arenaviruses are enveloped with a bisegmented RNA ambisense genome. The natural host of LCMV is the house mouse (Mus domesticus, Mus musculus), but ~5% of humans are seropostitive for LCMV and is known to cause severe meningoencephalitis in humans (Armstrong and Lillie, 1934; Jahrling et al. 1992). Over a 17 year span, from 1941 to 1958, LCMV was thought to contribute to ~10% of patients with aseptic meningitis (Meyer et al., 1960), showing that LCMV is a relevant human pathogen. LCMV uses the α-dystroglycan receptor to gain entry into cells (2010). There are several research strains of LCMV. The ones used in this thesis include LCMV Armstrong and clone 13. LCMV Armstrong is a polyclonal virus isolated from a monkey infected with an isolate from a woman who was thought to have St. Louis encephalitis in 1933 (Armstrong and Lillie, 1934; Welsh and Seedhom, 2008). LCMV clone 13 is a clonal strain isolated from a mouse persistently infected with LCMV Armstrong (Ahmed et al., 1984) and has three amino acids distinct from the Armstrong strain (Matloubian et al., 1990; Salvato et al., 1991). Two of these mutations enhance the affinity of the LCMV clone 13 for the α-distroglycan receptor allowing it to systemically infect mice and the other mutation is in the RNA polymerase and allows for faster viral replication (Matloubian et al., 1990; Salvato et al., 1991).

When B6 mice are infected with LCMV intraperitoneally (ip) there is a strong induction of type I IFN that peaks day 2-3, which correlates with NK cell activation (Biron et al., 2002). In mice infected with the Armstrong strain of LCMV,

Table 1. Mouse T cell epitopes

LCMV CD8 T cell epitopes		
GP33-41	KAVYNFATC	Db
GP34-41	AVYNFATC	Kb
GP92-101	CSANNSHHYI	Db
GP118-125	ISHNFCNL	Kb
GP276-286	SGVENPGGYCL	Db
NP205-212	YTVKYPNL	Kb
NP396-404	FQPQNGQFI	Db
LCMV CD4 T cell epitopes		
GP61-80	GLKGPDIYKGVYQFKSVEFD	IAb
NP309-328	SGEGWPYIACRTSIVGRAWE	IAb

PICV CD8 T cell epitopes		
NP38-45	SALDFHKV	Kb
NP122-132	VYEGNLTNTQL	Db
NP205-212	YTVKFPNM	Kb
VV CD8 T cell epitopes		
A11r198-206	AIVNYANL	Kb
B8R20-27	TSYKFESV	Kb
E7r130-137	STLNFNNL	Kb
A47L138-146	AAFEFINSL	Kb

virus is cleared from all organs by day 8 and the CD8 T cell response peaks between day 8 and 9 followed by the peak of the CD4 T cell response between day 9 and 11 (De Boer et al., 2003; Varga and Welsh, 1998; Wherry et al., 2003a). The LCMV-specific CD8 T cell response in naïve adult B6 mice is predictable among individuals and maintained throughout the life span of the mouse (Ahmed and Gray, 1996; Homann et al., 2001; Tough and Sprent, 1994; Zimmerman et al., 1996). There are two co-dominant responses against the LCMV epitopes NP396 and GP33/34. The epitope GP276 induces an intermediate dominance and there are 24 subdominant responses (Kotturi et al., 2007). In this thesis I am only interested in the subdominant responses to NP205, GP118 and GP92, which have been shown to be crossreactive with VV A11R (**table 1; Figure 1.5**) (Cornberg et al., 2007; Kim et al., 2005). There is no difference in the kinetics of viral clearance in mice deficient of B cells, suggesting that antibodies play little role in a primary infection (Cerny et al., 1988). The neutralizing antibody response for LCMV is generated later in infection between 1 and 3 months and mediates faster viral clearance during a secondary challenge (Battegay et al., 1993).

LCMV contains epitopes that are known to be crossreactive with PICV, VV and IAV (**Figure 1.5**) (Brehm et al., 2002; Chen et al., 2001; 2003; Cornberg et al., 2006; 2007; Kim et al., 2002; Wlodarczyk et al., 2013). In this thesis LCMV is used in every model of infection to study how the balance of viral load and the efficiency of the immune response determines immunopathology.

PICV and PICV immune response

Pichinde virus (PICV) is also an arenavirus, but as a new world arenavirus it is distantly related to LCMV with only 48% homology (calculated using William Pearson's Lalign program, see materials and methods). It was isolated from the Tomes's rice rat (Oryomys albigularis) and is not associated with human disease, but seroconversion has occurred in humans. New world arenaviruses are divided into three clades: A, B and C (Bowen et al., 1997). Clade C is similar to old world arenaviruses in that it also uses the alpha-dystroglycan receptor to enter cells (Spiropoulou et al., 2002). Clade B, which contains numerous hemorrhagic viruses, uses the transferrin receptor to enter cells (Flanagan et al., 2008). However, at this time the receptor for Clade A new world arenaviruses, including PICV, is unknown. The AN3739 strain of PICV was utilized for this thesis. This strain of PICV does not cause mortality in B6 mice even at high doses. Similar to LCMV, PICV is a strong type I IFN inducer (Marshall et al., 2010). The CD8 T cell immunodominance hierarchy consists of one dominant response NP38, and two subdominant responses NP122 and NP205 (Brehm et al., 2002) (**Table 1**). Typically PICV needs to be administered at a dose 400-fold higher than LCMV (10^7 PFU) to induce a cytolytic CD8 T cell response (Selin et al., 1994). There are no neutralizing antibodies to PICV and no known CD4 T cell epitopes.

PICV is known to be crossreactive with LCMV and VV (Chen et al., 2012; Cornberg et al., 2006; Kim et al., 2005; Selin et al., 1994; 1996) (**Figure 1.5**). PICV is used with LCMV in this thesis to study the immunoprotection mediated by co-infection in the presence of a crossreactive response and to study neonatal immune responses and heterologous immunity.

VV and the immune response to VV

VV is a large DNA virus from the poxvirus family. The original host for VV is unknown. The receptor(s) for VV are also unknown, but due to its ability to infect multiple cell types and species it is thought that VV has multiple receptors. The work done in this thesis used the WR strain of VV. Unlike LCMV and PICV, VV is not an inducer of type I IFN. VV employs multiple mechanisms to block the ability of cells to produce IFN. For example the C6 protein of VV has been found to block type I IFN production by inhibiting the activation of the transcription factors IRF3 and IRF7 (Unterholzner et al., 2011). Typically VV is cleared by day 7 post infection and the T cell response peak at day 6 post infection. VV has several documented CD4 and CD8 T cell epitopes (Sette et al., 2009) (**table 1**). The major immunodominant CD8 T cell epitope is $B8R_{20\text{-}27}$ with several subdominant epitopes including $A47L_{138\text{-}146}$, $E7R_{130\text{-}137}$ and $A11R_{198\text{-}206}$, which will be examined in this thesis (**table 1**). VV induces a strong antibody response leading to a potent neutralizing antibody response.

Previous immunization with several different pathogens provides protection against VV challenge including LCMV, PICV, IAV, MCMV and BCG (**Figure 1.5**) (Chen et al., 2001; Cornberg et al., 2007; Mathurin et al., 2009; Selin et al., 1998). In this thesis VV is used to challenge LCMV-immune mice and examine how this model of beneficial heterologous immunity can be altered if mice are immunized as neonates.

LCMV and VV model of heterologous immunity

Previous immunization with LCMV protects mice from lethal VV challenge (Chen et al., 2001) and mediates a 10-300-fold reduction in VV load (Selin et al., 1998). Both LCMV memory CD4 and CD8 T cells are required in adoptive transfer studies to mediate protection from VV infection (Selin et al., 1998). LCMV-specific memory cells are reactivated and proliferate after VV infection (Kim et al., 2002; Selin et al., 1994). After VV infection there are several LCMV-specific memory responses that expand. The most common crossreactivities include NP205, GP34 and GP118 (**Figure 1.5**) (Chen et al., 2001; Cornberg et al., 2007; Kim et al., 2002; 2005). By using NP205 in a blast search against the genome with H2-k^b binding motifs the crossreactive VV-A11R epitope was identified (Cornberg et al., 2007). T cell lines from LCMV-immune splenocytes stimulated with VV-A11R peptide were found to be crossreactive with LCMV NP205, GP118 and GP34 (Cornberg et al., 2007). The most common

crossreactivities in the cell lines were to GP118 and GP34 (Cornberg et al., 2007). Which crossreactive memory LCMV-specific response is reactivated upon VV infection is determined by the unique TCR repertoire, or private specificity of the individual mouse. After VV challenge, hosts that received LCMV-immune splenocytes from the same donor had an expansion of the same epitope-specific crossreactive response (Kim et al., 2005).

In addition to the immunoprotection mediated by this crossreactivity, some LCMV+VV mice develop enhanced immunopathology compared to naïve mice infected with VV. After an intraperitoneal VV challenge LCMV-immune mice develop AFN of the abdominal/epididymal fat pads, similar to the human panniculitis condition erythema nodosum (**Figure 1.7**) (Selin et al., 1998). In an analogous fashion, if VV is given intranasally LCMV-immune mice develop bronchus-associated lymphoid tissue (BALT) and bronchiolitis obliterans (Chen et al., 2003). Both erythema nodosum and bronchiolitis obliterans occur in humans of unknown etiology, but have been associated with viral infections and vaccinations. For example, erythema nodosum has been associated with HBV infection (Rogerson and Nye, 1990).

LCMV memory T cells that mediate protective immunity after VV challenge are also responsible for the enhanced immunopathology. Transfer of T cells from LCMV-immune donors into naive hosts resulted in lower viral titers, but also increased AFN compared to naive hosts that received naive donor cells (Selin et al., 1998). Furthermore, there was no correlation between the VV load in a

Figure 1.7

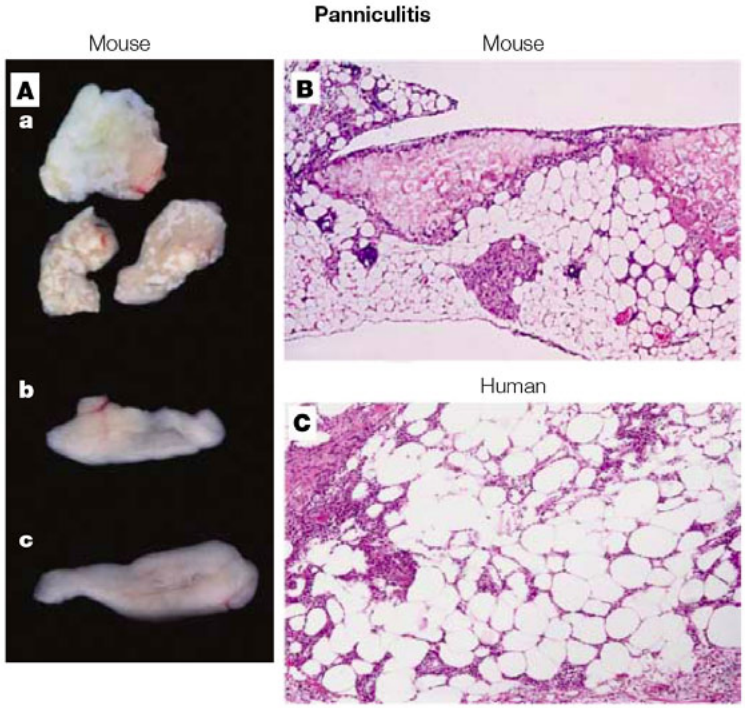


Figure 1.7. Fat pathology comparison in both humans and mice. A. Epididymal fat pads from (a.) LCMV-immune mice on day 5 post VV challenge show acute fatty necrosis (AFN). Fat pads from (b.) naïve mice on day 5 post VV infection and (c.) LCMV-immune mice are healthy showing no necrosis. **B.** Histology of epididymal fat pad from an LCMV-immune mice challenged with VV with large areas of necrosis and mononuclear infiltrates, or panniculitis. **C.** Histology of human skin samples of patients with erythema nodosum, a form of panniculitis, with similar histological features (Courtesy of Bruce Smoller). Image taken from Welsh RM and Selin LK. No one is naïve: Significance of heterologous T cell immunity. 2002. Nature Reviews Immunology. 6:417-426 (2002). Copyright license number 3196040973332.

mouse and the level of AFN (Nie et al., 2010). AFN was found to be mediated by IFNγ- and TNFα-dependent upregulation of Fas on adipose cells, which interacted with FasL on the surface of activated crossreactive CD8 T cells and caused necrosis of the fat pads (Siwei Nie, unpublished data). The severity of AFN in an individual LCMV-immune mouse challenged with VV was found to be dependent on the crossreactive TCR repertoire of that individual due to private specificity (Nie et al., 2010). LCMV-immune splenocytes from a single donor transferred into two naive hosts resulted in a similar severity of AFN in both hosts after VV challenge (Nie et al., 2010). This model of heterologous immunity was used to examine if immunization with LCMV as a neonate would induce the same protective crossreactive responses after VV challenge as mice immunized as adults.

LCMV and PICV model of heterologous immunity

LCMV-immune adult mice challenged with PICV had immunoprotection compared to naive mice challenged with PICV with a 1-log reduction in splenic viral titer (Selin et al., 1998). This was also true for the reverse, PICV-immune mice challenged with LCMV, but there was a more modest reduction of only 0.5 logs PICV in the spleen (Selin et al., 1998). In this model of heterologous immunity there is only one cross-reactive epitope, NP205 (**Figure 1.5**) (Brehm et al., 2002; Chen et al., 2012). LCMV and PICV share six out of the eight amino

acids in this epitope (**Figure 1.5**) (Brehm et al., 2002). After a primary infection of either LCMV or PICV, NP205 was a subdominant response, but after the heterologous viral challenge the NP205-specific response expanded and became the immunodominant CD8 T cell response in some mice (Brehm et al., 2002). Similar to the LCMV+VV model, transfer of LCMV-immune splenocytes into naïve hosts mediates a reduction of PICV viral load in the spleen and fat pad (Selin et al., 1998). However, depletion of CD4 or CD8 T cells before transfer ablates any protection (Selin et al., 1998). Using a mutant strain of LCMV Armstrong, which has a leucine to alanine mutation at position 212 that prevents the presentation of the NP205 epitope (L212A variant), there is no longer immunoprotection after PICV challenge in either the fat pad or the spleen (Chen et al., 2012).

The expansion of the crossreactive NP205-specific response during sequential infection skewed the immunodominance hierarchies for both LCMV and PICV, with NP205-specific T cells dominating in most mice (Brehm et al., 2002). Within the NP205-specific CD8 T cell population in sequentially infected mice there was also narrowing of the Vβ repertoire (Cornberg et al., 2006). In LCMV-immune mice the majority of NP205-specific cells use Vβ 16. While Vβ 16 is also present in the NP205-specific population after PICV infection, it is co-dominant with Vβ 5.1/5.2 (Cornberg et al., 2006). After a homologous challenge, there is a slight narrowing of the repertoire, but after a heterologous challenge there is significant narrowing of the repertoire and alteration of Vβ usage. For

example, PICV-immune mice challenged with LCMV can skew to Vβ 5.1/5.2, 16, 7 or 12 (Cornberg et al., 2006).

This skewing of the NP205 Vβ repertoire in PICV+LCMV-immune mice permanently alters the memory repertoire and has detrimental effects during a subsequent PICV rechallenge (PICV+LCMV+PICV) (Chen et al., 2012). PICV+LCMV-immune mice challenged with PICV have a higher probability of developing AFN (Chen et al., 2012). During an acute PICV infection 0 out of 25 mice developed AFN and only 2 out of 10 PICV-immune mice developed AFN during PICV rechallenge. However, 74% (17/23) of PICV+LCMV-immune mice got AFN when they were re-exposed to PICV (Chen et al., 2012). This enhanced disease severity is attributed to the crossreactivity of the NP205 epitope between LCMV and PICV (Chen et al., 2012). If PICV-immune mice are challenged with the L212A mutant virus, which lacks the NP205 response, and then rechallenged with PICV (PICV+L212A+PICV) the incidence of AFN drops significantly to 34% (9/24). This suggests that beneficial crossreactivities can become detrimental depending on the context of the infection.

A variation of this model was used to study the effects of simultaneous co-infection rather than sequential infection with these two viruses containing a crossreactive epitope on immunoprotection during a homologous rechallenge. Also, neonates were immunized with PICV and challenged with LCMV as adults to study the effect of the maturity of the immune response during infection on crossreactivity.

Thesis Objectives

After viral infection CD8 T cell responses are induced to clear the virus and maintained in memory to protect against reinfection. CD8 T cells employ many functions to decrease viral load including proinflammatory anti-viral cytokines, such as IFNγ and TNFα. They also employ several cytotoxic functions to kill virus-infected cells, such as FasL expression and the release of perforin and granzymes. Their ability to produce chemokines to recruit many types of immune cells and to make proinflammatory cytokines, as well as lyse infected cells makes CD8 T cells, if not controlled, capable of inducing severe immunopathology and death. Therefore, my hypothesis is that a balance exists between viral load and efficiency of the immune response, specifically the T cell response, which determines the disease outcome of the host after infection. If there is an inefficient immune response it would allow for virus to linger in peripheral tissues, which would recruit increased numbers of activated cytotoxic CD8 T cells, which would recruit other immune cells, such as neutrophils and monocytes and lead to increased immunopathology. In this thesis 4 models were examined where the efficiency of the immune response was predicted to be less than optimum: **1.** mice were co-infected with two viruses containing a crossreactive epitope, **2.** the inoculating dose of a virus that can induce clonal exhaustion was administered at different doses, **3.** neonatal mice, which have

immature immune systems, were immunized with arenaviruses and **4.** a beneficial model of heterologous immunity was examined in neonatal mice. My thesis is presented in five parts:

Chapter III: Co-infection alters CD8 T cell immunity and immunopathology

This chapter tests the hypothesis if the competition between the immune responses to two viruses, LCMV and PICV, which contain a crossreactive epitope, administered simultaneously will lead to alterations in the CD8 T cell response, and if these alterations can affect the disease outcome in viral clearance or immunopathology upon homologous challenged with either virus.

Chapter IV: Clonal exhaustion as a mechanism to protect against severe immunopathology and death from an overwhelming CD8 T cell response

In this chapter mice were infected with 3 different doses (low, intermediate and high) of LCMV clone 13, a virus that is known to induce clonal exhaustion when administered at a high dose. We will test the hypothesis whether decreasing the inoculating dose would change the clonal exhaustion or functional capacity of the CD8 T cells and impact the severity of immunopathology. In other words does clonal exhaustion act as a mechanism to prevent death from T cell-mediated immunopathology, with some rapidly replicating viruses.

Chapter V: T cell mediated mortality in neonatal mice infected with LCMV

This chapter tests the hypothesis whether an immature, Th2-skewed neonatal immune system can control infection with the arenavirus LCMV and by what mechanism.

Chapter VI: Heterologous immunity is a mechanism for protection in neonatal mice: LCMV+VV

This chapter tests the hypothesis that neonatal mice develop equivalent CD8 T cell crossreactivity and T cell-mediated immunoprotection in the LCMV+VV model of heterologous immunity as adult mice.

Chapter VII: Neonates control PICV infection and show CD8 T cell immunodominance hierarchy skewing after LCMV challenge

This chapter tests the hypothesis whether the immature immune system of neonatal mice can control infection with a less virulent arenavirus, PICV. Along wit the hypothesis that the immaturity of the neonatal immune system at the time of PICV infection may alter crossreactive responses in mice challenged with LCMV as adults.

Chapter 2: Methods

Mice

Male C57BL/6J (B6, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5-6 weeks of age. For neonatal studies females at 8-10 weeks of age were used for breeding. Neonates were used at the indicated ages. All mice were maintained under specific pathogen free conditions at the Department of Animal Medicine, University of Massachusetts Medical School. This study was done in compliance within the guidelines of our Institutional Animal Care and Use Committee.

Viruses

LCMV (Armstrong strain and clone 13 strain) and PICV (AN3739 strain) stocks were propagated in BHK21 baby hamster kidney cells (Selin et al., 1994). The L212A LCMV Armstrong variant was made using reverse genetics by Juan Carlos de la Torre (Chen et al., 2012). This alanine mutation at position 212 of the nucleoprotein disrupts MHC class I binding for the NP205 epitope and results in ablation of this CD8 T cell response. Vaccinia virus (WR stain) was propagated on NCTC-L929 or Hela cells (Selin et al., 1994). To control for culture contaminants supernatants from PICV-infected BHK or VV-infected NCTC-L929 were purified through a sucrose gradient and diluted in Hank's balanced salt solution (HBSS), and LCMV was diluted >40-fold in HBSS.

Infections

Adult mice 6-8 weeks old were immunized intraperitoneally (ip) with $5x10^4$ plaque-forming units (PFU) of LCMV Armstrong (wildtype or L212A variant) and/or $2x10^7$ PFU of PICV. At 6 weeks post-immunization mice were considered immune. Control mice were left uninoculated or inoculated with HBSS. Control mice were always age and sex matched to the experimental group and kept in the same specific pathogen free housing. Immune mice were challenged with either $2x10^6$ PFU LCMV clone 13 intravenously (iv), $2x10^7$ PFU of PICV ip or $1x10^6$ PFU of VV ip. For LCMV clone 13 infections mice were inoculated iv with low dose, $2x10^4$ PFU; medium dose, $2x10^5$ PFU; and high dose, $2x10^6$ PFU of LCMV clone 13. Neonatal mice were infected with 5, 50 or 500 PFU of LCMV Armstrong ip or $4x10^4$, $4x10^5$ or $4x10^6$ PFU of PICV ip.

Peptides

LCMV-specific peptides: NP396-404 (FQPQNGQFI; Db), GP33-41 (KAVYNFATC; Db), GP34-41 (AVYNFATC; Kb), GP276-286 (SGVENPGGYCL; Db), NP205-212 (YTVKYPNL; Kb) and GP118-125 (ISHNFCNL; Kb)

PICV-specific peptides NP38-45 (SALKFHKV; Kb) NP122-132 (VYEGNLTNTQL; Db) NP205-212 (YTVKFPNM; Kb)

VV-specific peptides: B8R20-27 (TSYKFESV; Kb), E7R130-137 (STLNFNNL; Kb), A47L138-146(AAFEFINSL; Kb) and A11R198-205 (AIVNYANL; Kb) (**Table 1.1**)

Peptides were at 90% purity by reverse phase-HPLC from 21st Century Biochemicals (Marlboro, MA).

CD4 and CD8 T cell depletion

Adult mice were injected ip on days -1 and 7 of LCMV clone 13 infection with 100μg of anti-CD8 antibody (clone 2.43). Neonates were injected with 50μg in 50μl of anti-CD4 (clone GK1.5) anti-CD8 (clone 2.43) antibodies or IgG control LFT2 on days 0, 4 and 8 of LCMV infection.

Viral titer

LCMV, PICV and VV viral titers were determined by plaque assay. Spleens, fat pads, kidneys, brains or lungs were homogenized in 1mL and livers were homogenized in 2mL in adult mice. In neonates liver, kidney and brain were homogenized in 0.5mL of complete RPMI media containing 10% heat-inactivated fetal bovine serum (FBS), 50units/mL penicillin and 50μg/mL streptomycin. Homogenized samples were spun at 2,000 rpm for 20 minutes and then supernatants were frozen down at -80°C. On the day of the plaque assay samples were thawed and serially diluted 1:10 in complete MEM containing 10% heat-inactivated FBS, 50units/mL penicillin and 50μg/mL streptomycin and 100μl of diluted sample was used to infect a monolayer of vero cells in 1mL of complete MEM in 6-well plates. Plates were incubated for 1.5 hours at 37°C and overlayed

with 4mL of 1:1 EMEM containing 10mL of heat-inactivated FBS, 25 units/mL penicillin and 50ug/mL streptomycin :1% agarose in water. Samples were stained at 48 hours for VV, 3 days for PICV and 4 days for LCMV with 2ml of 1:1 EMEM: 1% agarose in water and 0.015mg/ml of 1% Neutral red in PBS. Plaques were counted at 72 hours for VV, day 4 and 5 for PICV and day 5 and 6 for LCMV. To determine LCMV viral load in the co-infected mice, a plaque assay was performed as above for LCMV but stained with 1ml of 1% iodonitrotetrazolium chloride (INT) in PBS on day 4 after plaque assay. The chemical stains uninfected and PICV-infected cells, thereby allowing for specific identification and enumeration of LCMV plaques alone (Cooper, 1959; Logan et al., 1975). Viral loads are shown as log10 PFU/mL.

ALT assay

Alanine transferase (ALT) assay was purchased from D-Tek LLC (Bensalem, PA). 15µl of frozen serum was thawed and mixed with 150µl of reconstituted substrate and absorbance was read at 340nm at 0, 1, 2 and 3 minutes immediately afterwards. ALT concentration was calculated as the average change in absorbance/minute *1768. Data are shown in international units/liter.

Leukocyte Preparation

Peripheral blood (100-300µl) was treated with 3mL of 0.84% NH_4Cl and incubated for 10 minutes at 37°C. Blood was then washed with complete RPMI.

Spleens from mice were ground spleens between two pieces of fine mesh with the back of a syringe and strained through fine mesh. Samples were then treated with 2mL 0.84% NH_4Cl to lyse red blood cells. Mice were profused with 10mL of HBSS prior to isolation of brains to remove peripheral blood that may contaminate samples. Brains of mice were processed through mesh similar to spleens, spun down and resuspended in 30% percoll in RPMI. Samples were then underplayed with a 70% percoll in RPMI and spun at 500 g for 30 minutes at 18°C with no deceleration brake. Lymphocytes were collected from the 30/70 interface.

Intracellular cytokine staining

Single cell suspensions were prepared from peripheral blood, spleens or PEC from mice, counted, plated at $2x10^6$ cells/well and stimulated with 1μM of peptide along with golgi plug (brefeldin A) and recombinant human IL-2 for 4.5 hours at 37°C in complete RPMI. Cell were treated with Fc receptor blocking antibody (BD Pharmigen clone 24.G2) and stained for CD8 (BD Pharmigen clone 53-6.7), CD44 (biolegend clone IM7 and ebioscience clone IM7) and PD-1 (BD Pharmigen clone J43) in FACS buffer consisting of HBSS with 2% heat-inactivated FBS. Samples were then treated with cytofix/cytoperm and stained with IFNγ (BD Pharmigen clone XMG1.2) and TNFα (BD Pharmigen clone MP6-XT22) in PermWash (BD Pharmigen) diluted 1:10 in water. Samples were

collected on a LSRII (BD Pharmigen) and analyzed with FlowJo software (Tree Star Inc.).

Vβ staining

Vβ staining was done on both unstimulated cells or peptide stimulated cells after 4.5 hour incubation. After treatment with 24.G2 to block Fc receptors Vβ monoclonal antibodies were added to the panel of surface stains. In separate wells cells were stained for Vβ 2- PE (clone B20.6), Vβ 3-PE (clone KF25) and Vβ 5.1/2-FitC (clone MR9-4), Vβ 4-PE (KT4) and Vβ 9-FitC (MR1O-2), Vβ 6-PE (clone RR4-7) and Vβ 12-FitC (clone MR11-1), Vβ 7-PE (clone TR310) and Vβ 8.1/2-FitC (clone MR5-2), Vβ 8.3-PE (clone 1B3.3) and Vβ 13-FitC (clone MR12-3), Vβ 10b-PE (clone B21.5) and Vβ 14-FitC (clone 14-2), Vβ 11-PE (clone RR3-15) and Vβ 17a (clone KJ23). All Vβ antibodies were purchased through BD Biosciences.

Surface and tetramer staining

Single cell suspensions were prepared from peripheral blood or spleens from mice. Samples were then treated with 0.84% NH_4Cl to lyse red blood cells. Samples were treated with Fc receptor blocking antibody (BD pharmigen clone 24.G2) and then stained with PE labeled tetramers in 100µl of FACs buffer. Surface stains including KLRG1 (eBioscience clone 2F1), IL7R (eBioscience clone A7R34), CD62L (BD pharmigen and clone mel-14), CD44 (biolegend clone

IM7 and ebioscience clone IM7) and CD8 (BD Pharmigen clone 53-6.7) were then added. Samples were then treated with cytofix and samples were collected on a LSRII (BD Pharmigen) and analyzed with FlowJo software (Tree Star Inc.).

Cytometric bead array

Mouse inflammation cytometric bead array was purchased from BD Pharmingen. Briefly, 10µl of thawed serum was incubated with 10µl of capture bead mixture and 10µl of PE-detection reagent and incubated for 2 hours at room temperature. Samples were collected on a LSRII (BD Pharmigen) and analyzed with FCAP Array software (BD Pharmigen).

Type I IFN Bioassay

Serum was isolated from mice at 24, 48 and 72 hours post-infection. Serum was serially diluted and added to NCTC-L929 cells in a 96-well flat bottom plate. The following day $2x10^4$ PFU of VSV was added to each well. Two days after the addition of virus, the dilution of serum at which 50% of the cells were protected was determined.

AFN scoring

The level of acute fatty necrosis (AFN) was scored visually to assess disease severity on a scale from 0 to 8. Level 1 and 2 represent very mild to mild AFN with a few small necrotic spots on one or both abdominal fatpads; level 3 and 4

denote mildly moderate to moderate AFN with larger necrotic spots on one or both abdominal fatpads; levels 5 and 6 represent moderately severe to severe necrosis with very large patches; level 7 represents very severe AFN with extensive necrosis of the majority of the fat pads; level 8 represents moribund necrosis to which the animal can not survive.

Histology

Lungs, livers and fat pads from mice were collected, fixed in 10% neutral buffered formaldehyde and paraffin-embedded. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and analyzed microscopically by a pathologist.

Lung histology scoring

Scoring of lung pathology was graded based on a scale outlined below and based on previously published studies (Chen et al., 2003; Wlodarczyk et al., 2013). Scoring of lung pathology was graded by one pathologist, who was blinded in regards to treatments the mice had received. Lungs were scored on a scale of 0-5 (0, within normal limits; 1, mild interstitial mononuclear infiltrates, disorganized BALT, perivascular edema; 2, moderate interstitial mononuclear infiltrates, small amount of organized BALT, pulmonary edema; 3, moderate interstitial mononuclear infiltrates, pulmonary edema, enhanced organized BALT, mild consolidation; 4, severe interstitial mononuclear infiltrates, greatly enhanced

pulmonary edema, enhanced organized BALT, moderate consolidation and moderate necrotizing bronchiolitis; 5, severe interstitial mononuclear infiltrates, greatly enhanced pulmonary edema, enhanced organized BALT, severe consolidation, severe necrotizing bronchiolitis and vasculitis involving more than half of the lung.)

Fat pad histology scoring

Abdominal fat pads were fixed in 10% neutral buffered formaldehyde and paraffin embedded. Tissue sections were stained with H&E and analyzed microscopically. Fat pad pathology was blindly graded by a pathologist based on the distribution and severity of disease from 0 to 6 (0, within normal limit; 1, mononuclear infiltrate; 2, mononuclear infiltrate with small areas of necrosis; 3, greater than 10 areas of necrosis less than 1/25 of visual field; 4, areas of necrosis that are 1/10 of visual field; 5, areas of necrosis that are ½ of visual field; 6, areas of necrosis that are greater than ½ of visual field.)

Analysis of sequence similarity between viruses

The sequence similarity between measles and mumps viruses and LCMV and PICV were determined using the William Pearson's Lalign program (ch.EMBnet.org). Global alignments were done on the L and S segments for LCMV (L segment NC_004291 and S segment NC_004291) and PICV (L

segment NC_006439 and S segment NC_006447). Whole genome alignments were done for measles virus (NC_001498) and mumps virus (AB744048).

Statistics

Descriptive statistics are expressed as mean +/- standard error of the mean. Standard error of the mean is displayed in all figures where relevant unless stated differently in figure legends. Statistical analysis was done using the Student's T test when comparing two groups and ANOVA when comparing three or more. Mortality curves were analyzed with log rank test using Prism software (Graphpad software, La Jolla Ca). *p<0.05, **p<0.01,***p<0.001 ****p<0.0001

Chapter 3: Viral Co-infection alters CD8 T cell immunity and Immunopathology

Antiviral immunity is predominately studied in the context of infection with a single pathogen, although simultaneous infection with two or more microorganisms is a common occurrence. Simultaneous co-infections occur when pathogens share the same route of transmission, such as insect vectors or contaminated blood products. Multiple bites from virus-infected insect vectors (e.g. mosquitoes) can transmit a simultaneous co-infection. Additionally, mosquitoes can be co-infected and transmit multiple viruses (Chamberlain and Sudia, 1957; Lam and Marshall, 1968). During a 2006 Dengue virus outbreak in India, 19% of patients were co-infected with multiple serotypes of Dengue virus and a higher percentage of the patients with Dengue hemorrhagic fever were co-infected with multiple dengue virus strains (Bharaj et al., 2008). Another study found that 13% of patients admitted to the hospital during the 2009 H1N1 IAV pandemic were co-infected with at least one other respiratory virus (Esper et al., 2011). Interestingly, patients co-infected with IAV and coronavirus or respiratory syncytial virus had enhanced severity of symptoms over patients infected with only IAV (Esper et al., 2011; Palacios et al., 2009). Used hypodermic needles and contaminated blood products may also trigger co-infections when they harbor more than one virus. 90-95% of intravenous drug users infected with human immunodeficiency virus (HIV) are also infected with HCV (Maier and Wu,

2002), with some of these patients possibly contracting both viruses at the same time. HIV/HCV co-infection is associated with faster progression to HCV-mediated liver disease and increased risk of cirrhosis compared to patients infected with only HCV (Singal and Anand, 2009). Simultaneous co-infection with hepatitis B and D viruses, which is more common in intravenous drug users, is also more frequently associated with fulminant hepatitis compared to sequential infection (Grabowski and Wedemeyer, 2010). These studies indicate that co-infections can cause increased severity of disease and show the importance of understanding how co-infections differ from single virus infections.

Despite the apparent detriments afforded by co-infection with multiple pathogens, children are routinely exposed to multiple antigens simultaneously through combination vaccines and simultaneous administration of multiple vaccines. Typically, physicians and parents do not condone more than three injections within a single doctor's visit (Meyerhoff et al., 2003; Woodin et al., 1995). However, CDC protocols allow for children to receive up to 9 vaccine injections containing 13 different vaccines at their 12-15 month doctor's visit (Kroger et al., 2011). One major disadvantage of simultaneous vaccination is the possible occurrence of vaccine interference, where one vaccine dampens the response to a second vaccine (Vidor, 2007). For example, in Nigerian children the simultaneous administration of the measles vaccine with the smallpox, yellow fever and the combination diphtheria, pertussis and tetanus vaccines resulted in a 89% to 70% decrease in measles seroconversion rates (Ruben et al., 1973).

Due to the paucity of identified human T cell epitopes in the majority of vaccines it is not clear how concurrent administration of multiple vaccines may affect T cell immunity in humans. It is unknown if cellular immunity is reduced by vaccine interference or if there are other qualitative changes caused by co-infection, where two distinct immune responses are competing within the same host. Using a mouse model, where virus-derived T cell epitopes are better defined, we questioned how simultaneous co-infection would alter the developing virus-specific T cell responses.

In the current study, mice were co-infected with two distantly related non-cytolytic arenaviruses: LCMV and PICV. Protective immunity to LCMV and PICV is predominantly mediated by virus-specific T cell responses with limited contributions from neutralizing antibodies (Cerny et al., 1988; Walker et al., 1984). During sequential infections with LCMV and PICV there is protective heterologous immunity. (Brehm et al., 2002; Chen et al., 2012; Cornberg et al., 2006; Selin et al., 1998). This alteration in disease outcome is dependent on a cross-reactive CD8 T cell epitope, NP205-212, which differs in only 2 of 8 amino acids between the two viruses. Naïve mice immunized with PICV generate a low frequency of memory CD8 T cells specific for NP205 and these T cells are reactivated during subsequent LCMV challenge, resulting in an altered immunodominance hierarchy and faster viral clearance than that seen in naïve mice challenged with LCMV (Brehm et al., 2002; Cornberg et al., 2006; Selin et al., 1998). A similar event occurs in LCMV-immune mice challenged with PICV.

During a heterologous viral challenge, only the crossreactive cells are re-activated and expand, skewing the population and causing a narrowing of the TCR repertoire. This narrow TCR repertoire may be more efficient at clearing virus early, but during persistent viral infections this may allow for viral immune escape (Cornberg et al., 2006).

This study investigated whether co-infection with two live viruses would alter the characteristics of the CD8 T cell response to each virus and subsequently alter immune protection and severity of disease compared to single virus-immune mice during homologous challenge. Because these two viruses contain a cross-reactive epitope this model was also useful for directly examining the influence of T cell crossreactivity on disease outcome upon challenge following co-infection.

A. Co-immunized mice showed reduced protective immunity compared to single virus-immune mice after challenge.

To determine immune protection after co-immunization, naïve mice were immunized with the Armstrong strain of LCMV, PICV or both simultaneously. After six weeks mice had cleared both viruses and were considered immune and challenged with the clone 13 strain of LCMV or PICV. The clone 13 strain of LCMV was used because it replicates more extensively due to the attachment protein binding more avidly to its receptor (Ahmed et al., 1984).

As expected, LCMV-immune mice were protected after LCMV clone 13 challenge compared to naïve mice, as evidenced by the significant reduction in serum viral load at day 2-3 post challenge (**Figure 3.1A**). Compared to LCMV-immune mice where 60% (6/10) of the mice had completely cleared virus, virus was cleared in only 23% (3/16) of the co-immunized mice. ($p<0.05$ by Fischer's exact test) (**Figure 3.1A**). However, both LCMV- and co-immunized mice were able to clear LCMV clone 13 from the serum by day 5 post challenge (data not shown). In a similar fashion, after PICV challenge 92% (11/12) of PICV-immune mice cleared virus by day 3 post challenge while only 20% (3/15) of co-immunized mice could clear virus ($p<0.0004$ by Fischer's exact test). However, by day 5 post challenge virus could not be detected in either PICV- or co-immunized mice (data not shown). Collectively, these results demonstrated that co-immunized mice were not as efficient at clearing virus compared to LCMV or PICV single-immune mice, suggesting that the memory responses in co-infected mice were not as efficient at clearing a secondary challenge.

B. Co-immunized mice had increased immunopathology during challenge compared to single virus-immune mice.

Increased and prolonged virus replication, even by a few days, may lead to enhanced recruitment and expansion of activated memory T cells and cause collateral cellular damage or immunopathology (Selin et al., 2011).

Figure 3.1

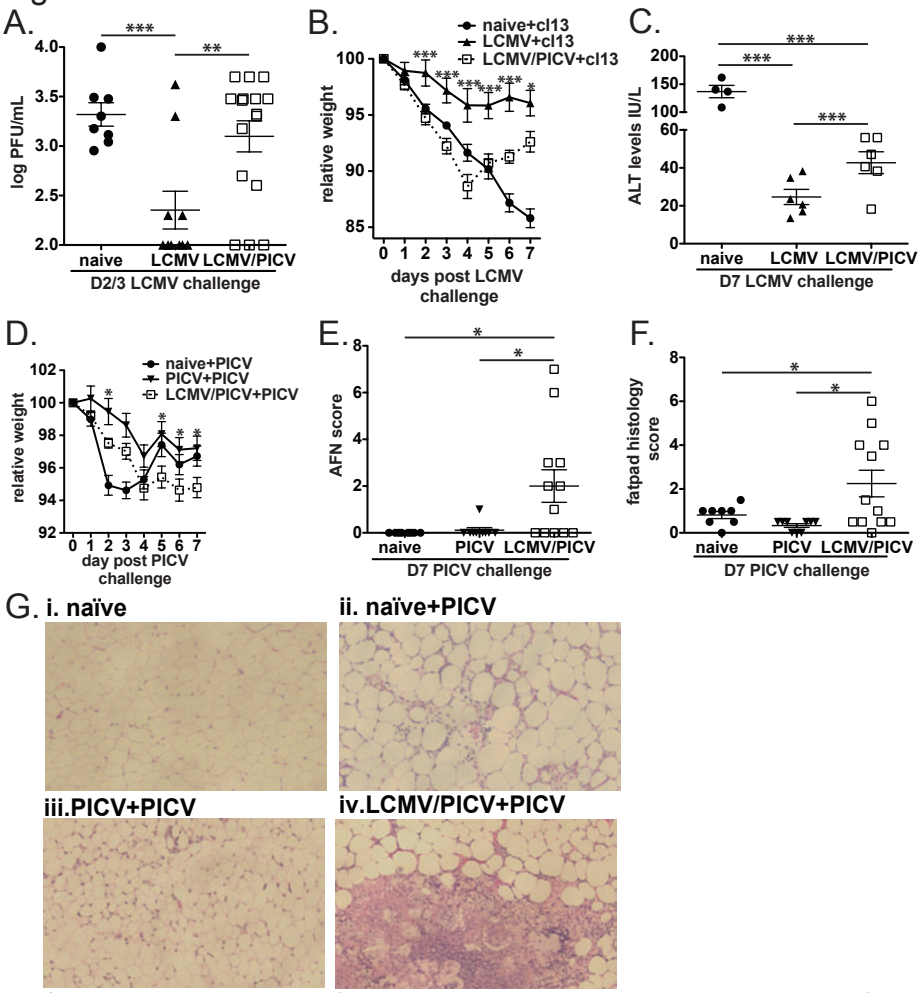


Figure 3.1. Co-immunized mice had significantly increased viral loads and immunopathology after challenge. **A.-C.** Naïve, LCMV- and LCMV/PICV co-immunized mice were challenged with $2X10^6$ PFU LCMV clone 13 i.v. **A.** Viral loads were determined in serum at days 2-3 post-challenge. Data was pooled from three similar experiments. **B.** Mice were weighed daily, and relative weight was calculated from day 0. n=3-8 mice/group. Data are representative of three similar experiments. Statistics compare LCMV- and co-immunized mice. **C.** ALT levels were determined on day 7 post LCMV clone 13 challenge in the serum. n = 5 mice/group. Data were pooled from two similar experiments. **D.-G.** Naïve, PICV- and co-immunized mice were challenged with $2X10^7$ PFU of PICV i.p. **D.** Mice were weighed daily and relative weight was calculated from day 0. n=7-12 mice/group. Statistics show differences between PICV- and co-immunized mice. Data are pooled from two similar experiments. **E.** Acute fatty necrosis (AFN) score of abdominal/epididymal fat pad and **F.** fat pad histopathology scores were determined on day 7 post PICV-challenge. n=8-12 mice/group. Data are pooled from two similar experiments. Histopathology and AFN scoring system outlined in Materials and Methods. **G.** Fat pad sections from naïve (**G.i.**), naïve+PICV (**G.ii.**), PICV+PICV (**G.iii.**) and co-infected+PICV (**G.iv.**) were stained with H&E at day 7 of PICV-challenge. **G.i.** naïve mice showed normal fat pad structure. **G.ii.** naïve+PICV mice showed focal patches of mononuclear infiltrates. **G.iii.** PICV+PICV mice showed mild mononuclear infiltrates around the periphery. **G.iv.** LCMV/PICV+PICV mice showed severe necrosis of up to 50% of the fat pad. *p<0.05, **p<0.01,***p<0.001 ****p<0.0001

LCMV challenge. The high dose of LCMV clone 13 used in this study induces transient weight loss in naïve mice during the first week of infection. Mice will eventually undergo clonal exhaustion of the T cell response and do not develop severe immunopathology (Stamm et al., 2012; Waggoner et al., 2012). To determine if co-immunized mice were developing enhanced immunopathology compared to LCMV-immune mice, weight loss was measured after LCMV challenge (**Figure 3.1B**). Naïve mice infected with LCMV clone 13 progressively lost 15% of their body weight by day 7, whereas LCMV-immune mice were largely protected from weight loss. Interestingly, co-immunized mice lost significantly more weight than LCMV-immune mice. Co-immunized mice lost up to 15% of their body weight during the first 4 days of challenge. During this time, co-immunized mice were similar to naïve mice challenged with LCMV clone 13, with no significant difference in their relative weight. These data suggest that co-immunized mice were deficient in immunological protection from LCMV clone 13-induced weight loss during this time period. In contrast to acutely infected naïve mice, co-immunized mice began to regain weight after day 4, such that co-immunized mice had a greater relative weight at day 6 ($p<0.004$) and day 7 ($p<0.002$).

LCMV clone 13 infection has previously been associated with lung and liver pathology (Pellegrini et al., 2011; Waggoner et al., 2012), so to more specifically assess immunopathology we measured liver damage by quantifying the liver enzyme alanine transaminase (ALT) after LCMV challenge (**Figure**

3.1C). Clinically, elevated ALT levels are diagnostic of hepatocellular injury (Sheehan and Haythorn, 1979). After LCMV challenge, naïve mice had the highest ALT levels at day 7 compared to either of the immune groups. Co-immunized mice had higher ALT levels than LCMV-immune mice, indicating enhanced liver damage in co-immunized mice during LCMV clone 13 challenge.

PICV challenge. Co-immunized mice also had enhanced immunopathology during challenge with PICV compared to PICV-immune mice (**Figure 3.1D**). Resembling LCMV-immune mice challenged with LCMV clone 13, PICV-immune mice had the least relative weight loss during PICV challenge. Naïve mice infected with PICV had a substantial drop in relative weight by day 2 post challenge, but by day 7 were approximately equal to PICV-immune mice (**Figure 3.1D**). Co-immunized mice lost weight gradually until day 4, but unlike the naïve group, co-immunized mice did not regain weight by day 7 post challenge. Unlike LCMV clone 13 infection, PICV infection was not found to induce liver damage as measured by ALT levels. However, in sequential heterologous challenge models, mice can develop severe panniculitis (Chen et al., 2012; Nie et al., 2010; Selin et al., 1998), a necrosis of the abdominal fat also known as acute fatty necrosis (AFN). The most common form of human panniculitis is erythema nodosum, which affects subcutaneous fat and can occur following certain acute viral infections or vaccinations (Maggiore et al., 1983; Smoller et al., 1990). After PICV challenge we assessed panniculitis of abdominal/epididymal fat pads. (**Figure 3.1E-G**). Naïve mice did not develop

AFN, and only 1 out of 9 PICV-immune mice had very mild AFN (AFN score = 1) (**Figure 3.1E**). Co-immunized mice had a range of AFN scores. Two co-immunized mice had severe AFN (AFN score = 6 and 7), five mice had mild to moderate AFN (AFN score = 1-3) and five mice had no AFN. To further assess severity of pathology we also scored histological sections of the fat pads (**Figure 3.1F&G**). Naïve mice challenged with PICV displayed distinct focal areas of mononuclear infiltrates within the fat pads. PICV-immune mice presented with mild mononuclear infiltrates located only along the periphery of the fat pad. In contrast, co-immunized mice on average demonstrated more severe and variable panniculitis (**Figure 3.1F**) wherein the most affected mice had severe necrosis covering more than half of the fat pad (**Figure 3.1G**). These data show that co-immunized mice had significantly enhanced immunopathology compared to single virus-immune mice during both LCMV and PICV challenges. These data, along with the increased viral load in co-immunized mice compared to single virus immunized mice, suggest that there was a difference in the memory response in co-immunized mice.

C. Co-infected mice had variable and unpredictable epitope-specific CD8 T cell responses after primary infection.

During a viral infection, T cells respond to a limited number of viral epitopes. The different epitope-specific T cell populations can be arranged into a hierarchy based upon the size of the response, also known as an

immunodominance hierarchy. The immunodominance hierarchy is controlled by several factors, including precursor frequency, antigen-MHC class I binding affinity and stability and the ability of the antigen-presenting cell to effectively process and present antigen (Yewdell, 2006). We questioned if co-infection would affect the development of two different virus-specific CD8 T cell responses within the same host. Specifically, we wondered if co-infection would alter the immunodominance hierarchies for the individual viruses. In addition, we questioned if there were any changes in the cross-reactive NP205-specific response, as both viruses present a variant of this epitope.

As demonstrated previously, naive mice infected with a single virus developed predictable and reproducible CD8 T cell immunodominance hierarchies that are stable for the life of the host (Ahmed and Gray, 1996; Homann et al., 2001; Tough and Sprent, 1994; Zimmerman et al., 1996) unless sequentially infected with another pathogen (Selin et al., 1996; 1999). In agreement with those findings, mice immunized with LCMV exhibited the strongest CD8 T cell responses to the co-dominant epitopes NP396 and GP33/34 (**Figure 3.2A**) and weaker responses against the subdominant and cross-reactive epitope, NP205. PICV immunization of naïve mice produced a strong response to the dominant PICV epitope NP38 and a smaller response to the subdominant NP205 epitope (**Figure 3.2A**). In contrast to the single virus-infected mice, the highly predictable immunodominance hierarchy against each

Figure 3.2

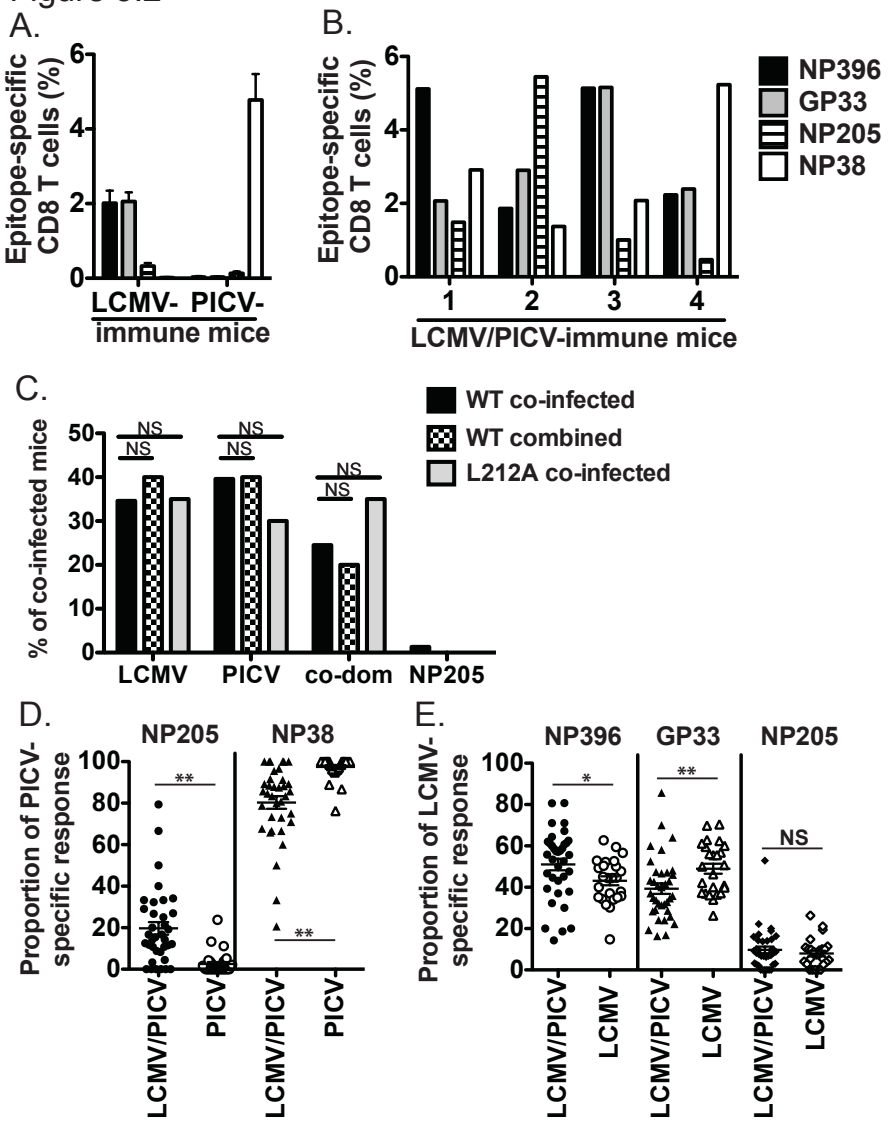


Figure 3.2. Co-infected mice had altered and variable CD8 T cell immunodominance hierarchies after primary infection. Peripheral blood lymphocytes were isolated from LCMV-, PICV-, and co-immunized mice and epitope-specific CD8 T cells were determined by ICS assay for IFNγ production. **A.** LCMV- and PICV-immune mice. n=25 mice/group from five similar experiments. **B.** Four individual co-immunized mice representative of immuno-dominance patterns observed in 15 similar experiments. **C.** Frequency of mice that had a dominant response to either LCMV or PICV epitopes, co-dominant responses to both LCMV and PICV epitopes or a dominant response to the crossreactive epitope, NP205. wt co-infected n=164 from 15 similar experiments. wt combined n=15 from two similar experiments. L212A co-infected n=20 from four similar experiments. **D.-E.** The proportional response for each epitope was determined for each virus. **D.** PICV epitope-specific proportions were calculated to NP38 and NP205. **E.** LCMV epitope-specific proportions were calculated to NP396, GP33 and NP205. n=25-36 mice/group from 7-8 experiments pooled with a line to represent the mean of each group. *p<0.05, **p<0.01

virus was perturbed in co-immunized mice, with significant individual variation between mice (**Figure 3.2B**). In a single experiment examining four co-immunized mice, the hierarchies of two mice were predominantly directed against immunodominant LCMV-specific epitopes (NP396 and GP33/34) whereas each of the other mice had a dominant response against either PICV NP38 or the cross-reactive NP205 epitope (**Figure 3.2B**). This latter response was unexpected, since immunodominance by NP205-specific CD8 T cells has not been observed in mice infected with either LCMV or PICV alone (Brehm et al., 2002). To see if the CD8 T cell responses in co-infected mice were skewed in general to one virus over the other, mice were categorized by which virus dominated the CD8 T cell response (**Figure 3.2C**). For example of the four individual co-immunized mice in **figure 3.2B**, mouse 1 & 3 are skewed to LCMV, while mouse 2 skewed to PICV and mouse 4 skewed to NP205. The majority of co-infected mice had a dominant response to only one of the viruses. The LCMV-specific response was the stronger in 37% of co-infected mice, while 40% had a dominant PICV-specific response. In 22% of co-infected mice there was a co-dominant LCMV- and PICV-specific response. These data were from mice co-infected with two separate virus injections, but similar variance in immunodominance hierarchy and dominance was found when LCMV and PICV were combined in the same needle before injection (**Figure 3.2C**). These data suggest that there is greatly increased variability between individual co-

immunized mice and that a normally subdominant cross-reactive T cell response can be a dominant response during simultaneous viral infections.

To understand how this variability may impact the viral clearance and immunopathology of co-infected mice during a challenge, virus-specific immunodominance hierarchies were examined (**Figure 3.2D&E**). Three epitopes for LCMV (NP396, GP33 and NP205) and two epitopes for PICV (NP38 and NP205) were examined in co-infected mice compared to single virus-infected mice in terms of their proportion of the total virus-specific response. Compared to PICV-immune mice, co-infection predominantly resulted in a skewed PICV-specific response (**Figure 3.2D**). Co-infected mice had a larger fraction of PICV-specific T cells that responded to the cross-reactive, subdominant NP205 epitope rather than NP38. Moreover, there was tremendous variation within the NP205-specific response between individual co-infected mice. The proportional NP205-specific response ranged from 0.0-23.8% of the PICV-specific CD8 T cells in PICV-immune mice, but in co-infected mice there was a greater range from 0.0-79.4%.

The LCMV-specific immunodominance hierarchy was also skewed in co-immunized mice (**Figure 3.2E**). Co-infected mice had a higher proportion of NP396-specific T cells and a lower proportion of GP33/34-specific T cells compared to LCMV-immune mice. In contrast to the PICV-specific response, no difference was found in the NP205-specific responses between co-infected and LCMV-immune mice. It is important to note that whereas NP396- and GP33/34-

specific responses are co-dominant in LCMV-immune mice, the proportion of NP396-specific cells is significantly ($p<0.003$) greater than the proportion of GP33/34-specific cells among co-infected mice. Collectively, there were proportional differences within both the LCMV- and PICV-specific responses of co-immunized mice compared to their single-immune counterparts, yet the cross-reactive NP205 response was only increased within the PICV-specific immunodominance hierarchy.

D. NP205-specific memory T cells mediated enhanced immunopathology in co-infected mice during PICV challenge.

The cross-reactive NP205-specific response between LCMV and PICV mediates immune protection during sequential heterologous infections (LCMV+PICV or PICV+LCMV) (Brehm et al., 2002; Chen et al., 2012; Cornberg et al., 2006; Selin et al., 1998). However, this same cross-reactive response can also mediate immunopathology in PICV-immune mice challenged with PICV when there is an intervening LCMV infection (PICV+LCMV+PICV) (Chen et al., 2012). We questioned if the increased cross-reactive response within the PICV-specific hierarchy would correlate with enhanced immunopathology in co-infected mice during PICV challenge. Weight loss (**Figure 3.3A**) and severity of panniculitis (**Figure 3.3C&E**) after PICV challenge correlated with the proportion of memory NP205-specific CD8 T cells in the peripheral blood of co-immunized mice prior to challenge with PICV. By comparison, there was no correlation

Figure 3.3

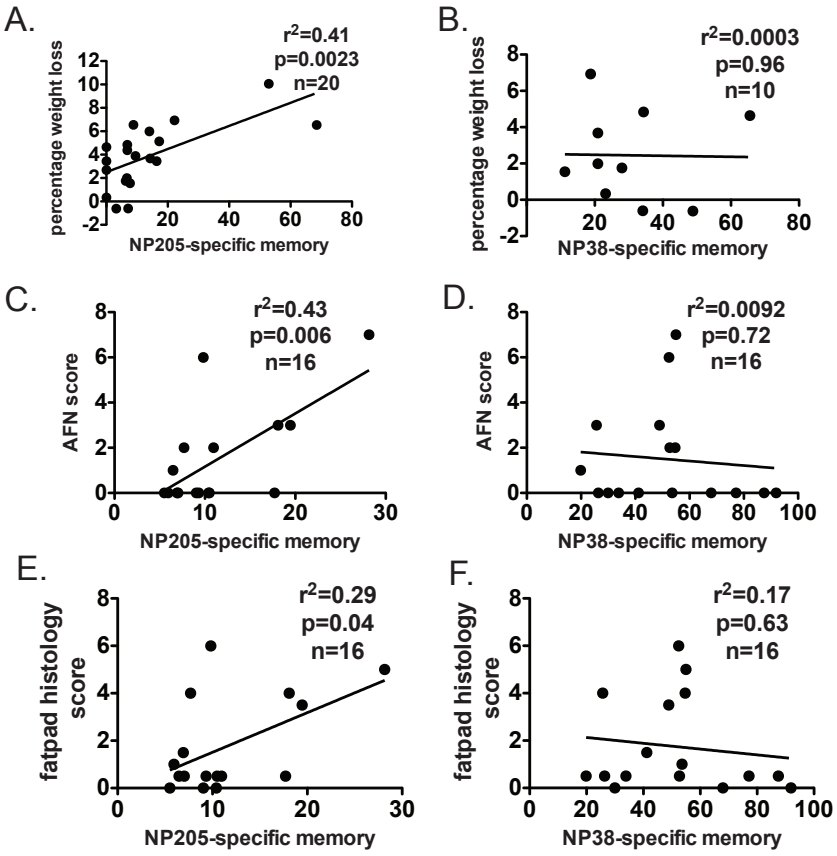


Figure 3.3. NP205-specific memory proportions prior to PICV challenge directly correlated with severity of immunopathology in co-infected mice after PICV challenge. Linear regression analyses comparing the proportion of memory cells specific to the cross-reactive NP205 (**A, C and E**) or the non-cross-reactive NP38 (**B, D and F**) epitope to the severity of disease as indicated by percentage of weight loss (**A&B**), AFN score (**C&D**) or fat pad histopathology score (**E&F**).

between the proportion of non-cross-reactive NP38-specific memory cells and any measure of PICV-induced immunopathology (**Figure 3.3B, D&F**). To directly test whether the NP205-specific response was involved in induction of immunopathology, we utilized a LCMV variant that does not induce a NP205-specific response. The LCMV variant virus (rL212A) harbors a leucine to alanine mutation at position 8, one of the MHC class I binding residues in this peptide (Chen et al., 2012). Use of this mutant negates the cross-reactive response between LCMV and PICV. Co-infection with the rL212A variant and wildtype PICV resulted in a similar variation in the immunodominance hierarchy as found with wildtype LCMV co-infection, suggesting that the cross-reactive response is not mediating this variability between individuals (**Figure 3.2C**). After PICV challenge, mice co-immunized with PICV and the rL212A variant underwent significantly less weight loss than the mice co-infected with PICV and wildtype LCMV (**Figure 3.4A**). Of note, weight loss in rL212A co-infected mice challenged with PICV mirrored that of the PICV-challenged, PICV-immune mice. rL212A co-infection also prevented panniculitis and widespread necrosis of fat pads associated with PICV challenge of wildtype co-infected mice (**Figure 3.4B-D**). These results demonstrate that responses to the cross-reactive NP205-specific response mediated the increased immunopathology in co-infected mice upon PICV challenge.

Figure 3.4

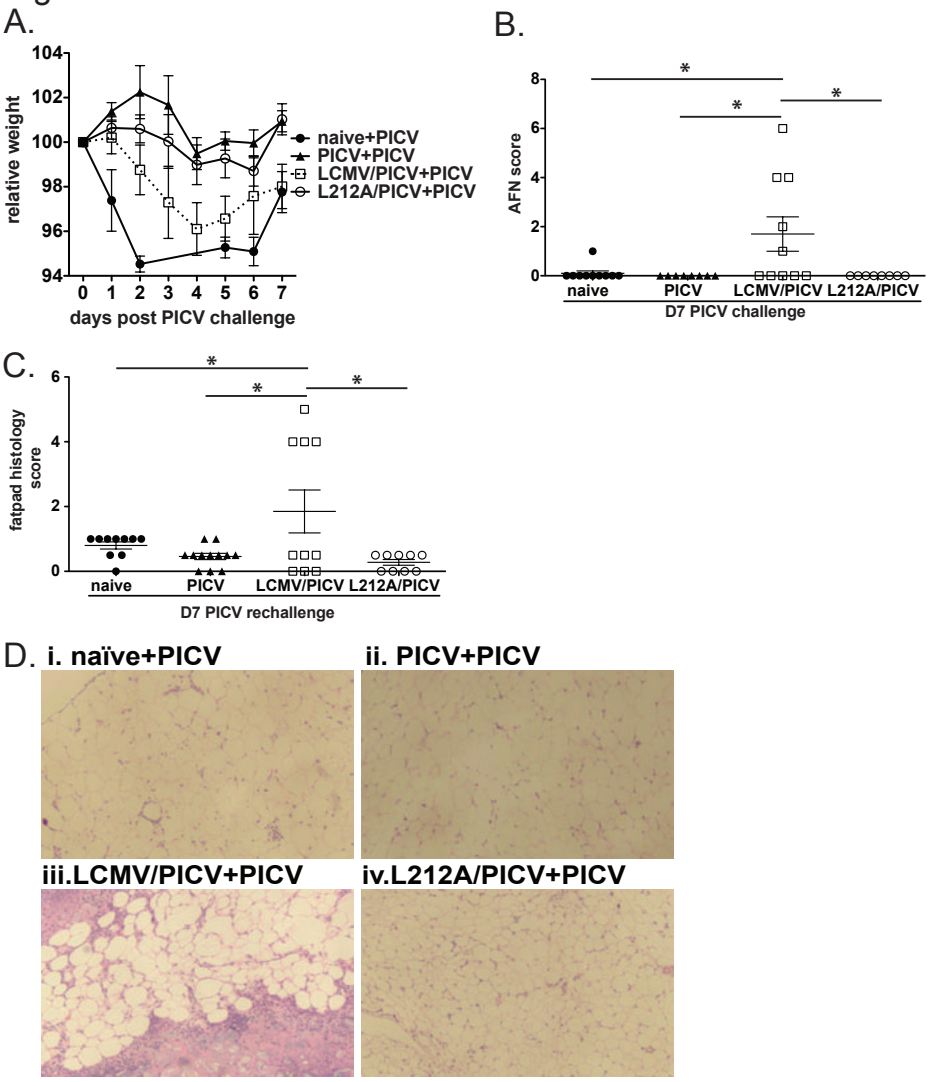


Figure 3.4. NP205 memory response mediates immunopathology in co-immunized mice during PICV challenge. A. Naïve, PICV-, wt LCMV/PICV-, and NPL212A/PICV-immune mice were challenged with $2x10^7$ PFU PICV i.p. Mice were weighed daily and relative weight was calculated from day 0. n=7-10 mice/group. Data are from two similar experiments pooled. Statistics show differences between wildtype and L212A co-immunized mice **B.** AFN score of fat pads and **C.** Fat pad histopathology scores were determined on day 7 post PICV-challenge. Data are from two similar experiments pooled. **D.** Fat pad sections were stained with H&E. (**D.i.**) Naïve+PICV showed focal patches of mononuclear infiltrate. (**D.ii.**) PICV+PICV showed very mild mononuclear infiltrate. (**D.iii.**) wt LCMV/PICV+PICV mice showed severe necrosis. (**D.iv.**) L212A/PICV+PICV displayed mild mononuclear infiltration. *p<0.05

E. Co-infected mice had a decreased number of LCMV virus-specific CD8 T cells after primary infection.

In contrast to PICV challenge, T cell cross-reactivity did not play a major role in the enhancement of immunopathology in co-infected mice challenged with LCMV clone 13 (data not shown). We questioned whether there was a generalized defect in the LCMV-specific memory CD8 T cell response in co-infected mice after primary infection. At the peak of the CD8 T cell response, the magnitude of the entire CD8 T cell response in LCMV-infected mice was 10-fold greater than that in PICV-infected mice (**Figure 3.5A**). The concurrent administration of PICV with LCMV caused a 4-fold reduction in the size of the LCMV-specific response compared to LCMV-infected mice. In contrast, there was no difference in the size of the PICV-specific response between PICV- and co-infected mice at day 8 post infection.

In the memory phase, 8 weeks post infection, LCMV- and PICV-immune mice had more LCMV- or PICV-specific cells, respectively, compared to co-infected mice (**Figure 3.5B**). These results show that LCMV and PICV co-infection resulted in smaller memory pools specific to each virus. As the number of virus-specific CD8 T cells is known to correlate with protection (Cerwenka et al., 1999; Hamada et al., 2009; Zinkernagel and Welsh, 1976), this significant reduction in the size of the LCMV-specific memory response may be responsible for reduced viral clearance and enhanced immunopathology.

Figure 3.5

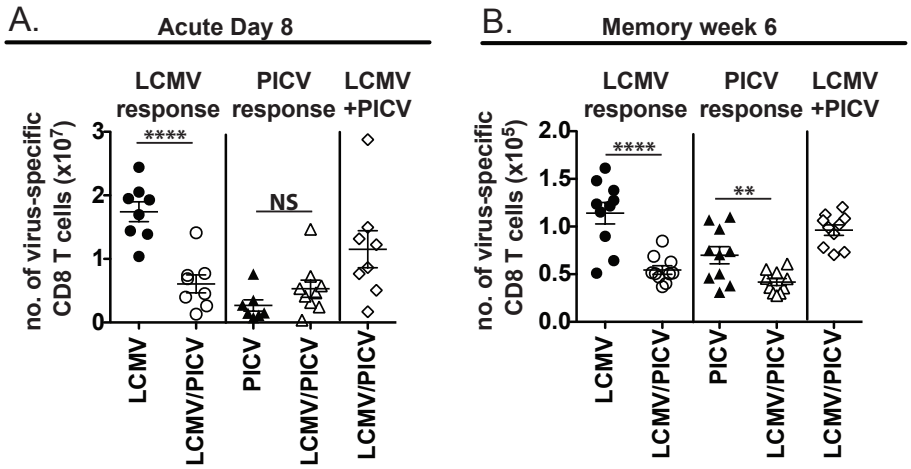


Figure 3.5. Co-infected mice had a smaller LCMV-specific CD8 T cell response after primary infection. On day 8 (**A.**) and 8 weeks post infection (**B.**) the total number of virus-specific CD8 T cells were determined by IFNγ production in an ICS assay. **A.** n=3-4 mice/group. Data are representative of three similar experiments. **B.** n=10 mice/group. Data are pooled from two similar experiments. LCMV+PICV response represents the summation of the LCMV and PICV responses in co-infected mice. *p<0.05, **p<0.01,***p<0.001 ****p<0.0001

F. Co-infected mice had fewer LCMV-specific CD8 T cells with an effector phenotype during both the acute and memory phases after primary infection.

Because co-infected mice had fewer LCMV-specific memory cells (**Figure 3.5A&B**), we assessed whether these cells would also have phenotypic differences consistent with altered differentiation. At the peak of a primary LCMV infection, up to 60% of the LCMV-epitope-specific effector cells became killer cell lectin-like receptor subfamily G member 1 (KLRG1)-positive and IL-7 receptor (IL7R/CD127)-negative (**Figure 3.6A&B**). This phenotype is indicative of short-lived effector cells, which have a greater probability of dying during the contraction phase (Hand et al., 2007; Joshi et al., 2007). In co-infected mice, this population (KLRG1+IL-7R-) was significantly reduced, 12 and 16% amongst NP396- and GP33-tetramer reactive CD8 T cell populations, respectively, compared to LCMV-infected mice (**Figure 3.6B**). The frequency of LCMV-specific CD8 T cells that were KLRG1-IL7R+ memory precursor effector cells was significantly higher in co-infected mice than their LCMV-single infected counterparts (**Figure 3.6C**). Interestingly, there was no difference in the differentiation of these two effector populations in PICV NP38-specific CD8 T cells between PICV- and co-infected mice (**Figure 3.6A-C**).

Co-immunized mice also had a less effector-like LCMV-specific CD8 T cell response in the memory phase. We measured tetramer-reactive LCMV-specific CD8 T cells for effector-like memory (CD62L lo KLRG1+IL7R-) and central-like memory (CD62L hi KLRG1- IL7R+) phenotypes (**Figure 3.6D&E**). The normal

Figure 3.6

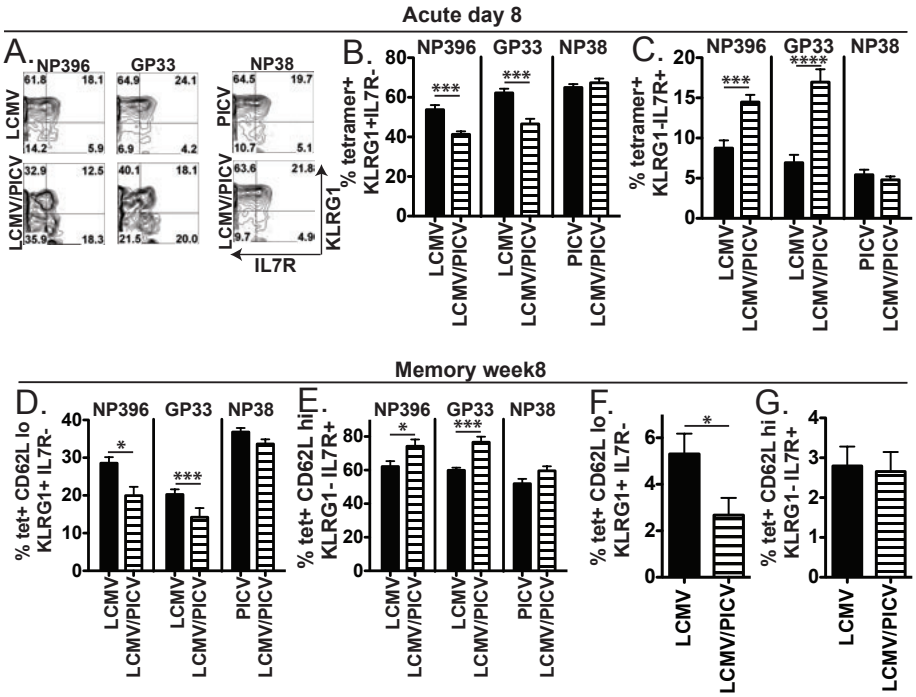


Figure 3.6. Decreased LCMV-specific effector and effector memory responses in co-infected mice after primary infection. A-G. Peripheral blood lymphocytes were isolated and stained with tetramers and antibodies specific for CD8, KLRG1, IL7R and CD62L. Data was pooled from two experiments. n=10 mice/group **A.** Percentages of KRLG1 and IL7R gated on CD8+tetramer+ cells. Representative of three similar experiments. **B-C.** Day 8 post infection gated on CD8+tetramer+ cells B. KLRG1+IL7R-. **C.** KLRG1-IL7R+. **D-E.** At 8 weeks post infection CD8+tetramer+ cells were gated on **D.** CD62L lo KLRG1+IL7R- and **E.** CD62L hi KLRG1-IL7R+. The total number of LCMV-specific response was calculated for **F.** CD62L lo KLRG1+IL7R- cells and **G.** CD62L hi KLRG1-IL7R+ cells at 8 weeks post infection. *p<0.05, **p<0.01,***p<0.001 ****p<0.0001

LCMV-epitope-specific memory response consists of 20-30% effector-like memory cells (CD62L lo KLRG1+IL7R-) and ~60% central-like memory cells (CD62L hi KLRG1-IL7R+). However, in the context of a co-infection central-like memory cell frequencies were increased by 15% and there was a coincident 6 and 9% reduction in the frequency LCMV NP396- and GP33-specific effector-like memory CD8 T cells, respectively. Similar to the acute time point, in the memory phase there was no phenotypic differences in the PICV-NP38-specific cell populations between PICV- and co-immunized mice. We calculated the total number of CD8 T cells specific to LCMV immunodominant epitopes NP396 and GP33 with either a central- or effector-like memory phenotype. Interestingly, there was a significant 50% reduction in the number of effector-like memory cells (CD62L lo KLRG1+IL7R-) in co-infected mice, but no difference in the number of central-like memory cells (CD62L hi KLRG1- IL7R+) between co-immunized and LCMV-immune mice (**Figure 3.6E&F**). These data show that in co-infected mice a smaller proportion of LCMV NP396- and GP33-specific CD8 T cells have an effector-like phenotype at both the peak of the T cell response and in memory.

G. Infectious LCMV load is similar in LCMV- and co-infected mice during primary infection.

Decreased antigen load and shortened durations of antigen presentation can lead to a smaller effector T cell response (Badovinac et al., 2002; Kaech and Ahmed, 2001; Prlic et al., 2006; Schiemann et al., 2003; van Heijst et al., 2009).

Therefore, we examined whether LCMV antigen load was reduced in co-infected mice compared to LCMV-infected mice as a possible explanation for the reduced size of the LCMV response in co-infected mice. However, there was no difference in LCMV viral load in the spleen between LCMV- and co-infected mice on days 3, 5 or 7 after primary infection (**Figure 3.7A**). Viral load was highest in both groups on day 3. By day 5 both groups were controlling virus and by day 7 and at day 15 LCMV was cleared below the limit of detection in both groups. These results show that there is no difference in LCMV viral load in the spleen where immune responses are being generated.

H. Innate cytokine and chemokine production peaks earlier in PICV- and co-infected mice during primary infection.

In addition to viral load, the cytokine milieu early during infection is an important factor in the shaping of virus-specific T cell responses (Cui and Kaech, 2010; Curtsinger and Mescher, 2010; Welsh et al., 2012). Inflammatory cytokines, such as type I IFN, have been found to affect proliferation and differentiation of effector CD8 T cells during LCMV infection (Curtsinger and Mescher, 2010; Marshall et al., 2011; Welsh et al., 2012). We examined whether co-infection altered the kinetics or composition of the LCMV-induced cytokine environment.

LCMV infection induced a strong type I IFN response that peaked between days 2 and 3 post-infection (**Figure 3.7B**) similar to previous studies

Figure 3.7

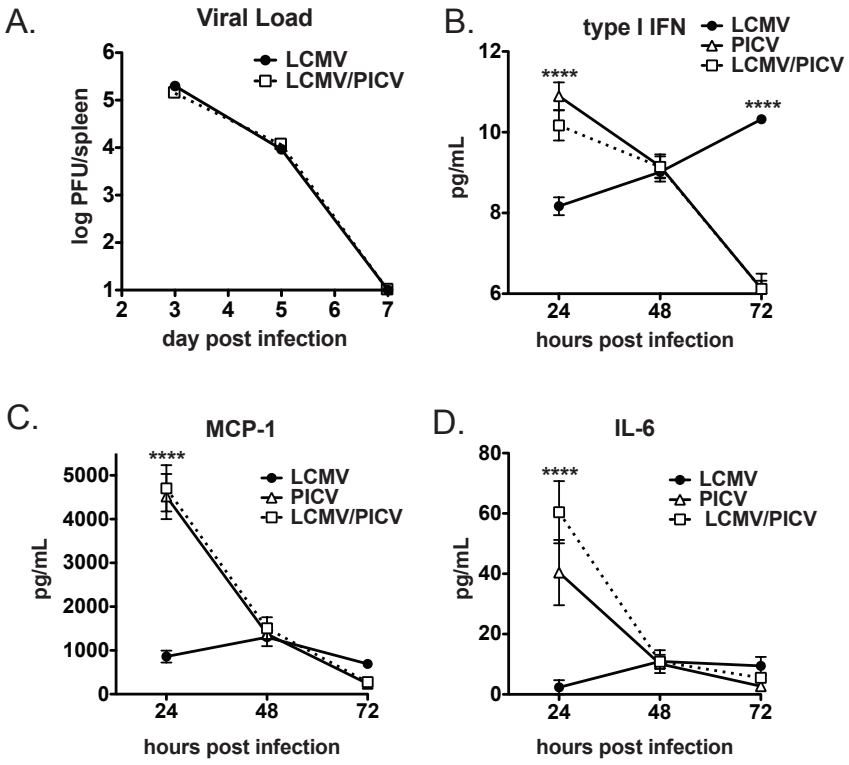


Figure 3.7. Early cytokine responses in co-infected mice are significantly different than in LCMV-infected mice during primary infection. A. Splenic viral load was determined by INT stained plaque assay in LCMV- and co-infected mice after primary infection. n=4-5 mice/group. Data are representative of two similar experiments. **B.** Functional type I IFN was measured in the serum of LCMV-, PICV- and co-infected mice by bioassay. n=3-14 mice/group. Data are pooled from 4 similar experiments. **C.** MCP-1 and IL-6 levels were determined in the serum by cytometric bead array. n=15 mice/group from 3 pooled experiments.

(Hinson et al., 2010; Li et al., 2013; Zuniga et al., 2008). PICV triggered a rapid type I IFN response, which peaked 24 hours after infection and then rapidly tapered off. Interestingly, the kinetics of type I IFN induction in co-infected mice mirrored that of PICV-infected mice and was different than that of LCMV-infected mice at 24 and 72 hours post infection.

There were also differences in other inflammatory proteins at 24 hours post infection (**Figure 3.7C&D**). While levels of IL-6 and MCP-1 were low 24 hours after an LCMV infection, they were greater than 5-fold higher in PICV- and co-infected mice, which both had similar levels of cytokines production (**Figure 3.7C&D**). At 48 and 72 hours, both MCP-1 and IL-6 were low in all groups with no differences. During primary infection within co-infected mice the PICV-induced innate response is present, not the LCMV-induced response, suggesting that this altered cytokine environment may be altering proliferation and differentiation of the LCMV-specific CD8 T cells.

Chapter Summary

In this chapter we show that co-immunization with LCMV and PICV resulted in decreased immune protection and enhanced immunopathology after challenge with either virus. This data would suggest that during co-infection the developing immune responses are less efficient at clearing reinfection. Persisting virus lingering in the peripheral tissues may cause recruitment of effector T cells

and the development of immunopathology. After primary co-infection there were several differences in the CD8 T cell responses compared to mice infected with a single virus. Co-infected mice had increased variability in the CD8 T cell immunodominance hierarchies and a dramatic decrease in the overall size of the LCMV-specific CD8 T cell response. This variability in the immunodominance hierarchies of co-infected mice resulted in an enhanced cross-reactive response in some mice that mediated enhanced immune-mediated fat pad pathology during PICV challenge. During an LCMV challenge, higher viral loads and increased liver pathology in co-infected mice was associated with a reduction in LCMV-specific CD8 T cells memory cells, specifically cells with an effector memory-like phenotype. These data suggest that the magnitude and character of memory CD8 T cell responses generated in response to simultaneous co-infections differed substantially from those induced by single immunization.

Chapter 4: Clonal exhaustion as a mechanism to protect against severe immunopathology and death from an overwhelming CD8 T cell response.

Because of the ability of viruses to infect cells, peptide cleavage products from many of their encoded proteins get incorporated into nascent class I MHC molecules and get presented at the cell surface to CD8 T cells bearing TCR specific for the peptide-MHC complex. As a result, viral infections frequently stimulate very potent class I-restricted CD8 T cell responses capable of perforin- or FasL-dependent cytotoxicity, as well as IFNγ and TNFα production. Indeed, CD8 T cells are essential regulators of viral infection, playing important roles in the clearance of virus-infected cells and sometimes causing damaging immunopathology (Doherty and Zinkernagel, 1974; Gilden et al., 1972a; 1972b; Nathanson and Cole, 1971). The relative balance between protective immunity and immunopathology often determines the fate of the virus-infected host (Selin et al., 2011). Classic examples are that of LCMV, where the same clone of T cells responsible for viral clearance can mediate a severe leptomeningitis if the virus is replicating in the brain (Doherty and Zinkernagel, 1974; Gilden et al., 1972b). The pathology that is induced by T cells during an acute infection most likely results from the inflammatory conditions brought about by the presence of high numbers of T cells lysing infected tissues via perforin and FasL, producing pro-inflammatory cytokines, including TNF, and chemokines which recruit even more cells. Important factors in how rapidly virus is cleared include the initial viral

load, rate of viral replication and the efficiency of the activated antigen-specific T cells. To understand how these factors mediate immunopathology mice were inoculated with three different doses of LCMV clone 13. Due to mutations LCMV clone 13 can systemically infect mice and replicate faster than the Armstrong strain from which it was isolated (Matloubian et al., 1990; Salvato et al., 1991). We hypothesize that infection with lower doses of virus that are easily controlled by the T cell response, there will be little immunopathology, but as the viral load increases there may be a shift in the balance between antigen load and efficiency of the T cell response leading to enhanced pathology until the T cell response becomes exhausted.

A. Medium dose LCMV clone 13 led to increased immunopathology and mortality.

B6 mice were infected intravenously with low dose (2 X 10^4 PFU), medium dose (2 X 10^5 PFU) and high dose (2 x 10^6 PFU) LCMV clone 13 iv There was significantly higher mortality in the medium dose group, with only 25% of mice surviving at Day 13 (**Figure 4.1A**). Both the medium and high dose groups experienced a rapid and significant weight loss of greater than 20% beginning at day 6 after infection, with the high dose group starting to recover after day 10 while the medium dose group not recovering even by day 13 (**Figure 4.1B&C**). All of the mice that survived the medium dose infection were persistently infected

Figure 4.1

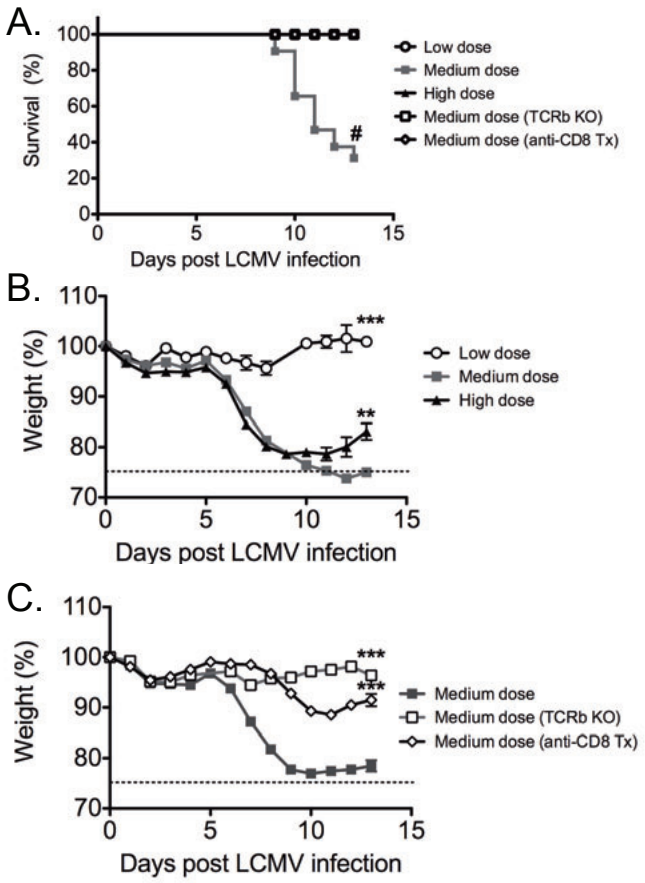


Figure 4.1. Balance between viral load and CD8 T cell response determines disease outcome to LCMV clone 13 infection. A. Increased mortality in C57BL/6 mice infected with medium dose of LCMV clone 13 (2 X 10^5 PFU) i.v. as compared to low dose (2 X 10^4 PFU) or high dose (2 X 10^6 PFU) depleted of CD8 T cells with anti-CD8 mAb or TCRβ KO mice were protected from lethal effect of medium dose (# p=.05; n=11-33 mice per group). (Mantel-Cox Mortality, # p<0.002) **B.** Increased weight loss in both medium dose and high dose LCMV clone 13 infected mice, but high dose began to regain weight at day 13 while medium dose do not. (medium vs high, ** p=.008; medium vs low, *** p=.0002; n=11-12 mice per group).**C.** Mice depleted of CD8 T cells with anti-CD8 mAb or TCRβ KO mice were protected from weight loss during medium dose (medium vs TCRβ KO, *** p=.0001; medium vs CD8 depleted, *** p=.0002; TCRβ KO vs CD8 depleted *** p=.003, n=9-22 mice per group).

with LCMV, similar to the high dose group. The dramatic immunopathology was mediated by the T cell response, as demonstrated by the fact that TCRβ KO mice infected with the medium dose did not have any significant weight loss or death, in contrast to WT controls (**Figure 4.1A&C**). The TCRβ KO mice had a slightly higher viral load than the WT controls and yet did not have pathology (log10 PFU/spleen: B6 5.7±0.13 SEM; TCRβ KO 6.3±0.13; n=5/group; p<.02). The CD8 T cells were the major T cell population involved in mediating this effect, as demonstrated by depletion of CD8 T cells with mAb. B6 mice depleted of CD8 T cells prior to infection with the medium dose of LCMV also had significantly less weight loss and decreased mortality than the WT controls (**Figure 4.1C**) without a significant difference in viral load in the spleen (log10 PFU/spleen: B6 4.9±0.5 SEM; anti-CD8 tx 5.3±0.07 n=5-9/group).

B. Medium dose LCMV clone 13 infection caused a partial clonal exhaustion of the LCMV-specific CD8 T cell response.

Persistent infection leads to a disruption of the normal immunodominance hierarchy and function of CD8 T-cell responses during high dose LCMV clone 13 infection (Wherry, 2011; Wherry et al., 2003a). CD8 T-cell functional impairment occurs in a hierarchical fashion in chronically infected mice. Production of IL-2 and the ability to lyse target cells in vitro are the first functions compromised, followed by the ability to make TNFα, while IFNγ production is most resistant to functional exhaustion. Antigen appears to be the driving force for this loss of

function, since a strong correlation exists between the viral load and the level of exhaustion (Mueller and Ahmed, 2009). Epitopes presented at higher levels in vivo result in physical deletion, such as NP396, while those presented at lower levels, such as GP33 and NP205, induce functional exhaustion. Data would suggest that antigen levels drive the hierarchical loss of different CD8 T cell effector functions during chronic infection, leading to distinct stages of functional impairment and eventually to physical deletion of virus-specific T cells. Thus, the functionality of the epitope–specific CD8 T cells in the three groups of mice was determined by intracellular cytokine staining (ICS) for the presence of IFNγ- and/or TNFα-producing cells and calculating the ratio of IFNγ+TNFα+/IFNγ+ cells at days 7 and 13 post infection. The high dose clone 13-infected mice had significant exhaustion of NP396-, GP33 and NP205-specific responses, as defined by loss of the double positive IFNγ/TNFα producing cells by day 13 (**Figure 4.2A-C**). The medium dose mice had evidence of only partial exhaustion of their CD8 T cell response in this same time frame. They had significantly more of the double positive IFNγ/TNFα producing T cells by all three epitope-specific CD8 T cell responses on day 7 and day 13 than did high dose-infected mice, although significantly less than the low dose group. Expression of the prototypic cell surface marker of exhaustion, PD-1 (Barber et al., 2006; Sharpe et al., 2007; Wherry, 2011) on epitope-specific CD8 T cells at day 10 post infection was intermediate in the medium dose as compared to the high and low dose groups, consistent with partial exhaustion (**Figure 4.2D**). These results would suggest

Figure 4.2

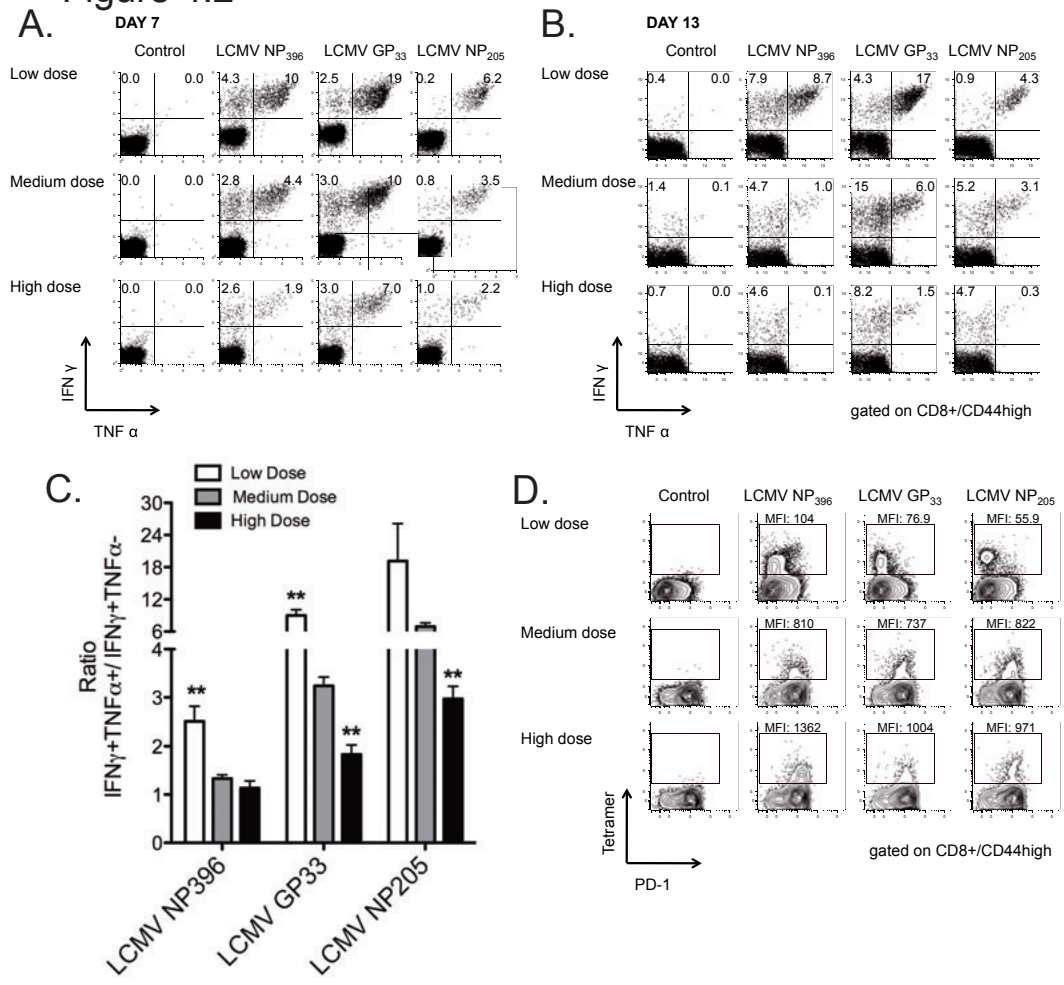


Figure 4.2. Partial clonal exhaustion in the LCMV clone 13 medium dose infected mice. The functionality of the epitope–specific CD8 T cells in the three groups of mice was determined by ICS staining for the presence of IFNγ and/or TNF-producing cells and calculating the ratio of IFNγ+TNF+/IFNγ+ cells at days 7 (**A.**) and 13 (**B.**) post infection. The high dose clone 13- infected mice had significant exhaustion of NP396-, GP33 and NP205-specific responses as defined by loss of double producing INFγ/TNF cells by day 13. **C.** The medium dose mice had evidence of only partial exhaustion of their CD8 T cell response in this same time frame. They had greater numbers of IFNγ/TNF producing cells by all three CD8 epitope-specific responses on day 7 and day 13 than high dose mice, although significantly less than the low dose group. (NP396: low vs medium, high vs low; GP33: low vs medium, medium vs high, high vs low; NP205: medium vs high, high vs low, ** $p<0.01$; n=5-7 mice/group). **D.** CD8 expression of cell surface marker PD-1 on epitope-specific cells at day 10 post infection was intermediate in the medium dose as compared to the high and low dose groups, consistent with partial exhaustion. This is representative of two similar experiments.

that at the medium dose of virus the viral load is high enough to result in a partial exhaustion phenotype (Wherry et al., 2003b) and these sub-optimally functioning CD8 T cells are able to mediate the induction of severe immunopathology leading to death.

C. Mice infected with medium dose LCMV clone 13 had increased lung and liver immunopathology

The lung immunopathology in the surviving medium dose mice as assessed by histology demonstrated severe pulmonary edema and interstitial infiltrates with consolidation, enhanced bronchus associated tissue (BALT), and necrotizing bronchiolitis (**Figure 4.3A**). Using our established method of scoring lung pathology (Chen et al., 2003; Wlodarczyk et al., 2013) the medium dose mice had significantly more pathology than either the high and low dose mice or the TCRβ KO and the CD8 T cell depleted mice, all of which had minimal interstitial infiltrates, consistent with this being CD8 T cell-mediated pathology (**Figure 4.4A**). The livers of the medium dose mice also had significantly increased liver pathology (**Figure 4.3B**) as demonstrated by increased liver enzyme, alanine aminotransferase (ALT) **(Figure 4.4B)**, in their serum as compared to low dose, TCRβ KO, and CD8 T cell-depleted mice. Histological sections of the liver showed serious bridging necrosis in the medium dose mice (**Figure 4.3B**), while the high and low dose mice as well as the TCRβ KO and CD8 depleted medium dose mice had only periportal and sinusoidal inflammatory

Figure 4.3

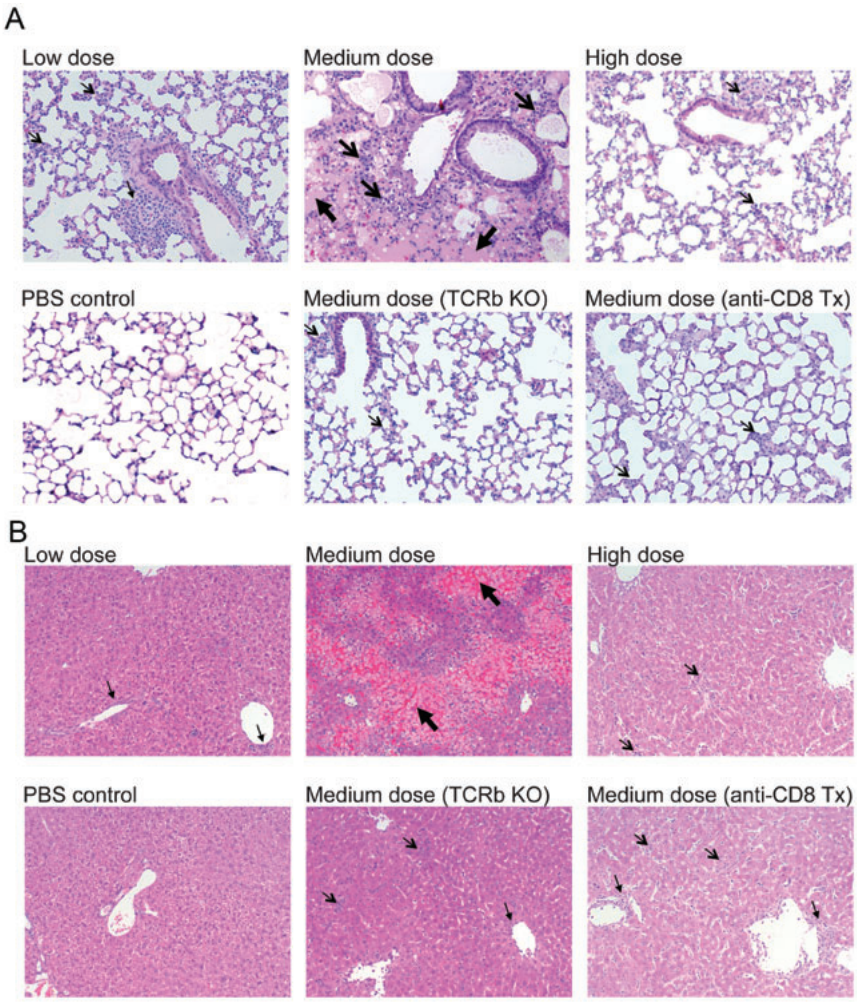


Figure 4.3. Enhanced immunopathology in the lung and liver during medium dose LCMV clone 13 infection. A. Low dose LCMV clone 13 infection i.v. induces mild interstitial infiltrates as demonstrated in histology (H&E stain) sections of the lung at day 13. Medium dose LCMV clone 13 infection i.v. induces severe pulmonary edema, interstitial infiltrates with consolidation, enhanced bronchus associated tissue (BALT), and necrotizing bronchiolitis. High dose LCMV clone 13 infection i.v. induced minimal pathology with few interstitial infiltrates. Mice depleted of CD8 T cells with anti-CD8 mAb or TCRβ KO mice were protected from severe lung pathology having only minimal interstitial infiltrates. **B.** Histological sections of the liver (H&E stain) showed serious bridging necrosis in the medium dose LCMV clone 13 infected mice at day 13, while the high and low dose mice as well as the TCRβ KO and CD8-depleted medium dose mice had only periportal and sinusoidal inflammatory infiltrates. Arrows indicate the areas of significant pathology.

Figure 4.4

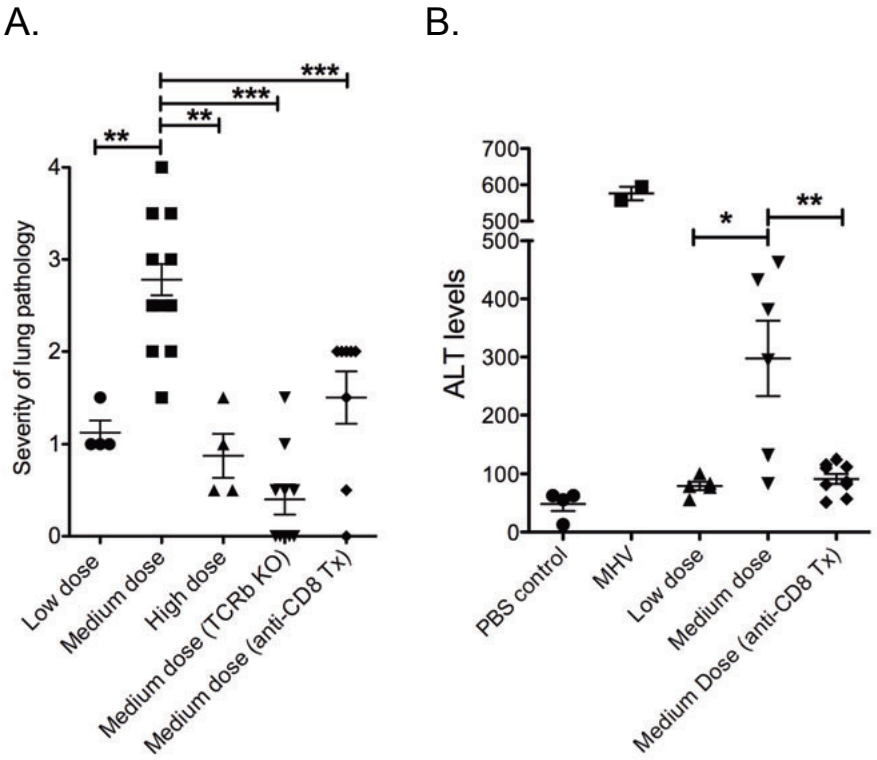


Figure 4.4. Increased pathology in the lung and liver during medium dose LCMV clone 13 infection. A. The severity of the lung pathology was scored using our established histology scale. The lungs of mice infected with LCMV clone 13 medium dose i.v. had significantly more severe lung pathology than low dose or high dose infected mice. This pathology was abrogated by anti-CD8 mAb treatment or by infecting TCRβ KO mice. (** p=.003, *** p<0.001; n=4-9 mice/group) **B.** Severity of liver pathology was assessed by measuring the levels of the liver enzyme ALT (see Methods). ALT levels were significantly increased in medium dose infected mice as compared to low dose. Treatment with anti-CD8 at the time of infection with medium dose prevented this increase in ALT levels. Positive control for this ALT assay is day 13 mice infected with mouse hepatitis virus (MHV) (N=2). (* p=.02, ** p=.008; n=6-10 mice/group). These data represent two separate experiments pooled.

infiltrates. The architecture of lymph nodes of mice infected with LCMV clone 13 was found to be essentially normal at all three doses. In other words, there was recruitment of lymphocytes in T cell zones and increased numbers of germinal centers with infection, but there was no overall destruction or necrosis of the lymphoid structures as has been reported for high dose LCMV-WE infection (Ludewig et al., 1999; Zinkernagel et al., 1999) (data not shown).

Chapter Summary

Using three different inocula of LCMV clone 13 these results showed completely different outcomes. Mice given low doses of LCMV clone 13 developed a strong effector CD8 T cell response, which cleared the virus. If the viral load becomes high very rapidly it can result in clonal exhaustion of the T cell response and viral persistence associated with little immunopathology. However, if the mice were given an intermediate dose of LCMV clone 13, the immune response was able to develop and exhausted more slowly, leaving time for massive collateral damage and resulting in increased mortality with severe lung and liver necrosis.

Chapter 5: Neonates are highly susceptible to T cell mediated immunopathology and mortality after LCMV infection

Both neonatal humans and mice are found to be highly susceptible to increased mortality after infection. Since, neonates lack any protective immunological memory they receive many vaccines. There are numerous differences in the innate, humoral and cellular arms of the neonatal immune system and these differences understandably result in altered immune responses to both vaccination and infections (**Figure 1.6**).

Newborn mice infected at day 1 of age with LCMV undergo central tolerance and deletion of their LCMV-specific T cells, which leads to persistent live-long infection (Hotchin and Cinits, 1958; Volkert and Larsen, 1964). Only one study has been done to examine T cell responses to LCMV infection in older neonates (Belnoue et al., 2007). Using day 14 of age Balb/c infants they found that LCMV WE strain was cleared with delayed kinetics and little immunopathology (Belnoue et al., 2007). To understand how the differences in the innate and adaptive arms of the immune system would alter CD8 T cell priming we proposed to study the day 7 neonatal mice during infection with the nonlytic virus LCMV. This age was chosen as the newborn mouse is immunologically much more immature than a human newborn and is at greater risk of tolerance induction. In fact at day 7 the mouse immune system is more similar to human newborns, including similar IFNγ production to BCG and similar

TCR repertoires (Adkins et al., 2004). Throughout the remainder of this thesis the term “neonatal mouse” refers to day 7 of age, unless otherwise stated. We hypothesized that day 7 B6 neonates will have altered viral clearance and pathology as compared to adults upon LCMV (strain Armstrong) infection due to there decreased innate and adaptive responses.

A. Day 7 neonates were susceptible to T cell-mediated immunopathology

Adult B6 mice infected with the normal dose of LCMV Armstrong $5x10^4$ PFU ip clear virus within one week, develop a strong CD8 T cell response and have minimal immunopathology. The Belnoue et al. study (Belnoue et al., 2007) that infected infant, day 14 of age, Balb/c mice with LCMV WE found a dose-dependent IFNγ response of LCMV-specific CD8 T cells, with a 30 PFU infection inducing a lower response than 100 PFU. As LCMV Armstrong, unlike the WE strain, does not cause immunopathology or clonal exhaustion in adult B6 mice we began our studies with a slightly higher dose than the previous study. After infection with 500 PFU of LCMV Armstrong ip in day 7 of age neonates, surprisingly over 80% of pups succumbed to infection (**Figure 5.1A**). When pups were infected with a log less virus, 50 PFU, 80% of pups still died. At 5 PFU of LCMV Armstrong there was a significant increase in survival, with only 64% of pups dying. However, the kinetics of mortality in pups infected with 500 PFU, 50 PFU, or 5 PFU were the same with mice succumbing to infection between days

Figure 5.1

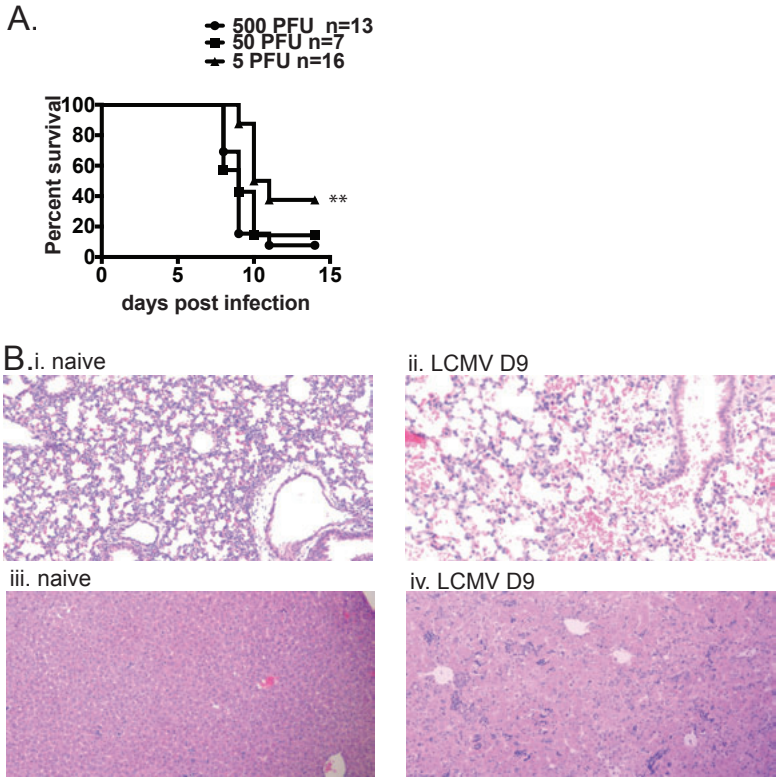


Figure 5.1. Neonatal mice developed severe immunopathology and death after LCMV infection. A. Neonatal mice at day 7 of age were infected with 5, 50 or 500 PFU of LCMV Armstrong ip. Survival was assessed to day 14 post infection. n=7-16 mice/group from two similar experiments. **B.** Lung (**i & ii**) and liver (**iii & iv**) sections were stained with H&E from naïve day 12 of age neonates (**i & iii**) and neonates infected with LCMV and harvested at day 9 of infection (**ii & iv**). Representative organs from 4 similar experiments.

7-11 post infection (**Figure 5.1A**). Histology of lungs and livers in surviving infected neonates displayed enhanced mononuclear infiltrates at day 9 post infection, the time when the majority of the neonates were dying and also the peak of the CD8 T cell response in adult LCMV infection (**Figure 5.1B**). The death rate and immunopathology of vital organs found in neonates infected with low doses of LCMV Armstrong resembled that observed in adult male mice infected with an intermediate dose of the more virulent LCMV clone 13 when given iv (Waggoner et al., 2012) (Chapter 4). An intermediate dose of LCMV clone 13 iv resulted in 75% mortality, which appeared to be mediated by T cell responses, as TCRβ ko mice and mice depleted of CD8 T cells resulted in 100% survival of mice (**Figure 4.1A**). With this intermediate dose of LCMV clone 13 there was also severe liver and lung pathology in the surviving mice (**Figure 4.3**). We initially questioned if neonates were dying of a similar mechanism as the adult mice receiving an intermediate dose of LCMV clone 13. When TCRβ ko neonates were infected with 50 PFU of LCMV Armstrong they had 100% survival as compared to wildtype neonates, which had only ~20% survival (**Figure 5.2A**). To further examine which subset of T cells was causing death, pups were depleted of either CD4 or CD8 T cells (**Figure 5.2B**). Pups depleted of CD4 T cells showed no difference in either the kinetics or the frequency of mortality (**Figure 5.2B**). Depletion of CD8 T cells significantly reduced mortality to only 55% (**Figure 5.2B**). Furthermore, the death found in neonates was not due to viral load, as TCRβ ko mice at day 9 post infection had 6 logs of virus in kidney,

Figure 5.2

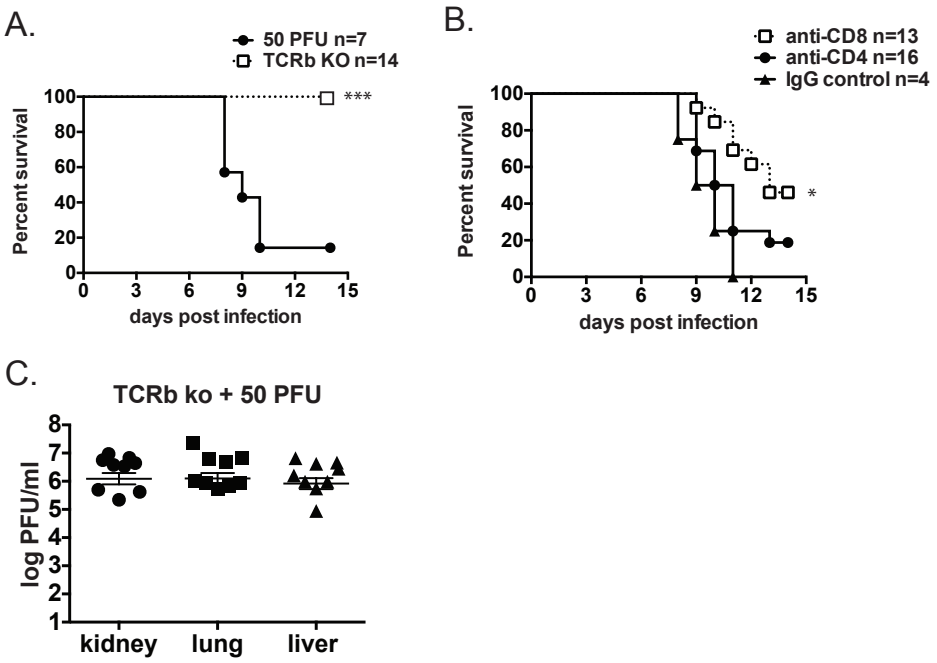


Figure 5.2. Neonates died from T cell-mediated immunopathology. A. wt and TCRβ ko neonates were infected with 50 PFU of LCMV ip and survival was monitored until day 14 post infection. Data are from two similar experiments. wt mice were also shown in figure 5.1 A. **B.** B6 neonates infected with 5 PFU of LCMV ip were treated with 50μg of either anti-CD8, anti-CD4 or IgG control on days 0, 4 and 8 post infection. **C.** Viral load in TCRβ neonates was determined in kidney, lung and liver on day 9 post infection by plaque assay. Data are from two similar experiments.

lung and liver, and 100% of these mice survived until the end of the experiment at day 14 post infection (**Figure 5.2C**). We found these results surprising because neonates are known to have poor inflammatory responses and smaller T and B cell pools compared to adults (Ridge et al., 1996; Siegrist and Aspinall, 2009). We questioned how much smaller the CD4 and CD8 T cell responses were in uninfected day 7 neonates as compared to 6 week old adult mice. At 1 week of age uninfected mice had reduced frequencies and total numbers of CD4 and CD8 T cells compared to 6 week old adults (**Figure 5.3 A-D**). CD4 T cell frequencies are on average 1.8% in 1 week old neonates and expand 12-fold by week 6 of age when mice are considered to be adults (**Figure 5.3C**), similar to previous findings (Hassett et al., 1997). The CD8 T cell population consists of only 1% of the spleen at day 7 and increases to 13% by week 6 (**Figure 5.3A**). By total number there is a 38-fold reduction in CD8 T cells in spleens of neonatal mice (**Figure 5.3B**). Therefore, it was surprising that after LCMV infection neonates could mount a CD8 T cell response strong enough to mediate death.

B. Neonates developed strong CD8 T cell responses with altered immunodominance hierarchies

Immunopathology in the adult mice infected with the intermediate dose of LCMV clone 13 iv was found to be due to a partial, rather than complete, clonal exhaustion of the CD8 T cell response. Complete exhaustion, as seen in adult mice infected with high dose LCMV clone 13 iv, resulted in little pathology and persistent infection (**Figure 4.1-4.4**). At this high dose, high antigen load causes

Figure 5.3

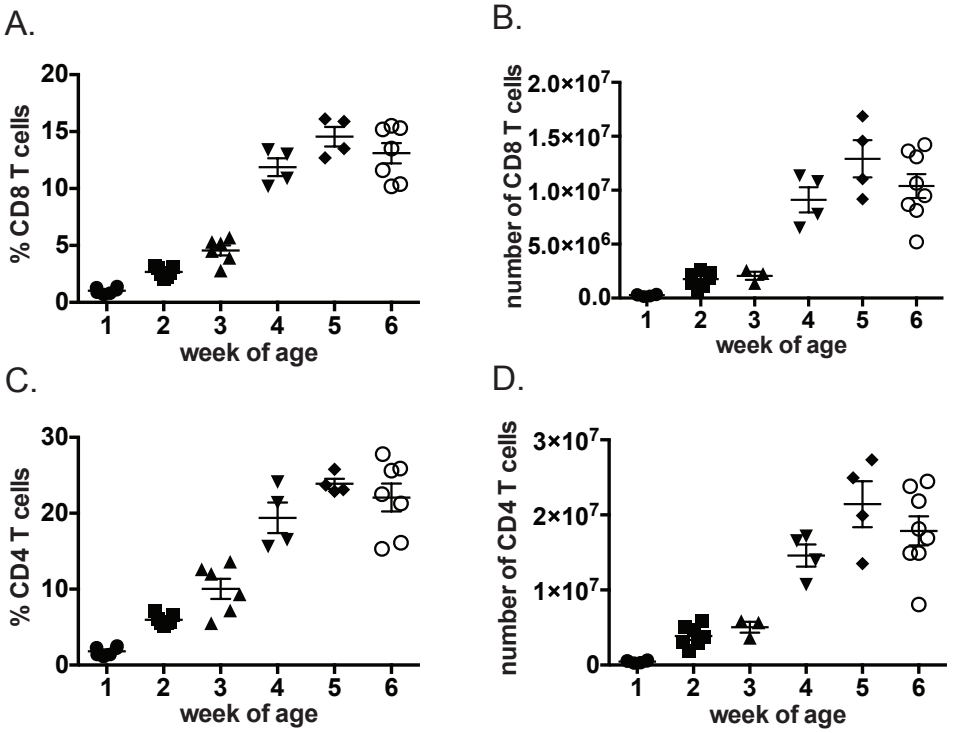


Figure 5.3 Naive neonates had decreased frequencies and total numbers of CD4 and CD8 T cells. At 1, 2, 3, 4, 5 and 6 week(s) of age the frequency (**A & C**) and total number (**B & D**) of splenic CD8 (**A & B**) and CD4 (**C & D**) T cells were examined by surface staining in naïve mice. Data are from 1-2 similar experiments with at least 3 mice/group.

overstimulation of the CD8 T cells leading to a stepwise loss of function ending in apoptosis. At the intermediate dose antigen load is high enough to induce a partial clonal exhaustion with a decrease of the high affinity LCMV-specific response NP396 (**Figure 4.2**). This causes a skewing of the immunodominance hierarchy resulting in a dominant GP33-specific response with decreased cytotoxicity and TNFα production by these cells, but still strong IFNγ production (**Figure 5.4A**). Due to the similar mortality rates we questioned if neonates were also undergoing a partial clonal exhaustion.

Examination of the T cell response in neonates at day 14 post infection showed a similar change in the immunodominance hierarchy, as intermediate LCMV clone 13 in adults, with a loss of NP396-specific CD8 T cells (**Figure 5.4B**). To determine functionality of this population we examined cytokine production and found that as opposed to the adult model of intermediate LCMV clone 13, neonatal CD8 T cells were able to produce both IFNγ and TNFα upon restimulation at both day 9 and 14 post infection (**Figure 5.4C**). Another indicator of exhaustion is the prolonged expression of the CD28-family inhibitory molecule, PD-1, on the surface of activated CD8 T cells. The peak of PD-1 expression on LCMV-specific CD8 T cells in adult mice was prior to day 9, after which PD-1 is downregulated during low dose LCMV Armstrong infection, as previously reported (**Figure 5.4D**) (Barber et al., 2006). Similar to adults PD-1 expression on activated CD8 T cells was downregulated by day 9 post infection. By day 14 post infection LCMV-specific CD8 T cells in neonates were PD-1 negative. These

Figure 5.4

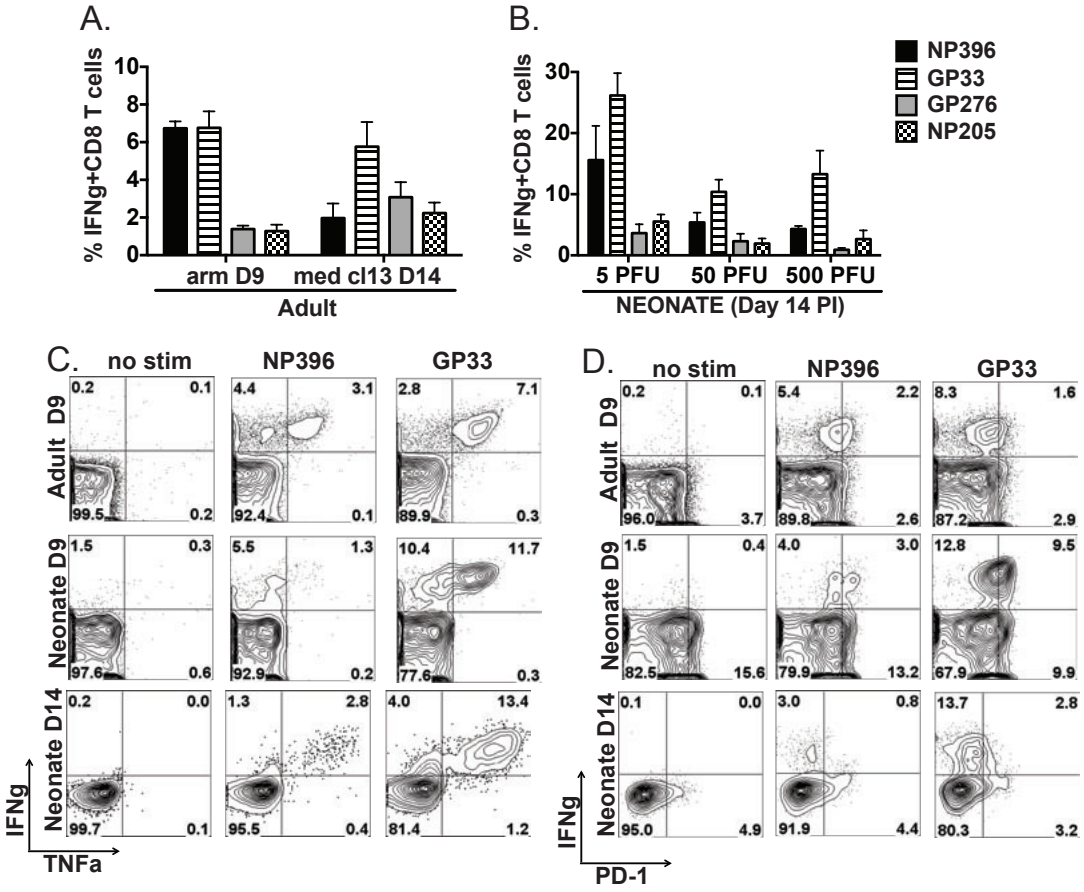


Figure 5.4 Neonates had altered CD8 T cell immunodominance hierarchies, but were not undergoing clonal exhaustion. A. Adult mice were immunized with either LCMV Armstrong or an intermediate dose of LCMV clone 13 and harvested on day 9 or day 14 post infection, respectively. Immunodominance hierarchies were determined by IFNg production by CD44 hi CD8 T cells after peptide stimulation. Data are from 5-7 mice in 2-3 similar experiments. **B.** Neonates were infected with 5, 50 or 500 PFU of LCMV Armstrong and immunodominance hierarchies were determined on day 14 post infection. n= 2-4 mice/group. Data are representative of 1-3 experiments. **C.** Representative facs plots of IFNg vs TNFa gated on CD8+ CD44hi splenocytes in an adult infected with LCMV Armstrong at day 9 post infection and a neonate infected with 500 PFU of LCMV Armstrong at day 9 and 14 post infection. Data are representative of 2-4 experiments. **D.** Representative facs plots of IFNg vs PD-1 gated on CD8+ CD44hi splenocytes in an adult infected with LCMV Armstrong at day 9 post infection and a neonate infected with 500 PFU of LCMV Armstrong at day 9 and 14 post infection. Data are representative of 2-4 experiemnts.

data would collectively suggest that neonatal CD8 T cells were not undergoing partial clonal exhaustion due to high antigen load.

As clonal exhaustion is a mechanism for protection from potentially lethal infections (Chapter 4) we questioned if neonates were unable to clonally exhaust. Neonates were infected with an adult dose of high dose of LCMV clone 13 ip and monitored for death. These pups all survived until day 14 post infection and examination of CD8 T cell responses showed complete clonal exhaustion by loss of IFNγ and TNFα production (**Figure 5.5**). These data suggest that at very high infecting doses with a virus known to induce clonal exhaustion, LCMV clone 13, neonates can exhaust, and as in adult mice this does not happen at low doses of LCMV Armstrong.

C. Neonates had protracted viral load and delayed LCMV-specific CD8 T cell responses

Since neonatal mice infected with low doses of virus were not becoming partially clonally exhausted like the medium dose LCMV clone 13 adult model, we questioned when they cleared virus and how this compared to adult mice. In adult mice infected with LCMV Armstrong, although virus replicates predominantly in the spleen and lymph nodes it has been reported to infect other organs including the liver, kidney and fat pads (Wherry et al., 2003a) (data not shown). In these studies in adult mice in the kidneys LCMV replicated to low levels and peaked with ~4 logs of virus on day 4 post infection after which

Figure 5.5

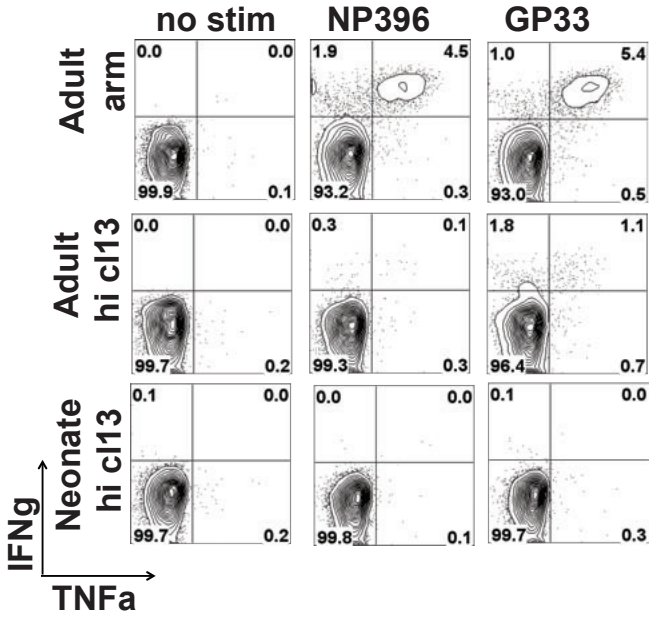


Figure 5.5. Neonatal CD8 T cells could clonally exhaust. Adult mice were infected with either LCMV Armstrong ip or high dose LCMV clone 13 iv and neonatal mice were infected with the same high dose of LCMV clone 13 ip. On day 14 post infection IFNg and TNFa production was examined after peptide stimulation. Data are representative of two similar experiments.

viral load decreased and was cleared by day 15. Virus is cleared from spleen and liver by day 8 with a peak in viral load at day 4 (Wherry et al., 2003a). In neonatal mice infected with either 5 or 50 PFU of LCMV the clearance of virus was protracted compared to adults (**Figure 5.6 A&B**). In neonatal mice LCMV replicated in all organs examined: the kidney, spleen, fat pads, lung and liver peaking at ~5 logs, one log higher than reported in adult mice (**Figure 5.6 A&B** and data not shown). In the kidney of neonates infected with 5 or 50 PFU of LCMV the viral load peaked days 5-9, rather than on day 4 as seen in adult mice, after which viral loads decreased. The majority of neonates infected with 5 PFU cleared virus in the kidney by day 14 post infection, but neonates infected with 50 PFU, even though viral load was decreased, still had ~3 logs of virus at day 14 post infection (**Figure 5.6A**). In the liver the viral load peaked at day 6-7 post infection, rather than day 4, and declined thereafter, but was not cleared by day 8 as in adults (**Figure 5.6B**). By day 14 post infection, virus was cleared in all organs in the majority of neonates infected with either 5 or 50 PFU of LCMV (**Figure 5.6B**). These data show that viral load was protracted in neonatal mice, even during infection with very low doses of LCMV Armstrong.

In adult mice the peak of the CD8 T cell response, which is at day 9 post infection, correlated with the clearance of virus on days 7-8 (Wherry et al., 2003a). We questioned if the peak of the CD8 T cell response in neonates would be delayed due to their protracted viral clearance. The frequency of LCMV-

Figure 5.6

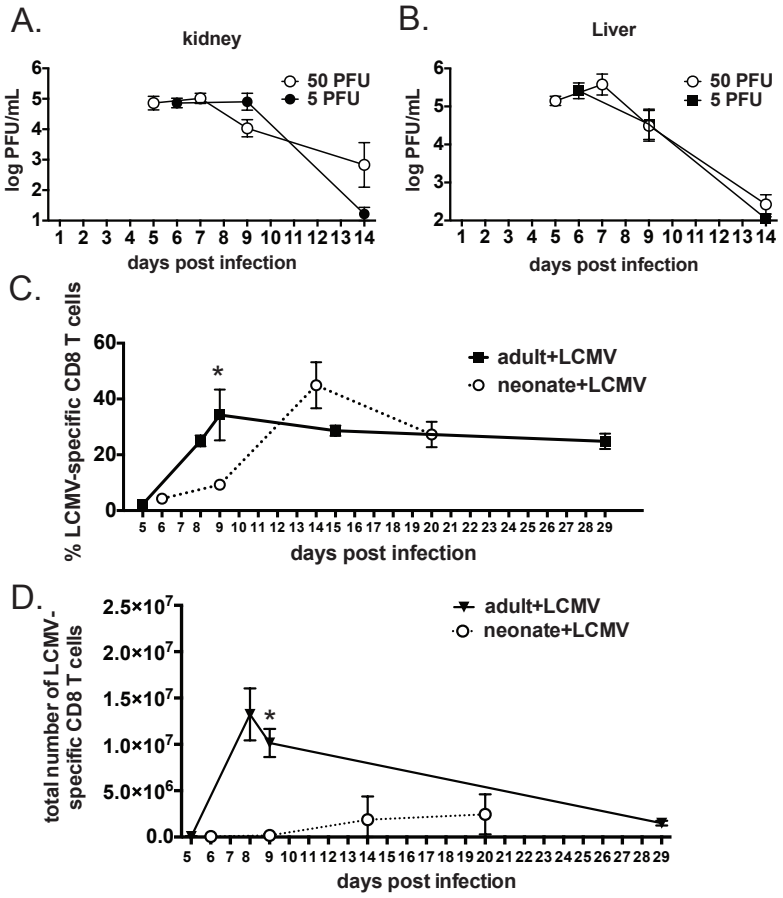


Figure 5.6 Neonates cleared virus by two weeks post infection and had delayed T cell kinetics. Neonates were infected with 5 or 50 PFU of LCMV Armstrong ip and viral load in the **A.** kidney and **B.** liver were determined at multiple time points. n=4-10 mice/group. Data are from 2 similar experiments. Neonates were infected with 5 PFU and adult mice were infected with $5x10^4$ PFU of LCMV armstrong ip. On various days post infection the **C.** frequency and **D.** total number of LCMV-specific CD8 T cells. n=3-12 mice/group. Data are from two similar experiments.

specific CD8 T cells peaked later in neonates than in adults during LCMV infection (**Figure 5.6C**). In adults the frequency of LCMV-specific CD8 T cells increased between days 5-9 to make up more than 30% of the CD8 T cell response (**Figure 5.6C**). In neonates the LCMV-specific cells expanded to a similar frequency, but this did not occur until after day 9, with the peak of the response being at day 14. By total number the LCMV-specific CD8 T cell response in adult mice peaked at day 8 instead of day 9, and this may be due to the contraction phase initiating on day 9 post infection (**Figure 5.6D**). In neonates there were significantly lower numbers of LCMV-specific CD8 T cells throughout infection compared with adult mice (**Figure 5.6D**). In neonates there was an increase in the number of LCMV-specific cells between day 9 to 14 post infection, but the number of cells did not contract after this time point. The number of LCMV-specific CD8 T cells was still high at day 20 post infection (**Figure 5.6D**). This may be due to residual virus in some mice or contraction may occur differently in a maturing mouse. These data show that the kinetics of both viral clearance and LCMV-specific CD8 T cells are delayed in neonates.

D. Neonates had replicating virus and LCMV-specific CD8 T cells in their brains and mortality was perforin-mediated

Since neonatal mice infected with LCMV Armstrong were not undergoing partial clonal exhaustion like the intermediate dose adult mice infected with LCMV clone 13, I looked for other models of LCMV that induced mortality.

Studies from mice infected with LCMV intracranially found adult mice succumb to T cell-mediated death starting at day 6 post infection (Cole et al., 1971; 1972; Gilden et al., 1972b; Hotchin, 1962) I questioned whether virus was replicating in the brains of neonates and infiltrating T cells were inducing immunopathology leading to death. Low levels of virus were found in the brain on day 5 post infection of neonates, by day 7 viral load had increased and by day 9 post infection there were 4 logs of virus in the brain (**Figure 5.7A**). LCMV Armstrong infection in adult mice do not normally gain access to the mature CNS. However, one study did report that very minimal virus could be found in the brain of some LCMV Armstrong-infected adult mice, but this could be due to contaminating peripheral blood (Wherry et al., 2003a). In our study mice were perfused during harvest to exclude this possibility. Viral load in the brain was high during the time when neonates were dying during infection. Since CD8 T cells were mediating the mortality of neonates infected with LCMV we questioned if T cells were infiltrating the CNS to clear virus and inducing death. Activated CD8 T cells were found in the brain of neonates on day 9 post infection (**Figure 5.7B**). These CD8 T cells were found to be antigen specific responding to stimulation in an ICS assay with the epitopes NP396 and GP33 with IFNγ production (**Figure 5.7B**). Infiltration of CD8 T cells into the CNS may be playing a major role in the death of neonates during LCMV infection.

In the intracranial model of LCMV infection in adult mice CD8 T cells are essential for lethal disease

Figure 5.7

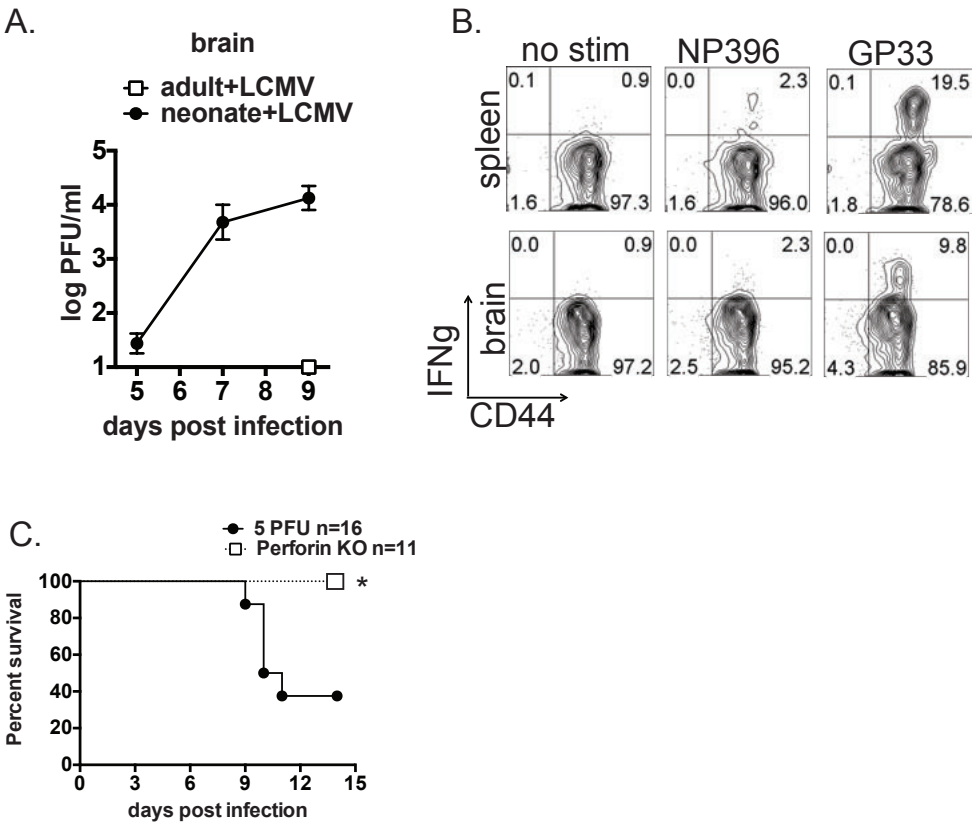


Figure 5.7. Neonates had virus replicating and LCMV-specific CD8 T cells in their brains and were dying from a perforin-dependent mechanism. A. Neonates were infected with 50 PFU of LCMV Armstrong ip and the viral load in their brain was determined on day 5, 7 and 9 post infection. The viral load of adult mice infected with 5x104 PFU of LCMV Armstrong was determined on day 9 post infection. neonates n=4-5 mice/group. adult n=2 mice at day 9 only. Data are from two similar experiments. **B.** Representative facs plots for IFNg production by CD8 T cells in response to peptide stimlation was examined in both the spleen and brain on day 9 post infection of neonatal mice. Representative from two similar experiments. **C.** wt and perforin ko neonates were infected with 5 PFU of LCMV armstrong ip and survival was monitored until day 14 post infection. Data are from two similar experiments. wt mice are also shown in figure 5.1 A.

(Cole et al., 1972; Doherty and Zinkernagel, 1974; Nansen et al., 1998; Storm et al., 2006). However, the direct cytotoxic functions of CD8 T cells have been found to not be required for mortality as the release of chemokines can still attract monocytes and neutrophils into the CNS and induce death (Kim et al., 2009). In order to determine which functions of CD8 T cells were required for T cells to mediate mortality in neonatal mice we used a number of genetically modified mouse models. T cells have several cytokine and cytolytic functions that can mediate cell death and thus immunopathology. Fas-Fas ligand (FasL) interactions can induce cell death via caspase 8 activation. Pups lacking FasL, infected with 5 PFU of LCMV showed no difference in the kinetics of death or the development of immunopathology (data not shown). Likewise, when FasL deficient pups were treated with anti-IFNγ and soluble TNF receptor (etanercept) to block cytokine signaling there were no differences in mortality (data not shown) indicating Fas-FasL, IFNγ and soluble TNF do not play a major role in mortality in neonates infected with LCMV. CD8 T cells can also lyse virus-infected cells directly by cytolytic properties. CD8 T cells contain perforin and granzymes. These enzymes are released within close proximity to a target cell and induce pores in the cellular membrane and activate caspase 3- and 7-mediated apoptosis. We questioned if T cell-mediated immunopathology would be blocked if pups lacked perforin. Perforin KO pups were infected with a low dose of 5 PFU of LCMV and had 100% survival (**Figure 5.7C**). These data

suggest that in neonates CD8 T cells mediated severe immunopathology that resulted in death in the majority of pups through perforin-mediated lysis of cells.

D. Maternal antibody protected neonates from death by mediating faster viral clearance

Previous studies have found that neonates from LCMV-immune mothers can mediate protection from lethal infections (Baldridge and Buchmeier, 1992). LCMV-immune mothers could protect day 10 of age pups from a lethal intracranial infection. We questioned if passive immunity would also be a method to increase survival of day 7 neonates infected with LCMV Armstrong. Female mice were immunized with LCMV Armstrong between 6-8 weeks of age considered immune 6 weeks post infection, after which they were used for breeding. Neonates infected with 50 or 500 PFU of LCMV Armstrong were completely protected from death when mice were followed until day 14 post infection (**Figure 5.8A**) and had minimal lung and liver infiltrates (**Figure 5.8B**). Neonates from LCMV-immune mothers cleared virus from the kidneys by day 9 post infection, while neonates from naïve mothers still had ~3 logs of virus at day 14 post infection (**Figure 5.8C &D**). These data suggest that passive immunity could control viral replication early in infection, possibly compensating for the immature innate immune system, allowing the neonatal CD8 T cell response to control infection without inducing massive collateral damage.

Figure 5.8

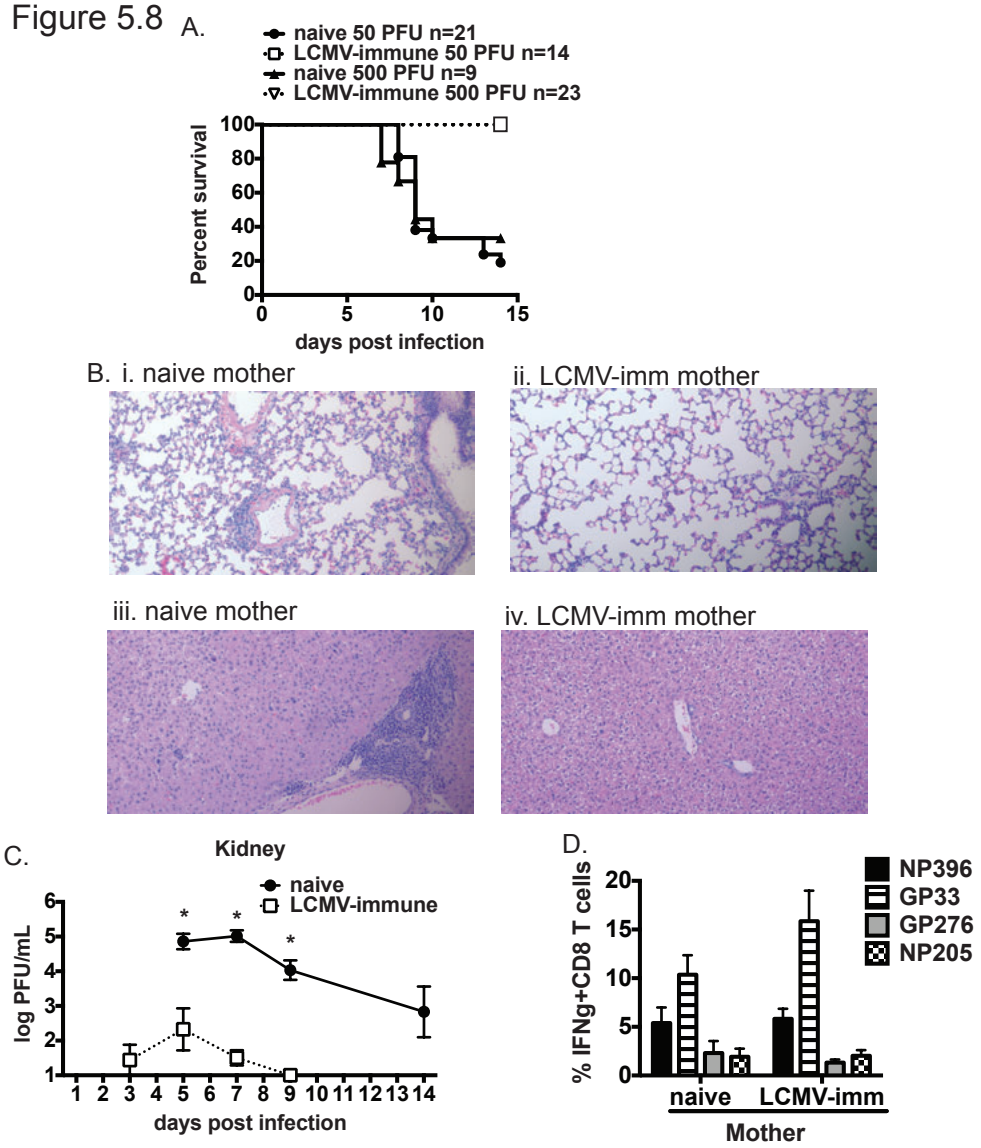


Figure 5.8 Passive immunity protected neonates by mediating faster viral clearance. Neonates from naïve or LCMV-immune mothers were infected with 50 or 500 PFU of LCMV Armstrong ip. **A.** Mortality was monitored until day 14 post infection. Data are from two similar experiments. **B.** Lung (**i & ii**) and liver (**iii & iv**) sections were stained with H&E on day 14 post infection from neonates infected with 500 PFU of LCMV Armstrong ip from naïve (**i & iii**) and LCMV-immune (**ii & iv**) mothers. Sections are representative of two similar experiments. **C.** Viral load was determined in the kidney on various days post infection from neonates with naïve or LCMV-immune mothers infected with 50 PFU of LCMV. n=4-5 mice/group. Data are from 2 similar experiments. **D.** CD8 T cell immunodominance hierarchies were examined by IFNg production after peptide stimulation on day 14 post infection with 50 PFU LCMV from neonates with naïve or LCMV-immune mothers. n=3-9 mice/group. Neonates from naive mothers are also shown in figure 5.4B. Data are representative of 3 similar experiments.

Studies have found that maternal antibody blocked the development of cellular and humoral responses to the live-attenuated measles vaccine (Premenko-Lanier et al., 2006). Normally, the measles vaccine is administered at 9 months of age. However, Peter Aaby's group showed that an additional earlier vaccination at 4.5 months, during the presence of maternal antibody, enhanced survival (Martins et al., 2008). We questioned if LCMV was cleared in neonates from LCMV-immune mothers too quickly and not allowing for the development of a CD8 T cell response. At day 14 post infection CD8 T cell immunodominance hierarchies were similar in the neonates from LCMV-immune mothers and naïve mothers both in frequency of responses and both had the low NP396-specific CD8 T cell response shown earlier (**Figure 5.8D**).

E. Neonates had altered naïve Vβ repertoires and highly variable antigen-specific Vβ usage

We questioned at what age does the LCMV-specific immunodominance hierarchy of a young mouse resemble that of an adult. To compare to the day 7 neonate, mice were infected at day 12, 18, 20 and 22 of age and immunodominance hierarchies were examined at day 9 post infection. Interestingly, mortality was only found in mice infected at day 7 of age (data not shown). The decreased NP396-specific population was found in mice infected at day 12, 18 and 20, but at day 22 there was a shift in dominance and the NP396-specific response is greater than the GP33-specific response by both frequency

Figure 5.9

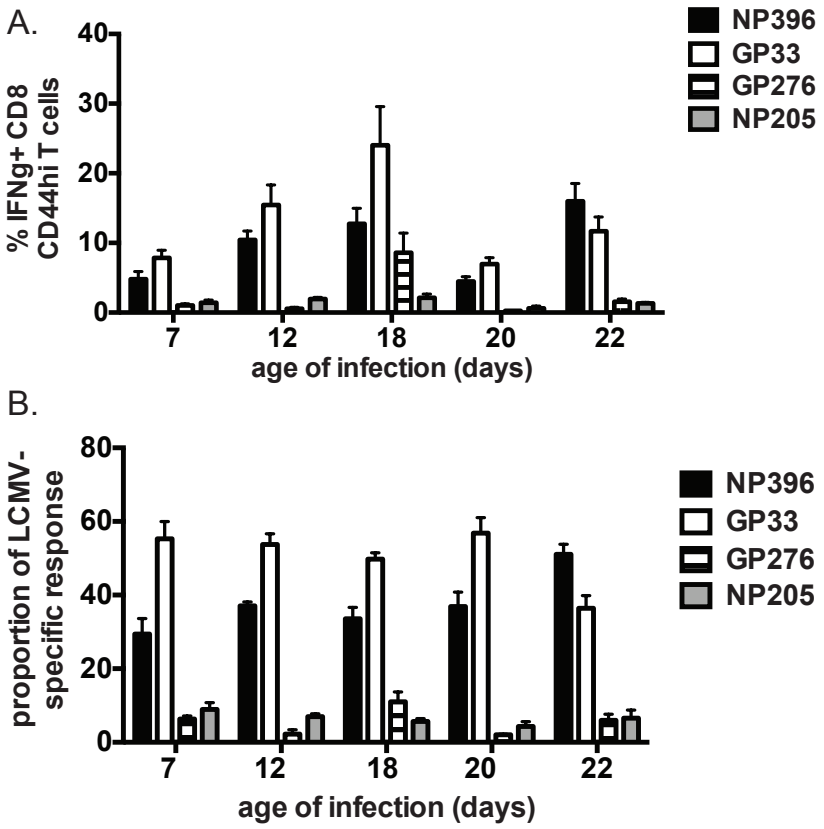


Figure 5.9 Neonatal CD8 T cell immunodominance hierarchies were age-dependent. Mice were infected with 50 PFU of LCMV Armstrong ip at day 7, 12, 18, 20 and 22 of age and immunodominance hierarchies were determine by IFNg production after peptide stimulation on day 9 post infection and represented as frequency (**A.**) or total number (**B.**) n= 3-13 mice/group. Data are representative of 2 similar experiments.

and proportion (**Figure 5.9A&B**). These data would suggest there is a change that occurs in the immune responses of mice at three weeks of age that leads to an increased NP396-specific response. I questioned if there was any evidence that the limited TCR repertoire, due to delayed expression of TdT, at day 7 of age may be mediating this change over time in the NP396-specific response. I found that there were significant differences in both CD4 and CD8 T cell Vβ repertoires in B6 neonates. In neonatal uninfected mice in CD8 T cells there were significantly decreased frequencies of Vβ 5.1/2, 8.1/2, 8.3, 9 and 10 families and increased frequencies of Vβ 11 and 13 (**Figure 5.10A**). The CD4 T cell repertoire of neonates also had decreases in the frequency of Vβ 8.1/2, 8.3 and 10 families and increases in the Vβ 11 suggesting that there were major differences in these Vβ families between neonates and adults (**Figure 5.10B**). There were also increases in the Vβ 6 and 12 CD4 T cell populations in neonates (**Figure 5.10B**). These numerous differences in Vβ repertoire distribution in neonates suggest that neonates would also show different Vβ responses for antigen-specific populations.

In adult mice infected with LCMV Armstrong the Vβ repertoire of the NP396-specific population includes a dominant public Vβ 8.1/2 usage in all mice followed by subdominant usage of Vβ 6, 9, 12 and 13 (**Figure 5.10C**) (Blattman et al., 2000). There is variation, or private specificity, between individuals on the subdominant Vβ responses (Blattman et al., 2000). The Vβ repertoire of NP396-specific cells was found to be significantly different than in adults, and there was

Figure 5.10

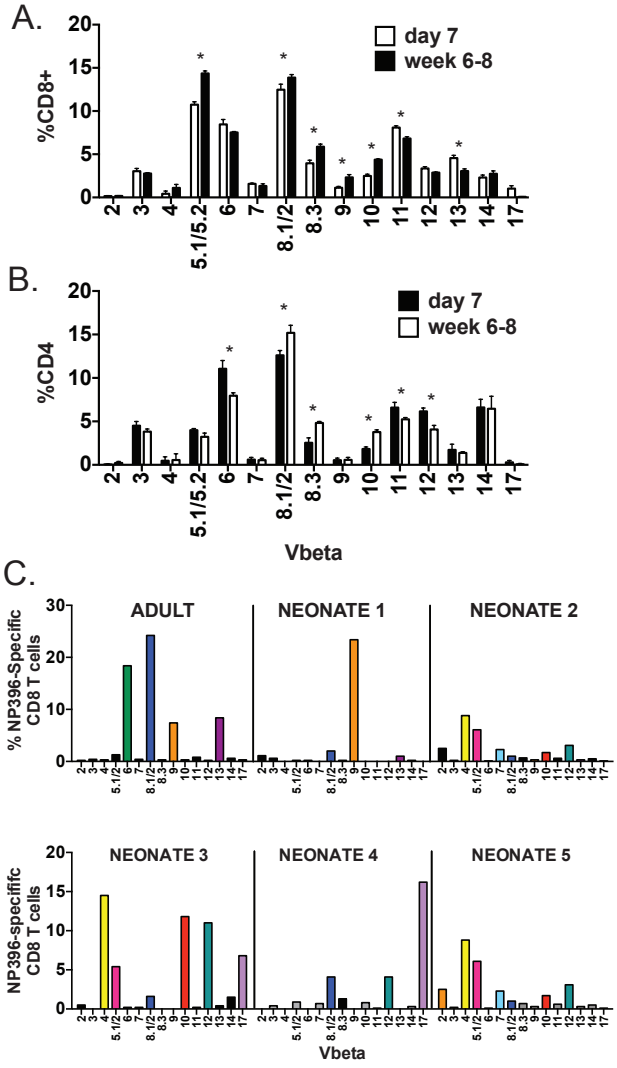


Figure 5.10. Neonates had altered T cell vbeta repertoires. The vbeta repertoires of CD8 (**A.**) and CD4 (**B.**) T cells were analyzed by monoclonal antibody staining in naïve neonatal and adult mice. n=5-7 mice/group from two similar experiments. **C.** On day 9 of LCMV infection, the vbeta repertoire of NP396-specific CD8 T cells from the spleens of adult and neonatal mice were determined by monoclonal antibody staining. Data is representative from 3 similar experiments.

a high degree of variability, or private specificity, between individual neonates, a finding which did not occur in adults (**Figure 5.10C**). For example, one pup had co-dominance of Vβ usage of 4, 10 and 12, whereas another pup had a more monoclonal response with only a Vβ 9 usage (**Figure 5.10C**). This variability between individuals demonstrates increased private specificity of the T cell repertoires in neonatal mice compared to adults. Even though neonates are genetically identical they have different random insertions in the CDR3 region, thereby causing unique TCR repertoires in each mouse, known as private specificity.

The variability in the TCR repertoire is due in part to the enzyme TdT, which introduces random nucleotides into the CDR3 region during T cell development. In mice TdT expression in the thymus occurs between days 4 to 5 with longer CDR3 regions being detected in single positive thymocytes by day 8 of age (Bogue et al., 1991). I found that naïve adult TdT ko B6 mice had an altered CD8 T cell Vβ repertoire distribution (**Figure 5.11A**). TdT ko mice had an increased usage of Vβ 8.1/2 and 8.3 and an decreased usage of Vβ5.1/2. The decreased usage of Vβ5.1/2 was also found in naïve day 7 neonates, but neonates had a reduction in the Vβ usage for Vβ 8.1/2 and 8.3 (**Figure 5.10A**). After LCMV infection adult TdT ko mice developed altered immunodominance hierarchies as measured by both frequency and proportional data (**Figure 5.11B&C**). Compared to wildtype adult mice, TdT ko adult mice had a significant reduction in the frequency of not only NP396-specific cells, but also GP33-

Figure 5.11

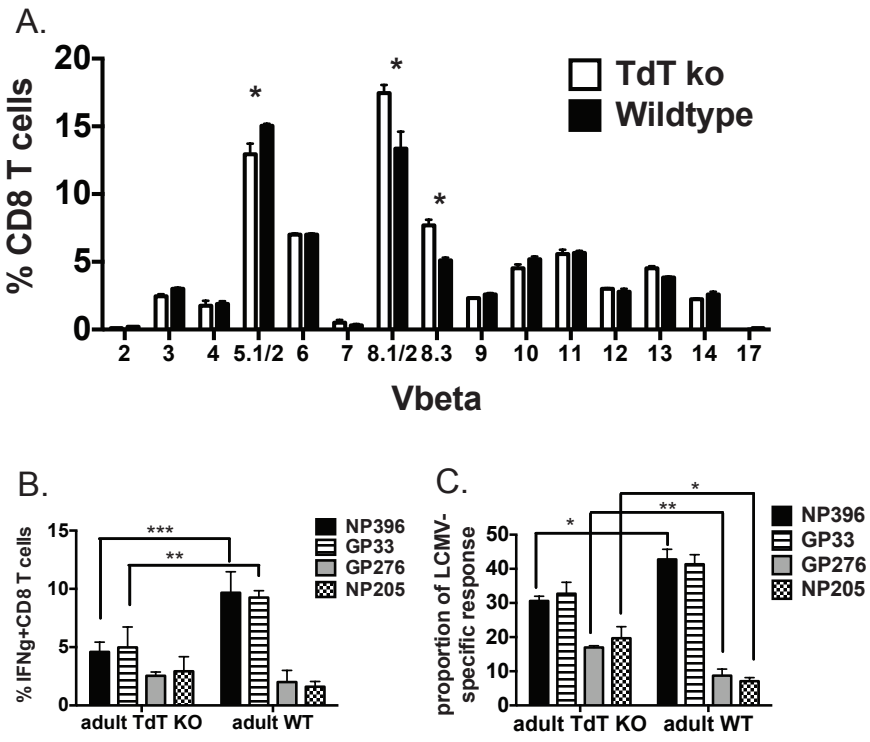


Figure 5.11 TdT deficient mice had altered CD8 T cell vbeta repertoires and LCMV-specific immunodominance hierarchies. A. The CD8 T cell Vbeta repertoires of naive adult TdT ko and wildtype mice were determined by monoclonal antibody staining. n=2-3 mice/group from a single experiment. **B. & C.** Adult TdT ko and wild-type mice were infected with LCMV and on day 9 post infection CD8 T cell immunodominance hierarchies were determined **B.** frequency and **C.** proportion. n=5-7 mice/group from two similar experiments.

specific cells. There were no differences in GP276- or NP205-specific cells (**Figure 5.11B**). By proportion, TdT ko mice had a significant reduction in NP396-specific T cells with no difference in the GP33-specific population (**Figure 5.11C**). Interestingly, the subdominant epitopes, GP276 and NP205, made up a greater proportion of the response in TdT ko mice compared with wildtype adult mice (**Figure 5.11C**). The reduction in the NP396-specific response in TdT ko adult mice was similar to that found in wildtype neonatal mice infected with LCMV (**Figure 5.4B**), but there was also a reduction in the GP33-specific response that was not found in day 7 neonates. Interestingly, TdT ko adult mice had an increased usage of Vβ 8.1/2, which is the dominant Vβ found in the NP396-specific population of LCMV-infected mice, and still had a decreased response to this epitope. These data suggest that the CDR3 region may be playing a greater role in antigen-specific responses than just the Vβ repertoire. However, these data collectively show that there were differences in the repertoire of neonates, both in the naïve populations and the antigen-specific responses.

F. Neonates have decreased type I IFN responses

In addition to the differences found in the neonatal TCR repertoire we questioned if differences in the innate responses of neonates could be responsible for the altered immunodominance hierarchies. A reduction in the NP396-specific CD8 T cell population has also been found in IRF7 KO adult mice.

Figure 5.12

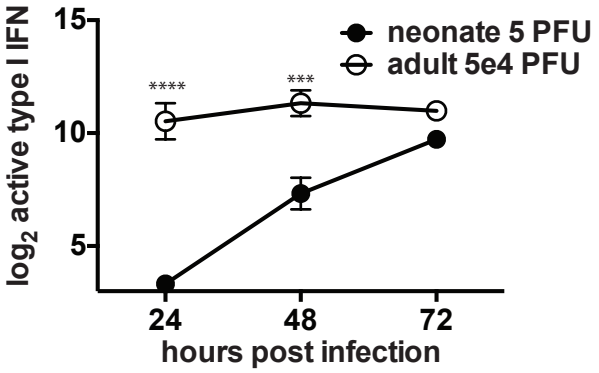


Figure 5.12 Neonates had delayed type I IFN responses after LCMV infection. Type I IFN was measured in the serum of neonates and adult mice at 24, 48 and 72 hours post infection by type I IFN bioassay. n=3-7 mice/group. Data are from three similar experiments.

IRF7 is a key transcription factor in the induction of type I IFN after LCMV infection (Zhou et al., 2012). Since type I IFN is required for CD8 T cell proliferation and differentiation during LCMV infection (Welsh et al., 2012), we measured type I IFN by bioassay after LCMV infection in neonates compared to adults. We found that at 24 hours post infection type I IFN levels were below the limit of detection in our assay (**Figure 5.12**). At 48 hours type I IFN was detected, but at significantly lower levels in neonates. By day 3 post infection neonatal type I IFN levels were similar to adults. Delayed type I IFN induction is not surprising and is known to occur in neonates (Kollmann et al., 2012). Type I IFN controls anti-viral responses, and delayed expression would allow unchecked viral replication in neonates and could be contributing to the generation of altered immunodominance hierarchies by a yet unknown mechanism.

G. Female pups are protected from death but only on the Balb/c background

Different strains of mice are known to have different susceptibilities viral infection. Balb/c mice are known to be more sensitive to Th1-inducing viruses. Balb/c mice have a higher affinity IL-4 receptor that skews immune responses to Th2 (Schulte et al., 1997). We questioned if Balb/c mice would be more resistant to neonatal LCMV infection, as they may not develop such a strong Th1/CTL immune response. We found that Balb/c infected neonates died at a similar rate as B6 neonates (**Figure 5.12A**). However, on the Balb/c background

Figure 5.13

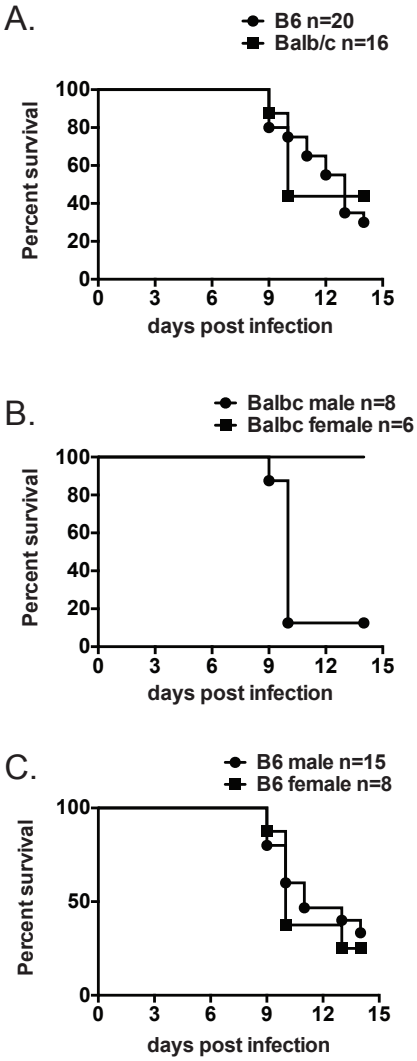


Figure 5.13 Sex differences mediated survival of Balb/c female mice after LCMV infection. A. Mortality of B6 and Ba b/c neonates after infection with 5 PFU of LCMV Armstrong ip was monitored until day 14 post infection. **B.** Mortality of males and females were examined for Balb/c (**B.**) and B6 (**C.**) strains of mice.

all female mice survived and only males succumbed to infection. Hormonal differences that exist between males and females have long been known to alter immune responses. In B6 neonates there was no difference between males and females.

Chapter Summary

The data in this chapter show that day 7 neonates were highly susceptible to T cell-mediated perforin-dependent immunopathology and death after LCMV infection. Death was not due to viral load alone, as mice lacking both CD4 and CD8 T cells had high viral loads in several organs, but retained a 100% survival rate. Passive immunity through LCMV-immune mothers could mediate faster viral control in neonates and also result in 100% survival. LCMV-specific CD8 T cell responses in neonates were similar to adults in frequency, but significantly reduced in total number. CD8 T cell immunodominance hierarchies in neonates resembled adults infected with an intermediate dose of LCMV clone 13, in that neonates had decreased NP396-specific populations. However, neonates differed from LCMV clone 13-infected adults as they did not develop partial clonal exhaustion. Analysis of the Vβ distribution of the CD4 and CD8 T cell populations in uninfected neonates showed significant differences in the frequency of several Vβ including those that are commonly used in the NP396-specific Vβ repertoire of adult mice. Examination of the NP396-specific Vβ repertoire of neonates showed that they were both significantly different from the adult repertoire, but

there was also a high degree of variability between individuals, which is not seen in adults. TdT ko adults also had a reduction in the NP396-specific response, further supporting the role of the narrowed repertoire in the altered immunodominance hierarchies of neonates. In addition to the altered CD8 T cell response we showed that there was a severely delayed type I IFN response in neonates.

Chapter 6: Heterologous immunity is a mechanism for protection in neonatal mice

Human newborns and infants receive multiple vaccines to protect them against infection. Previously, vaccinations have been given with the thought of protecting against specific pathogens. However, several non-specific effects of immunization have been observed. Children that receive the BCG or measles vaccine, which are live attenuated vaccines, are non-specifically protected from death from other pathogens besides tuberculosis or measles virus (Aaby et al., 1995; Roth et al., 2005). Furthermore, this protection is lost and children are actually more susceptible to death if they are subsequently given the combination diphtheria, tetanus and pertussis (DTP) vaccine (Aaby et al., 2007). Heterologous adaptive and innate immunity may be mediating these "non-specific" effects of vaccines.

One model of protective heterologous immunity is LCMV-immune mice challenged with VV. Previous immunization with LCMV can protect mice from a lethal challenge with VV (Chen et al., 2001; Cornberg et al., 2007; Selin et al., 1998). Adoptive transfer of LCMV-immune splenocytes into naïve mice protects during a VV challenge (Selin et al., 1998). However, if the LCMV-immune splenocytes are depleted of either CD4 or CD8 T cells, protection is lost (Selin et al., 1998). Crossreactive CD8 T cell lines, that were generated by stimulating LCMV-immune splenocytes with VV epitopes, can protect naïve mice from VV challenge (Cornberg et al., 2007; 2010). During VV challenge of LCMV-immune

mice there is an expansion of several different LCMV epitope specific populations. Some individual LCMV-immune mice show an expansion of LCMV-NP205-specific CD8 T cells, while others have a crossreactive LCMV NP118- or GP34-specific response (**Figure 1.5**) (Kim et al., 2005). Private specificity, or the unique TCR repertoire of individual mice, mediates which crossreactive response will expand during VV challenge (Kim et al., 2005). The TCR repertoires of neonatal mice are less diverse than that of adults (**Figure 5.10**) (Adkins, 2005). We questioned if this altered T cell repertoire would result in altered protection in this model of beneficial heterologous immunity if mice were immunized with LCMV as neonates. Furthermore we questioned if the altered neonatal repertoires may impact the crossreactive patterns that are found when these same mice are infected with VV as young adults 6 weeks later.

A. Mice immunized with LCMV as neonates show immune protection after VV challenge

Since neonates from naïve mothers have ~80% mortality after LCMV infection (**Figure 5.1**) I decided to use neonates from LCMV-immune mother which are protected from death (**Figure 5.8**). Neonates were immunized with 50 PFU of LCMV ip at day 7 of age and allowed to mature to 7 weeks of age, after which they were challenged with VV. On day 6 post VV challenge mice were harvested, and the viral load in the fat pads was determined by plaque

assay (**Figure 6.1A**). Similar to adult mouse studies, there were significantly lower viral loads in both males and females that were immunized with LCMV at day 7 of age. These data suggest that heterologous immunity is also a mechanism of protection when mice are immunized as neonates.

B. Males immunized as neonates develop enhanced panniculitis during VV challenge

Adult male LCMV-immune mice challenged with VV develop immunopathology of the fat pads called AFN. The IFNγ and TNFα produced by crossreactive memory cells recruited to the fat pad causes the upregulation Fas on adipose tissue (Siwei Nie, unpublished data). Fas expression on the surface of adipocytes interactions with FasL on CD8 T cells and results in patches of necrosis on the fat pads. AFN can vary in severity from mild to moribund. The severity of pathology is also determined by the private specificity of the crossreactive memory T cell population, as demonstrated in adoptive transfer experiments (Nie et al., 2010). At the dose of VV used for these experiments AFN is not directly induced by viral lysis of cells. Uninfected adult and neonatal male mice challenged with VV have mild levels of AFN (score 0-3) (**Figure 6.1B**). With previous immunization with LCMV, AFN levels are increased to moderate (score 4-6) and severe (score 7-8). Interestingly, LCMV-immune female mice do not develop AFN after VV challenge (**Figure 6.1B**). The mechanism for this sex

Figure 6.1

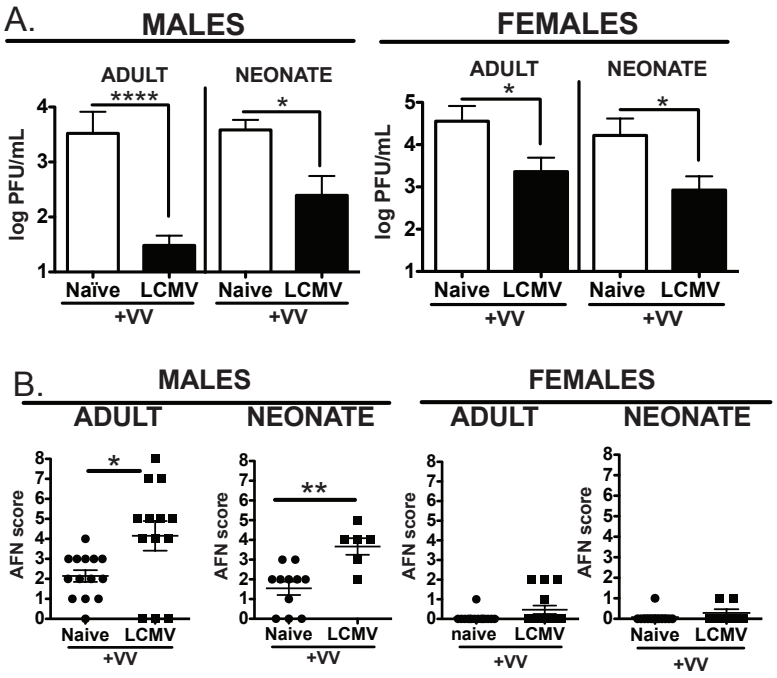


Figure 6.1. Mice immunized with LCMV as neonates develop immune protection and immunopathology after VV challenge. Male and female mice were immunized with LCMV at either day 7 of age (neonate) or at 6 weeks of age (adult). Neonates were immunized with 50 PFU in 50ul ip while adults were given 5x104 PFU in 100ul ip. After six weeks mice were considered immune and all mice challenged with 1x106 PFU of VV in 100ul ip. On day 6 post VV challenge **A.** viral load was determined in fat pads by plaque assay and **B.** panniculitis was scored on a scale from 0-8. Data is from three similar experiments comparing adults n=13-20 mice/group and neonates n=6-12 mice/group. Statistical significance was determined using the Student's T test. *p<0.05, **p<0.01, ****p<0.0001

difference is currently unknown. When both males and females were immunized with LCMV as neonates and challenged with VV, AFN was only found in males, similar to that found in adults. Uninfected male neonatal mice developed mild AFN with a significant increase in LCMV-immune mice (**Figure 6.1B**). These data suggest that the mechanisms for AFN are maintained in mice immunized as neonates.

C. VV responses are similar in mice immunized with LCMV as neonates and adults

After infection with VV, mice develop CD8 T cell responses to several VV-specific antigens. These responses are predictable and consistent between individual mice. Naïve mice infected with VV produced a dominant response to B8R with subdominant A47L-, E7R- and A11R-specific responses (**Figure 6.2**). The A11R-specific response is known to be crossreactive between LCMV and VV as LCMV-immune splenocytes can expand in culture to A11R stimulation and produce IFNγ and TNFα and stain with both tetramers (Cornberg et al., 2007; 2010). We questioned if mice were immunized with LCMV as neonates would this alter their crossreactive CD8 T cell responses to VV compared with mice immunized as adults. There were no differences in the VV-specific immunodominance hierarchies between mice immunized with LCMV as neonates or adults (**Figure 6.2**). The only significant difference that was

Figure 6.2

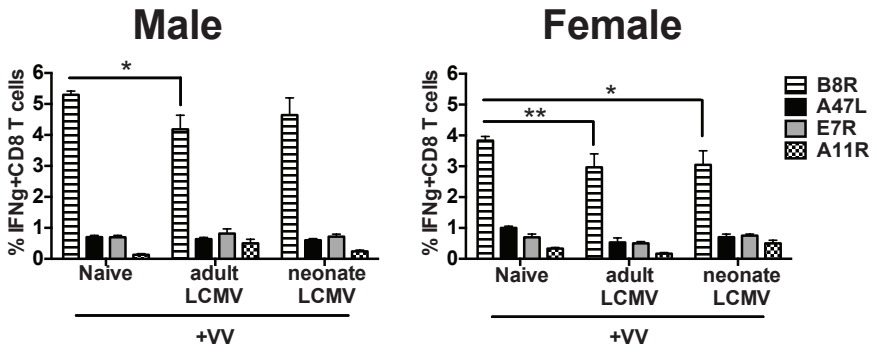


Figure 6.2. VV-specific CD8 T cell responses are similar between mice immunized as neonates or adults after VV challenge. On day 6 post VV challenge, VV-specific CD8 T cell responses were determined in mice immunized with LCMV as neonates (day 7 of age) or adults (week 6 of age) or naive controls. IFNg production was assessed after stimulation with VV epitopes B8R, A47L, E7R and A11R. Data is representative of two similar experiments. n=2-5 mice/group.

observed was a decreased response to the dominant B8R epitope in both male and female mice immunized as adults, and female, but not male mice immunized as neonates. (**Figure 6.2**). This reduction may be due to the more rapid clearance of VV by the memory CD8 T cell responses.

D. Mice immunized as neonates have similar numbers of LCMV-specific memory, but altered immunodominance hierarchies

The size of the CD8 T cell memory population has been found to determine the level of protective immunity in homologous challenge models (Cerwenka et al., 1999; Hamada et al., 2009). Because neonates have reduced T cell numbers (Ridge et al., 1996) (**Figure 5.3**) I questioned if mice immunized as neonates would have fewer LCMV-specific CD8 T cells compared to mice immunized as adults. Surprisingly, we found that there was no difference in the number of memory LCMV-specific CD8 T cells in the spleens between these groups six weeks post LCMV infection (**Figure 6.3A**). The increase in the LCMV memory frequency and the later dominance of NP396 most likely relates to the slow clearance of LCMV over more than 14 days as compared to adults.

In LCMV-immune mice challenged with VV there are multiple LCMV-specific memory populations that can recognize VV antigens and expand. Most commonly these are GP33/34-, NP205- and GP118-specific populations (Kim et al., 2005). We compared immunodominance hierarchies in mice immunized as neonates to adults and found that there were several differences even before VV

Figure 6.3

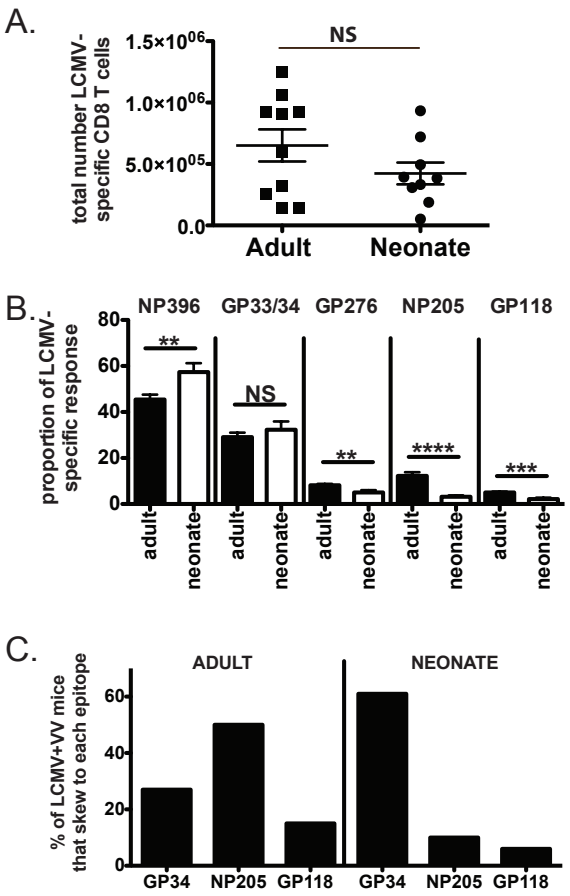


Figure 6.3. Mice immunized with LCMV as neonates had similar number of LCMV-specific CD8 T cells, but altered immunodominance hierarchies and skewing after VV challenge. At 6 weeks post LCMV-immunization the **A.** total number of LCMV-specific CD8 T cells and **B.** LCMV CD8 T cell immunodominance hierarchies were determined in the blood or spleen mice immunized as a neonate or an adult. Data is from three similar experiments. n=25-29 mice/group **C.** To determine skewing of the immunodominance hierarchies due to crossreactivity LCMV-immune mice were bleed before VV challenge and immunodominance hierarchies from this time point were compared to immunodominance hierarchies from day 6 post VV challenge. These show the frequency of mice skewing to each LCMV epitope. Data are representative of three similar experiments. n=26 adults n=39 neonates. NS= not significant, **p<0.01, ***p<0.001, ****p<0.0001

challenge (**Figure 6.3B**). There was a higher proportion of NP396-specific cells in mice immunized as neonates that was surprising because this population was decreased at day 9 and 14 post LCMV-infection (**Figure 5.4**). There was no difference in the proportion of GP33/34-specific cells between neonates and adults. However, there was a reduced proportion of the crossreactive, NP205- and GP118-specific cells (**Figure 6.3B**). Therefore, the LCMV memory of mice immunized as neonates is altered in the epitope-specific hierarchy as compared to mice immunized as adults. We questioned if this could influence the pattern of crossreactive LCMV responses, which expand during VV infection.

In mice immunized with LCMV as an adult and challenged with VV the most common crossreactivity that arises is NP205 (Kim et al., 2005). Expansion of NP205-specific memory cells is found in about 50% of LCMV-immune mice after VV challenge, with 27% of mice skewing towards GP33/34-specific cells and 15% towards GP118-specific cells (Kim et al., 2005). By assessing immunodominance hierarchies before and after VV challenge we can determine which crossreactive response expanded in individual mice. Mice immunized as neonates showed a different skewing pattern than mice immunized as adults (**Figure 6.3C**). The most common crossreactivity was GP33/34, which occurred in 61% of mice with 10% expanding NP205- and 6% expanding GP118-specific responses. The decreased usage of NP205- and GP118-specific memory responses during VV challenge may be due to their decreased frequency and

perhaps altered TCR repertoire since NP205- and GP118-specific populations were diminished compared to mice immunized as adults.

Chapter Summary

In this chapter we show that the age of immunization does not alter the immune protection and immunopathology that can occur in the beneficial LCMV+VV model of heterologous immunity. Mice immunized as neonates with LCMV have lower viral loads and some male mice develop panniculitis. The VV-specific CD8 T cell response that develops in these mice is unchanged by the age of LCMV infection, suggesting no dramatic alterations in the immune systems functionality. Considering the differences in neonatal and adult LCMV-specific responses it was not surprising to find that the LCMV-specific crossreactive responses utilized by mice immunized as neonates differed from mice immunized as adults. This may be due to precursor frequency as there was a significant decrease in the NP205-specific memory proportion in mice immunized as neonates even before VV challenge, or it may be due to TCR repertoires as neonatal mice develop different TCR repertoires to adults. These data demonstrate that even though there are altered crossreactivities in mice immunized as neonates, the principles of heterologous immunity are maintained in the immature neonatal immune system, and most likely contribute to the beneficial “non-specific” effects of vaccines.

Chapter 7: CD8 T cell immunity and heterologous immunity in neonatal mice infected with PICV

Neonatal mice are sensitive to infection in general, but also to infection with nonlytic viruses, like LCMV, that can quickly replicate within a host (chapter 5). PICV is distantly related to LCMV, and they share 48% sequence homology (see methods for sequence analysis), but PICV does not replicate as well in adult B6 mice. To obtain an optimum CD8 T cell response adult mice need to be infected with $2x10^7$ PFU of PICV ip, which is 400-fold higher than the optimum dose of LCMV Armstrong. PICV provides protective heterologous immunity to both LCMV and VV infections (Brehm et al., 2002; Chen et al., 2012; Selin et al., 1998). Expanded populations of the crossreactive NP205-specific cells induce faster clearance of heterologous viruses but also cause a skewing of both the LCMV- and PICV-specific CD8 T cell immunodominance hierarchies, with most mice developing a dominant or co-dominant NP205-specific response. We questioned whether neonatal mice, which succumb to LCMV infection, would be able to clear PICV. Furthermore, we questioned if mice immunized with PICV as neonates would show expansion of the crossreactive NP205-specific population thus altering the PICV-specific immunodominance hierarchy after LCMV challenge due to crossreactivity.

A. Neonatal mice control PICV infection without mortality

I questioned if neonatal mice that are highly sensitive to LCMV would be able clear PICV without mortality. Day 7 neonatal mice were infected with $4x10^4$ PFU of PICV ip, and 100% of mice survived. The dose of PICV was increased to $4x10^5$ and $4x10^6$ PFU, and still 100% of pups survived (data not shown), suggesting that PICV can not replicate to as high levels in neonates, as was found with LCMV infections, allowing the CD8 T cell response to clear virus without inducing severe immunopathology. To determine the kinetics of viral clearance in neonatal mice compared to adults PICV titers were determined in multiple organs over time. In the fat pads of adult mice PICV replicated well, reaching ~3 logs during the early phase of infection, day 2, after which viral load declined and was cleared by day 7. In neonatal mice viral load in the fat pad remained high at ~3-4 logs until day 7 post infection after which it declined and virus was cleared between day 8-11 (**Figure 7.1A**). In adult mice PICV does not grow well in the kidney. Low levels of replicating virus can be detected in the kidney between days 3-5. Interestingly, in neonatal mice virus replicated to over three logs of virus in the kidney and followed a similar kinetics of clearance as the fat pad (**Figure 7.1A**). These data show that even though neonatal mice do not die from PICV infection, nor do they achieve the same levels of virus as LCMV, they still have protracted viral clearance, a hallmark of infection in neonates.

Figure 7.1

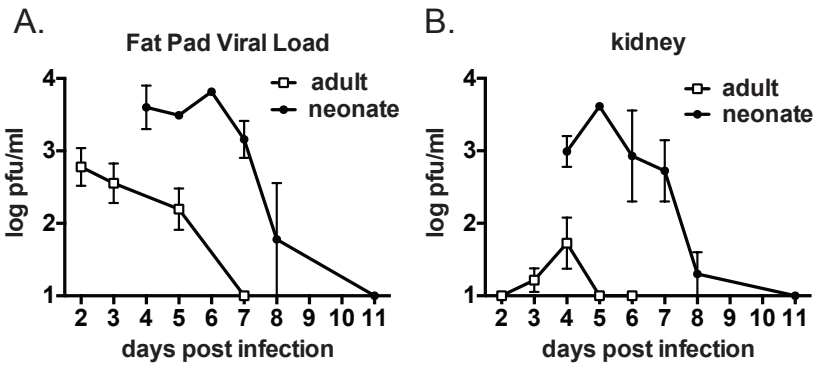


Figure 7.1. Viral clearance is protracted in neonatal mice compared to adults after PICV infection. Neonatal (day 7 of age) and adult (week 6 of age) were infection with PICV. Neonates were infected with $4x10^4$ PFU ip while adults received $2x10^7$ PFU ip. Viral load was determined by plaque assay at numerous days post infection in **A.** fat pad and **B.** kidney. Neonates n=2 mice/time point. Adults n=4-13 mice/time point.

B. Neonatal mice are capable of producing adult-like CD8 T cell responses

Since CD8 T cells mediate PICV clearance, we examined how the CD8 T cell responses in neonates compared to that of adult mice after PICV infection. The overall size of the PICV-specific response was significantly reduced in neonatal mice infected with $4x10^4$ PFU, but by increasing the infecting dose to $4x10^5$ and $4x10^6$ PFU neonatal mice produced PICV-specific responses that were similar in frequency to adults (**Figure 7.2A**). The total number of responding CD8 T cells in neonates was still significantly lower than adult mice (**Figure 7.2B**).

To more closely examine the PICV-specific CD8 T cell response, immunodominance hierarchies were examined for three epitopes. At day 8 post infection in adult mice the NP38-specific response dominated, followed by an intermediate NP122-specific response and a very subdominant NP205-specific response (**Figure 7.2C**). At low dose there was a significant reduction in the frequency of NP38-specific cells compared to adult mice but, by increasing the infecting dose to $4x10^5$ and $4x10^6$ PFU, the NP38-specific response could reach adult levels (**Figure 7.2C**). Similar to the CD8 T cell responses in LCMV-infected neonates, there was a difference in PICV-specific immunodominance hierarchy with a loss of NP122-specific T cell response (**Figure 7.2C**). Even with increased doses this response did not develop (**Figure 7.2C**).

These experiments were performed on day 8 post infection because this is the known peak of the CD8 T cell response in adult mice after PICV infection.

Figure 7.2

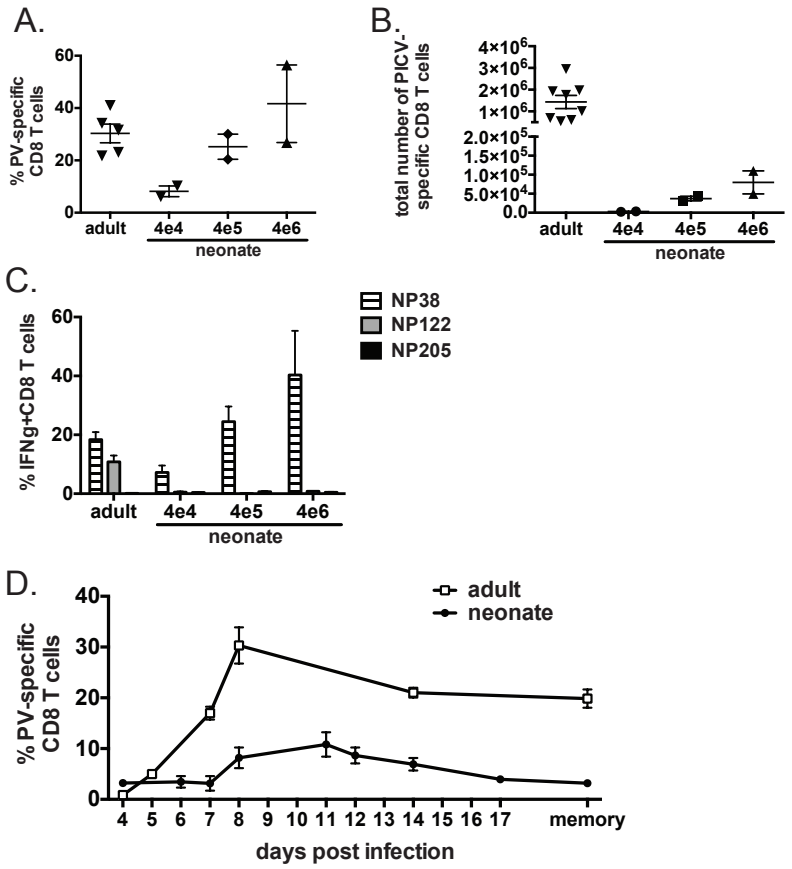


Figure 7.2. Neonates infected with PICV can produce adult-like CD8 T cell responses with delayed kinetics and alter immunodominance hierarchies. Neonatal mice were infected with $4x10^4$, $4x10^5$ or $4x10^6$ PFU of PICV and compared to adult mice infected with $2x10^7$ PFU on day 8 post infection for **A.** frequency and **B.** total number of PICV-specific CD8 T cells. Neonate data is from a preliminary experiment. Adult data is representative from several similar experiments **C.** Immunodominance hierarches were also determined in these groups of mice by IFNg production after peptide stimulation. Neonate data is from a preliminary experiment. n=2 mice/group. Adult data is representative from several similar experiments n=4 mice/group. **D.** The kinetics of PICV-specific CD8 T cell responses were determined for neonates infected with $4x10^4$ PFU of PICV and compared to adult PICV-infected mice. Neonate data is preliminary with n=2-7 mice/group. Adult data is representative from several similar experiments n=2-12 mice/group.

However, based on the viral load data we know that it takes longer for neonates to clear PICV infection. We questioned if the peak of the CD8 T cell response would be delayed in neonates, similar to that found in LCMV-infected neonates. Examining the frequency of PICV-specific CD8 T cells through the course of infection we observed that in adult mice the peak of the response was at day 8 after which the response declined into memory (**Figure 7.2D**). In neonates we found that the highest frequency of PICV-specific cells was found at day 11 post infection, suggesting there was a delay in the response (**Figure 7.2D**). These results show that neonates can produce adult-like responses to PICV infection, but still have the hallmarks of delayed viral clearance and reduced number of CD8 T cells and altered immunodominance hierarchies.

C. Neonates immunized with PICV show adult-like expansion of the crossreactive NP205 response leading to altered immunodominance hierarchy after LCMV challenge

After immunization with PICV adult mice develop a subdominant response to the crossreactive epitope, NP205 (Brehm et al., 2002). This small epitope-specific response, usually less than 1% of the memory CD8 T cell pool, can expand after LCMV challenge and cause faster viral clearance, narrowing of the NP205-specific TCR repertoire, alteration in the immunodominance hierarchy and can allow for viral escape (Brehm et al., 2002; Cornberg et al., 2006; Selin et al., 1998). In a preliminary experiment we questioned could the crossreactive

NP205 response expand upon subsequent challenge with LCMV and alter the immunodominance hierarchies of mice immunized with PICV as neonates. Mice immunized as neonates developed strong responses to the dominant NP38 epitope, a lower NP122-specific response than adults and a similarly low frequency of the NP205-specific response in memory (**Figure 7.3**). After challenge with LCMV there was an expansion of the NP205-specific memory response and at day 7-8 this response dominated in all of the mice (**Figure 7.3**). These data demonstrate that, like adult cells, crossreactive CD8 T cells produced during a neonatal infection can expand during a subsequent heterologous infection and altered immunodominance hierarchies.

Chapter Summary

This chapter summarizes preliminary data on a model of neonatal infection with a second less virulent arenavirus, PICV. After infection of day 7 neonates with PICV there was no mortality even at a high dose of $4x10^6$ PFU. Even at this high dose, PICV did not replicate to the same levels as LCMV Armstrong or remain as protracted. Neonates could successfully clear PICV, but with delayed kinetics compared to adult mice. The CD8 T cell responses in neonates were adult-like in frequency, but not total number, and peaked later than adults. The immunodominance hierarchies in neonates were altered with a loss of the NP122-specific response. When mice immunized with PICV as

Figure 7.3

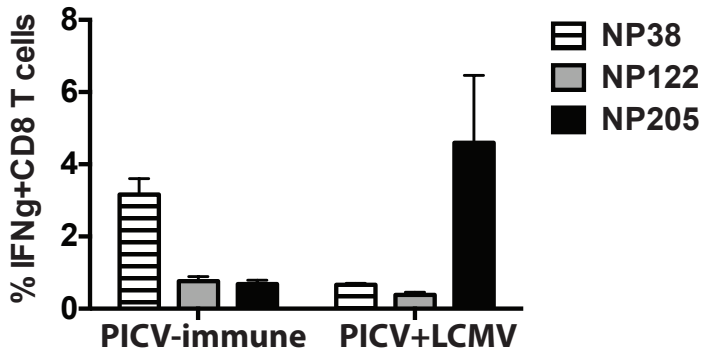


Figure 7.3. Mice immunized with PICV as neonates and challenged with LCMV demonstrate skewing of the immunodominance hierarchy and expansion of the crossreactive response. Mice immunized with 4x104 PFU of PICV at day 7 of age were challenged with 5x104 PFU of LCMV Armstrong six weeks later. Immunodominance hierarchies were determined on day 7-8 post challenge. Data is from two similar experiments with 6-13 mice/group.

neonates were challenged with LCMV there was a skewing of the immunodominance hierarchy such that the crossreactive NP205-specific response expanded, similar to that found in mice immunized with PICV as adults. Whether this mediates protective heterologous immunity still needs to be tested.

Chapter 8: Discussion

The induction of T cell-mediated immunopathology is one of the consequences of the host's immune system attempting to clear a viral infection. Immunopathology can be induced during the reactivation of memory cells or during a new naïve response. T cells employ several mechanisms to induce immunopathology, such as the production of cytokines like IFNγ and TNFα, which activate and recruit other immune cells to the site of infection or the production of cytotoxic factors such as FasL, perforin and granzymes. My major hypothesis is that the difference between an immune response that causes severe pathology versus one that induces minimal pathology is the efficiency at which the immune response can clear antigen and quickly shut down. If a pathogen is cleared quickly there is limited cellular damage, but an infection that is allowed to replicate, for a prolonged period, requires lysis of a greater number cells to clear infection and a longer amount of time for T cells to amplify an immune response. This thesis used four different models, each defining a different mechanism to study the balance between viral load and the efficiency of the immune response and their subsequent affect on disease outcome.

In the first model we studied the effect of competition between immune responses to two viruses, LCMV and PICV, which contained a crossreactive epitope, NP205 (Chapter 3) (**Figure 8.1**). Co-infection caused the LCMV-specific CD8 T cell response to become smaller in size. Specifically there was a

Figure 8.1

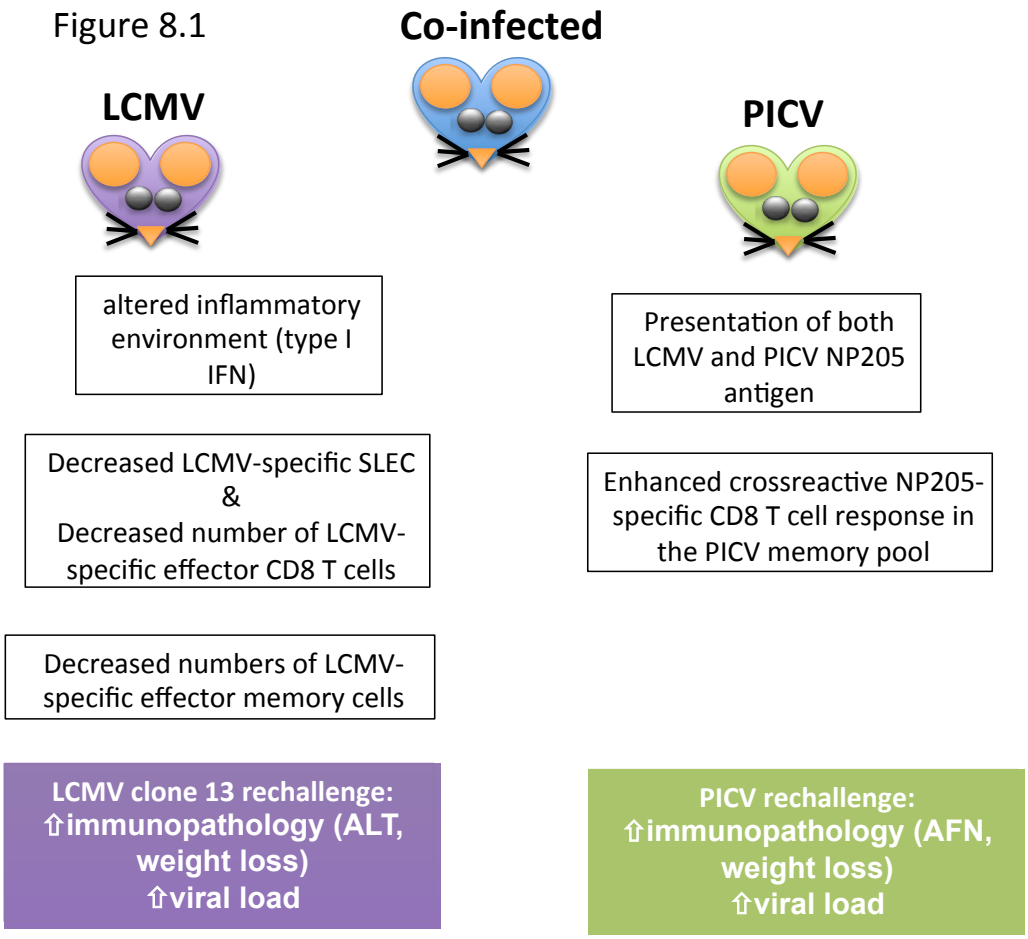


Figure 8.1. Co-infection led to enhanced immunopathology during rechallenge due to reduced effector memory cells and enhanced crossreactivity. Left side: LCMV clone 13 mechanism. Compared to mice infected with LCMV alone, co-infected mice had an inflammatory environment early during infection, specifically with an earlier peak in type I IFN. This may have played a role changing CD8 T cell differentiation resulting in co-infected mice having a smaller LCMV-specific effector pool at the peak of the T cell response with a smaller frequency of SLEC. After contraction these differences resulted in co-infected mice having a smaller LCMV-specific effector memory pool. We believe these differences between LCMV and co-infected lead to the enhanced immunopathology found during LCMV rechallenge. **Right side: PICV mechanism.** Compared to PICV only infected mice, co-infected mice had an enhanced memory proportion of the crossreactive epitope NP205 prior to PICV rechallenge. This increase in the NP205-specific response was most likely due to the presentation of both LCMV- and PICV-NP205 during co-infection. The NP205-specific response was found to directly mediate immunopathology during PICV rechallenge.

reduction in the LCMV-specific effector, but not the central, memory pool (**Figure 3.5-3.6**). Upon rechallenge with LCMV clone 13, co-immunized mice had enhanced immunopathology, as measured by increased ALT levels and greater weight loss, with higher viral loads compared to mice immunized with LCMV alone (**Figure 3.1**). Co-infection also led to an increase in the crossreactive memory pool, in some mice. The frequency of which directly correlated with fat pad pathology and weight loss during PICV rechallenge. Ablation of the NP205-specific response using a mutant virus resulted in minimal immunopathology (**Figure 3.2-3.4**). By administering two viruses simultaneously, the competition between the immune responses to these viruses decreased the efficiency of the memory CD8 T cell immune response to both, such that during a rechallenge with either virus, replicating virus was not cleared as effectively as from a LCMV- or PICV-immune mouse and increased the chance of developing immunopathology in some mice (**Figure 8.2**).

In a second model, by using three different doses of the highly virulent LCMV clone 13, a virus known to replicate to such high levels it can result in clonal exhaustion, we shifted this balance (**Figure 8.3**). During a low dose infection with LCMV clone 13, mice developed a strong, functional CD8 T cell response that cleared virus after approximately one week, causing minimal immunopathology. During a high dose infection LCMV clone 13 induced clonal exhaustion of the LCMV-specific CD8 T cell response, with a loss of IFNγ and

Figure 8.2

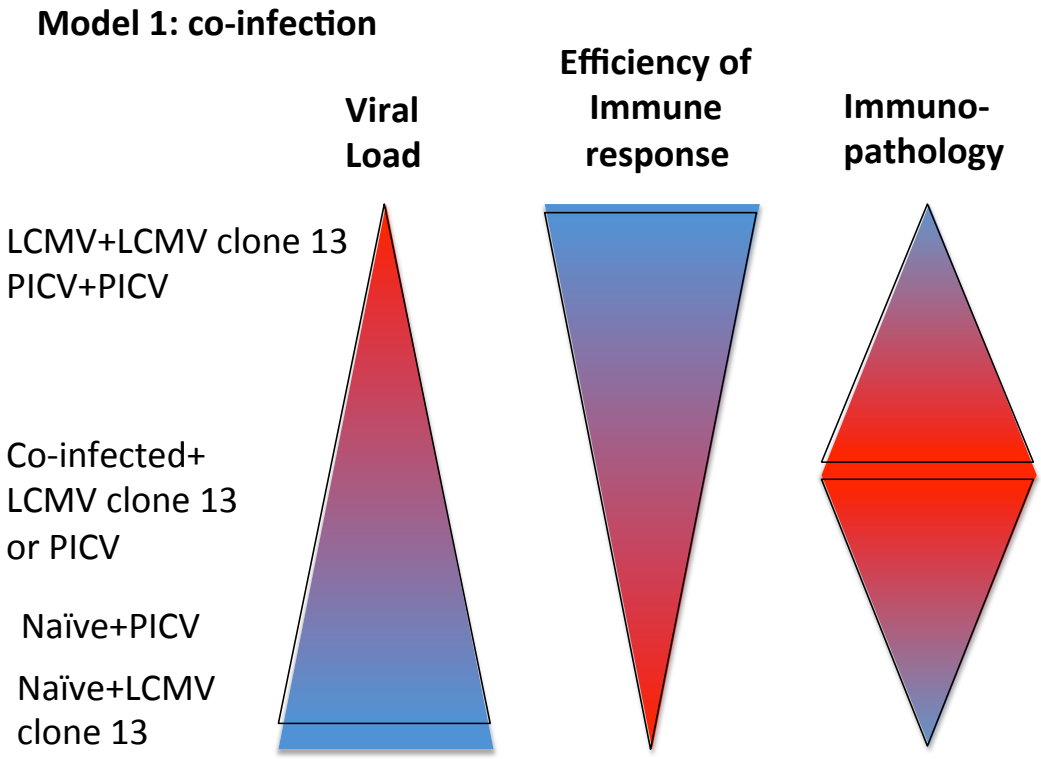


Figure 8.2 Decreased effector memory and enhanced crossreactivity in co-immunized mice led to increased viral load and immunopathology. This diagram is representative of the respective differences in viral load, efficiency of the immune response and the resulting immunopathology between homologously rechallenged single virus immune mice (LCMV+ LCMV clone 13 and PICV+PICV), co-infected and naive mice challenged with either LCMV clone 13 or PICV. The less effective memory responses generated during co-infection could not clear the rechallenge virus as well as the memory population in LCMV- or PICV-immune mice and resulted in enhanced immunopathology. Naïve mice had no immunological memory and had higher viral load with little immunopathology.

Figure 8.3

Model 2: partial clonal exhaustion

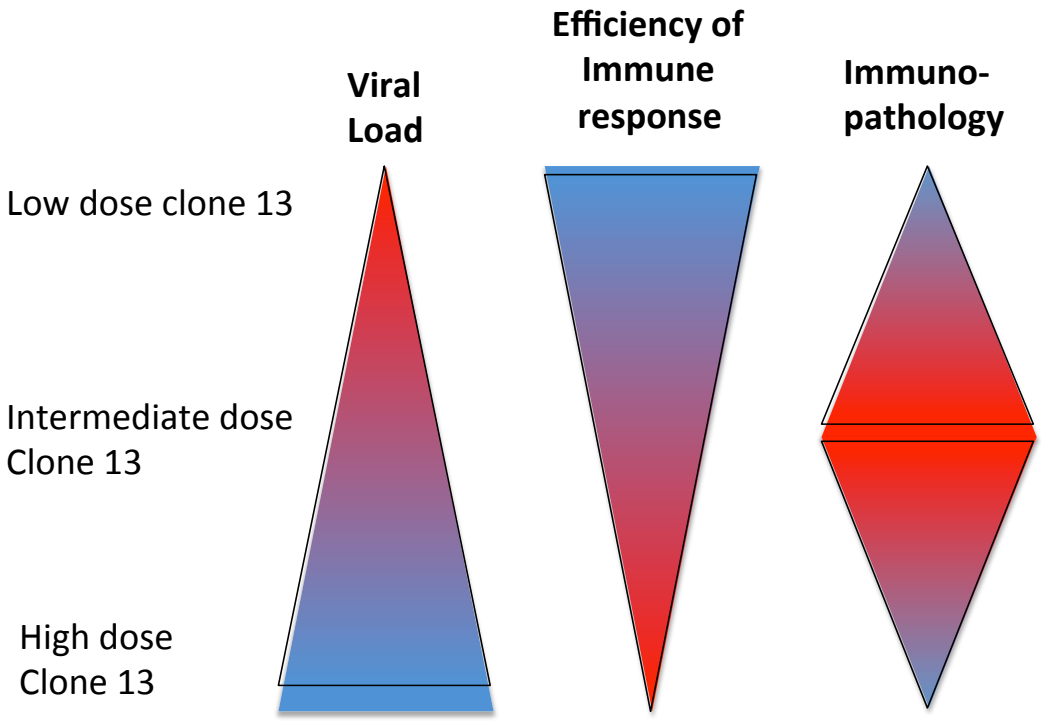


Figure 8.3 The partial clonal exhaustion of the CD8 T cell response during an intermediate dose of LCMV clone 13 infection resulted in a less efficient T cell response and severe immunopathology. The balance between viral load and T cell response determined the disease outcome of mice inoculated at three different doses of virus. A low dose of LCMV clone 13 resulted in a strong T cell response that quickly cleared virus and caused little immunopathology. At a high dose of LCMV clone 13, the T cell response was clonally exhausted and caused to limited immunopathology, but is persistent virus infection. An intermediate dose of LCMV clone 13 induced an inefficient partially clonally exhausted immune response and mice develop severe immunopathology with up to 75% mortality.

TNFα production by the NP396-specific population, and TNFα from the other epitopes (**Figure 4.2B**). In this instance clonal exhaustion or loss of a functional T cell response in the presence of an overwhelming viral load was beneficial to the host as there is little immunopathology. The price, however, was viral persistence. However, an intermediate dose of LCMV clone 13 was found to cause severe immunopathology of the lung and liver and death in up to 75% of mice (**Figure 4.1, 4.3 & 4.4**). Upon examination of the T cell response in mice infected with an intermediate dose of LCMV clone 13 we found partial clonal exhaustion, where the NP396-specific response was not lost, but the TNFα:IFNγ ratio shifted had so these cells are less effective. (**Figure 4.2**). This partial exhaustion caused a moderate reduction in the efficiency of the T cell response, but not to the level of complete exhaustion as found during high dose LCMV clone 13 infection, resulting in severe T cell-mediated immunopathology as they were unable to clear the virus (**Figure 8.3**).

In a third model, we used neonatal mice known to have an immature, less efficient immune system to examine the balance between viral load and efficiency of the T cell response and its effect on disease outcome. Using the LCMV Armstrong system, where adult mice have minimal immunopathology and a functional CD8 T cell response that clears virus quickly, we infected day 7 neonatal mice and found severe immunopathology and ~80% mortality (**Figure 5.1 & 8.4**). Our data would suggest that delayed immune responses, particularly early production of type I IFN, which directly control viral replication, allowed

Figure 8.4

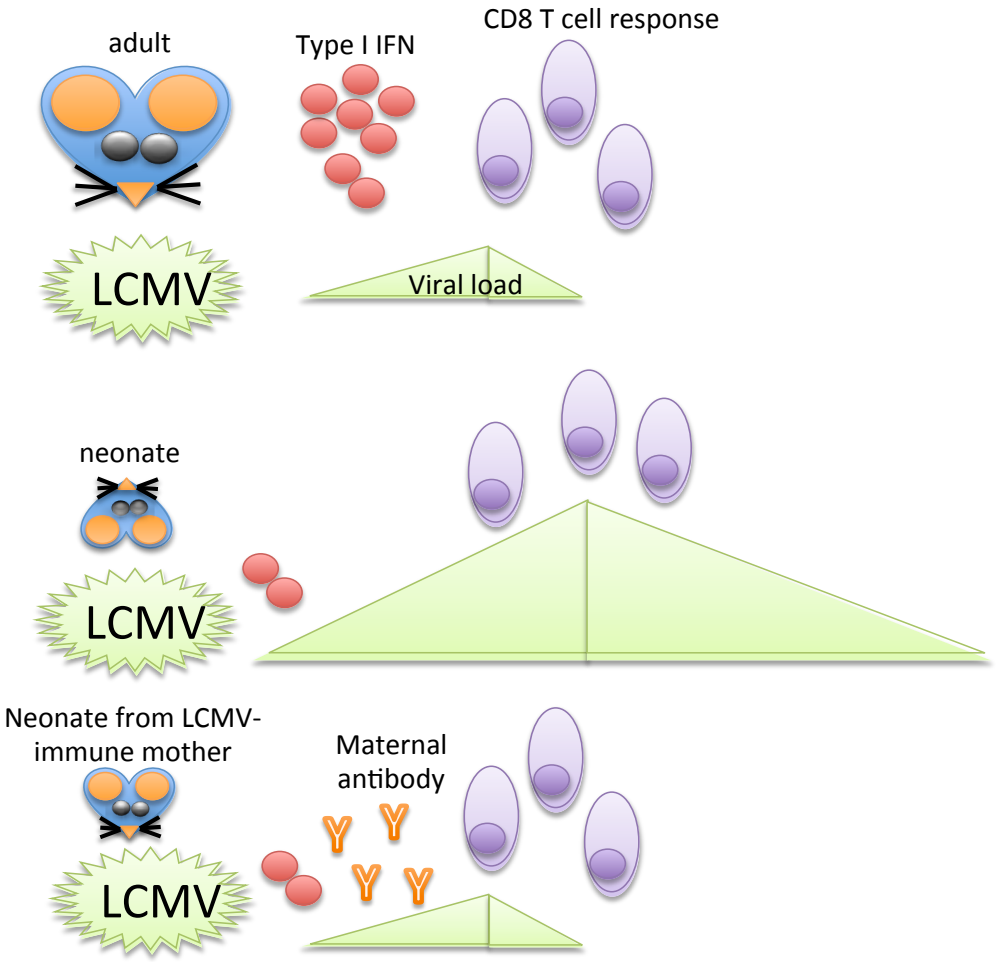


Figure 8.4. Neonates had immature innate immune responses (type I IFN) that allowed for greater LCMV replication and enhanced T cell-mediated immunopathology compared to adult mice. Top: In adult mice infected with LCMV Armstrong there is a strong type I IFN response that induces an anti-viral state and keeps viral replication in check until the CD8 T cell response can clear infection. **Middle:** Neonates from naïve mothers have poor innate responses which may be allowing for extensive viral replication and dissemination of virus to areas, such as the CNS. The developing CD8 T cell response induces severe perforin-mediated immunopathology leading to death in ~80% of neonates. Surviving neonates have protracted viral clearance. **Bottom:** The immaturity of the neonatal innate response can be compensated for by passive maternal antibody from LCMV-immune mothers. Neonates from LCMV-immune mothers had faster viral clearance and 100% survival.

LCMV to disseminate into the immature CNS in neonatal mice (**Figure 5.7**). In adult mice, LCMV Armstrong does not normally replicate in the CNS during an ip infection. In neonates the high viral load did not cause clonal exhaustion, allowing cytotoxic CD8 T cells to enter the immature CNS and induce perforin-mediated immunopathology and death (**Figure 5.7**). Neonatal mice were capable of undergoing clonal exhaustion under the right circumstances, such as a high dose LCMV clone 13 infection which replicates very rapidly early to high levels (**Figure 5.5**). We further perturbed the balance by infecting neonates of LCMV-immune mothers, which were able to clear virus faster than neonates from naïve mothers and had 100% survival rate with minimal lung and liver pathology (**Figure 5.8**). When early viral load was controlled, such as in adult mice infected with LCMV Armstrong or neonates from LCMV-immune mothers, an efficient CD8 T cell response cleared virus quickly with little resulting immunopathology (**Figure 8.5**). When clonal exhaustion occurred, either in neonates or adults infected with a high dose of LCMV clone 13, there was also minimal immunopathology due to the lack of a cytotoxic T cell response that could induce cellular damage (**Figure 8.5**). Using a second less virulent arenavirus, PICV, we found an adult-like T cell response that did not induce mortality or immunopathology due to the slower replication of PICV and relatively normal rate of clearance. Without widespread, high viral loads T cells did not induce severe immunopathology in neonates infected with PICV (**Figure 8.5**).

Figure 8.5

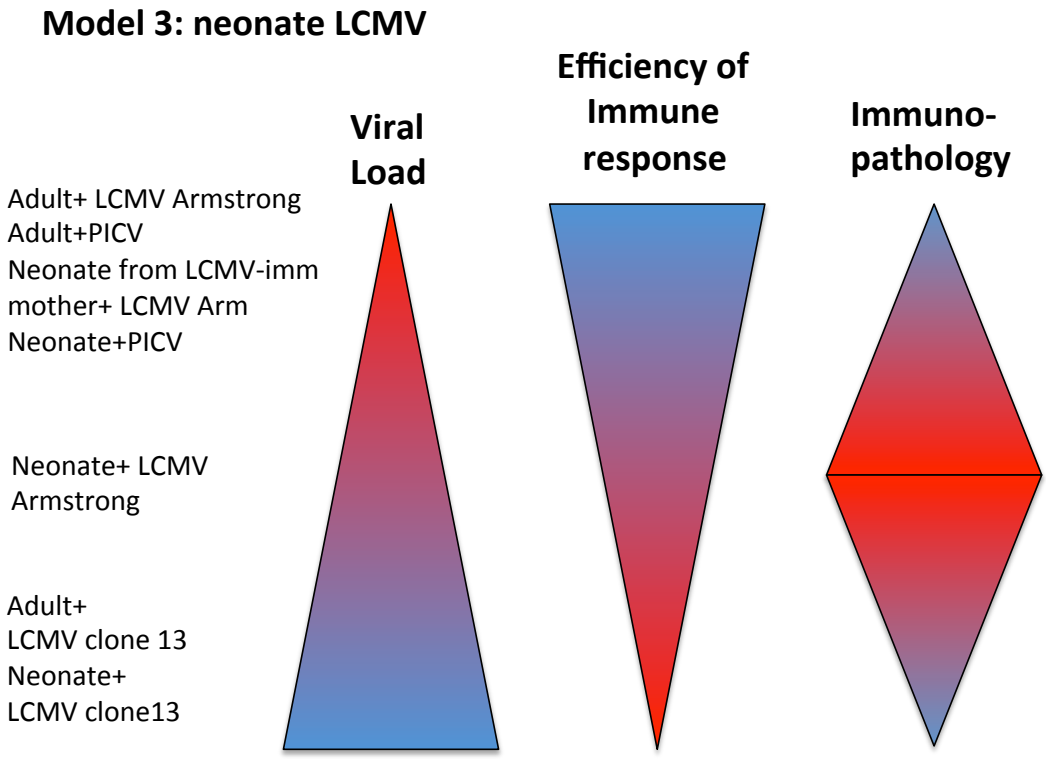


Figure 8.5 Neonatal mice infected with LCMV have a strong T cell response, but poor innate response that can not control viral replication early during infection leading to perforin-mediated T cell-dependent immunopathology. Adult mice infected with either LCMV Armstrong or PICV have efficient innate and T cell responses that cleared virus and caused little pathology. Similarly, neonates infected with PICV controlled viral replication and had 100% survival. However, the majority of neonates infected with LCMV Armstrong died from T cell-dependent perforin-mediated immunopathology. This is due to poor control of viral replication early during infection leading to widespread viral infection, specifically to the brain, which may play a role in death. Even though neonates have poor innate responses they can become clonally exhausted during high dose LCMV clone 13 infection, similar to adult mice. After clonal exhaustion mice are persistently infected with minimal immunopathology.

To further understand the role of memory development and immunopathology in neonatal mice we used a fourth model examining beneficial heterologous immunity. During a VV challenge, LCMV-immune mice immunized as neonates were compared to mice that were immunized as adults. Mice previously immunized to VV have been shown to have sterilizing immunity during VV rechallenge due to the high efficiency of their VV-specific memory responses resulting in very limited immunopathology (**Figure 8.6**). Naïve mice challenged with VV have no immunological protective immunity, resulting in high viral load. However, naïve mice developed VV-specific CD8 T cell responses that cleared virus within ~1 week, and those mice developed little T cell-mediated immunopathology. LCMV-immune mice, immunized as either neonates or adults, have crossreactive memory responses that aid in clearing virus faster than a naïve response, but slower than a VV-specific memory response during VV challenge. However, due to the slower rate of VV clearance, the less efficient crossreactive memory cells have time to amplify the immune response in the fat pads, where VV grows preferentially to high doses. This increased IFNγ and TNFα produced by the memory cells leads to increased Fas expression on adipocytes and Fas-FasL dependent necrosis (Nie et al., 2010). This model of heterologous immunity illustrates the balance of memory T cell responses, either homologous (VV+VV), crossreactive (LCMV+VV) or primary (naïve+VV) in the development of immunopathology.

Figure 8.6

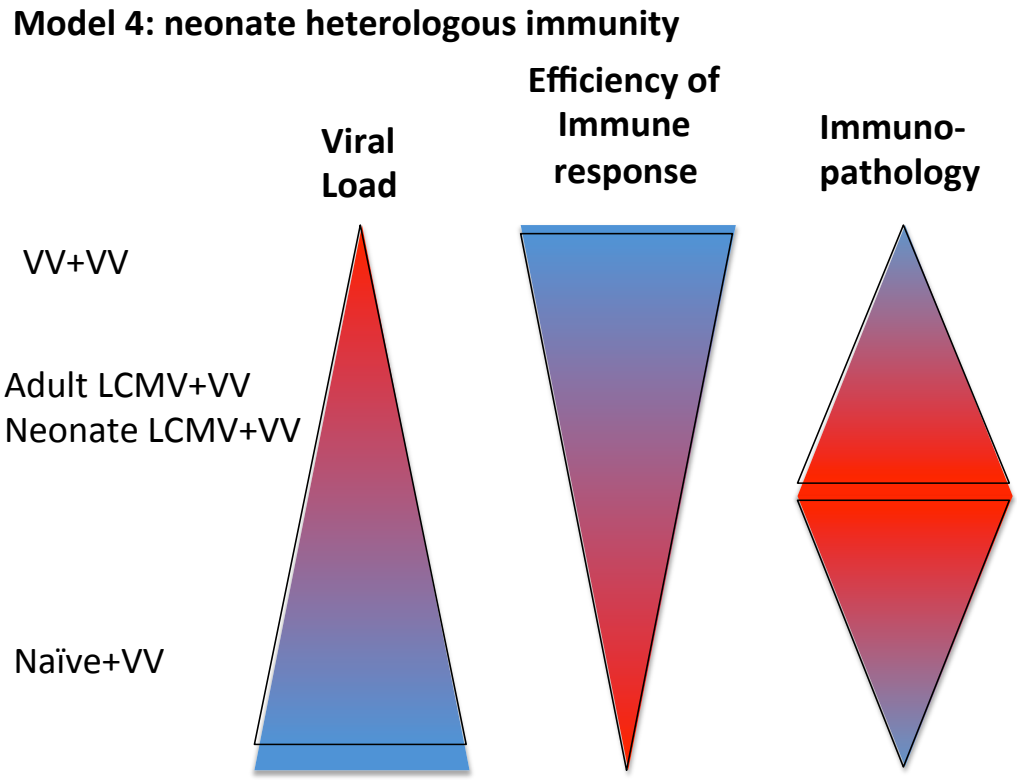


Figure 8.6. Crossreactive memory responses mediated immunoprotection in mice immunized with LCMV as neonates during VV challenge, but also induced AFN. Naïve mice challenged with VV had higher viral load early during infection because they lacked immunological memory, but there was also no T cell memory response to cause immunopathology. VV-immune mice had sterilizing immunity, clear virus quickly and did not develop immunopathology. LCMV-immune, either immunized as neonates or adults, had crossreactive memory responses that led to lower viral loads than naïve mice during VV challenge, but not lower than VV-immune mice. This slower viral clearance of LCMV-immune mice allowed for inefficient crossreactive T cells to amplify the immune response in the fat pads where VV replicates well and led to Fas-FasL dependent AFN.

Model 1: Co-infection reduced effector memory and enhanced crossreactivity

Co-infection with two viruses containing a cross-reactive epitope can lead to increased disease severity and delayed viral clearance upon re-exposure to either of the immunizing viruses. Furthermore, this could be correlated with at least **two different mechanisms**, one mechanism for each of the two virus rechallenges. During PICV rechallenge, an **increased inefficient crossreactive CD8 T cell response** contributed to enhancement of immunopathology in co-infected mice, while protection against LCMV clone 13 rechallenge was reduced in co-infected mice coincident with a **reduction in the number of LCMV-specific effector-memory cells**. In both viral rechallenge models, an ineffective memory T cell response in co-infected mice facilitated increased viral replication for a longer period of time, possibly leading to enhanced and prolonged accumulation of secondary effector T cells in the tissues. This may in turn be associated with increased collateral damage in the form of immunopathology.

Crossreactive responses have been shown to mediate severity of immunopathology in models of heterologous immunity. In IAV-immune mice challenged with LCMV, there is significant lung pathology induced by the crossreactive IAV-specific memory responses to PB1 and PA224 (Wlodarczyk et al., 2013). These responses are crossreactive with the LCMV epitopes GP34 and GP276, respectively. IAV-immune mice with the greatest magnitude of crossreactive responses developed the most severe lung pathology after LCMV

challenge. In fact, there was a direct correlation between the severity of pathology and the frequency of the crossreactive responses prior to LCMV challenge. Ablation of the crossreactive responses by either peptide tolerization or use of viral mutants resulted in decreased lung pathology (Wlodarczyk et al., 2013). We found a similar reduction of the fat pad immunopathology when we co-infected mice using the mutant LCMV (L212A), which lacks the crossreactive NP205 epitope. Homologous memory responses can result in protective immunity and quickly clear virus before immunopathology can develop. Crossreactive memory responses are more likely to clear virus slower and less efficiently. Slower viral clearance allows for greater viral replication allowing for greater recruitment and amplification of the crossreactive memory responses leading to greater production of IFNγ, TNFα and cytotoxic factors resulting in the enhanced immunopathology observed in some instances of heterologous immunity.

The crossreactive NP205-specific response is responsible for both protection and the induction of immunopathology during sequential infections with LCMV and PICV. PICV-immune mice challenged with LCMV clear virus more quickly than naïve mice acutely infected with LCMV due to the rapid expansion of PICV-NP205-specific memory CD8 T cells that are not present in naïve mice (Brehm et al., 2002; Cornberg et al., 2006; Selin et al., 1998). PICV-NP205-specific memory cells are at a higher precursor frequency and lower activation threshold than naïve LCMV-specific T cells and are localized within the

periphery. However, when PICV-immune mice are rechallenged with PICV after an intervening LCMV infection (PICV+LCMV+PICV), a NP205-specific memory T cell response of higher affinity to LCMV will most likely be selected for. This is not ideal for PICV protection. Consequently, this crossreactive T cell response is no longer beneficial to the host during a subsequent PICV rechallenge, but contributes to the development of severe panniculitis in 60% of the mice (Chen et al., 2012). During simultaneous co-infection with LCMV and PICV, NP205 epitopes from both viruses were most likely presented. Since there is no difference in the proportion of NP205-specific cells within the LCMV-specific immunodominance hierarchy in co-infected mice, we believe that the increase in NP205 in the PICV-specific immunodominance hierarchy is due to the addition of the LCMV-NP205-specific cells. Therefore, the NP205-specific response in co-infected mice may have a repertoire where an inappropriate crossreactive response dominated during the PICV rechallenge and caused severe immunopathology in some mice similar to the pervious study in heterologous immunity.

During LCMV clone 13 challenge of co-infected-immune mice, no correlation between immunopathology and the crossreactive NP205-specific response was found. This would be consistent with the NP205 response in co-infected mice being predominantly directed against the LCMV-NP205 epitope, which represents an appropriate NP205-specific response for control of a LCMV clone 13 challenge (Chen et al., 2012). It has previously been shown that the

number of virus-specific memory cells positively correlates with protection from lethal infection (Cerwenka et al., 1999; Hamada et al., 2009). We believe that the smaller LCMV-specific memory population in co-infected mice was an important contributor to enhanced immunopathology. Co-immunized mice may take longer to control viral replication in peripheral organs, such as the liver, allowing for increased infiltration of activated T cells and enhanced immunopathology. Interestingly, memory CD8 T cells with heightened expression of CD62L have previously been shown to mediate protection during systemic infections of high dose LCMV clone 13 in adoptive transfer experiments (Nolz and Harty, 2011). Effector memory cells are purportedly activated quickly after re-infection and contribute to early control of viral replication (Bachmann et al., 1997). In a VV skin infection model, effector memory cells were found to be the major protective memory cell subtype (Jiang et al., 2012). In the current study, there was no difference in the number of central memory cells between LCMV- and co-infected mice, but co-infected mice had significantly fewer effector memory cells. These data suggest that, if there is no difference in the number of central memory cells, an increased number of effector memory cells can enhance protection during a LCMV rechallenge.

T cell differentiation and proliferation are shaped by several factors, including antigen load and cytokine milieu (Cui and Kaech, 2010; Curtsinger and Mescher, 2010). LCMV- and co-infected mice were infected with the same dose of LCMV Armstrong and exhibited similar kinetics of viral clearance. In contrast,

there were differences in type I IFN, MCP-1 and IL-6 between LCMV- and co-infected mice, while co-infected mice mirrored PICV-infected mice. The infection environment has been altered in other studies by use of recombinant viruses. For example, one group found that an IAV expressing LCMV-GP33 mediated a smaller GP33-specific response than wildtype LCMV, which had a reduced KLRG1 expression (Mueller et al., 2010). This suggests that the PICV-induced early innate inflammatory environment is altering the differentiation and proliferation of the LCMV-specific CD8 T cell response. More specifically, type I IFN has been found to play a major role in the proliferation and survival of CD8 T cells during an LCMV-infection. The levels of type I IFN were similar between LCMV- and co-infected mice, but followed different kinetics. In LCMV-infected mice type I IFN peaks between 48 and 72 hours post infection, while in co-infected mice the peak is expedited to 24 hours post infection. Pre-exposure of virus-specific CD8 T cells to type I IFN can result in poor proliferative responses of CD8 T cells upon encounter with cognate ligand (Marshall et al., 2011). Thus, the early bath of type I IFN that the LCMV-specific cells receive in co-infected mice may be altering the normal LCMV-specific differentiation and proliferation.

Another possible explanation for the decreased size of the LCMV-specific memory response in co-infected-immune mice may be an unknown factor that controls the size of the T cell compartment during primary infections. In memory, co-infected-immune mice have smaller LCMV- and PICV-specific responses than LCMV- and PICV-immune mice, respectively. However, the total virus-specific

response (LCMV+PICV response) in co-infected-immune mice is similar to the LCMV-specific response in LCMV-immune mice, suggesting that LCMV alone induces a strong T cell response that fills up the available space in a naïve mouse. When co-administered with PICV, there is a reduction in the LCMV-specific response to make room for the PICV-specific response, resulting in a smaller memory pool in co-infected-immune mice. Several labs have studied the changes in the size of the memory T cell pool over sequential infections (Masopust et al., 2007; Selin et al., 1996; 1999). However, in the current study the decreased LCMV-specific memory population in co-infected-immune mice was generated during a primary infection and was not a product of sequential infection induced attrition.

There are several alterations in both the early innate and adaptive immune responses during a co-infection, which may not be predicted from studying individual virus infections. These alterations resulted in a less efficient CD8 T cell response and led to enhanced disease severity upon rechallenge. This strongly suggests that it would be important to have a better understanding of human T cell responses to vaccines that are given concurrently, such as the live-attenuated measles, mumps, rubella vaccine, to optimize vaccine strategies. With the increase in the number of vaccines available, use of combination vaccines and simultaneous administration of vaccines has become necessary to accommodate all the required immunizations into routine doctor visits and maintain vaccination rates. Similarly, the reuse of intradermal needles and bites

from insect vectors can also result in a co-infection. As antibody responses generally correlate with protection, the majority of the time antibody titers are used to determine vaccine efficacy. However, live-attenuated vaccines will also induce T cell responses, and their impact on subsequent immunity either beneficial or detrimental, has largely been neglected. The T cell epitopes for the majority of vaccines are currently unknown, severely limiting the understanding of human T cell responses to vaccines. These data suggest that a better understanding of human T cell responses to vaccines is necessary to optimize immunization strategies. Furthermore, diagnosis and treatment of co-infections are more difficult than single virus infections, so studying how the immune system responds to multiple pathogens simultaneously may aid in design of the treatments for HIV/HCV co-infected patients.

Model 2: Clonal exhaustion protected mice from T cell mediated mortality during viral infection

Altering the infection dose with a rapidly replicating virus that can cause clonal exhaustion, such as LCMV clone 13, can also shift the balance and result in enhanced immunopathology. When a mouse is infected with a high dose of LCMV clone 13 the viral load becomes so high that there is a complete clonal exhaustion of the CD8 T cell response. During clonal exhaustion there is a stepwise loss of function in CD8 T cells, where cytotoxic function is lost then cytokine production and finally these cells die by apoptosis (Wherry, 2011).

Clonal exhaustion of the LCMV-specific CD8 T cell response results in viral persistence with very limited pathology (Ahmed et al., 1984; Welsh and McNally, 1999; Zajac et al., 1998; Zhou et al., 2004). Likewise, mice infected with a low dose of LCMV clone 13 developed minimal pathology, as virus was cleared quickly by a fully functional CD8 T cell response. However, when mice were infected with an intermediate dose of LCMV clone 13 we found extensive lung and liver pathology that resulted in death of up to 75% of the animals. This pathology was found to be T cell-mediated. Compared to the low dose infection, the CD8 T cell response after the intermediate dose infection is less efficient at clearing virus due to its partial clonal exhaustion, where the majority of the LCMV-specific CD8 T cells are no longer cytotoxic and produce limited amounts of TNF. With this less efficient CD8 T cell response, LCMV clone 13 is allowed to replicate to higher levels in essential organs. The balance between the T cells inducing immunopathology and the rate at which the remaining T cell response undergoes complete clonal exhaustion determines the survival of the individual mouse **(Figure 8.2)**.

The limited pathology that occurred after high dose infection suggests that extensive clonal exhaustion may be a beneficial immune mechanism that prevents death from an overwhelming CD8 T cell response. The medium dose mice that survived eventually underwent complete clonal exhaustion of their immune response and became persistently infected, much like the high dose-infected mice. Persistent LCMV clone 13 infection is ultimately cleared in B6

mice by day 60, and epitope-specific CD8 T cells return, except for the high affinity NP396 T cells, which have been completely eliminated by apoptosis,(Wherry, 2011; Zajac et al., 1998; Fuller and Zajac, 2003) in the presence of functional LCMV-specific CD4 T cells (Fuller et al., 2004; Yi et al., 2010).

Clonal exhaustion is predicted to be an evolutionary mechanism for survival during infection with fast replicating viruses (Wherry, 2011). Clonal exhaustion is found to occur in some patients infected with HIV, HCV and HBV, but the mechanism for this is not clear. Furthermore, patients that are infected with HBV displayed a range of disease severity ranging from an acute resolving infection to a chronic persistent infection or fulminant hepatitis (Kondo et al., 2004) and PD1 expression on virus-specific CD8 T cells has been correlated with the outcome of HBV (Zhang et al., 2011). PD1 expression increased on HBV-specific CD8 T cells early after infection and HBV clearance correlated with loss of PD1 expression (Zhang et al., 2011). Similar to these human studies, PD1 expression correlates with viral clearance in mice acutely infected with LCMV Armstrong (Barber et al., 2006). PD-1 expression on LCMV-GP276-specific CD8 T cells is high at day 6, but by day 8 when the virus has been cleared from most organs PD1 expression has returned to naïve-like levels (Barber et al., 2006). During high dose clone 13 infection PD-1 expression is induced early, but is sustained (Barber et al., 2006). In HBV patients, delayed PD1 expression on HBV-specific CD8 T cells was associated with delayed exhaustion of the CD8 T

cell response and subsequent acute liver failure. These data suggest that in mice infected with an intermediate dose of LCMV clone 13 there may be a delay in upregulation of PD1 on the surface of virus-specific cells.

Another study examined different dose inoculations with LCMV clone 13 and found that different doses induced different levels of weight loss (Stamm 2012). Similar to this study, they found that an intermediate dose of virus caused the most severe pathology (Stamm 2012). This group proposed that CD4 T cells were inducing this wasting phenotype found in mice infected with the intermediate dose (Stamm 2012). These results are similar to ours in that we found TCRβ KO mice, which lack both CD4 and CD8 αβ T cells, have decreased pathology. However, it has previously been shown that CD4 help is required for CD8 T cell function, and without it cells are more likely to undergo clonal exhaustion and be unavailable to mediate pathology (Matloubian 1994 and Bettegay M 1994). A deeper look into this model has shown that NK cells act as a rheostat and control CD8 T cell exhaustion. (Waggoner 2012). NK cells can kill activated CD4 T cells, resulting in the loss of CD4-help and exhaustion of CD8 T cells. Therefore, depletion of NK cells from mice infected with the intermediate dose of LCMV results in a fully functional CD8 T cell response, that does not develop the partially exhausted phenotype, leading to viral clearance with limited pathology. When mice infected with a high dose of LCMV clone 13 were depleted of NK cells they shifted down in the paradigm and did not undergo complete clonal exhaustion, but only a partial exhaustion and developed severe pathology

similar to untreated mice infected with the intermediate dose of LCMV clone 13 (Waggoner et al., 2012).

These studies suggest a counter intuitive treatment strategy for other lethal arenaviruses, such as Lassa virus. Perhaps patients infected with fast replicating viruses should be treated short-term with anti-CD3 (Keymeulen et al., 2005; Chatenoud, 2009) or anti-CD8, as their immunopathology and death may be T cell mediated and preventable. Generalized immunosuppressive therapy such as corticosteroids may have been tried in the past and are being used during acute IAV H1N1 infection in patients with severe lung disease, but these treatments would lead to suppression of all aspects of the immune response, leaving the host helpless. Just focusing on the CD8 T cell responses or even particular CD8 functions such as blocking FasL or INFγ may improve outcome without disabling the complete immune system.

Model 3: Neonatal susceptibility to LCMV infection is mediated by perforin-dependent CNS pathology

Our data show that neonatal mice infected with LCMV at day 7 of age were highly susceptible to perforin-mediated T cell-dependent death. Furthermore, the presence of replicating virus and virus-specific CD8 T cells in the CNS during the days of greatest mortality suggests that neuropathology is a major mechanism of death. The age of infection is important in understanding the disease outcome as 12 day old infant mice did not die from low dose LCMV

Armstrong infection (data not shown). Alternatively, the day 7 neonates could be protected by passive maternal antibody, which would be able to mediate viral control early after infection. Our data from this study suggests that the limited or delayed innate immune response of day 7 neonates allows for enhanced viral replication and results in greater immunopathology from CD8 T cells clearing wide spread viral infection.

It is well documented in humans that newborns are more susceptible to viral infections, such as herpes simplex virus (HSV), due to their reduced ability to induce a type I IFN response (Kollmann et al., 2012). Newborns to infants 2 months in age are at the highest risk of severe HSV infection and susceptibility correlates with low TLR-mediated type I IFN production (Kollmann et al., 2012). In our study we found that type I IFN production was delayed in neonates after LCMV infection (**Figure 5.12**). Neonates had undetectable type I IFN levels at 24 hours post infection and reduced levels at 48 hours post infection. It was not until 72 hours post infection that adult-like levels were detected in neonates (**Figure 5.12**). This delayed response may have allowed dissemination of virus into organs where LCMV Armstrong is not normally found in adults, such as the CNS (**Figure 5.7A**) (Wherry et al., 2003a) and led to T cell-mediated death.

The intracranial model of LCMV infection has been extensively studied in both adults and day 1 neonatal mice (Evans et al., 2002). After intracranial infection of adult mice, LCMV replicates in the chorioid plexus, ependymal and meninges of adult mice, with 90% of the virus leaving the CNS and replicating in

the periphery (Storm et al., 2006). In the spleen CD8 T cells are primed to develop into effector cells. After infection cells of the CNS release low levels of chemokines, such as CCL5 (RANTES) and CXCL10 (IP10), that draw in T cells to the brain (Asensio and Campbell, 1997). Likewise, astrocytes within the CNS can release growth factors, such as VEGF, that cause vascular permeability of the blood brain barrier (BBB) (Argaw et al., 2009). The BBB is composed of endothelial cells connected by tight junctions and separates circulating blood from the central nervous system. Historically, the BBB was thought to be immature in neonates and allowed for the passage of dyes from the blood stream into the brain (Behnsen G 1927). However, several studies have reexamined the concentration of dyes, such as Evan's blue and trypan blue used to examine BBB permeability, in humans, rats, rabbits and mice and showed that there was no passage of dye into the brain of neonates suggesting that the BBB is fully formed at this stage of development (Grontoft, 1954; Millen and Hess, 1958; Moos and Møllgård, 1993). However, the sensitivity to permeabilization in neonates may be different than that found in adults, making them more prone to CNS T cell-mediated immunopathology.

Immunocompetent adult mice infected intracranially with LCMV undergo 100% T cell-dependent mortality between day 6-8 post infection (Cole et al., 1972; Doherty and Zinkernagel, 1974; Nansen et al., 1998; Storm et al., 2006). The mechanism for death in this model is complex as blocking CD8 T cell function using several different knock out mice, including mice lacking perforin or

granzymes, still resulted in death due to the chemokine recruitment of monocytes and macrophages to the CNS (Kim et al., 2009). We found 80% mortality of neonates infected with LCMV ip (**Figure 5.1A**). However, by blocking cytolytic function of CD8 T cells, 100% of neonates survived the end of the experiment at day 14 post infection (**Figure 5.7C**). In adult mice infected intracranially with LCMV Armstrong perforin deficient mice survived 3-5 days longer than wildtype controls, but this was attributed to delayed recruitment of cells to the CNS (Storm et al., 2006). It is currently unknown if perforin is required for CD8 T cells to enter the CNS during LCMV infection of neonates. Furthermore, the requirement of monocytes and neutrophils that occurs in adults may not occur in neonates due to altered chemokine production or chemokine receptor expression.

Generally, LCMV Armstrong when administered ip is not thought to replicate in the CNS. However, one study has reported very low levels of virus can be found in the brains of some mice infected with LCMV Armstrong ip at day 5 post infection and the presence of small populations of GP33-tetramer positive CD8 T cells (Wherry et al., 2003a). After iv infection with LCMV clone 13 over 6 logs of virus can be found in the brain until one month after infection and ~10% of the CD8 T cells isolated from the brain were GP33-tetramer positive, but the function of these T cells was not assessed (Wherry et al., 2003a). Since during persistent infection with LCMV clone 13 the CD8 T cell response undergoes clonal exhaustion, with a loss of cytotoxcity, this may prevent CD8 T cell-mediated CNS pathology. In neonatal mice even though viral load was very high

clonal exhaustion did not occur during low dose LCMV Armstrong infection, and this allowed for a fully functional T cell response to attack the CNS and induce death (**Figure 5.4**). It is currently unknown if viral replication in the CNS and CD8 T cell infiltration are playing a role in the enhanced immunopathology found in adult mice infected with an intermediate dose of LCMV clone 13, but this is a strong possibility.

Clonal exhaustion of CD8 T cell responses is due to over activation of the cells through extensive exposure to antigen during some fast replicating viral infections. Neonatal mice infected with 500 PFU of LCMV Armstrong do not lose production of TNFα or retain PD-1 expression giving no indications of exhaustion (**Figure 5.4**). When neonates are infected with a high dose of LCMV clone 13 ip there is complete clonal exhaustion of the LCMV-specific CD8 T cell response and 100% survival (**Figure 5.5**). Whether low doses of LCMV clone 13 infection result in clonal exhaustion or mortality of neonates is unknown at this time. Furthermore, it is also unknown if neonates given a higher dose of LCMV Armstrong would undergo clonal exhaustion and survive. There may be other factors besides initial viral dose that are playing a role. LCMV clone 13 has a higher affinity for the alpha-dystroglycan receptor and a point mutation in the RNA polymerase that result in infection of a greater number of cell types and faster viral replication (Matloubian et al., 1990; Salvato et al., 1991). Furthermore the induction of type I IFN has recently been linked with the clonal exhaustion found during LCMV clone 13 infection (Teijaro et al., 2013; Wilson et al., 2013).

Infection with high dose LCMV clone 13 induces strong IFNβ responses within the first 24 hours post infection, which is not found during LCMV Armstrong infection (Teijaro et al., 2013; Wilson et al., 2013). Neonates had poor type I IFN responses during the first 48 hours of LCMV Armstrong infection (**Figure 5.12**), and this may be part of the explanation for why neonates do not undergo clonal exhaustion and survive. It is unknown if day 7 neonates produce stronger type I IFN responses after high dose clone 13 infection, where they did clonally exhaustion.

Tissue-dependent restrictions on viral replication promote LCMV Armstrong replication in neuronal cells over LCMV clone 13 (Dockter et al., 1996). Isolation of viral clones, from either the brain or spleen, of mice infected as day 1 newborns with LCMV Armstrong revealed different tropisms (Ahmed et al., 1984). Virus isolated from the brain and used to infect adults caused an acute infection with a strong induction of a CD8 T cell response, while a splenic viral isolate caused persistent infection and clonal exhaustion (Ahmed et al., 1984). Day 1 newborns infected with LCMV clone 13 have replication of LCMV clone 13-like clones in their brain (Dockter et al., 1996). However, if clone 13 was mixed with LCMV Armstrong prior to infection, LCMV Armstrong preferentially grows in the CNS, out-competing LCMV clone 13 (Dockter et al., 1996). If the differential tissue-specific preferences of LCMV clone 13 and Armstrong affects the T cell mediated death of neonates is unknown at this time.

Model 4: Neonatal repertoire alters immunodominance hierarchies and crossreactivities

After infection with either LCMV or PICV, neonatal mice developed CD8 T cell immunodominance hierarchies that are altered from hierarchies that develop in adult mice infected with either of these viruses. LCMV-infected neonates have decreased frequencies of NP396-specific CD8 T cells, while after PICV infection neonates do not mount a response to the NP122 epitope. These alterations between adults and neonates could be from a combination of several factors including TCR repertoire, Tregs suppression of immune responses, or the ability of APC in neonates to process and present the same epitopes as adults.

The TCR repertoire in newborn mice is limited to the VDJ segments encoded in the germline. Between days 4 and 5 of age the enzyme TdT is expressed in the thymus of mice (Bogue et al., 1991). This enzyme introduces non-coding-nucleotides into the junctions between the VDJ segments and exponentially increases the diversity of the TCR repertoire in an individual. By day 8 of age elongated CDR3 regions, which contain the junctions between the D and J segments, are found in single positive thymocytes (Bogue et al., 1991). We found that mice that were infected at day 7 of age had altered immunodominance hierarchies at day 9 post infection with either LCMV or PICV (**Figure 5.4 & 7.2**). At this age the TCR repertoire of mice is more diverse than a newborn, but is still significantly different compared to adults, as shown by the differences in Vβ repertoire (**Figure 5.10**). Specifically, we found that the Vβ repertoire in the

NP396-specific population of neonatal mice was highly variable both from the predictable Vβ usage of adult mice and between individual mice, with each individual neonatal mouse having a different Vβ repertoire in their NP396-specific CD8 T cell population. This variability between neonates indicates the development of private specificity, or unique repertoires that are actively developing in individual neonates. In adult mice the NP396-specific response has a very high affinity response to the peptide MHC complex compared to other responses such as GP33 (van der Most et al., 1998; Wherry et al., 2003a). Naïve neonates have a significant reduction in the Vβ 8.1/2-specific CD8 T cell population at day 7 of age. This is the dominant Vβ that appears in the NP396-specific population in adult mice (**Figure 5.10C**) (Blattman et al., 2000). This suggests that the high affinity Vβ 8.1/2 response does not develop in mice until later. Adult TdT ko mice had a similar reduction in the NP396-specific response as neonates, further suggesting that the limited TCR repertoire of neonates may be playing a role in the decreased NP396-specific responses (**Figure 5.11**). However, since uninfected TdT ko adult mice actually had an increase in their Vβ8.1/2 usage (**Figure 5.11**) and still had a loss in their NP396-specific response after LCMV infection, it may also suggest that the Vβ usage has less of an impact on the hierarchy development, and it may be more mediated by the specific CDR3 region generating high affinity clones.

Besides the intrinsic properties of the TCR, other factors such as antigen processing and presentation may be mediating the reduced NP396-specific

response found in LCMV-infected neonates. It has been reported that the NP396-specific response is dependent on cross presentation (Alatery et al., 2010). Cross presentation is the process of antigen presentation where antigen is taken up by the APC through pinocytosis or phagocytosis of dying cells and loaded into MHC class I molecules in the endosome. One study used in vitro stimulation of T cell lines with L929 that were incubated with UV-treated LCMV-infected HEK293 cells to test the cross presentation of LCMV epitopes (Alatery et al., 2010). HEK293 cells cannot directly present antigen to the CD8 T cell lines and the UV-treatment killed all replicating LCMV. Therefore, LCMV peptides must be picked up by the L929 cells and cross presented to the CD8 T cell lines. In this study NP396 was cross presented at higher levels than GP33 (Alatery et al., 2010). Studies have found that CD8α+ DC are the major subset of APC that cross present antigen to CD8 T cells in several different viral infections, including LCMV (Belz et al., 2005). The inability of neonatal mice to cross present NP396 may be another possible explanation for the decreased NP396-specific response after LCMV infection. By day 7 of age neonatal mice have adult-like splenic architecture and ratio of DC: T cells (Sun et al., 2003). However, it is unknown if they have the same ability to cross present during viral infection as the inflammatory environment plays a big role in DC maturation and neonates have poor innate responses. Altering the inflammatory environment by the addition of the TLR 2 and 3 agonists, Pam3CysSerLys4 and polyinosinic polycytidine acid (poly IC), to LCMV infection in adult mice also resulted in a decrease in only the

NP396-specific response (Siddiqui and Basta, 2011). The concurrent administration of both TLR agonists with LCMV decreased the ability of APC to cross present antigen (Siddiqui and Basta, 2011). Since cross presentation may play a greater role in the immunodominance of the NP396-specific response it was reduced compared to mice infected with LCMV alone.

There may also be T cell-intrinsic differences between neonates and adults that lead to altered immunodominance hierarchies. One study found that RSV infection in day 7 neonates led to a similar reduction in an immunodominant epitope (Ruckwardt et al., 2011). In this study it did not appear to relate to TCR repertoire suggested by the fact that TdT ko mice did not show the same reduction in the immunodominant epitope. To examine if the priming environment of the neonate was responsible for the decreased immunodominant response, adult CD8 T cells were transferred into neonates and their immunodominance was examined after infection. Adult cells that responded to infection within the neonatal host could develop the normal adult hierarchy. The conclusion of this study was that there were additional CD8 T cell-intrinsic defects in neonatal T cells that caused the loss of the immunodominant epitope-specific response. Therefore, besides TCR repertoire and antigen presentation, there could be other unknown defects that contribute to the decreased NP396- and NP122-specific responses in neonatal mice after LCMV or PICV infection.

Model 4: Heterologous Immunity, crossreactivity and neonatal T cells

Mice immunized as neonates with LCMV and challenged with VV or immunized with PICV and challenged with LCMV showed similar traits of crossreactivity and heterologous immunity. In both of these models of protective heterologous immunity memory cells can crossreact with antigens from the challenging virus and mediate faster viral clearance of a heterologous virus. Compared to mice immunized with LCMV as adults, mice immunized as neonates showed altered crossreactivities when challenged with VV. In mice immunized as adults with LCMV and then challenged with VV the most commonly used crossreactive response is NP205. However, GP33/34 crossreactivity was found in the majority of mice immunized as neonates. Possible reasons for this difference are the TCR repertoire of NP205-specific cells in neonates is less crossreactive to VV or the higher precursor frequency of memory GP33/34-specific cells in mice immunized as neonates gives that population an advantage during VV challenge.

The TCR repertoire, specifically the Vβ repertoire of crossreactive CD8 T cells is associated with disease outcome in heterologous infections (Cornberg et al., 2007; 2010). This was demonstrated using adoptive transfer of CD8 T cell lines derived from different LCMV-immune mice stimulated with the VV epitope A11R into naïve hosts. Most of these crossreactive CD8 T cell lines mediated faster viral clearance and decreased weight loss after VV challenge, but some

did not (Cornberg et al., 2010). Protection was associated with higher avidity to VV-A11R and a less diverse, more oligoclonal Vβ repertoire (Cornberg et al., 2010). It is unknown if protective crossreactive responses between LCMV and VV are due to specific high affinity Vβ populations, but this is a likely possibility. This would then suggest that the limited TCR repertoire of the neonate may not allow for these high avidity crossreactive responses in the NP205-specific memory pool that occurs commonly in adult mice.

T cells from mice deficient in TdT, similar to newborn mice, have been reported to have increased crossreactivity or promiscuity of their TCR allowing them to respond to a greater number of antigens than wildtype T cells (Gavin and Bevan, 1995). T cell clones isolated from TdT deficient and wildtype mice specific to the IAV epitope NP366 were screened against a library of random peptides coated targets and TdT ko clones were able to recognize a greater number of peptides (Gavin and Bevan, 1995). The role of TdT is to insert non-template encoded nucleotides into the CDR3 region. As TdT expression increases in newborn mice the CDR3 region becomes longer (Bogue et al., 1991). However, increased crossreactivity did not correlate with length of the CDR3 region in this study (Gavin and Bevan, 1995). One possible explanation for this increased promiscuity of the TdT ko TCR is that it makes more interactions with the α-helices of the MHC molecule instead of the epitope (Gavin and Bevan, 1995). This is supported by a study that found positive thymic selection, where T cells interacting with self peptide-MHC complexes are promoted to survive, was

increased in TdT ko mice (Gilfillan et al., 1994). This increased crossreactivity may allow the limited TCR repertoire in the periphery of neonates to respond to a wide range of pathogens. This type of crossreactivity that may be less dependent on TCR-epitope interactions is not found in middle-aged humans (Naumov et al., 2008) (Anna Gil, unpublished data). In HLA-A2 individuals the IAV M1-specific response is found to be more crossreactive if the CDR3 region contains multiple glycines/alanine runs, as this would allow the TCR to be more flexible to interact with a greater range of epitopes. In elderly individuals, who are more reliant on their crossreactive memory pools, there is an increase in the CDR3 length of their M1-specific CD8 T cell response (Anna Gil, unpublished data). In contrast to the neonatal T cell response, which contains no memory T cells, the majority of the middle-aged and elderly human T cell pool is composed of memory cells. Both of these situations benefit from increased crossreactive responses to recognize pathogens, but with age and numerous sequential infections with unrelated pathogens there is a selection for the longer CDR3 regions containing glycine runs that may provide a more flexible, higher affinity response to common pathogens that we are frequently exposed to, such as IAV.

Mice immunized with LCMV as neonates had altered immunodominance hierarchies as compared with mice immunized with LCMV as adults (Figure 6.3) with reduced the memory precursor frequency to GP276, NP205 and GP118. In mice immunized as adults NP205 was the most common crossreactive response followed by GP34 and GP118, but in neonates GP34 was more often

crossreactive. The lower memory precursor frequency of NP205- and GP118-specific cells in mice immunized as neonates may be playing a role in the altered crossreactivities during VV challenge. This reduction in the size of the NP205- and GP118-specific cell populations may be promoting the GP34 crossreactivity as the smaller populations may not be able to compete.

Sex differences mediate altered immunopathology in Balb/c mice, but not B6

Immunological sex differences arise due to differential hormonal regulation between males and females (Klein, 2012). In B6 neonatal mice infected with LCMV Armstrong there was no difference in mortality between males and females (**Figure 5.13C**). However, in Balb/c neonates, females had 100% survival after infection with 5 PFU of LCMV, while the vast majority of males succumb to infection (**Figure 5.13B**).

Sex differences are also found in the Balb/c model for myocarditis after Coxsackie virus infections (Huber et al., 1982). This model recapitulates human sex differences found in myocarditis as it affects mostly males or pregnant females in the 3rd trimester or postpartum time period (Woodruff, 1980). In the mouse model the protective effects found in females are attributed to higher estrogen levels. Estrogen receptors are expressed on numerous immune cells including NK cells, T and B cells, macrophages and dendritic cells (Robinson and Klein, 2012). T cells express the estrogen receptor and the effects of estrogen

signaling are concentration dependent. Low to normal levels of estrogen result in Th1 priming of T cells, while higher doses result in Th2 skewing of the T cell response and humoral responses. Generally, males have lower levels of estrogen and produce cellular immune response, while females have higher levels of estrogen and better humoral immunity (Grossman et al., 1983; Styrt and Sugarman, 1991). In Balb/c mice infected with Coxsackie virus males had Th1 skewed immune responses with CD4 T cells producing IFNγ and IL-2 and IgG2a antibody responses. Females in the same study developed CD4 T cells producing IL-4 and IL-5 and IgG1 antibody responses (Huber and Pfaeffle, 1994). These results were hormone dependent as the addition of testosterone to females or estrogen to males could invert the skewing of the immune response. Estrogen can directly alter IFNγ expression because there is an enhancer element in the IFNγ gene (Robinson and Klein, 2012). Low doses of estrogen produce IFNγ production in T cells along with increased MAP kinase signaling and Tbet expression (Robinson and Klein, 2012).

Coxsackie virus-induced myocarditis is T cell-dependent immunopathology (Huber and Lodge, 1984), similar to what we found in neonates infected with LCMV. Ablation of T cell responses decreases disease severity in both models. It is currently unknown if increasing the estrogen levels in B6 neonatal mice play a protective role and result in decreased immunopathology after LCMV infection or the treatment of Balb/c female neonates with androgens would induce increased immunopathology and death.

Strain-specific differences between B6 and Balb/c mice were also playing a role in immunopathology induced by LCMV in neonates. In B6 neonates males and females both died with the same kinetics and developed the same pathology, but Balb/c females survived, while the males did not (**Figure 5.13**). The Balb/c strain of mice is known to be more Th2-prone due to the increased affinity of the IL-4 receptor alpha chain for IL-4 (Schulte et al., 1997). The characteristics of Balb/c females to be more Th2-skewed based upon strain differences and enhanced estrogen levels may be the deciding factor in the survival of female Balb/c mice during LCMV infection.

Summary

Understanding how the dynamics of the T cell response are changed by the context of the infection and how this changes the resulting immunopathology is important in developing protective immunological memory. In this thesis I utilized several models to change the efficiency of the immune response and induced T cell mediated immunopathology. Co-infections are commonly acquired from multiple mosquito bites or combination vaccines with several live-attenuated viruses. This thesis shows that co-infections are drastically different than single virus infections in the early innate, effector and memory phase of the infection and understanding how different viruses impact each other is critical to vaccine design and treatment of co-infected patients. Several human pathogens can cause clonal exhaustion. Using a murine model of clonal exhaustion we showed

how dose, or antigen load, can change the functionality of the T cell response and the developing immunopathology. This study has implications for understanding why some patients develop severe immunopathology and others do not. Understanding neonatal immunity is important for vaccine design and treatment of early childhood infections. We showed that for some quickly replicating viruses, such as LCMV, the lack of early viral control can lead to widespread infection and severe T cell-mediated immunopathology. Maternal immunizations to provide passive immunity or type I IFN treatments may provide the support the neonatal immune system needs to control infections or enhance vaccination. Furthermore, the benefits of heterologous immunity were found to be maintained in mice immunized as neonates. Understanding how beneficial crossreactivity, or “non-specific immunity”, can protect against unrelated pathogens may be an additional mechanism used to guard neonates against lethal infections.

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