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Regulation of Immune Pathogenesis by Antigen-Specific CD8 T Cells Following Sequential Heterologous Infections: A Dissertation

Alex T. Chen
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Regulation of Immune Pathogenesis by Antigen-Specific CD8 T Cells Following Sequential Heterologous Infections

A Dissertation Presented

By

Alex TY. Chen

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 9, 2010

Immunology and Virology
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Regulation of Immune Pathogenesis by Antigen-Specific CD8 T Cells Following Sequential Heterologous Infections

A Dissertation Presented
By
Alex. TY. Chen

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April 9, 2010
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I would like to express my gratitude towards my mentor, Dr. Raymond Welsh, for his un-wavering support over the course my thesis research. Ray has been a great mentor and has guided me along the path to scientific research, and I feel very fortunate to be one of his students. I would like to thank Dr. Liisa Selin for many stimulating scientific discussions following my serendipitous entry into the often mind-bending world of T cell cross-reactivity and heterologous immunity. I would like to thank Dr. Michael Brehm for all the help he provided me when I first came to the lab. Another person I like to thank is Dr. Eva Szomolanyi-Tsuda for our earlier collaboration on the role of gamma-delta T cells on polyomavirus-induced tumorigenesis. Finally, I would like to thank Drs. Ken Rock, Masanori Terajima, Alan Rothman, and Linda Cauley for serving as members of my thesis defense committee. I also like to thank Keith Daniels for providing technical support and to our lab manager Carey Zammitti for keeping the lab under control.

I would like to thank all the past and present members of the Welsh, Selin, and Szomolanyi-Tsuda lab. It has been a great pleasure working with all of you. My life in the lab would never have been so fun and exciting if it weren’t for all you guys. Those great moments that we share will always be with me forever.

Finally, I like to thank my family for all of their support. Thank you mom and dad for never questioning where my interest lies. Thank you Amy and Andy, for cheering me on during the bad times. I could never have done this without your full support.
Previously, our lab demonstrated that heterologous immunity could result in either gain or loss of protective immunity and alteration in immune pathology following infection by a second un-related pathogen. One of the prototypical models to study T cell-mediated heterologous immunity involves two distantly related arenaviruses, namely lymphocytic choriomeningitis virus (LCMV) and Pichinde virus (PV). Each virus encodes a cross-reactive CD8 epitope that has six out of eight in amino acid (aa) similarity with respect to its counterpart at the position 205-212 of the nucleoprotein (NP205). Heterologous challenge between LCMV and PV results in 1) expansion of the cross-reactive NP205-specific CD8 T cell responses and alteration of the immunodominance hierarchy and 2) partial protective immunity (heterologous immunity).

Our lab showed that cross-reactive NP205-specific CD8 T cell receptor (TCR) repertoires become extremely narrowed following a heterologous challenge between LCMV and PV. Therefore, I questioned if LCMV NP205 epitope escape variants could be isolated during a dominant but narrowed cross-
reactive NP205-specific CTL response. In the first part of my thesis, I describe the isolation of a LCMV NP-V207A CTL escape variant in vivo using PV-immune animals challenged with LCMV clone 13. The LCMV NP-V207A variant contains a point mutation, which results in the switching of valine to alanine at the third non-anchoring residue of the LCMV NP205 CD8 epitope. Immunization of mice with the LCMV NP-V207A variant results in a significantly diminished cross-reactive NP205-specific CD8 T cell response. This suggests that the point mutation is responsible for the loss in the immunogenicity of the LCMV NP205 CD8 epitope. In addition, an in vitro rescued(r) recombinant LCMV variant (r/V207A) that encodes the original mutation also induces a highly diminished cross-reactive NP205-specific CD8 T cell response in mice. In agreement with the result obtained from the intracellular cytokine assays (ICS), MHC-Ig dimers loaded with the LCMV NP205 (V-A) peptide could only detect a minute population of cross-reactive NP205-specific CD8 T cells in mice infected with r/V207A variant virus. All the data indicate that the point mutation results in a significant loss in immunogenicity of the LCMV NP205 CD8 epitope.
So far, no direct link between the cross-reactive NP205-specific CD8 T cells and heterologous immunity had been established in this system. Therefore, we immunized mice with either LCMV WT or the LCMV NP-V207A variant virus and showed that a significant loss of heterologous immunity is associated with the group immunized with LCMV NP-V207A variant virus. Again, r/V207A-immune animals also displayed a significant loss in heterologous immunity following PV challenge. This suggests that the cross-reactive NP205-specific CD8 T cells mediate the majority of heterologous immunity between LCMV and PV in vivo. In comparison to the PV-immune control group, PV clearance kinetics mediated by the cross-reactive NP205-specific CD8 T cells were significantly delayed. Finally, these data also suggest that bystander activation plays very little role in heterologous immunity between LCMV and PV.

Many studies in murine systems and humans suggest that cross-reactive T cells are often associated with immune pathology. We showed that in mice that were sequentially immunized with PV and LCMV (PV+LCMV WT double immune mice), there was a development of a high incidence and high level of immune pathology known as acute fatty necrosis (AFN) following a final PV
challenge. The data suggest that these cross-reactive NP205-specific CD8 T cells might play an important role in immune pathogenesis. Therefore, we asked if the cross-reactive NP205-specific CD8 T cells play a role in immune pathogenesis by comparing the incidence of AFN between the (PV+LCMV WT) and the (PV+LCMV NP-V207A) double immune mice following a final PV challenge. In agreement with our hypothesis, the result showed the (PV+LCMV NP-V207A) double immune mice developed a significantly lower incidence of AFN compared to the (PV+LCMV WT) double immune mice. However, linear correlation studies comparing the frequency of different antigen-specific CD8 T cell populations within the (PV+LCMV WT) double immune mice before challenge and the severity of AFN following the PV challenge suggest that two opposing antigen-specific CD8 T cell populations are involved in determining the final outcome of the immune pathology. The PV NP38-45-specific CD8 T cell response (PV NP38) appears to be more protective than the cross-reactive NP205-specific CD8 T cell response. In addition, a positive linear correlation between the ratio of cross-reactive NP205 to PV NP38 and the severity of AFN seem to suggest that these cross-reactive populations are important contributors
to immune pathogenesis. Peptide titration studies examining the functional avidities to different antigenic specificities suggest that both populations consist of high avidity TCR and peptide MHC (TCR:pMHC) interactions. However, skewing within the cross-reactive NP205 specific CD8 T cell response towards the LCMV NP205 epitope response in one of the (PV+LCMV WT) double immune mice suggests that cross-reactive NP205 specific CD8 T cells could constitute a sub-optimal response to a PV challenge.

In summary, I questioned what might be some of the immunological consequences of heterologous immunity in this model. First of all, we have established a direct link between the cross-reactive NP205-specific CD8 T cell response and heterologous immunity in LCMV and PV. Second of all, I demonstrated that a LCMV NP205 epitope escape variant could be selected \textit{in vivo} under the conditions of heterologous immunity. In addition, I showed that PV clearance kinetic was significantly delayed in cross-reactive NP205-mediated heterologous immunity as compared to homologous challenge. Finally, we demonstrated that cross-reactive NP205-specific CD8 T cells could play an important role in immune pathogenesis in this model. However, correlation data
indicate that two opposing antigen-specific CD8 T cell populations could ultimately decide the outcome and magnitude of immune pathology in each individual mouse. All the data presented above strongly suggest that the cross-reactive NP205 CD8 T cells play a crucial role in immune pathology in this model system by 1) interfering with the regular establishment of immunodominance hierarchy orders, or 2) exhibiting a sub-optimal protective immunity due to the nature of the cross-reactive epitope.
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<tbody>
<tr>
<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>AFN</td>
<td>Acute fatty necrosis</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<td>APLs</td>
<td>Altered peptide ligands</td>
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<td>CDRs</td>
<td>Complementary determining regions</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<td>CTL</td>
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<td>EBV</td>
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<td>Intracellular cytokine staining</td>
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<td>Interleukin-2</td>
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<td>i.p.</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NP</td>
<td>Nucleoprotein</td>
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<td>NK</td>
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<td>Peptide MHC</td>
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Chapter I

Introduction

A. T cell Cross-Reactivity

Memory lymphocytes represent an important hallmark of our mammalian adaptive immunity. Unlike naïve lymphocytes that require a certain number of cell divisions to acquire necessary effector functions, memory lymphocytes are able to respond to the same antigenic determinants in a swift manner, thereby dramatically slowing down the progress of re-infection (Veiga-Fernandes et al., 2000). Historically, cross-reactive antibodies have been suggested in the modulation of viral infections and autoimmune diseases. For example, a phenomenon called “original antigenic sin” was first described where cross-reactive antibodies from a previous influenza A viral strain (IAV) could bias the development of more protective neutralizing antibodies from a newly infecting strain (Virelizier et al., 1974). In addition, non-neutralizing antibodies specific to one serotype of dengue virus have been suggested to mediate “immune enhancement” by binding to a second dengue serotype and facilitate infection
(Morens and Halstead, 1990). Heterologous immunity mediated by the cross-reactive T cell responses has been shown to lead to either accelerated or delayed viral clearance and altered immune pathology (Chen et al., 2003; Selin et al., 1998). Thus, the study of cross-reactive T cell response is crucial in understanding the nature of diseases as well as future vaccine designs.

T cell receptors (TCRs) are important in recognition of antigens that are synthesized inside a cell, and they share a very similar structure with immunoglobulin made by B cells. A typical TCR is composed of alpha and beta chains. Each chain is composed of a variable and a constant region. Like B cells, the diversity of the T cell repertoire derives from the ability to undergo somatic recombination between different segments of the variable regions (Chien et al., 1984). Given the constraint on the overall size of the immune system to generate sufficiently diverse TCR repertoires to recognize all the potential epitopes, a TCR needs to be both specific and degenerate at the same time (Wilson et al., 2004). It has been calculated that in order to have a murine T cell repertoire that can recognize potentially any given foreign epitopes, a single TCR must be able to recognize up to $10^5$ to $10^6$ peptide-MHC combinations (Mason,
1998). Otherwise, the murine immune system would need to be significantly larger if it is to follow the “one clone, one antigen” rule to obtain a naïve T cell pool that can recognize potentially any given epitope. Therefore, T cell “poly-specificity” must be an essential feature of the TCR repertoire in which a single TCR can be degenerate enough to recognize myriads of epitopes while maintaining its ignorance to self-antigens (Wucherpfennig et al., 2007). Finally, a balance between TCR specificity and degeneracy is the key aspect in studying the T cell cross-reactive response.

T cell cross-reactivity can be categorized into different levels. Due to the inherent cross-reactivity of the TCR with the self-peptide MHC (pMHC), allo-reactive T cells in the recipients usually present a significant tissue transplantation barrier (Archbold et al., 2008; Molina and Huber, 1990). In fact, it has been estimated that approximately 1% to 10% of the naïve CD8 T cells are allo-specific (Brehm et al., 2007; Suchin et al., 2001). Virus-specific CD8 T cells generated following a viral infection displayed a strong association with allograft rejections (Brehm et al., 2005; Welsh et al., 2000). A similar report also suggests that CMV-specific CD8 T cells in human can also cross-react with allo-pMHC,
which might be one of the reasons why CMV-infection is often associated with high incidence of graft-rejections (Gamadia et al., 2004).

In addition, cross-reactive T cells are often major participants in autoimmune diseases. T cell epitope “molecular mimicry”, a term that is often used to describe foreign pathogenic epitopes that closely resemble self-peptides, has been postulated to be one of the mechanisms of autoimmune diseases. For example, a study by Croxford et al suggested that molecular mimicry could play a critical role in the induction of autoimmune disease in the MS model. They showed infection of mice with a non-pathogenic strain of Theiler’s murine encephalomyelitis virus encoding a Haemophilus influenzae epitope, which shares 6/13 amino acid (aa) similarity with the dominant myelin proteolipid protein epitope (PLP 139-151), induced disease in the CNS (Croxford et al., 2006). In humans, epitope molecular mimicry between cardiac myosin and the streptococcal M protein has been implicated in the pathogenesis of rheumatic heart disease by cross-reactive T cells (Ellis et al., 2005). Finally, a mouse dopamine beta-mono-oxygenase peptide, which shares 44% in aa sequence similarity with LCMV immunodominant peptide GP33-41 (GP33), has been
suggested as a cross-reactive self-ligand (Ohteki et al., 1999). Furthermore, there is also evidence showing that Epstein-Barr nuclear antigen (EBNA)-specific T cells from Epstein-Barr virus (EBV) infected individuals could also contribute to the development of multiple sclerosis by cross-reacting with myelin antigen (Lünemann et al., 2008).

The third category of T cell cross-reactivity involves antigenic determinants derived from either related or un-related pathogens. For example, CD8 T cell cross-reactivity has been observed between two epitopes from the middle T antigen in polyomavirus (Wilson et al., 1999). The authors suggest that this immunodominant, broad cross-reactivity could play a role in prevention of CTL escape variants and tumor escape variants. In addition, CD8 T cell cross-reactivity has also been reported between two epitopes from different proteins from the same virus (Kuwano et al., 1991). Specifically, my thesis work will focus on T cell cross-reactivity between two distantly related viruses. This type of T cell cross-reactivity could have significant impact on the overall composition of the host immune repertoire and bears significant implications on the future of vaccine design.
Cross-reactive CD8 epitopes between LCMV and PV have been previously identified. Heterologous challenge between LCMV and PV results in modulation of the immunodominance hierarchy (Brehm et al., 2002). The LCMV NP205 (YTVKYPNL) and PV NP205 (YTKFPMN) epitopes are both K\textsuperscript{b}-restricted and differ only in two of the aa anchoring residues. The T cell cross-reactivity between these two distantly related viruses has been shown to be bi-directional, highlighting the structural similarities between these two epitopes.

The crystal structures of the LCMV and PV NP205 peptide-MHC complexes were solved by our collaborators in Australia. A comparison of the overall structure between the LCMV and PV NP205 peptide complexes suggest that there are changes in the conformation of the amino terminal residues (P1-P4), with a marked change in P4 Lys (K) (Fig 1.1). Further analysis suggests that there is a significant movement at the amino terminus of the LCMV NP205 epitope as compared to the PV NP205 epitope (Fig 1.1). Overall, PV NP205 seems to be more stable (Fig 1.1). A closer look at the anchoring residue at P5 supports the hypothesis that PV NP205 might be more stable. The P5 Phe (F) of the PV NP205 epitope fits very nicely to the anchoring pocket (Fig 1.1). On the other
hand, the hydroxyl group at the end of Tyr (Y) from the LCMV NP205 epitope pushes the residues in the anchoring pocket away from the peptide residue, which results in changes in the P4 Lys conformation (Fig 1.1). Thus, despite the high aa sequence similarity between the LCMV and PV NP205 CD8 epitopes, the T cell repertoires that are required to recognize each distinct epitope might be quite different. In fact, we have learned that the majority of the LCMV NP205-specific CD8 T cells have a preferential usage of Vb16, whereas the PV NP205-specific CD8 T cells share between Vb5.1/5.2 and Vb16 usage (Cornberg et al., 2006). In the LCMV and vaccinia virus (VV) model, a VV cross-reactive epitope has also been identified based on the concept of molecular mimicry to the LCMV NP205 epitope (Cornberg et al., 2007). The VV-encoded, subdominant K\(^b\)-restricted epitope called A11R 198-206 (AIVNYANL) is 50% in aa sequence similarity to the LCMV NP205 epitope.

The T cell cross-reactivity between LCMV and VV involves multiple LCMV-specific memory populations. The cross-reactive expansion of NP205-specific CD8 T cells are observed most frequently (50%), followed by GP34-41-specific CD8 T cell populations (23%), and GP118-125 specific CD8 T cell
populations (15%) among the different LCMV-immune mice challenged with VV (Selin et al., 2006). This reflects the private TCR repertoire of each individual host, a term we refer to as private specificity (Kim et al., 2005; Lin and Welsh, 1998). It has been shown that even in mice that share the same genetic background, their naïve T cell repertoires are not completely identical (Bousso et al., 1998). Therefore, private specificity reflects the variations in length and sequence diversity within the complementary determining region 3 (CDR3) due to a random insertion of p and n nucleotides during end joining and could play an important role in heterologous immunity (Kedzierska et al., 2008).
H2-K^b Crystal structures

Fig 1.1 Crystal structures of the LCMV and PV NP205-212 peptide bound K^b complex. The arrows indicate the position of Lysine (K) aa residue.
B. Heterologous Immunity

Heterologous immunity is a term that describes either an enhancement or delay in viral clearance and subsequent alteration in immune pathology associated with an immune host upon infection with a related or unrelated pathogen. Previously, our lab showed that the memory cytotoxic T lymphocytes (CTLs) from LCMV-immune mice acutely challenged with either PV or VV are able to lyse either the target cells infected with the original LCMV or the second challenging virus, suggesting that the established LCMV-specific memory populations can be highly cross-reactive (Selin et al., 1994). Subsequently, our lab questioned the role these cross-reactive memory CD8 T cells play in a heterologous virus challenge. Our lab found that there was a significant reduction in the challenge virus titer (10 to 100 fold reduction) in the spleens and abdominal fat pads when LCMV-immune hosts were challenged with either PV or VV as compared to naïve mice (Selin et al., 1998). Furthermore, adoptive transfer studies showed that both CD4 and CD8 memory populations were required for the heterologous immunity in this system (Selin et
These studies suggest that cross-reactive T cells might be mediating heterologous immunity in the two systems.

The LCMV+VV system also demonstrates an interesting aspect of heterologous immunity. The partial protection mediated by the cross-reactive expansion of the memory populations can lead to an altered immune pathology. LCMV-immune hosts challenged with VV develop severe inflammation in the abdominal fat pads that is characterized by white necrotic lesions and T cell infiltrates in a pathology known as acute fatty necrosis (AFN) (Selin et al., 1998; Yang et al., 1985). This resembles a similar immune-mediated pathological condition in humans known as erythema nodosum, a form of panniculitis. Thus, heterologous immunity is a balance between protection and immune pathology.

T cell cross-reactivity has been found between human papillomavirus type16 E7 and coronavirus NS2 protein (Nilges et al., 2003). In addition, cross-reactive memory CD8 T cells specific to either EBV or hepatitis C virus (HCV) are also present in certain human populations that have been previously infected with IAV (Clute et al., 2005; Wedemeyer et al., 2001). Our lab also showed that HLA-A2-restricted IAV matrix protein M1-specific T cells generated in bulk culture
are cross-reactive to an EBV BMLF1 epitope, an early EBV protein required for the virus lytic cycle. In two of the subjects, this cross-reactive expansion \textit{in vitro} accounted for nearly one third of the BMLF1-specific responses. This finding suggests that cross-reactive T cells could contribute to the characteristic lymphoproliferation that one often witnesses in EBV-associated infectious mononucleosis. However, the two cross-reactive epitopes share 33\% in aa similarity, demonstrating that in certain cases aa sequence similarity alone could not account for this cross-reactivity. On the other hand, the T cell cross-reactive epitopes between IAV (NA 231-239) and HCV (NS3 1073-1081) share a high level of aa similarity (7/9 aa similarity) (Wedemeyer et al., 2001). Ferrari et al reported the potential immunological consequence of this T cell cross-reactivity when they screened a group of patients with acute HCV infection. When they stimulated the blood samples from each patient using a HCV peptide library to examine the cellular immune response, two of the patients with the most severe disease known as fuminant hepatitis exhibited extreme dominant responses to the HCV NS3 1073-1081 epitope, which is known to be cross-reactive to IAV NA231-239 (Urbani et al., 2005). These strong cross-reactive T cell responses in
two of the patients correlated with delayed viral clearance and altered immune pathology.

Dengue virus is a member of the flavivirus family and is prevalent in the tropics and causes significant morbidity and mortality in individuals that have secondary infections with one of the four heterologous strains (Sangkawibha et al., 1984). Enhancement of antibody-mediated uptake of the virus has been proposed to be a plausible mechanism for the high viremia observed in patients infected with different serotypes (Morens and Halstead, 1990). On the other hand, it has been hypothesized that cross-reactive T cells that recognize highly similar antigenic epitopes between different strains could be the mediators of dengue hemorrhagic fever or dengue shock syndrome. For example, studies have shown that CD8 memory T cells from one serotype infection could be activated by another serotype encoding a highly cross-reactive epitope. In addition, activation of these CD8 T cells by the cross-reactive peptides often leads to a different cytokine profile compared to the wild type peptide stimulation (Imrie et al., 2007; Zivny et al., 1999). Recently, studies in patients suggested that high affinity T cell populations could undergo massive apoptosis (activation-
induced cell death) following a secondary heterologous dengue virus infection (Dong et al., 2007; Mongkolsapaya et al., 2003). In addition, the remaining low avidity cross-reactive CD8 T cells have been shown to produce large amounts of pro-inflammatory cytokines upon re-stimulation. In conclusion, all the studies suggest that T cell-mediated heterologous immunity is often associated with a loss of protective immunity and altered pathology.

C. Immunodominance Hierarchy and T Cell Cross-Reactivity

The phenomenon known as “immunodominance hierarchy” refers to the distinct and stable hierarchical patterns in the frequency of antigen-specific CD8 T cell responses following an infection. For example, LCMV infection in B6 mice always results in the domination by the GP33 and NP396-404 (NP396)-specific CD8 T cell responses, which together could account for more than 50% of the LCMV-specific CD8 cells at peak of the response. These dominant responses are usually accompanied by other epitope-specific CD8 T cell responses that are lower in frequency. For example, LCMV NP205-specific CD8 T cell responses typically account for roughly 2% to 3% of the total LCMV-
specific CD8 responses at day eight post-infection. On the other hand, infection of B6 mice with PV, a new world arenavirus, results in a dominant PV NP38-specific CD8 T cell response followed by a subdominant PV NP205-specific CD8 T cell response. One of the interesting aspects of T cell cross-reactivity between LCMV and PV is the alteration of the immunodominance hierarchy by the subsequent heterologous viral infection (Brehm et al., 2002). Infection of either LCMV-immune or PV-immune mice with their respective heterologous virus results in a massive increase in frequency of the subdominant cross-reactive NP205-specific CD8 T cells. All the experiments so far suggest that multiple factors are involved in the generation of immunodominance hierarchy. For example, the antigen expression level, stability of the MHC complex and peptide affinity, and TCR repertoire can all play in concert in determining the final outcome (Chen et al., 2000). A finer aspect of the immunodominance hierarchy might involve immunodomination of the subdominant clones by the more immunodominant clones through mechanisms that are not fully understood. One of the hallmarks of memory T cells is their ability to respond to their cognate antigens and proliferate in a rapid fashion without the requirement of co-
stimulatory signals (Veiga-Fernandes et al., 2000). Therefore, one possibility is that the cross-reactive memory T cells have the proliferative advantage and can outcompete their naïve counterparts through direct competition with antigen presenting cells (APCs). This would go in line with the observation that immunodominance can be removed by expression of the subdominant epitope on separate APCs (Roy-Proulx et al., 2001). Therefore, immunodominance by cross-reactive memory T cells could play a role in the alteration of immunodominance hierarchy. In fact, adoptive transfer of carboxyfluorescein succinimidyl ester (CFSE)-labeled memory lymphocytes revealed that the cross-reactive NP205-specific CD8 memory populations preferentially expanded upon heterologous virus challenge, indicating their proliferative advantage. Whether these cross-reactive memory T cells directly compete for the interaction with antigen presenting cells remain to be studied. Therefore, the cross-reactive T cell response is usually accompanied by the modulation of immunodominance hierarchy and could lead to the change in viral clearance and immune pathology.
D. T Cell Private Specificity

In most cases, the specificity and magnitude of cross-reactive T cell responses depend on the availability of the TCR repertoire that is present within each individual mouse. This is a term we referred as private TCR repertoire or private specificity. It has been shown that even in genetically identical animals, their TCR repertoires are not absolutely identical (Bousso et al., 1998). This is due to the random insertion and deletion of nucleotides of the CDR3 region. For example, the magnitude of the cross-reactive NP205-specific CD8 T cell expansion in LCMV-immune mice can be quite varied in each individual mouse upon PV challenge (Cornberg et al., 2006). Another striking example of how private TCR repertoire can govern the specificity of the cross-reactive T cell responses is seen in LCMV-immune mice following VV challenge. Adoptive transfer experiment using different LCMV-immune donor splenocytes showed that either a cross-reactive NP205, GP34, or GP118 would respond following VV challenge, depending on the endogenous TCR repertoire of the LCMV-immune mouse (Kim et al., 2005). Therefore, the private specificity of a given antigen-specific T population can have a dramatic impact on the quality and magnitude of
the cross-reactive T cell responses and could result in different disease outcomes (Nie et al., 2010).

**E. TCR Repertoires and Viral CTL Escape Variants**

The TCR repertoire is a direct reflection of the diversity of the T cell clones that are specific for a given antigenic epitope. Different antigen-specific T cell clones display a preferential usage at the variable region of the TCR beta chain (Vβ usage), which can be readily tested by a panel of commercially available monoclonal antibodies against different murine Vβs. For example, roughly about 35% of the LCMV-induced CD8 T cell populations use Vβ 8.1 (Lin and Welsh, 1998). However, as many crystal data suggest, the diversity of an antigen-specific T cell population lies within the CDR3 regions of the variable region of alpha and beta chain of the TCR (Vα and Vβ, respectively) (Bjorkman, 1997; Garcia et al., 1998; Turner et al., 2006). Most importantly, the CDR3 region of the Vβ chain appears to be the most critical determinant of the overall TCR repertoire in certain models, since this region plays a crucial role in antigen recognition (Ishizuka et al., 2008). Two of the most commonly employed
methods for measuring TCR repertoire within antigen-specific clones are TCR spectra typing and direct sequencing of the CDR3.

The reason why some antigenic epitopes induce a diverse TCR repertoire while others induce a restricted oligo-clonal TCR repertoire is still not well understood. One study suggests that featureless “vanilla” epitopes tend to select a less diverse TCR repertoire with public Vβ usage whereas epitopes with prominent features tend to select for a more diverse TCR repertoire (Turner et al., 2005). The overall virus-specific TCR repertoire in LCMV infection appears to be stable (Blattman et al., 2000; Lin and Welsh, 1998). Both reports conclude that there is no significant change (skewing) in overall antigen-specific TCR repertoire from the acute phase into the memory phase. However, there are certain situations in which antigen-specific TCR repertoire could undergo a clonal change. First of all, several labs observed that under chronic infections, the antigen-specific TCR repertoires undergo constant selection (Chen et al., 2001b; Day et al., 2007; Lin and Welsh, 1998). Second of all, the overall TCR repertoire also narrows in the naïve T cells that undergo homeostatic proliferation (La Gruta et al., 2000), and it can lead to skewing of the Vβ usage and reduction in
heterologous immunity (Lin et al., 2008). Finally, we showed that T cell cross-reactivity between two distantly-related viruses (LCMV and PV) can also lead to the narrowing of its cross-reactive TCR repertoires, providing the third scenario (Cornberg et al., 2006). Most of the studies agree that there is a positive correlation between diversity of the antigen-specific TCR repertoire and immune control of infectious organisms. In another words, the diversity within the CDR3 region represented by different CTL clones is critical in recognizing potential antigenic variants. This TCR repertoire diversity is especially important in controlling organisms such as RNA viruses with inherently high mutation rates (Dockter et al., 1996; Kuntzen et al., 2007). A good example which illustrates this biological principle is the rapid generation of LCMV GP33 escape variant(s) in P14 transgenic mice, which express essentially a fixed TCR combination specific for LCMV GP33-41 epitope (Pircher et al., 1990). Thus, a limited TCR repertoire specific to an immunodominant epitope dramatically increases the odds of generating viral escape variants (Charini et al., 2001; Meyer-Olson et al., 2004).
Viruses and our immune system have co-evolved for millions of years. Viruses have developed a myriad of immune-evasion strategies in order to successfully replicate inside a cell and propagate. For example, a large DNA virus such as VV encodes many gene products that can interfere with the innate immune system signaling such as interferon alpha/beta (IFN α/β) and IL-18 pathways (Moss, 2001). Other viruses that belong to the herpes virus family evade immune detection by establishing latency (Cunningham et al., 2006). For RNA viruses such as LCMV that contain relatively small genomes, the viruses take advantage of the high mutation rate due to the low fidelity of the RNA-dependent RNA polymerase (Sevilla and de la Torre, 2006). As such, arenaviruses exist as viral quasispecies that enable them to adapt quickly to different selective pressures (Dockter et al., 1996). For example, an LCMV Armstrong strain variant that has the ability to cause persistent infection in mice was isolated in the spleens of neonatal mice (Ahmed et al., 1984). In addition, several LCMV CTL epitope escape variants have been selected under either in vitro conditions using CD8 T cell lines and in vivo in transgenic mice expressing specific Vα and Vβ TCR combinations (Aebischer et al., 1991; Pircher et al.,
1990). As predicted, all the point mutations occur within the particular CD8 epitope sequences that were selected against. These variant CD8 epitopes, also referred as altered peptide ligands (APLs), usually differ in aa sequence in certain key positions. Two major mechanisms account for the lack of CD8 T cell recognition. First of all, the switch of an aa residue that contains side chains responsible for recognition can result in loss of CTL recognition (Velloso et al., 2004). On the other hand, mutation at anchoring residues responsible for the binding to the MHC could result in complete loss of recognition since the variant APL loses its binding ability (Moskophidis and Zinkernagel, 1995).

In summary, the overall diversity of the antigen-specific TCR repertoire is crucial in recognizing potential CTL escape variants. T cell cross-reactivity between LCMV and PV not only results in alteration of immunodominance hierarchy but an extensive narrowing of the cross-reactive TCR repertoire. Under such a dominant but narrowed T cell response, we predicted that LCMV NP205 epitope escape variants could be selected in vivo. Finally, isolation of LCMV NP205 epitope escape variants could provide further understanding regarding the nature of CTL escape variants, T cell cross-reactivity and
heterologous immunity. Therefore, the first objective of my thesis work is to isolate different kinds of LCMV NP205 escape variants under heterologous virus challenge conditions \textit{in vivo}.

F. TCR Structure, MHC Recognition, and Antigen Presentation

The T cell component of adaptive immunity requires a different way to present the foreign peptides. It is now clear that T cells recognize foreign antigens as short stretches of peptides in the context of the major histocompatibility complex (MHC) (Bouvier and Wiley, 1994; Rudensky et al., 1991; Zinkernagel and Doherty, 1974). For class I MHC presentation (MHC I), all the nascent peptides made in cells are cleaved enzymatically by the proteasomal machinery in the cytoplasm into small fragments, trans-located into the lumen of the endoplasmic reticulum (ER) via the TAP transporter, and bound to class I MHC to start the antigen presentation process on the cell surface (Bai and Forman, 1997; Shepherd et al., 1993). The class II (MHC II) antigen presentation pathway for CD4 T cells is different and involves acquisition of extracellular proteins through the phagosomal compartment. The proteins are
proteolytically cleaved in the endosomes into small fragments and presented via class II MHC (MHC II) on the cell surface (Deussing et al., 1998). The majority of the T cells in humans and mice express receptors composed of alpha and beta subunits. Both the alpha and beta subunits are composed of variable and constants regions (Garboczi et al., 1996). The variable region of the alpha chain consists of re-arranged variable (V), and joining (J) regions. On the other hand, the variable region of the beta chain consists of re-arranged V, diversity (D), and J regions. Three CDRs within the variable region of each subunit are important for MHC binding and peptide recognition. The first and second CDRs are encoded within the TCR V segment, whereas the CDR3 forms the junctional diversity region following the V(D)J recombination (Rowen et al., 1996). Therefore, the CDR3 loops are highly diverse in sequence and length and are responsible for interaction with bound peptides (Garcia et al., 1998).

One of the important questions in modern immunology has been on how the mammalian adaptive immune systems discriminate “self” from “non-self”. The “clonal selection theory” postulated by Burnet has provided a great framework in explaining how the mammalian immune systems achieve such exquisite
selection (Burnet, 1962). The T cell selection process takes place in the thymus and ensures that each thymocyte express the proper combination of the alpha and beta subunits of the TCR in order to recognize peptide-bound MHCs in the periphery (Carlyle and Zúñiga-Pflücker, 1998). Briefly, during the positive selection process, each thymocyte must display a correctly recombined TCR beta chain paired with a surrogate alpha chain to ensure the proper recognition of the self-peptide-MHC (Dudley et al., 1994; Petrie et al., 1993). This is followed by a negative selection process, which ensures that thymocytes with high affinities to the self-peptides are eliminated. Therefore, thymic selection plays an important role in the elimination of self-reactive T cells.

A number of crystal structure studies on the TCRs and MHC complexes have yielded exciting clues about the nature of antigen recognition. The current consensus indicates that the Vα and Vβ domains are in close contact with the amino and carboxy termini of the peptide, respectively (Ding et al., 1998; Reinherz et al., 1999). In addition, the highly diverse CDR3 loops are generally located at the center of the MHC groove for peptide recognition. It is important to note that there could be some exceptions to this generalized observation as
more TCR-pMHC crystal structures are solved. Another important feature of TCR recognition is its plasticity (Wilson et al., 2004). Evidence has shown that a substantial conformational change could take place upon TCR binding to the pMHC, suggesting that a given TCR has the ability to adapt to various terrains on the MHC (Garcia et al., 1998).

As increasing numbers of cross-reactive TCR:pMHC crystal structures are being solved, five different models of TCR cross-reactivity have been proposed. The “induced fit” model highlights the structural adjustment that could take place for TCR:pMHC binding while the “differential docking” model proposes that difference in docking orientation could play a role in T cell cross-reactivity (Colf et al., 2007). The “molecular mimicry” model suggests that a high peptide structural similarity can also result in cross-recognition (Harkiolaki et al., 2009). On the other hand, the “structural degeneracy” model suggests that an epitope that lacks any specific features might actually result in un-expected T cell cross-reactivity (Li et al., 2005). Finally, an interesting model termed “antigen-dependent tuning of pMHC flexibility” proposes that different peptides can induce
conformational changes on a MHC to form an important base for TCR cross-reactivity (Borbulevych et al., 2009).

G. LCMV: A Prototypical Arenavirus

LCMV belongs to the family of arenaviridae, owing to its grain-like (sandy) morphology under the electron microscope. So far, the family of arenaviridae consists of 22 members that are divided into two groups based on their antigenic properties (Charrel and de Lamballerie, 2003). The Tacaribe serocomplex, also known as the New World group, contains viruses that are indigenous to the Americas (Emonet et al., 2006). One example of a New World arenavirus is PV. PV can cause fatality in certain strains of hamsters. On the other hand, the Lassa-lymphocytic choriomeningitis serocomplex are known as the Old World arenaviruses that are indigenous to Africa (Emonet et al., 2006). Guanarito, Junin, Lassa, Machupo, and Sabia are the 5 members that are able to cause hemorrhagic fevers in human (Charrel and de Lamballerie, 2003). Specific groups of rodents are the principal hosts of the arenaviruses. LCMV belongs to the Old World arenaviruses but is distributed world wide due to its
association with *Mus musculus*. Occasionally, accidental contact between the rodents and humans results in infection. In fact, LCMV was first isolated from a patient who died in the St. Louis encephalitis epidemic in 1933 (Buchmeier et al., 1980). The family of Arenaviridae are enveloped, containing two single-stranded RNA segments with unique ambisense coding strategy (Buchmeier et al., 1980). These two single-stranded RNA segments are appropriately named Large (L) and Small (S) segments based on their coding size. The L segment encodes a viral RNA-dependent RNA polymerase and a Z protein that has been suggested to interfere with type I interferon induction (Fan et al., 2010; Salvato et al., 1989). On the other hand, the S segment contains the viral structural proteins such as nucleoprotein (NP) and envelope glycoprotein complex (GPC), which can be subdivided into GP1 and GP2 (Riviere et al., 1985).

LCMV has been a major workhorse in modern immunology. For example, the study of mice congenitally infected with LCMV provided the foundation for immune central tolerance mechanisms and thymic selection (Traub, 1939). The rule of TCR-MHC restriction was also discovered in the LCMV system. Several characteristics of the LCMV model have allowed it to become one of the best
models to study host cellular immunity to viral infections. First of all, the RNA genome of LCMV is relatively small and compact compared to other larger RNA and DNA viruses (contains only 4 known gene products). Therefore, the relatively simple genome combined with the fact that LCMV naturally infects mice comprises a very robust system. This allowed tremendous epitope mapping efforts to be carried out in the early years and enabled the scientific communities to study the interaction between virus and T cell using well defined CD8 and CD4 epitopes in this system. Second of all, the non-lytic nature of LCMV infection allows scientists to clearly distinguish between viral versus immune-mediated pathology (Oldstone and Dixon, 1970). Third, all the RNA viruses exhibit extreme adaptability to the host immune response by virtue of low fidelity RNA dependent RNA polymerase activity. Therefore, the dynamic interplays between the virus and the host responses can be greatly appreciated in the LCMV system.
H. Host Immune Responses to an Acute LCMV Infection

A viral infection represents an intricate balancing act between an organism that is trying to hijack the host cell machinery for self-propagation verses a host immune surveillance system that is designed to recognize and eliminate any foreign organisms. In an immuno-competent mouse, intra-peritoneal (i.p.) inoculation of $10^2$ to $10^5$ plaque-forming units (pfu) of LCMV induces a very strong antigen-specific CD8 T cell response. In the initial stage of infection, type I interferon is induced within 24 hours as part of the innate response and reaches its peak at 72 hours. At the same time, activated natural killer (NK) cells can be found, due to type I interferon induction or target recognition (Orange and Biron, 1996; Welsh, 1978). Although natural killer (NK) cells are clearly activated during an acute LCMV infection, there is no indication so far that NK cells play a role in innate resistance to LCMV infection (Bukowski et al., 1983a; Welsh and Kiessling, 1980). Instead CTL functions represent a major mechanism for controlling of the virus (Kägi et al., 1995). LCMV is able to grow in many tissues, such as blood, liver, spleen, lymph node, thymus, lung, kidney and brain (Buchmeier et al., 1980). The LCMV virus titer peaks at around
four to five days after infection and the virus becomes undetectable in the blood after eight days following infection. The rapid decline in virus titer correlates with the rise of the CTL responses. The level of the antigen-specific CD8 T cell response reaches its peak eight days following an acute LCMV infection. An immunodominance hierarchy can be observed in which different antigen specific CD8 T cell responses are arranged in a predictable manner. Following the peak of the CD8 response, more than 90% of highly activated CD8 T cells undergo programmed cell death (Razvi et al., 1995). This immune-silencing state is thought to be the major mechanism to transition the immune system back to a normalized state.

Although the total number of CD4 T cells does not increase dramatically, the frequency of virus-specific CD4 T cells does increase during an acute LCMV infection. At the peak of the CD4 response, greater than 10% of the CD4 T cells that are specific for LCMV peptides produced interferon gamma (IFNγ) by intracellular cytokine staining assay (ICS) (Varga and Welsh, 1998).

Studies have found that non-neutralizing (complement fixing) antibodies could be detected as early as four days following an LCMV infection and could
play a role in controlling virus spread (Geschwender et al., 1976). Interestingly, free neutralizing antibodies that target surface glycoproteins (GP1 and GP2) did not seem to be involved in controlling LCMV infection because they remain undetectable for long periods of time after infection (Kimmig and Lehmann-Grube, 1979).

In summary, an acute LCMV infection in B6 mice is primarily controlled by the hosts’ antigen specific CD8 T cell responses. A stable CD8 immunodominance hierarchy can be found at the peak of the response eight days following infection. There is no indication of NK cell involvement in resistance to LCMV. However, NK cells are clearly activated upon LCMV infection. Free complement-fixing antibodies can be found early in the LCMV infection. However, neutralizing antibodies, which are thought to be more important in controlling the virus titer, do not appear until three to four months after infection. Taken these all together, CTL effector activities are absolutely essential in controlling the LCMV infection.
I. CD8 T Cell Response to Persistent LCMV Infection

The emergence of an LCMV Armstrong variant called clone 13 in Balb/c carrier mice further demonstrated the adaptability of the viruses that belong to this arenavirus family (Ahmed and Oldstone, 1988; Ahmed et al., 1984). When immunized at a high dose through the intravenous route (i.v.), LCMV clone 13 is able to cause a persistent infection in adult mice. A comparison of the RNA genome sequences between the LCMV Armstrong strain and the LCMV clone 13 strain has revealed 5 separate single nucleotide mutations; two are found in the S RNA segment and three are found in the L RNA segment (Salvato et al., 1991). Two out of five mutations affect the protein coding sequence. The first one is the lysine to glutamine at aa 1079 of the polymerase gene. The second one is the phenylalanine to leucine at amino aa 260 in the GP1 protein on the viral surface. Since the persistent phenotype is strongly associated with the mutation in GP1, this suggests that a difference in binding affinity and viral entry might account for this phenotype (Salvato et al., 1991). Oldstone et al confirmed this by first identifying alpha-dystroglycan as the cellular ligand for LCMV GP1 binding. In addition, they found that the binding
affinity to alpha-dystroglycan of the GP1 from the persistent phenotype was at least 1000 fold higher than the acute phenotype (Sevilla et al., 2000).

The LCMV Armstrong variant clone 13 provides a useful model to study CD8 T cell responses to persistent viral infections. Initially, a high viral load can be detected in various organs such as blood, liver and lung. Then the virus load subsides and is maintained at a low but detectable level. The virus can persist in a host for several months. Since antigen specific CD8 T cells are important in the clearance of LCMV, the failure to clear the virus is due to the loss of CD8 T cell effector functions (Zajac et al., 1998). This CD8 “clonal exhaustion” is associated with excess viral replication and high antigen load characterized by a progressive loss of different effector cytokines (IL-2<TNF<IFNγ) (Fuller et al., 2004). Furthermore, different types of clonal exhaustion can be observed between different antigen specific CD8 T cell populations. For example, GP33-specific CD8 T cells become anergized, whereas NP396-specific CD8 T cells are completely deleted at the end stage of the clonal exhaustion process, as they are unable to be detected by NP396-specific tetramers (Fuller et al., 2004).
J. Thesis Objective

Heterologous immunity is a balance between partial protective immunity and immune pathology. Previously, our lab identified two highly cross-reactive NP205-specific CD8 epitopes between LCMV and PV. In addition, heterologous challenge between LCMV and PV resulted in partial protection when compared to naïve controls. The overall objective of my thesis work is aimed at delineating in greater detail the mechanistic relationship between cross-reactive T cells and heterologous immunity using this system. Most importantly, I will utilize this LCMV+PV heterologous challenge system to ask why and how the cross-reactive T cells are often associated with immune pathology.

Objective 1. Isolation of LCMV NP205 escape variants under a dominant but narrowed cross-reactive NP205-specific TCR repertoire. In order to examine the relationships of T cell cross-reactivity and heterologous immunity in this system, a variant virus that contains either a non-immunogenic form or a knockout version of the cross-reactive NP205 epitope would be highly desirable. The finding that cross-reactive NP205 populations undergo a selective
expansion, which results in narrowing of their TCR repertoire following heterologous challenge led us to believe that there is a reasonable chance to isolate LCMV NP205 epitope escape variants in vivo.

**Objective 2.** No formal studies have been done to establish the direct link between the cross-reactive NP205-specific CD8 populations and heterologous immunity between LCMV and PV. In addition, some reports suggest bystander T cell activation, whereby T cells are activated by the cytokine milieu in a MHC-independent manner, as a potential mechanism for heterologous immunity. To find out whether cross-reactive NP205-specific CD8 T cells are mediators of heterologous immunity between LCMV and PV, I will compare the level of heterologous immunity between LCMV WT and LCMV NP-V207A-immune animals following PV challenge. We hypothesize that if the cross-reactive NP205-specific CD8 T cells were mediators of heterologous immunity in this model, we would observe a significant loss of heterologous immunity in the LCMV NP-V207A-immunized mice challenged with PV.
Objective 3. Our data showed that the establishment of a large cross-reactive NP205-specific CD8 T cell populations in mice that received PV and LCMV immunization in a sequential manner could often cause more harm than good when the hosts received PV as a final challenge. These mice developed a high incidence of AFN. Therefore, we questioned the role the cross-reactive NP205-specific CD8 T cells may play in immune pathogenesis. I will compare the incidence of AFN between the (PV+LCMV WT) and (PV+LCMV NP-V207A) double immune mice following PV re-challenge. If our hypothesis is correct, we should expect a significant lower incidence of AFN in the (PV+LCMV NP-V207A) double immune mice challenged with PV. This finding could have important implications for the tailoring of the immune response in future vaccine designs.
Chapter II
Material and Methods

A. Mice

C57BL/6 (B6, H2-K\textsuperscript{b}) male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.SJL-ptprca (it.1) congenic male mice (Ly5.1) were purchased from Taconic Labs (Germantown, N.Y). All the mice were used at 2-12 months of age and maintained under specific pathogen-free conditions at UMMS. All animal work was reviewed and approved by the UMMS IACUC committee.

B. Virus stocks and infection protocol

LCMV (Armstrong strain and clone 13), LCMV NP-V207A, \textit{in vitro} rescued LCMV variants (r/LCMV WT and r/V207A), and PV (AN3739 strain) were propagated in BHK-21 cells as described (Selin et al., 1994). To avoid immune responses generated to bovine serum following sequential infections, PV was purified by sucrose density gradient ultra-centrifugation and diluted in HBSS before immunization (Selin et al., 1998).
For primary infections, mice were immunized i.p. with $5 \times 10^4$ plaque forming units (pfu) of LCMV and $2 \times 10^7$ pfu of purified PV. Mice were considered immune 6 weeks after immunization. For homologous challenge, PV-immune mice were challenged i.p. with $2 \times 10^7$ pfu of purified PV. For heterologous challenge, PV-immune mice were challenged i.p. with $5 \times 10^4$ pfu of LCMV clone 13. LCMV-immune mice were challenged i.p. with $2 \times 10^7$ pfu of purified PV. For generation of (PV+LCMV WT) and (PV+LCMV NP-V207A) double immune mice, PV-immune animals were challenged i.p. with $1 \times 10^6$ pfu of LCMV WT or LCMV NP-V207A variant.

C. Isolation of the LCMV CTL escape variant

For isolation of an epitope escape variant, PV-immune mice were inoculated with $2 \times 10^6$ pfu of LCMV clone 13 i.v., and virus was isolated from the kidney 8 months later. LCMV plaques were excised and used to infect MC57G cell monolayers in 12-well plates. After 48 hours total cellular RNA was isolated, reverse transcribed using a poly-T primer, and the NP205 region of the cDNA was PCR amplified using 0.3μM each of flanking primers 5’-GGTCCTCGC-TGTGTGCTTGGCTTGA-3’ and 5’-TGGGGAGGCTCAGTGCAGAAGAAC-3’, using
pfx polymerase from invitrogen corporation (Carlsbad, CA). The PCR conditions were: (a) 94°C for 2 minutes; (b) 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 60 seconds (repeated 30 times); (c) 72°C for 60 seconds. DNA sequencing was done at the UMMS sequencing facility with custom primers (5 pmol/μl), 5’-CACCAAGACTAAAGTTATAGCCAG-3’ and 5’-AGGGTGCAAGTGGTGTGGTAAGAG-3’. The following murine β-actin primers were used: forward 5’-CGAGGCCCGACAGCAAGAGAG-3’; reverse 5’-CGGTTGGCCTTAGGGTTCAG-3’.

D. MHC-Ig dimer

Soluble dimeric mouse H2-K^b-Ig fusion proteins (MHC-Ig dimer) were purchased from BD Bioscience (San Diego, CA). The LCMV WT NP205-212 and LCMV NP205-212 (V-A) peptides (1mg/ml) were incubated with the MHC-Ig dimer at an 800 to 1 molar ratio and recombinant human beta-2 micro-globulin (0.15μg/μg of dimer) from BD Bioscience at 4°C for 4 days to allow passive loading of the peptide. The final products were used for surface staining assays.
E. Synthetic peptides

LCMV peptides GP33-41 (KAVYNFATC), GP92-101 (CSANNSHYI), GP118-125 (ISHNFCNL), GP276-286 (SGVENPGGYCL), NP396-404 (FQPQNGQFI), NP205-212 (YTVKYPNL), NP205-212 (V-A) (YTAKPNL), L1878-1885 (GPFQSFVS), PV NP38-45 (SALDFHKV) and PV NP205-212 (YTVKFPNM) synthetic peptides were purchased from BioSource International or 21st Century Biochemicals with 90% purity.

LCMV peptides L156-163 (ANFKFRDL), L338-346 (RQLLNLDVL), L349-357 (SSLIKQSKF), L455-463 (FMKIGAHPI), L689-697 (KFMLNVSYL), L775-782 (SSFNNGT), L1428-1435 (NSIQRT), L1878-1885 (GPFQSFVS), and L2062-2069 (RSIDFERV) were kindly provided by Dr. Alessandro Sette from the La Jolla Institute for Allergy and Immunology (Kotturi et al., 2007).

F. Peptide/MHC stabilization assay

TAP-1 deficient RMA-S cells (Townsend et al., 1989) were seeded into 96-well U-bottom plates at 5x10^5 cells per well. Following an incubation in a 5% CO_2 incubator at 27°C for 4 hours, NP205 peptides were added at different concentrations and incubated overnight. The cells were then stained with mAb to
H2-K\textsuperscript{b} (clone AF6 88.5) conjugated with PE (BD Bioscience) and analyzed by FACS.

**G. Functional avidity assay**

Splenocytes (1x10\textsuperscript{6} cells/well) were placed in a 96-well plate with 0.2µl of Golgi Plug (BD Bioscience) and human recombinant IL-2 (10U/ml) (BD Bioscience). 10x serial dilutions of a given peptide were made (starting at 5µM) and added to individual wells. The plate was incubated at 37°C in 5% CO\textsubscript{2} for 5 hours followed by surface staining and ICS. A peptide titration curve was generated as % maximum IFN\textgamma release using the frequency of IFN\textgamma+, CD8α+ T cells stimulated with the highest concentration of peptide as 100%.

**H. Acute fatty necrosis scores**

The severity of AFN was scored based on the guidelines from a previous publication (Selin et al., 1998). (1-2) very mild to mild disease with a few white necrotic spots on one or both lower abdominal fat pads; (3-4) mildly moderate and moderate with larger patches of necrosis of the lower abdominal fat pads and extension into the upper left quadrant fat pad around the spleen; (5-6)
moderately severe to severe with very extensive large patches of necrosis on the lower abdominal fat pads and spotty fatty necrosis throughout omental fat pads as well as the splenic fat pad; (7) very severe disease with such severe fatty necrosis that the organs are adherent to each other.

I. Plaque assay

Organs were harvested and homogenized with a tissue homogenizer in 1 to 2 ml of tissue culture medium. The supernatants were collected and stored in an -80 °C freezer. ATCC Vero cells (2x10^5 cells/well) were used to titrate LCMV and PV (Bukowski et al., 1983b). The plates were stained with neutral red at either 3 (LCMV) or 4 (PV) days after the infection. The plaques were counted 24 hours after. The pfu was expressed as Log_{10} pfu/ml.

J. Isolation of lymphocytes from fat pad tissue

The infiltrating leukocytes in the fat pads were isolated by mincing and digesting with collagenase B (200 mg/ml) in MEM plus 4% BSA for 1 hour at 37°C, and then separated over Lympholyte®-M from Cederlane Laboratory (Burlington, ON). A standard peptide stimulation protocol is performed followed by surface staining and ICS.
K. Intracellular cytokine staining (ICS)

Leukocytes from spleens, blood and abdominal fat pads (1x10^6 cells/well) were stimulated with either medium or 1μM peptides containing 0.2μl of GolgiPlug and human recombinant IL-2 (BD Pharmingen) at 37°C for 5 hours. Intracellular cytokine staining was performed using a cytofix/cytoperm kit from BD Bioscience. Intracellular cytokine-producing cells were detected with allophycocyanin (APC)-conjugated anti-mouse IFNγ monoclonal antibodies (1:1000) (XMG1.2) and phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-mouse TNFα (1:200) (MP6-XT22).

L. Cell Surface and MHC-Ig dimer staining by flow cytometry

All the surface antibodies were used at a 1:200 dilution per well. Single-cell suspensions of splenocytes or blood lymphocytes were first incubated with anti-mouse CD16/CD32 Fc-block antibody (1μl/well) for 15 minutes on ice. Subsequently a cell surface staining procedure was performed using PerCP-Cy5.5 anti-mouse CD8α (clone 53-6.7) and FITC anti-mouse CD44 (clone IM7). The samples were incubated on ice for 20 minutes.
For MHC-Ig dimer studies, the splenocytes were first blocked with the Fc-block (1μl/well) for 15 minutes on ice. Subsequently, different amounts of MHC-Ig dimers loaded with different LCMV NP205 peptides were added to the samples and incubated for 1 hour on ice. This was then followed by the regular surface staining protocol.

**M. Generation of in vitro rescued LCMV variant viruses**

The generation of the rescued(r) recombinant LCMV (r/LCMV) *in vitro* has been reported (Sanchez and de la Torre, 2006). Briefly, the cDNAs derived from either the S and L segments of LCMV Armstrong strain were inserted separately into expression plasmids with a T7 promoter. In addition, 3 other plasmids were required. A plasmid expressing T7 polymerase, along with two other plasmids expressing either LCMV cis and trans activating factors were required. Finally, all five plasmids were transfected into BHK-21 cells and r/LCMV could be detected in the tissue culture supernatant.

The nucleotide sequence that contained the original point mutation was sent to our collaborators at Scripps Research Institute in La Jolla. The cloning and rescue of the recombinant LCMV NP-V207A (r/V207A) was done by Andrew
Trees, a student of the Immunology Summer Program, directed by Sebastien Emonet in Dr. Juan Carlos de la Torre’s laboratory. In addition, r/LCMV containing different versions of the anchoring residue mutation in the LCMV NP205 epitope were also generated.

N. Adoptive transfer experiments

LCMV-immune splenocytes (2x10⁷) expressing the Ly5.2 congenic marker were CFSE-labeled and injected i.v. (200ul) into congenic C57BL/6 mice (Ly5.1). One day after, the mice were challenged i.p. with 2x10⁷ pfu of PV. The spleens were harvested 8 days following infection and stimulated with different peptides for 5 hours. Standard surface staining and ICS were preformed.

O. Peptide:MHC crystal structure

The crystal structures of the LCMV and PV NP205 peptides:H2-Kb complexes were generated by Carole Guillonneau and Stephanie Gras under the guidance of Stephen Turner (Department of Microbiology and Immunology, The University of Melbourne, Parkville, Australia) and Jamie Rossjohn (The Protein Crystallography Unit, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Victoria, Australia), respectively.
P. Statistical analysis

Statistical analysis was performed using GraphPad Prism™ software (5.0b). Comparisons between two groups were performed using the unpaired Student’s t test (2-tailed). Comparisons between more than two groups were performed using one way ANOVA analysis (2-tailed). Pearson’s correlation test was used to measure the correlation between two independent variables. $P$ values less than 0.05 were considered statistically significant.
Previously, our lab showed that heterologous challenge between LCMV and PV could result in a selective expansion of the cross-reactive NP205-specific CD8 T cells. These cross-reactive NP205-specific CD8 T cells are oligo-clonal in nature. Since T cell oligo-clonality (narrowed TCR repertoire) had been correlated with an increase in incidence of viral escape in other systems (Kalams et al., 1994), we questioned if LCMV NP205 CTL escape variants could be generated in vivo as one of the immunological consequences following a heterologous challenge in this particular system. Based on all the previous knowledge that we learned from this heterologous system, I believed that there was a reasonable chance of success. First of all, the cross-reactive NP205-specific CD8 T cell responses dominate the immune response and dramatically change the immunodominance hierarchy following a heterologous challenge (Brehm et al., 2002). Second, there was a significant narrowing (skewing) in the cross-reactive NP205-specific CD8 T cell repertoire in vivo following a heterologous challenge (Cornberg et al., 2006). Finally, due to the low fidelity in
RNA dependent RNA polymerase, LCMV exists as quasispecies and is highly adaptable in nature (Sevilla and de la Torre, 2006). In fact, different LCMV CTL escape variants have been generated under either in vitro or in vivo conditions (Lewicki et al., 1995).

The first step to isolate LCMV NP205 CTL variants in vivo was to challenge PV-immune mice with LCMV clone 13 (LCMV WT) and wait for the onset of viral persistence (Fig 3.1). LCMV clone 13 is a genetic variant of LCMV Armstrong strain. In contrast to the LCMV Armstrong strain, the LCMV clone 13 is able to establish persistent infections when inoculated at high dose i.v. (2x10^6 pfu/mouse). Since viral fitness, pressure of the CTL selection, and many other factors all play a role in determining the outcome of the viral CTL escape selection, a LCMV clone 13 infection that causes a persistent infection is important. This prolonged viral replication could dramatically increase the odds of generating LCMV NP205 CTL escape variants in vivo. Following a period of viral persistence, peripheral organs were harvested at different time points for viral plaque assay. Subsequently, individual LCMV plaques were picked and screened for point mutations within the LCMV NP205 epitope region. Briefly, two
sets of primers specifically designed to amplify the LCMV NP205 region were used for PCR amplification and subsequent sequencing reactions. All the LCMV CTL escape variants previously isolated either in vitro or in vivo bear point mutation(s) within their respectively selected epitopes, indicating the direct relationship between the location of the mutation and the CTL selective pressure (Aebischer et al., 1991; Pircher et al., 1990). Therefore, I anticipated that LCMV NP205 CTL escape variants would contain mutation(s) in the LCMV NP205 epitope.
Persistent dose
LCMV Clone 13

PV-Immune

NP38
NP205
GP33
NP396

Domination of cross-reactive NP205-specific CD8 T cells with narrowed TCR repertoire in the presence of high viral antigen load

Selection of LCMV NP205-212 CTL escape variants

Figure 3.1  Schematics on *in vivo* generation of LCMV NP205-212 CTL escape variants.
A. Specific PCR amplification and sequencing of the LCMV NP205 epitope region

An *in vitro* viral culture system was set up to screen all the potential LCMV NP205 CTL escape variants. The LCMV Armstrong containing the wild type LCMV NP205 sequence was used as a positive control. Briefly, the MC57G cell line derived from B6 mice was infected with different LCMV plaque isolates. After 48 hours, total messenger RNA was isolated and reverse-transcribed into cDNA. The region that spanned the LCMV NP205 epitope was amplified by PCR. Subsequently, the PCR products were purified and sequenced with a different set of primers. Figure 3.2A shows the specific amplification of the region that encompasses the LCMV NP205 epitope in MC57G cell lines infected with LCMV Armstrong (lane 1). The size of the PCR product (~548bp) confirms the specificity of the primer pair used in the experiment. The internal controls using either commercially supplied RNA (Fig 3.2A, lane 7) or murine beta-actin controls indicate that there are no confounding factors in the reaction (Figure 3.2B). Finally, sequencing of the PCR product in either direction (forward and reverse) reveals unequivocally the wild type LCMV NP205 sequence in both
nucleotide level and aa levels (YTVKYPNL) (Figure 3.3). In addition, the sequence data matched specifically to the NP205 region of the LCMV nucleoprotein using the NIH nucleotide blast search program (data not shown). Therefore, we have established an *in vitro* system for screening of potential point mutations within the LCMV NP205 epitope region.
Figure 3.2  Specific amplification of the LCMV genome region encompassing the LCMV NP205-212 epitope. (A) The MC57G cell line was infected with either LCMV Armstrong or medium (negative control). 48 hours after, total RNA was isolated and reverse transcribed into cDNA. The cDNAs were then amplified by primers that flank the LCMV NP205-212 epitope region. The cDNAs were also amplified with beta-actin primers as an internal control. Lanes 3 and 7 represent commercially available Hela RNA amplified with primers provided. (B) Represents a re-analysis of the beta-actin controls.
Figure 3.3  Nucleotide sequence of the wild type LCMV NP205-212 epitope. The underline denotes the putative position of the anchoring residue positions in the context of H2-Kb.
B. The LCMV NP-V207A escape variant contains a variant NP205 epitope sequence that resembles the Lassa virus sequence in nature

In a group of PV-immune mice challenged with LCMV clone 13 (N=4), a LCMV NP205 epitope escape variant (LCMV NP-V207A) was isolated from the kidney of a test animal eight months after infection. The kidney could possibly represent the site of LCMV persistence, because they were the only organs that still harbored the virus (spleen and brain tissues). A single point mutation was detected within the third aa codon sequence (GTC to GCC) of the LCMV NP205 epitope. The frequency of the LCMV NP-V207A variant virus in the kidney of this particular host is approximately 20%, because one out of five viral clones contained the point mutation (figure 3.4). Moreover, no sequence mutation(s) was detected within the region encompassing the LCMV NP205 epitope from the rest of the animals (0/15). Finally, no additional mutation(s) were found in the remaining stretch of sequences that encompass the LCMV NP205 epitope (~250bp sequence) in the LCMV NP-V207A variant. This single point mutation predicts a change of the original valine (V) to alanine (A) at the third position of the LCMV NP205 epitope (Table 3.1). Based on the putative K\textsuperscript{b} binding motif
published by several labs, this is a non-anchoring aa residue mutation. However, the third aa residue in K\textsuperscript{b} has been indicated as an auxiliary anchoring residue, suggesting that it is also an important residue for binding and recognition (Hudrisier et al., 1997). Finally, the highly conserved nature of the LCMV NP205 epitope sequences from both the Old World and New World arenaviruses reveal an interesting aspect of this mutation (Table 3.1)
Figure 3.4 A single point mutation (*) within the LCMV NP205-212 epitope results in switch of valine to alanine at the 3rd non-anchoring amino acid residue in the LCMV NP-V207A variant virus. The underline denotes the putative position of the anchoring residue positions in the context of H2-Kb.
Comparison of the majority of NP205 sequences indicated that only three positions are subjected to variations among the different arenaviruses. Two of them are the putative anchoring residues at positions five and eight. The last variable is at position three, which is the auxiliary anchoring residue. Interestingly, the only variation for the third position so far is between either a valine or an alanine. While all the New World arenaviruses contain valine at the third position, the Old World arenaviruses contain either a valine (LCMV) or an alanine (Lassa virus and Mopeia virus). Thus, this V to A mutation in the LCMV NP-V207A escape variant reflects the specificity and delicate balance between viral fitness and host responses. Interestingly, the mutated LCMV NP205 epitope sequence now resembles the Lassa virus NP205 sequence in nature.
### Old World Arenavirus

<table>
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<tr>
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<td>LASSA</td>
<td>YTAKYPNT</td>
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<td>MOPEIA</td>
<td>YTAKYPNM</td>
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### New World Arenavirus

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</tr>
<tr>
<td>SABIA</td>
<td>YTVKFPNL</td>
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Table 3.1 High degree of conservation in the NP205-212 epitope sequences among the arenaviruses. The underline denotes the anchoring residues.
C. The variant NP205 epitope stabilizes $K^b$ \textit{in vitro} but elicits a significantly lower antigen-specific CD8 T cell response at physiological concentrations

Based on the putative sequence of the NP205 (V-A) epitope, the epitope should be able to bind to $K^b$. I set up an \textit{in vitro} MHC stabilization assay to address this question. Briefly, TAP-deficient RMA-S cells that have been shown to maintain a very low class I MHC expression on their surface due to a defect in peptide transport into the ER were used for the assay (Ljunggren et al., 1990). When peptide fragments that contain the putative binding motifs are added exogenously, an increase in class I MHC expression on the cell surface can be seen. Therefore, I compared the ability of the LCMV NP205 (YTVKYPNL) and the LCMV NP205 (V-A) (YTAKYPNL) peptides to stabilize $K^b$ \textit{in vitro}. Both LCMV NP205 and LCMV NP205 (V-A) peptides are able to stabilize $K^b$ at 100μM concentration, as shown by a significant increase in the mean fluorescence intensity (MFI~40.0) for antibody against $K^b$ (Figure 3.5A). The un-pulsed RMA-S cells indicate the basal level of antibody staining (MFI~10.0) (Figure 3.5A). Finally, the peptide titration curves suggest that both wild type and LCMV NP205 (V-A) peptides bind $K^b$ with similar affinity and kinetics (Figure 3.5B). Overall, these data suggest that the LCMV NP205 (V-A) peptide can bind to $K^b$ in a
similar manner as the wild-type LCMV NP205 peptide, and can probably be presented \textit{in vivo}. 
Figure 3.5  The LCMV NP205-212 (V-A) peptide maintains the ability to stabilize Kb in vitro. RMA-S cells were incubated with different concentrations of either LCMV NP205-212 or LCMV NP205-212 (V-A) peptide overnight. Cell surface staining was performed by using a monoclonal antibody against Kb. (A) represents the Kb expression on the RMA-S cells incubated with 100μM of indicated peptides. (B) represents the average titration curve between the two indicated peptides using duplicate samples. These are representative of two independent experiments.
Next, I wanted to know how efficient the LCMV NP205 (V-A) peptide was at stimulating either LCMV or LCMV+PV effector T cell responses. Both LCMV NP205 and LCMV NP205 (V-A) peptides are able to stimulate NP205-specific CD8 T cells under a 5000 nM saturating condition (Figure 3.6). However, at a concentration that mimics physiological conditions (10,000 fold lower), a significant difference in IFNγ production between the LCMV NP205 and NP205 (V-A) peptide-stimulated groups was observed. In figure 3.6B, a significant reduction in the antigen-specific CD8 T cell response (~80% reduction) to the LCMV NP205 (V-A) peptide can be seen using the PV+LCMV WT T cell effectors at peptide concentration of 0.5nM ($p=0.0045$). In addition, a similar reduction in IFNγ production can be seen when using the LCMV WT effectors (Figure 3.6A). However, this difference is not as great as the one using the PV+LCMV WT effectors (Figure 3.6B). Since the mutation within the LCMV NP205 epitope was selected by a very small group of cross-reactive NP205-specific CD8 T cells, it is possible that the primary LCMV NP205-specific CD8 T cell response that contains a more diverse repertoire is able to recognize this variant epitope more efficiently.
Overall, these data suggest that the variant NP205 peptide is able to stabilize K\textsuperscript{b} \textit{in vitro} and possibly be presented \textit{in vivo}. However, there is a significant reduction in the ability of the LCMV NP205 (V-A) peptide to stimulate LCMV or PV+LCMV WT CD8 effector T cells at lower peptide concentrations. This suggests that the LCMV NP205 (V-A) peptide could be much less immunogenic in nature.
Figure 3.6 The LCMV NP205-212 (V-A) peptide is significantly less efficient at stimulating antigen-specific CD8 T cell responses at lower diluted concentrations. Splenocytes from (A) acute LCMV WT infection and (B) PV-immune + acute LCMV WT were stimulated ex vivo with different dilutions of either LCMV NP205-212 or LCMV NP205-212 (V-A) peptide. After 5 hours, a standard ICS assay was performed and analyzed by FACs. The analysis was done by gating on CD8α+ lymphocyte populations. The % of IFNγ max was calculated by designating the response at 5000nM as 100%. *p<0.05, ***p=0.0045 by student’s t test. These are representative data of 3 mice in group A and 3 mice in group B.
D. Infection of B6 mice with the LCMV NP-V207A variant results in a significantly diminished cross-reactive NP205-specific CD8 T cell response

To determine the immunogenicity of the LCMV NP205 (V-A) peptide in vivo, we infected B6 mice with a low dose (5x10^4 pfu) of either LCMV WT or LCMV NP-V207A viruses and analyzed their acute responses eight days after infection. I found that mice immunized with the LCMV NP-V207A variant displayed a significantly diminished cross-reactive response (LCMV NP205, LCMV NP205 V-A, and PV NP205) as compared to the mice infected with LCMV WT (Figure 3.7). In contrast to a significant reduction in cross-reactive NP205-specific CD8 T cell responses in the LCMV NP-V207A-infected animals, all the other LCMV specific T cell responses were comparable in mice infected with either virus (Figure 3.7).
Figure 3.7 The LCMV NP-V207A variant virus induces a greatly diminished cross-reactive NP205-specific CD8 T cell responses. B6 mice were immunized i.p. with 5x10⁴ pfu of either (A) LCMV WT and (B) LCMV NP-V207A variant viruses. Spleens were harvested 8 days after infection. Standard ICS was performed following ex vivo stimulation for 5 hours at 37°C in 5% CO₂ with different sets of LCMV-specific CD8 peptides. The analysis was done by gating on CD8α+ lymphocyte populations. These are representative data for 2 mice in group A and 3 mice in group B. These are representative data of 5 experiments.
Figure 3.8 PV-immune mice immunized with the LCMV NP-V207A variant also display a greatly diminished cross-reactive NP205-specific CD8 T cell response. PV-immune mice were challenged i.p. with 5x10^4 pfu of either (A) LCMV WT or (B) LCMV NP-V207A variant virus. Spleens were harvested 8 days after infection. A standard ICS was performed following ex vivo stimulation for 5 hours at 37°C in 5% CO2 with different sets of LCMV-specific CD8 peptides. The analysis was done by gating on the CD8α+ lymphocyte populations. These are representative data for 2 mice in group (A) and 3 mice in group (B).
Since only a small percentage of CD8 T cells (0.7%) responded to the LCMV NP205 (V-A) peptide stimulation in the LCMV NP-V207A-infected mice (Figure 3.7B), this result suggests that the point mutation has rendered the LCMV NP205 (V-A) epitope much less immunogenic in nature. This is consistent with our hypothesis that a dominant cross-reactive NP205-specific CD8 T cell response would select an LCMV variant that expresses a variant epitope that might evade such an immune response. Similarly, PV-immune mice challenged with the LCMV NP-V207A virus exhibit very little increase in the frequency of the cross-reactive NP205-specific CD8 T cell responses eight days after infection as compared to PV-immune mice challenged with LCMV WT (Figure 3.8, 1% vs. 18% of the CD8 response, respectively). These data suggest that the NP205 variant epitope is less immunogenic and results in very little cross-reactive expansion upon PV challenge. However, it is also possible that the LCMV NP205 (V-A) epitope could induce a very different T cell functionality that we could not pick up using a standard ICS assay. Studies using MHC-Ig dimers loaded with the LCMV NP205 (V-A) peptide are described in the second part of chapter four to address this particular question.
E. Both the LCMV WT and LCMV NP-V207A variants display similar replication kinetics *in vivo* and maintain the ability to clonally exhaust T cells

Next, we compared the replication kinetics between the LCMV WT and the LCMV NP-V207A variant viruses. When mice were infected with a low dose (5x $10^4$ pfu) of either LCMV WT or LCMV NP-V207A virus, no significant difference in virus titer could be detected between the two groups in all the organs tested (abdominal fat pad and kidney) six days after infection (Figure 3.9). Most importantly, both the LCMV WT and LCMV NP-V207A variant viruses are rapidly cleared by day nine post-infection and remain undetectable in all the major organs tested in the immune hosts (Figure 3.9)
Figure 3.9  Similar replication kinetics between the LCMV WT and LCMV NP-V207A variants. B6 mice were immunized i.p. with 5x10^4 pfu of either LCMV WT (black bar) or LCMV NP-V207A variant (white bar). The fat pads (A) and kidneys (B) were harvested at the indicated days post-infection. A standard LCMV plaque assay was performed at the same day. The arrow represents the cut-off point of detection. The values are expressed as Log_{10}(pfu/ml) ± SD. These are representative of two independent experiments with 5 mice per group.
With the notable exception that a very small LCMV NP205-specific CD8 T cell response was detected in LCMV NP-V207A-infected mice, the overall magnitude as well as the kinetics of the antigen-specific CD8 T cell responses was very similar in mice infected with either LCMV WT or LCMV NP-V207A (Figure 3.10). For example, the overall antigen-specific CD8 T cell responses peaks at around day nine post-infection and undergoes a contraction phase to reach the immune stage in mice infected with either virus (Figure 3.10). Therefore, the point mutation in the subdominant NP205 epitope did not result in any noticeable perturbation in either viral replication or T cell activation kinetics in the LCMV NP-V207A-immune hosts.
Figure 3.10 Similar activation kinetics between (A) LCMV WT and (B) LCMV NP-V207A variant. B6 mice were infected i.p. with $5 \times 10^4$ pfu of either LCMV WT or LCMV NP-V207A variant virus. Spleens were harvested at different time points post-infection. A standard ICS was performed following *ex vivo* stimulation for 5 hours at 37°C in 5% CO$_2$ with different sets of LCMV-specific CD8 peptides. The analysis was done by gating on the CD8α+ lymphocyte populations. Each value represent the average of 3 mice per group ± SD. These are representative data of 2 independent experiments.
Since the LCMV NP-V207A was isolated in the LCMV clone 13 background, we asked whether the variant still retains the LCMV clone 13 phenotype \textit{in vivo}. Like the LCMV clone 13, the LCMV NP-V207A variant is able to induce T cell clonal exhaustion when inoculated at high dose ($2 \times 10^6$ pfu) i.v. (Figure 3.11). Similar to the original report (Fuller et al., 2004), two of the major immunodominant T cell responses become either anergic (GP33) or deleted (NP396) in mice infected with high dose variant (Figure 3.11). Again, these data point out that specifically the pressure of the cross-reactive NP205 CTL selection drove the point mutation in the LCMV NP-V207A variant and that it probably imparted a minimal effect on other sequences.
Fig 3.11. The LCMV NP-V207A variant virus maintains the ability to establish persistent infection and clonally exhaust T cells. B6 mice were immunized with either (A) 2x10^6 pfu, (B) 2x10^5 pfu or (C) 2x10^4 pfu of LCMV NP-V207A variant virus i.v. The spleens from each group of mice were harvested 14 days after infection and the standard ICS was performed following ex vivo stimulation for 5 hours at 37°C in 5% CO₂ with different sets of LCMV-specific CD8 peptides. The analysis was done by gating on CD8α+ lymphocyte populations. The data are representative of 3 mice per group.
Chapter Summary

T cell cross-reactivity between two distantly related arenaviruses, LCMV and PV, results in a dominant expansion of cross-reactive subdominant NP205-specific CD8 T cells upon heterologous challenge, and, importantly, a significant narrowing of the cross-reactive NP205-specific CD8 T cell repertoire was observed (Cornberg et al., 2006). We predicted that NP205 CTL escape variants could be generated as the result of a dominant but narrowed cross-reactive NP205-specific CD8 T cell responses upon heterologous challenge.

Indeed, a LCMV NP-V207A CTL escape variant was isolated in a PV-immune mouse challenged with the heterologous LCMV. A point mutation was detected within the nucleotide sequence encoding the LCMV NP205 epitope, resulting in a switch of valine to alanine at the non-anchoring third aa residue (Figure 3.4). The resulting LCMV variant NP205 sequence resembles Lassa virus sequences in nature (Table 3.1). Furthermore, the LCMV NP205 (V-A) peptide displays the ability to stabilize K^b as efficiently as the wild type NP205 peptide, but is significantly less efficient at stimulating LCMV NP205-specific T
cell responses at lower physiological conditions \textit{in vitro} (Figure 3.5 & 3.6). Most importantly, this LCMV NP205 (V-A) epitope seems to be much less immunogenic \textit{in vivo}, as mice infected with the LCMV NP-V207A variant virus display a greatly diminished NP205-specific CD8 T cell responses (Figure 3.7 & 3.8). However, the LCMV NP-V207A variant virus displays similar replication kinetics and antigen-specific T cell responses to other major LCMV epitopes \textit{in vivo} (Figure 3.9 & 3.10). In keeping with the original clone 13 background, the LCMV NP-V207A variant virus maintains its ability to induce T cell clonal exhaustion in its host \textit{in vivo} (Figure 3.11). Furthermore, no additional mutation(s) were found in the remaining stretch of sequences that encompass the LCMV NP205 epitope (~250bp sequence) in the LCMV NP-V207A variant. This suggests that the point mutation in the NP205 epitope was selected by a dominant cross-reactive NP205-specific CD8 T cells upon heterologous challenge and this point mutation did not seem to affect the general replication characteristics of the LCMV clone 13 strain. Finally, the data presented above suggest that the single point mutation within the LCMV NP205 epitope results in a significant reduction in its immunogenicity.
It was interesting to find that a single amino acid switch (V-A) could result in such a dramatic loss in immunogenicity (Fig 3.7). One recent report has shown a similar finding in which a mutation in the original epitope resulted in loss of immunogenicity. It has been suggested that virus utilized this strategy to exploit “hole” in hosts’ T cell repertoire (Wölfle et al., 2008). This would imply that the mutated epitope could possibly resemble self peptides/antigens of the host so that most of the T cells that are able to recognize this variant epitope are deleted during negative selection in the thymus.
Chapter IV  
T Cell Cross-Reactivity and Heterologous Immunity

Part I

Previous studies from our lab showed that heterologous immunity between different viruses and bacteria is dependent on the sequence and route of challenge (Chen et al., 2001a; Mathurin et al., 2009). For example, T cell-mediated heterologous immunity can be demonstrated between LCMV and PV in a bi-directional manner, as secondary challenge with the heterologous virus results in partial protective immunity without apparent immune-associated pathology (Selin et al., 1998). One of the key questions regarding T cell cross-reactivity and heterologous immunity has been whether the cross-reactive memory T cells are directly responsible for the heterologous immunity observed. A direct relationship between the cross-reactive NP205-specific CD8 T cells and heterologous immunity between LCMV and PV still remained to be established. Studies have suggested that bystander T cell activation plays very little role, if any, in an LCMV infection (Masopust et al., 2007; Zarozinski and Welsh, 1997). However, the possibility that memory T cells can be activated via the induction of
cytokines in a MHC-independent manner still remains a possible mechanism for heterologous immunity (Tough et al., 1996; Tsunoda et al., 2007). For example, one group showed that ovalbumin-specific memory CD8 T cells could confer protection via IFN-γ production in response to IL-12 and IL-18 following *Listeria monocytogenes* infection (Berg et al., 2003).

Previous experimental data have suggested a role of cross-reactive NP205-specific CD8 T cells in mediating heterologous immunity in this system. First of all, memory T cells are required for heterologous immunity between LCMV and PV, as adoptive transfer of LCMV-specific memory donor splenocytes depleted of either CD4 or CD8 populations failed to show heterologous protection (Selin et al., 1998). Thus far, no CD4 cross-reactive epitopes have been identified between LCMV and PV. Therefore, the exact role of CD4 T cells in heterologous immunity between LCMV and PV remains to be determined. Traditionally, CD4 T cells are regarded as “helper” T cells that are important to maintain a functional memory CD8 T cell response (Sun and Bevan, 2003; Sun et al., 2004). Therefore, one possibility is that CD4 depletion of the LCMV-immune mice could render the cross-reactive CD8 T cells functionally
compromised. On the other hand, CD4 T cells have been shown to possess cytotoxic effector functions, and they could play an important role in other heterologous immunity models (Jellison et al., 2005; Mathurin et al., 2009).

Second of all, the LCMV NP205 and PV NP205 epitopes share a high aa sequence similarity. In addition, previous studies from our lab have shown that these cross-reactive NP205-specific CD8 T cells exhibited a broad functional cross-reactivity and proliferated upon heterologous challenge and resulted in alterations of the immunodominance hierarchy (Brehm et al., 2002; Cornberg et al., 2006). Finally, a significant correlation between the level of cross-reactive NP205-specific CD8 T cell responses and heterologous immunity can be seen in the homeostatic proliferation model using LCMV and PV. Specifically, naïve T cells that had undergone homeostatic proliferation in a lymphopenic environment generated different magnitudes of the cross-reactive NP205-specific CD8 T cell responses following LCMV infection. Therefore, we asked if there is a correlation between the level of cross-reactive NP205-specific CD8 T cell responses generated in these mice and degree of heterologous immunity following PV challenge. I found a positive correlation between the magnitude of cross-reactive
NP205-specific CD8 T cell response in each individual mouse and the level of heterologous immunity to PV challenge (Lin et al., 2008).

However, the establishment of a direct link between cross-reactive memory T cell populations and heterologous immunity requires a direct knockout of the cross-reactive NP205-specific CD8 T cell responses. This could be accomplished by either 1) isolation or generation of a LCMV variant that contains an altered version of cross-reactive NP205 CD8 epitope or 2) in vivo depletion or anergy induction of the putative cross-reactive CD8 T cell populations.

Here, we hypothesized that the cross-reactive NP205-specific CD8 T cells are mediators of heterologous immunity between LCMV and PV. We reasoned that if the cross-reactive NP205-specific CD8 T cells were directly responsible for heterologous immunity, a significant loss of heterologous immunity would be observed in LCMV NP-V207A variant-immune mice following PV challenge. If our hypothesis is correct, this could be the first evidence that highlights the importance of cross-reactive memory T cells in mediating heterologous immunity in a well-characterized and controlled experimental model system.
A. Limited evidence on the development of additional cross-reactive CD8 responses in LCMV NP-V207A-immune mice challenged with PV

Before the question of whether the cross-reactive NP205-specific CD8 T cells were responsible for mediating heterologous protective immunity between LCMV and PV could be addressed, I wanted to know if any additional cross-reactive epitopes were present when we challenged LCMV NP-V207A-immune animals with PV. Therefore, splenocytes from either LCMV WT-immune or LCMV NP-V207A-immune mice were CFSE-labeled and adoptively transferred into a group of congenic mice (Ly5.1). Upon challenge with the heterologous virus (PV), cross-reactive memory T cells that express a different congenic marker (Ly5.2) can be monitored based on their proliferation (CFSE dilution) and antigen-specific cytokine responses.
Figure 4.1 Limited evidence for the development of additional cross-reactive CD8 responses in LCMV NP-V207A-immune mice challenged with PV. (A) LCMV WT-Immune splenocytes and (B) LCMV NP-V207A-Immune splenocytes were labeled with CFSE, adoptively transferred into congenic hosts, and infected i.p. with PV a day later or (C) remained uninfected. 8 days after PV infection, spleens from different groups of mice were harvested and stimulated ex vivo with a panel of LCMV-specific CD8 peptides. Standard ICS and FACs were performed. The analyses were done by gating on Ly5.2+, CD8α+ donor T Cell populations. These are representative data of 2 mice in group (A) and 4 mice in group (B).
I found an extensive proliferation (CFSE dilution) of the cross-reactive NP205-specific CD8 T cells in congenic hosts that received wild type LCMV-immune splenocytes eight days after PV challenge (Figure 4.1). However, very little proliferation of the cross-reactive NP205-specific CD8 T cells could be detected in the congenic hosts that received LCMV NP-V207A-immune splenocytes (Figure 4.1). This experiment supports the conclusion drawn from previous chapter that cross-reactive NP205-specific CD8 T cell responses generated by the LCMV NP-V207A variant virus are minimal. In addition, there was no evidence of proliferation from other major LCMV immunodominant and subdominant epitope responses (GP33, NP396, GP276, GP118, and GP92) in congenic hosts that received splenocytes from LCMV NP-V207A-immune animals following PV challenge (Table 4.1).
Table 4.1 Limited evidence for the development of additional cross-reactive CD8 responses in LCMV NP-V207A-immune mice challenged with PV. LCMV WT-Immune and LCMV NP-V207A-Immune donor splenocytes were labeled with CFSE and adoptively transferred into congenic hosts and infected i.p. with PV a day later. 8 days post PV infection, spleens from different groups of mice were harvested and stimulated ex vivo with a panel of LCMV-specific CD8 peptides. Standard ICS and FACs were performed. The analyses were done by first gating on Ly5.2+, CD8α+ donor T Cell populations, and then looking at the IFNγ+, CFSE low population. These data represent the average of 2 mice from each donor. The asterisk represents a statistically significant difference (P<0.05) between the LCMV WT-Immune and LCMV NP-V207A-immune group by student’s t test.
However, the LCMV NP-V207A-immune splenocytes did show an extensive proliferation (CFSE low) and polyclonal activation via CD3 stimulation (Figure 4.1). This could be attributed to either a small number of transferred splenocytes (2x10⁷ cells per recipient) or the response to newly characterized L epitopes from LCMV (Kotturi et al., 2007). Therefore, a series of experiments were conducted to screen for potential cross-reactive epitopes derived from the LCMV L protein. The preliminary experiments yielded a potential cross-reactive epitope LCMV L1878-1885-specific CD8 response, which displayed a similar frequency as the cross-reactive NP205-specific CD8 T cell response upon heterologous challenge (LCMV WT+PV) (Table 4.2). However, the fact that the response to LCMV L1878-1885 also coincided with the diminished cross-reactive NP205-specific CD8 T cell response in the LCMV NP-V207A-Immune mice challenged with PV led me to suspect possible peptide contamination (Table 4.2).
Table 4.2 Screening of potential cross-reactive CD8 epitopes between LCMV and PV. PV-immune animals were infected i.p. with 5x10⁴ pfu of either LCMV WT or LCMV NP-V207A variant virus. 8 days after, (A) splenocytes and (B) peritoneal exudate cells (PECs) were harvested and stimulated ex vivo with a panel of newly defined LCMV L CD8 epitopes. The data represent the average frequencies of IFNγ+, CD8α+ T cells ± SD. The asterisk indicates a statistically significant difference (P<0.05) between the PV+LCMV WT and PV+LCMV NP-V207A group.
It became apparent that the initial observation was an artifact, as newly synthesized L1878-1885 peptide from a different source induced only a minimal response (Figure 4.2). However, it was interesting to note that the overall LCMV-specific CD8 responses was significantly higher in the peritoneal exudate cells (PECs) of the PV-immune mice challenged with LCMV NP-V207A as compared to PV-immune mice challenged with LCMV WT. This demonstrates that the significant reduction in the cross-reactive NP205-specific CD8 T cell response could lead to the restoration of the original LCMV immunodominance hierarchy upon heterologous challenge of PV-immune mice (Table 4.2). Putting these all together, there do not seem to be any indication of additional cross-reactive responses in LCMV NP-V207A variant viruses following heterologous challenge of PV.
Figure 4.2  LCMV WT-immune donor splenocytes were CFSE-labeled and adoptively transferred into congenic hosts (Ly5.1+). The recipient mice were infected i.p. with 2x10^7 pfu of PV. 8 days later, splenocytes were harvested and stimulated ex vivo with peptides indicated above. Standard ICS and FACs analysis were performed. The frequencies represent the donor CD8 T cells (Ly5.2+, CD8α+). This is representative of 2 recipients from one donor.
B. The LCMV NP-V207A variant-immune mice are associated with significant loss of heterologous immunity to PV challenge

To test our hypothesis that cross-reactive NP205-specific CD8 T cells mediate heterologous immunity between LCMV and PV, mice immunized with either LCMV WT or LCMV NP-V207A variant were challenged with $2 \times 10^7$ pfu of PV. Experiments also included naïve and PV-immune control groups to compare the viral clearance kinetics between homologous and heterologous challenge. The level of heterologous protective immunity was assessed four days after PV challenge by monitoring the antigen-specific responses and the PV titer in different organs. This early time point highlights one of the important properties of memory T cells, in which they are able to respond to their cognate peptides in a swift manner without further co-stimulation (Veiga-Fernandes et al., 2000).

When we compared the PV titer between the naïve and heterologous challenge control (LCMV WT) four days following a PV challenge, we found a significantly lower PV titer in the heterologous challenge control than the naïve control in both the spleen and the abdominal fat pads (Fig 4.3 & 4.4). For example, the PV titer was about 40 times less in the spleens of the LCMV WT-immune group as compared to the naïve control in this particular experiment.
Similarly, the PV titer was 30 times less in the abdominal fat pads of the LCMV WT-immune group as compared to the naïve control \((p<0.0001)\) (Figure 4.4). Although the level of heterologous immunity in different organs can vary in different experiments, the LCMV WT-immune groups consistently displayed a significant reduction in PV titer as compared to naïve controls (Table 4.3). These data recapitulate our original observation showing heterologous immunity between LCMV and PV (Selin et al., 1998). On average, the LCMV WT-immune animal contains about 3 to 40 fold lower PV titer in either the spleen or the abdominal fat pad compared to the naïve control group four days following PV challenge (Table 4.3).
Figure 4.3  LCMV NP-V207A variant-immune mice are associated with a significant loss of heterologous immunity to PV. Naïve, LCMV WT-immune, and LCMV NP-V207A-Immune mice were challenged i.p. with $2 \times 10^7$ pfu of PV. 4 days after infection, spleens were harvested. The PV titer was titrated using the standard plaque assay protocol. The asterisk indicates a significant difference (P<0.05) between the indicated groups by one way ANOVA analysis. The data shown above represent the average of 5 mice per group ± SD. These are representative of six independent experiments.
Figure 4.4  LCMV NP-V207A variant-immune mice are associated with a significant loss of heterologous immunity to PV. Naïve, LCMV WT-immune, and LCMV NP-V207A-Immune mice were challenged i.p. with 2x10⁷ pfu of PV. 4 days after infection, abdominal fat pads were harvested. The PV titer was titrated using the standard plaque assay protocol. The asterisk indicates a significant difference (P<0.05) between the indicated groups by one way ANOVA analysis. The data shown above represent the average of 5 mice per group ± SD. These are representative of six independent experiments.
On the other hand, we observed a significantly higher level of PV titer in either the spleen or the abdominal fat pad of the LCMV NP-V207A-immune group as compared to the LCMV WT-immune group (Figure 4.3 & 4.4). These data indicate that there is a significant loss of heterologous immunity in the LCMV NP-V207A-immune group following a PV challenge. Specifically, the PV titer in the spleens of the LCMV NP-V207A-immune group is about 13 times higher than that of the LCMV WT-immune group ($p<0.05$) (Figure 4.3). Similarly, the PV titer in the abdominal fat pad of the LCMV NP-V207A-immune group is 20 times higher than that of the LCMV WT-immune group ($p<0.0001$) (Figure 4.4). In addition, the PV titer in the LCMV NP-V207A-immune group often reached the same level as the naïve control group in both spleens and the abdominal fat pads (Table 4.3). These data show that the LCMV NP-V207A-immune groups are associated with significant losses of heterologous immunity upon PV challenge.

In addition, these data also suggest that the cross-reactive NP205-specific CD8 T cells could be responsible for the most of the heterologous immunity between LCMV and PV. Finally, when we compared the kinetics of PV clearance between the homologous challenged group (PV+PV) and the heterologous challenge...
group (LCMV+PV), these data suggest that PV clearance kinetics are significantly delayed in cross-reactive NP205-specific CD8 T cell-mediated heterologous immunity as compared to the homologous challenge group. This might imply that the dominant PV NP38-specific CD8 T cells in the PV immune animals play a more important role in protection (Table 4.3, experiment 5 & 6).
### Experiments

<table>
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<tr>
<th>Experiments</th>
<th>N+PV (A)</th>
<th>PV+PV (B)</th>
<th>LCMV WT+PV (C)</th>
<th>LCMV NP-V207A+PV (D)</th>
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Table 4.3  LCMV NP-V207A variant-immune mice are associated with significant loss of heterologous immunity to PV. Naïve, PV-Immune, LCMV WT-Immune and LCMV NP-V207A-Immune mice were challenged i.p. with $2 \times 10^7$ pfu PV. The indicated organs were harvested 4 days post-infection and titrated by standard plaque assay. The values represent the logarithmic scale ($\log_{10}$) and are written as pfu/ml ± SD. Unless otherwise indicated by the numbers in the parentheses, the data represent the average of 5 mice per group in each independent experiment. The symbols represent statistically significant differences (p<0.05) between indicated groups by one way Anova analysis. N.D=not done.
On average, the frequency of the cross-reactive NP205-specific CD8 T cells in the LCMV WT-immune mice prior to heterologous infection accounted for 0.4% to 1% of the total CD8 responses by intracellular cytokine staining (ICS) (Figure 4.5, n=5). On the other hand, the frequency of the cross-reactive NP205-specific CD8 T cells in the LCMV NP-V207A-immune mice remains undetectable (Figure 4.5). This supports our original hypothesis that the cross-reactive NP205-specific CD8 T cells could be mediating heterologous immunity between LCMV and PV (Table 4.4). In some experiments, a significant difference in the GP33 and NP396-specific CD8 T cell frequencies can be found between the LCMV WT and the LCMV NP-V207A groups. However, these antigen-specific CD8 T cells have not been shown to be cross-reactive with PV (Table 4.4). On the same note, the PV NP38-specific CD8 T cell response remained undetectable at day four in all three groups (naïve, LCMV WT and LCMV NP-V207A) (Table 4.4). Finally, both the protection data and the antigen specific CD8 T cell frequency data support our hypothesis that the cross-reactive NP205-specific CD8 T cells mediate heterologous immunity between LCMV and PV.
Figure 4.5  Frequency of antigen-specific CD8 T cell responses in the LCMV WT and LCMV NP-V207A immune mice before PV challenge. Peripheral blood from either LCMV WT-Immune and LCMV NP-V207A-Immune mice were harvested and stimulated ex vivo with the indicated peptides. The standard ICS and FACs were performed. The values represent the % of IFNγ+, CD8α+ T cells ± SD. This experiment represent 5 mice per group. The asterisk indicates a significant difference (P<0.05) by student’s t test.
<table>
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<td>9.11±1.95</td>
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Table 4.4 Frequency of the antigen-specific CD8 T cells in the LCMV WT-Immune and LCMV NP-V207A-Immune mice following PV challenge. LCMV WT-Immune and LCMV NP-V207A-immune mice were challenged i.p. with 2x10^7 pfu of PV. Splenocytes were harvested 4 days following challenge and stimulated ex vivo with a panel of LCMV-specific CD8 peptides. Standard ICS and FACS analysis were performed. The numbers represent the average frequency of IFNγ+, CD8α+ lymphocytes ± SD (n=5 per group). The symbols (*) indicates a statistically significant difference (P<0.05) between the LCMV WT+PV and LCMV NP-V207A+PV using the CD8 peptide indicated. N.D=not done.
Part II

In part one of this chapter, I showed that LCMV NP-V207A variant-infected mice are associated with a significant loss of heterologous immunity to PV challenge. This suggests that the cross-reactive NP205-specific CD8 T cells are mediators of heterologous immunity between LCMV and PV. However, we wanted to examine this issue more closely. First, it is possible that the LCMV NP-V207A variant virus contains additional mutations other than the mutation in the NP205 epitope as compared to the LCMV WT virus. Second, the original heterologous protection experiments were performed in the LCMV Armstrong background (Selin et al., 1998), whereas the mutant virus was derived from the LCMV clone 13 background. Therefore, we also wanted to make sure that the loss of heterologous immunity observed with the LCMV NP-V207A variant is not a result of the virus strain tested. In order to address these questions, collaboration was initiated with Dr. Juan Carlos de la Torre at the Scripps Research Institute. The in vitro arenavirus rescue system that he developed provides a quick way to manipulate the LCMV genome through reverse genetic
techniques (Sanchez and de la Torre, 2006) (Figure 4.6). Using this system, we asked if the point mutation found within the LCMV NP-V207A could account for the same phenotype in the LCMV Armstrong background. In addition, a proper wild type control in LCMV Armstrong (r/WT) background is also generated in vitro.
1. Pol II expression plasmid for T7RP
2. Expression plasmids for L and NP
3. Expression plasmids containing L & NP trans-activating factors

1. YTVKYPNL (r/WT)
2. YTAKYPNL (r/V207A)
3. YTVKAAPNL (r/Y209A)
4. YTVKYPNAL (r/L212A)
5. YTVKAPNAL (r/Y209A,L212A)

*The rescued viruses are made in the LCMV Armstrong background

Figure 4.6  Schematic diagram depicting the *in vitro* generation of rescued LCMV variants (r/LCMV)
C. A single point mutation in the r/V207A variant results in a greatly diminished cross-reactive NP205-specific CD8 T cell response in B6 mice

Following the successful in vitro generation and sequence verification of the rescue variant that contains the original (V-A) mutation (r/V207A variant virus), the r/V207A variant virus was tested for in vivo response in B6 mice. I found that infection of B6 mice with the r/V207A variant virus resulted in greatly diminished cross-reactive NP205-specific CD8 T cell responses (LCMV NP205, PV NP205, and LCMV NP205 V-A) as compared to the r/WT control (p=0.0002) (Figure 4.7 & 4.8). The LCMV NP205-specific CD8 T cell response induced by r/WT virus accounted for 1.7% of the CD8 T cell response as detected by IFNγ ICS at day eight post-infection (Figure 4.7). On the other hand, the LCMV NP205-specific CD8 T cell response induced by the r/V207A variant accounted for only 0.4% of the CD8 T cell response by IFNγ ICS (Figure 4.7). The responses from two of the major LCMV CD8 epitopes (GP33 & NP396) were similar between r/WT and r/V207A-immunized group (Figure 4.7 & 4.8).
Fig 4.7  Single point mutation in the r/V207A variant results in a greatly-diminished cross-reactive NP205-specific CD8 T cell response in B6 mice. B6 mice were infected i.p. with $5 \times 10^4$ pfu of either (A) r/WT or (B) r/V207A variant virus. 8 days later, splenocytes from each group were harvested and stimulated \textit{ex vivo} with a panel of LCMV-specific CD8 peptides. Standard ICS and FACs analysis were performed. The numbers indicates the frequency of IFN\gamma+, CD8α+ lymphocytes. These are representative data of 3 mice per group.
Figure 4.8 Single point mutation in the r/V207A variant results in greatly diminished cross-reactive NP205-specific CD8 T cell response in B6 mice. B6 mice were infected i.p. with 5x10^4 pfu of either (A) r/WT or (B) r/V207A variant virus. 18 days later, peripheral blood from each group were harvested and stimulated *ex vivo* with a panel of LCMV-specific CD8 peptides. Standard ICS and FACs analysis were performed. The numbers indicate the frequency of IFNγ+, CD8α+ lymphocytes. These are representative data of 3 mice per group.
Figure 4.9  Minimal cross-reactive NP205 expansion in the r/V207A-immune mice upon PV challenge.  (A) r/WT-Immune and (B) r/V207A-immune mice were challenged i.p. with 2x10^7 pfu of PV.  Spleens were harvested 8 days post-infection and stimulated ex vivo with indicated peptides.  Standard ICS and FACs were performed.  The numbers represent the frequency of IFNγ+, CD8α+ T cells.  These are representative data of 2 r/WT-immune and 3 r/V207A-immune mice.
Furthermore, PV infection of the r/V207A-immune mice did not result in any sizeable expansion of the cross-reactive NP205-specific CD8 T cell responses (Figure 4.9). This small antigen-specific CD8 T cell response to its natural LCMV NP205 (V-A) ligand in the r/V207A variant infected mice could either be due to a loss in immunogenicity or an altered response that was not picked up by the IFNγ ICS assay. To address this concern, MHC-Ig dimer studies using either LCMV NP205 or LCMV NP205 (V-A) peptides were done to correlate with the ICS data. In the mice infected with the r/WT virus, similar frequencies of antigen-specific CD8 T cells were detected using either LCMV NP205 MHC-Ig dimer or LCMV NP205 (V-A) MHC-Ig dimer at the peak of the LCMV-specific response (2.0±0.1 vs. 1.8±0.25 respectively) (Figure 4.10 & 4.11).
Figure 4.10  Small percentage of LCMV NP205-212 WT MHC-Ig dimer reactivity in r/V207A-infected mice.  Day 8 splenocytes from either (A) r/WT or (B) r/V207A-infected mice were used.  Different amounts of LCMV NP205-212 WT MHC-Ig Dimer were used to test for the reactivity.  A modified surface staining protocol was described in material and methods.  The numbers represent the percentage of MHC-Ig dimer positive, CD8α+ T cells.
Figure 4.11 Small percentage of LCMV NP205-212 (V-A) MHC-Ig dimer reactivity in r/V207A-infected mice. Day 8 splenocytes from either (A) r/WT or (B) r/V207A-infected mice were used. Different amounts of LCMV NP205-212 (V-A) MHC-Ig Dimer were used to test for the reactivity. A modified surface staining protocol was described in material and methods. The numbers represent the percentage of MHC-Ig Dimer positive, CD8α+ T cells.
This is reminiscent of the peptide titration experiment comparing the efficiency between the LCMV NP205 and the LCMV NP205 (V-A) peptides in chapter one (Figure 3.6), where we initially observed a similar level of IFN-γ response using either peptides at an optimal (saturating) concentration range (5μM). On the other hand, both types of MHC-Ig dimers were able to detect only a very small frequency of the antigen specific CD8 T cells in mice infected with the r/V207A variant virus (0.2±0 vs. 0.2±0 respectively) (Figure 4.10 & 4.11). Therefore, a significantly diminished cross-reactive NP205-specific CD8 T cell response was not the result of a previously unidentifiable immune response, but a direct consequence of the loss in immunogenicity.

D. The r/WT and r/V207A variant viruses display similar activation and replication kinetics in B6 mice

To find out whether the r/WT and r/V207A viruses behave phenotypically in a similar fashion, time course experiments were conducted. No significant difference in virus titer could be detected between the r/WT and r/V207A infected groups three days post-infection (Figure 4.13). In addition, both groups cleared the virus by day eight post-infection, and no signs of viral persistence could be
found in the spleens and abdominal fat pads by plaque assay (Figure 4.13).

Again, the magnitude of the antigen-specific CD8 T cell response from both
groups of mice peaked at around day eight post-infection and decreased
substantially in the memory phase (Figure 4.12). Therefore, the point mutation in
the r/V207A virus does not seem to affect the general replication characteristics
of the LCMV Armstrong strain virus.
Figure 4.12  Similar T cell activation kinetics between the r/WT and r/V207A variants. B6 mice were inoculated i.p. with 5x10^4 pfu of either (A) r/WT or (B) r/V207A variant. Spleens were harvested at the indicated days post-infection and stimulated ex vivo with indicated LCMV-specific CD8 peptides. Standard ICS and FACs were performed. Each column represents the average frequency of LCMV-specific CD8 T cell response ± SD. The asterisk indicates a statistically significant difference (P<0.05) in the frequencies between the r/WT and r/V207A-infected mice. These are representative data of 3 mice per group.
Figure 4.13  Similar replication kinetics between the r/WT and r/V207A variants. B6 mice were immunized i.p. with $5 \times 10^4$ pfu of r/WT (dark grey bar) or r/V207A variant (grey bar). (A) Spleens and (B) fat pad were harvested at the indicated time points. A standard plaque assay was performed to titrate the virus. The data are presented as in $\log_{10}(\text{pfu/ml}) \pm \text{SD}$. The arrow indicates the cut-off point for the plaque assay. Theses are representative data of 3 mice per group.
E. Limited evidence on the development of additional cross-reactive CD8 responses in r/V207A-immune mice challenged with PV

Since the new system was generated in the LCMV Armstrong background, I wanted to know if there were any additional CD8 cross-reactive responses present in the r/V207A-Immune mice challenged with PV. When I compared the day eight antigen-specific CD8 responses from the acutely challenged groups (either r/WT or r/V207A) and the heterologous challenge groups (r/WT+PV and r/V207A+PV), there was very little indication of additional cross-reactive CD8 epitopes. As shown in figure 4.14 and 4.15, the peptide screening experiment using the majority of the known LCMV-specific CD8 epitopes plus the newly identified CD8 epitopes from the LCMV L protein suggest that the cross-reactive NP205-specific CD8 T cell response probably accounts for the majority of the cross-reactive response between LCMV and PV.
Figure 4.14 Limited evidence for the development of additional cross-reactive CD8 responses in r/V207A-immune mice challenged with PV (Part I). Naïve B6 mice were immunized i.p. with $2 \times 10^5$ pfu of either r/WT (black bar) or r/V207A (white bar). r/WT and r/V207A-immune mice were challenged i.p. with $2 \times 10^7$ pfu of PV (light grey and grey bars, respectively). 8 days later, spleens were harvested from each group and stimulated ex vivo with a panel of indicated LCMV CD8 peptides. Standard ICS and FACs were performed. The data represent the average frequency of IFNγ+, CD8α+ T cells. These are data of 2 mice per group.
Figure 4.15  Limited evidence for the development of additional cross-reactive CD8 responses in LCMV NP-V207A-immune mice challenged with PV (Part II).  Naïve B6 mice were immunized i.p. with 2x10^5 pfu of either r/WT (black bar) or r/V207A (white bar).
r/WT and r/V207A-immune mice were challenged i.p. with 2x10^7 pfu of PV (light grey and grey bars, respectively).  8 days later, spleens were harvested from each group and stimulated ex vivo with a panel of indicated LCMV CD8 peptides.  Standard ICS and FACs were performed.  The data represent the average frequency of IFNγ+, CD8α+ T cells.  These are data of 2 mice per group.
F. A significant loss of heterologous immunity in the r/V207A variant-immune mice upon PV challenge

The rescue LCMV Armstrong variant was generated to provide further evidence that a greatly diminished cross-reactive NP205-specific CD8 T cell response results in a significant loss of heterologous immunity to PV challenge in the LCMV NP-V207A-immune mice. I anticipated that a significant loss of heterologous immunity to PV would be observed in the r/V207A-immune mice challenged with PV. If this hypothesis is true, these data would indicate that the cross-reactive NP205-specific CD8 T cells are mediators of heterologous immunity between LCMV and PV. Therefore, I set up an identical heterologous challenge experiment using this rescued LCMV system. PV challenge of the r/V207A-immune mice resulted in significant loss of heterologous immunity in both spleens and fat pads (Figure 4.16). No statistically significant difference in PV titer was observed in the spleens and fat pads of the naïve control and the r/V207A-immune group (Figure 4.16). On the other hand, PV challenge of the r/WT immune mice resulted in approximately a 6.3 and 3.2 fold reduction in PV virus titer in the spleens and fat pads, respectively (Figure 4.16). Thus, these
data support the conclusion that cross-reactive NP205-specific CD8 T cell populations mediate a majority of the heterologous immunity between LCMV and PV.
Figure 4.16  Significant loss of heterologous immunity in the r/V207A-Immune mice upon PV challenge. Naïve, r/WT-Immune, and r/V207A-immune mice were challenged i.p. with $2 \times 10^7$ pfu of PV. 4 days later, spleens and fat pads from each group were harvested and titrated by standard plaque assay. The numbers are expressed in $\log_{10}$ (pfu/ml) ± SD. The asterisk represents a statistically significant difference ($p<0.05$) between the indicated groups by one way Anova analysis. These are data of 5 mice per group.
Chapter Summary

One of the important questions regarding T cell cross-reactivity and heterologous protective immunity is whether these memory T cell populations are directly involved in heterologous immunity. Here, I used the LCMV NP-V207A CTL escape variant that exhibited a greatly diminished cross-reactive NP205-specific CD8 T cell response to address this particular question. I found LCMV NP-V207A-immune mice are associated with a significant loss of heterologous immunity to PV (Figures 4.3 & 4.4). In addition, the overall magnitude (naïve vs. LCMV NP-V207A) of the loss of heterologous immunity in the LCMV NP-V207A-immune mice also suggested that the cross-reactive NP205-specific CD8 T cells account for the majority of the heterologous immunity. This observation is in agreement with our previous adoptive transfer experiments showing that there were limited indications of additional cross-reactive epitopes present even in the absence of greatly diminished cross-reactive NP205-specific CD8 T cell responses (Table 4.1). The fact that there is an extensive proliferation (CFSE low) of adoptively transferred splenocyte populations from LCMV NP-V207A
donors following PV challenge suggestes that there is a possibility of additional cross-reactive CD8 epitopes (Fig 4.1). However, one could argue that there are some technical issues involving the readout. First of all, a small population of cross-reactive T cells that undergoes few rounds of division could appear to be the major CFSE low population due to the small number of donor splenocytes transferred. Therefore, CFSE readout might lead to an over-estimation of the magnitude of the overall proliferative response. Second of all, the use of CD8a and Ly5.2 congenic marker for tracking of the donor population represents an over-estimation of the antigen-specific T cells since CD45.2 (Ly5.2) markers are expressed on virtually all leukocytes. For example, CD45 can be found on the surface of hematopoietic stems cells, NK cells, B cells and T cells (Cook et al., 1987; Ledbetter et al., 1985). In addition, CD8a can be found on the surface of dendritic cells and T cells (Tailor et al., 2006). Therefore, our data suggest that the cross-reactive NP205-specific CD8 T cells are responsible for the majority of heterologous immunity between LCMV and PV.

Although we feel confident that a greatly diminished cross-reactive NP205-specific CD8 T cell response with the LCMV NP-V207A variant is the
direct consequence of the point mutation within the LCMV NP205 epitope, it was difficult to prove that additional mutations didn’t exist in the LCMV NP-V207A variant. In addition, we also wanted to find out if the loss of heterologous immunity in the LCMV NP-V207A-immune mice could also be seen in a different LCMV background. Therefore, collaboration was initiated with Dr. Juan Carlos de la Torre at Scripps Research Institute, who specializes in rescuing LCMV entirely from plasmids in vitro using reverse genetics. The r/V207A variant virus that contains the original mutation exhibited the phenotype that we anticipated, as infection of B6 mice also results in a greatly diminished cross-reactive NP205-specific CD8 T cell response (Figures 4.7 & 4.8). In addition, the MHC-Ig dimer studies also support our overall conclusion that the mutated LCMV NP205 (V-A) is significantly less immunogenic (Figures 4.10 & 4.11). Finally, the heterologous challenge experiment using the rescued virus system displayed essentially the same result as the LCMV clone 13 system (Figure 4.16). Therefore, the loss of heterologous immunity to PV in the r/V207A-immune mice indicates that the cross-reactive NP205-specific CD8 T cells account for the majority of heterologous immunity between LCMV and PV. In addition, bystander T cell
activation does not seem to play a role in the heterologous immunity between LCMV and PV.
Chapter V  
Contribution of the Cross-Reactive NP205-Specific CD8 T Cells To Immune Pathogenesis Following Sequential Heterologous Infections

Our data from chapter four showed that the cross-reactive NP205-specific CD8 T cells could mediate the majority of heterologous immunity between LCMV and PV. In addition, I also found that PV clearance kinetics mediated by these cross-reactive NP205-specific CD8 T cells is significantly delayed in comparison to a homologous challenge (PV+PV) (Figure 5.1). These data suggest that the cross-reactive NP205-specific CD8 T cells might not be the default antigen-specific CD8 T cell populations for clearance of PV. Heterologous immunity is often associated with a partial reduction in viral replication and alteration in immune pathology. In both the systemic and respiratory models that involve LCMV and VV, cross-reactive memory CD8 T cells have been implicated in altered immune pathology at the site of infection (Chen et al., 2001a; Selin et al., 1998). Furthermore, IAV-immune animals challenged with either LCMV or murine cytomegalovirus virus (MCMV) had elevated viral titer and more severe clinical pathology (Chen et al., 2003). Similarly, cross-reactive T cells have been implicated in some of the clinical manifestations of infection in humans. For
example, it has been proposed that part of the clinical manifestations of dengue hemorrhagic fever or dengue shock syndrome might be due to memory cross-reactive CD8 T cells from one serotype that are able to recognize a slight variant form of the epitope (Imrie et al., 2007). In addition, T cell cross-reactivity between IAV and HCV has been hypothesized to be a mediator of severe liver pathology in some individuals acutely infected with HCV (Urbani et al., 2005). Finally, our group has also identified CD8 cross-reactive epitopes between IAV and EBV, and this T cell cross-reactivity has been suggested to contribute to lympho-proliferative disease following EBV infection (Clute et al., 2005).

Our data showed that in mice that are sequentially immunized with PV and then LCMV (double immune), they developed a high incidence and severity of AFN following a final PV challenge. In addition, in some cases a loss of protective immunity in these double immune mice was observed upon PV re-challenge. Since the T cell memory compartment in these double immune mice are dominated by cross-reactive NP205-specific CD8 T cells, they could constitute sub-optimal protection. Therefore, we hypothesized that a significant number of the sub-optimal cross-reactive NP205-specific CD8 T cells from the
double immune mice (PV+LCMV WT) could contribute to immune-pathology upon PV challenge.
Figure 5.1 Cross-reactive NP205-specific CD8 T cell-mediated heterologous immunity displays significantly delayed PV clearance kinetics compared to homologous challenge. Naïve, PV-immune, LCMV WT-Immune, and LCMV NP-V207A-Immune animals were challenged i.p. with $2 \times 10^7$ pfu of PV. (A) spleens and (B) fat pad from each group were harvested 4 days later and titrated by standard plaque assay. The values are expressed as $\log_{10} (\text{pfu/ml}) \pm \text{SD}$. The asterisk represents a statistically significant difference ($p<0.05$) between the indicated groups by one way Anova analysis. These data are from 5 mice per group.
The question of whether the subdominant NP205-specific CD8 T cell response can mediate protection as efficiently as other dominant epitopes was addressed by using both the minigene expression and the recombinant vaccinia systems (van der Most et al., 1998). Their results suggested that immunization with the subdominant LCMV NP205 epitope using either a peptide-conjugate or a recombinant vaccinia virus system can confer protection to a high dose LCMV clone 13 challenge, but the viral clearance rate is much slower than immunization with dominant LCMV epitopes (van der Most et al., 1998). Therefore, this suggests that even in a homologous challenge (LCMV + Clone 13), LCMV NP205-mediated viral clearance might be sub-optimal. However, this might not necessarily suggest that the NP205-specific CD8 T cells are inherently less efficient at killing a target cell, as low expression of its cognate peptide on the target cell could reduce their ability to kill it.

If our hypothesis is correct, a PV challenge of the (PV+LCMV-V207A) double immune mice, which contain a significantly smaller number of cross-reactive NP205-specific CD8 T cells, should have a significantly lower incidence and severity of AFN. This heterologous challenge system using LCMV and PV is
well suited for studying CTL-mediated immune pathology for the following reasons. First of all, most of the tissue pathologies in arenavirus infections are T cell-mediated. Second, a well-defined cross-reactive CD8 epitope and an LCMV-V207A variant that mimics the knockout serve as important tools in this system. Finally, no neutralizing antibodies have been found in PV-infected animals (Chanas et al., 1980; Trapido and Sanmartín, 1971). Therefore, neutralizing antibodies will not play a role in protection in this system.

A. Domination of the cross-reactive NP205-specific CD8 T cells in the (PV+LCMV WT) double immune mice

First we wanted to monitor the frequency of antigen specific CD8 T cells in the blood of the (PV+LCMV WT) and (PV+LCMV V207A) double immune mice before a final PV challenge. The two antigen-specific CD8 T cell responses that I was interested in were the cross-reactive NP205-specific CD8 T cell response and the immunodominant PV NP38-specific CD8 T cell response. The PV NP38-specific CD8 T cell response represents the dominant CD8 T cell response in B6 mice infected with PV. However, following a heterologous challenge with LCMV, a process known as memory cell attrition takes place via type I interferon
induction by the viral infection (Bahl et al., 2006; Selin et al., 1999). Thus, many of the non cross-reactive memory lymphocytes undergo attrition while the cross-reactive memory lymphocytes that survive attrition actively proliferate. This results in a highly disproportionate ratio between the cross-reactive NP205-specific CD8 T cells and PV NP38-specific CD8 T cells in the (PV+LCMV WT) double immune mice. As expected, the cross-reactive NP205-specific CD8 T cell response was significantly higher in the (PV+LCMV WT) double immune mice as compared to the (PV+LCMV-V207A) double immune mice ($p<0.0001$) (Figure 5.2).
Figure 5.2  Domination of the cross-reactive NP205-specific CD8 T cells in the (PV+LCMV WT) double immune mice. B6 mice were first immunized i.p. with 2x10^7 pfu of PV. Six weeks later, the PV-Immune mice were subsequently immunized i.p. with 1x10^6 pfu of either LCMV WT (black bar) or LCMV NP-V207A variant (white bar) and rested for another 6 weeks. Before the final challenge with PV, peripheral blood from these two groups were collected and stimulated ex vivo with a panel of indicated CD8 peptides. Standard ICS and FACs were performed. The data represent the average frequency of the IFNγ positive, CD8α+ T cells ± SD. The asterisk represents a statistically significant difference (p<0.05) between the indicated groups by student's t test. These are data from 5 mice per group and representative of 4 independent experiments.
The average frequency of the cross-reactive NP205-specific CD8 T cell responses in the (PV+LCMV WT) double immune mice by ICS was approximately 9.5%±6.0 (n=18) (Figure 5.2). This variation in cross-reactive NP205-specific CD8 T cell response reflects the private specificity of each individual mouse (Cornberg et al., 2006). Typically, PV NP205-specific CD8 T cells display a co-dominant Vβ usage of Vβ 5.1/5.2 and Vβ 16 in PV-immune mice. However, different variations of Vβ usage of the cross-reactive NP205-specific CD8 T cells can be found in each individual PV-immune mouse following a heterologous challenge with LCMV. Adoptive transfer experiments showed that recipients who received the same LCMV-specific memory donor splenocytes developed similar magnitude of cross-reactive NP205-specific CD8 T cell response following PV challenge, demonstrating that the private TCR repertoire can influence T cell clonal dominance and heterologous immunity. On the other hand, the frequency of the cross-reactive NP205-specific CD8 T cells in the (PV+LCMV-V207A) double immune mice was about 1.1%±0.8 (n=20) (Figure 5.2). We did not detect a significant difference in the frequency of the PV NP38-specific CD8 T cell response in both groups of mice in the majority of the
experiments (Table 5.1). Therefore, this non cross-reactive memory population in both groups of mice underwent attrition following heterologous immunization (Table 5.1). In addition, the frequencies of other dominant LCMV CD8 epitopes were very similar in both groups of mice (Table 5.1).
Table 5.1  Summary of antigen-specific CD8 T cell frequency (% of CD8+ T cells) in the (PV+LCMV WT) double-immune mice before final PV challenge. The values represent the frequency of IFNγ+, CD8α+ T cells ± SD. The astrisk (*) represents a statistically significant difference (p<0.05) between the two groups by student’s t test. Unless otherwise indicated by the number in the parentheses, each group contains 5 mice.

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B. The (PV+WT) double immune mice have a significantly higher occurrence of AFN as compared to the (PV+LCMV-V207A) double immune mice following PV challenge

When I compared the incidence of AFN between (PV+LCMV WT) and (PV+LCMV-V207A) double immune mice following a PV challenge, I found a significantly higher frequency of AFN in the (PV+LCMV WT) double immune mice ($p=0.0056$, Mann-Whitney U test) (Figure 5.3). The majority (74%) of the (PV+LCMV WT) double immune (n=23) mice displayed various clinical grades of AFN as compared to 38% in the (PV+LCMV-V207A) double immune mice (n=25) (Figure 5.3). In addition, the overall severity of AFN appeared to be higher in the (PV+LCMV WT) double immune mice challenged with PV (Figure 5.3).
AFN Scale

Figure 5.3 (PV+LCMV WT) double immune mice exhibit a significantly higher AFN occurrence compared to the (PV+LCMV NP-V207A) double immune mice after PV challenge. The (PV+LCMV WT) and (PV+LCMV NP-V207A) double immune mice were challenged i.p. with $2 \times 10^7$ pfu of PV. 4 days post-infection, the mice were sacrificed and the severity of AFN in the visceral fat pads was accessed according to the previously published scale (refer to material and method section). This is a compilation of 4 independent experiments. The $p$ value obtained was a comparison of the AFN scale between the two groups using the Mann Whitney U test.
Although the PV titer in the naïve group usually reached the level of $10^3$ or $10^4$ pfu/ml in both the spleens and the abdominal fat pads (Table 4.3), no AFN was detected four days post-infection (n=20). This illustrates that in a viral system where the virus itself is non-cytolytic, the immune system of the host plays an important role in pathology (Oldstone, 1970, p21619). It was interesting to note that no PV could be detected in either the spleens or the abdominal fat pads of the (PV+LCMV WT) and (PV+LCMV-V207A) double immune mice in all the experiments four days following the PV challenge (n=23 and 24 respectively). It is possible that the presence of both cross-reactive NP205 and non-cross-reactive PV NP38-specific CD8 T cells could change the PV clearance kinetics in both groups of double immune animals. Therefore, an earlier time point harvest might be required to detect PV titer in these two groups of animals. Overall, these data re-affirm our initial observation that there is a high incidence of AFN in the (PV+LCMV WT) double immune mice following a PV challenge. Most importantly, a significant difference in the frequency of AFN between (PV+LCMV WT) and (PV+LCMV-V207A) double immune mice suggests that these cross-reactive NP205-specific CD8 T cells are important contributors to immune
pathology upon PV challenge (Figure 5.3). Since a small cross-reactive NP205-specific CD8 T cell response could still be detected in the (PV+LCMV NP-V207A) double immune mice (Table 5.1), it could still be argued that an “altered” variant cross-reactive NP205-specific CD8 T cell response plays an un-anticipated role in mediating immune pathology. Therefore, generation of \textit{in vitro} rescued NP205 epitope variants that contain alanine mutation(s) at the anchoring residue positions is underway to address this question. We anticipate that alanine substitution at the \textit{K}^b-restricted anchoring residues of the LCMV NP205 epitope could result in loss of MHC binding. \textit{In vitro} MHC stabilization experiments using different variations of LCMV NP205 peptides will be described in section F of this chapter. Finally, generation of a rescued LCMV variant that results in a “complete NP205 knockout” phenotype would help to clarify the role of the cross-reactive NP205-specific CD8 T cells in immune pathogenesis.
C. Domination of the cross-reactive NP205-specific CD8 T cells in the pathological tissues

Except for the dramatic difference in the frequency of the cross-reactive NP205-specific CD8 T cell response, the overall antigen-specific CD8 T cell responses in either (PV+WT) and (PV+LCMV NP-V207A) double immune mice following PV challenge are very comparable (Table 5.2). This suggests that the cross-reactive NP205-specific CD8 T cell populations still dominate the response in the (PV+LCMV WT) double immune mice following PV challenge.

In order to establish more direct evidence that the cross-reactive NP205-specific CD8 T cells are involved in AFN, I wanted to find out if the cross-reactive NP205-specific CD8 T cells are present in large percentages in the abdominal fat pad of the (PV+LCMV WT) double immune mice after PV challenge. I compared the frequency of antigen-specific CD8 T cells at the pathological site between (PV+LCMV WT) and (PV+LCMV-V207A) double immune mice following a PV challenge. As shown in figure 5.4, the cross-reactive NP205-specific CD8 T cell response was significantly higher in the (PV+WT) than the (PV+LCMV-V207A) double immune mice in the abdominal fat pad at day four following a PV challenge (22.2%±7.6 vs. 2.6%±1.4, respectively, p=0.0018, n=4). In contrast,
the frequency of the PV NP38-specific CD8 T cells does not seem to vary too greatly in the abdominal fat pads of the (PV+LCMV WT) and the (PV+LCMV-V207A) double immune mice after a PV challenge (11.2%±4.4 vs. 16.4%±4.3, respectively, $p=0.15$, $n=4$). It is interesting to note that a small percentage of CD8α+, CD44+ lymphocytes displayed a highly activated phenotype in the abdominal fat pads, as shown by a spontaneous release of IFNγ without any peptide stimulation (no stimulation in Figure 5.4). Finally, the fact that only a very small frequency of antigen-specific CD8 T cells (LCMV NP205=4.1%±1.5 and PV NP38=3.4%±0.7, $n=5$) could be detected in the abdominal fat pads of the naïve control group four days after a PV challenge indicates the strong involvement of re-activating memory T cells in this pathology.
<table>
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<tr>
<th>Experiment</th>
<th>LCMV GP33-41</th>
<th>LCMV NP396-404</th>
<th>LCMV NP205-212</th>
<th>PV NP205-212</th>
<th>PV NP38-45</th>
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<tr>
<td>PV+LCMV WT</td>
<td>3.02±0.88</td>
<td>3.10±0.92</td>
<td>5.93±3.14 (*)</td>
<td>5.92±3.26 (*)</td>
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<td>PV+LCMV NP-V207A</td>
<td>5.20±3.86</td>
<td>2.50±1.71</td>
<td>1.03±0.53</td>
<td>1.05±0.52</td>
<td>3.77±1.33</td>
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<tr>
<td>PV+LCMV WT</td>
<td>4.20±0.79</td>
<td>2.93±0.73</td>
<td>3.97±1.95 (*)</td>
<td>4.11±1.98 (*)</td>
<td>2.52±0.52</td>
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<tr>
<td>PV+LCMV NP-V207A</td>
<td>4.15±1.15</td>
<td>3.51±1.01</td>
<td>0.89±0.19</td>
<td>0.95±0.30</td>
<td>2.91±1.18</td>
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<tr>
<td>PV+LCMV WT</td>
<td>7.01±3.09</td>
<td>5.51±0.83 (*)</td>
<td>4.55±2.15 (*)</td>
<td>4.57±2.16 (*)</td>
<td>4.93±1.14</td>
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<td>PV+LCMV NP-V207A</td>
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<td>0.87±0.36</td>
<td>0.81±0.36</td>
<td>4.67±0.98</td>
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<td>PV+LCMV WT (3)</td>
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<td>1.86±0.40</td>
<td>2.98±2.09</td>
<td>3.04±2.06</td>
<td>1.83±0.20</td>
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<td>1.03±0.41</td>
<td>3.83±3.19</td>
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<tr>
<td>PV+LCMV WT (4)</td>
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<td>3.47±1.36</td>
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<td>4.04±1.18 (*)</td>
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<tr>
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<td>0.38±0.14</td>
<td>0.34±0.16</td>
<td>2.22±1.12</td>
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Table 5.2  Summary of antigen-specific CD8 T cell frequency (% of CD8+ T cells) in the (PV+LCMV WT) double-immune mice following PV challenge. The values represent the frequency of IFNγ+, CD8α+ T cells ± SD. The astrisk (*) represents a statistically significant difference (p<0.05) between the two groups by student's t test. Unless otherwise indicated by the number in the parentheses, each group contains 5 mice.
Figure 5.4  Domination of the cross-reactive NP205-specific CD8 T cell response in the visceral fat pad of the (PV+LCMV WT) double Immune mice following PV challenge. The abdominal fat pads from either (A) {PV+LCMV WT}+PV or (B) {PV+LCMV NP-V207A}+PV mice were harvested and digested with a cocktail of enzymes. The lymphocytes from the digested tissues were isolated and stimulated ex vivo with the peptides indicated. Standard ICS and FACs analysis were performed. The numbers represent the frequency of IFNγ+, CD8α+ T cells. These are representative data of 4 mice per group.
D. Contribution of the cross-reactive NP205-specific CD8 T cells to immune pathogenesis

In comparison to the (PV+LCMV NP-V207A) double immune animals, the significantly higher occurrence of AFN and the dominating proportions of the cross-reactive NP205 CD8 T cell response in the (PV+LCMV WT) double immune mice following PV challenge supported our hypothesis that these cross-reactive NP205-specific CD8 T cells could contribute to immune pathology. To find further supporting evidence, a correlation study was conducted to look at the relationships between the magnitude of the cross-reactive NP205-specific CD8 T cell response and the severity of the immune pathology in the (PV+LCMV WT) double immune mice. Based on our current hypothesis, we expected a positive correlation between the magnitude of the cross-reactive NP205-specific CD8 T cell response and severity of the AFN in the (PV+LCMV WT) double immune mice.

When I examined each individual mouse in the (PV+LCMV WT) double immune group and looked at the linear relationship between the frequency of the cross-reactive NP205-specific CD8 T cells before the final PV re-challenge and
the severity of the AFN four days after the PV re-challenge, I found a weak positive correlation. However, the correlation was not statistically significant (Figure 5.5). The reasons could be the following. First of all, a natural variation in the magnitude of the cross-reactive NP205-specific CD8 T cell response known as private specificity in each individual mouse could dramatically impact the association. Second of all, the development of pathology could be a multi-cellular and multi-factorial process. Therefore, one might need to take into account the possibility that there are other cellular factors involved. Finally, it could be a combination of both factors mentioned above.
Figure 5.5 The magnitude of cross-reactive NP205-specific CD8 T cell response in the (PV + LCMV WT) double immune mice alone does not correlate with the severity of AFN. The antigen-specific CD8 T cell frequency from each individual (PV+LCMV WT) double immune mouse before the PV challenge was plotted against the severity of the AFN after PV challenge. The \( r^2 \) (goodness of fit) and \( p \) value were obtained using Pearson’s correlation test.

\[ r^2 = 0.024 \]
\[ p = 0.54 \]
The PV NP38-specific CD8 T cell response represents a dominant CD8 response following a PV infection, whereas the PV NP205 is subdominant. Following a homologous challenge (PV+PV), the frequency of the memory T cell populations are boosted accordingly and the hierarchy is maintained. However, following a heterologous challenge (PV+LCMV WT), the cross-reactive NP205-specific CD8 T cell population preferentially expands and dominates the response while the non cross-reactive PV NP38-specific CD8 T cells undergo attrition (Selin et al., 1999). Therefore, it is possible that a dramatic switch in the hierarchy (frequency) and interplay between these two CD8 T cell populations could ultimately decide the pathological outcome of the (PV+LCMV WT) double immune mice.

Therefore, I wanted to find out if there was any correlation between the PV NP38-specific CD8 T cells and the severity of the AFN in the same group of (PV+LCMV WT) double immune mice. Interestingly, there was a significant negative correlation ($p=0.02$, $n=18$) when I compared the frequency of the PV
NP38-specific CD8 T cell response to the severity of the AFN in the (PV+LCMV WT) double immune mice (Figure 5.6). In addition, if I took the ratio between the cross-reactive NP205-specific CD8 T cells and the PV NP38-specific CD8 T cells from each individual (PV+LCMV WT) double immune mouse and correlated them with the severity of AFN, a highly significant positive linear correlation could then be seen (Figure 5.7, p=0.004). This strongly suggests that PV NP38-specific CD8 T cell response could play a more protective role than the cross-reactive NP205-specific CD8 T cell response.
Figure 5.6 The magnitude of the PV NP38-specific CD8 T cell response in the (PV +LCMV WT) double immune mice inversely correlates with the severity of AFN. The PV NP38-specific CD8 T cell frequency from each individual (PV+LCMV WT) double immune mouse before the PV challenge was plotted against the severity of the AFN after PV challenge. The asterisk indicates a statistically significant correlation using Pearson’s correlation test ($p<0.05$).
Figure 5.7  The ratio of cross-reactive LCMV NP205/PV NP38 T cell frequencies in the (PV+LCMV WT) double immune mice positively correlates with the severity of AFN. The ratio between two antigen-specific CD8 T cell populations from each individual (PV+LCMV WT) double immune mouse before the PV challenge was plotted against the severity of the AFN after PV challenge. The asterisk indicates a statistically significant correlation using Pearson’s correlation test ($p<0.05$).
As a negative control, I used one of the non cross-reactive LCMV CD8 epitopes that would not play a role in response to PV. No significant correlation was found when I compared the frequency of the LCMV GP33-specific CD8 response in the (PV+LCMV WT) double immune mice to the severity of the AFN (Figure 5.8). In addition, I did not find any significant linear correlation between the GP33/NP38 ratio and the level of AFN (Figure 5.8). This suggests that the non cross-reactive GP33-specific CD8 T cells do not play a role in immune pathogenesis in the (PV+WT) double immune mice following a PV challenge.
Figure 5.8  (A) the frequency of the non-cross-reactive LCMV GP33-specific CD8 response, and (B) the ratio of non cross-reactive LCMV GP33 to PV NP38 in the (PV+LCMV WT) double immune mice do not correlate with the severity of AFN. The antigen-specific CD8 T cell frequencies from each individual (PV+LCMV WT) double immune mouse before the PV challenge were plotted against the severity of the AFN after PV challenge. The $r^2$ (best fit) and the $p$ values were obtained by Pearson’s correlation test.
Therefore, the induction of pathology might depend on either a direct or indirect interplay between these two CD8 T cell populations. Finally, the correlation data support our hypothesis that the cross-reactive NP205-specific CD8 T cells are important contributors to immune pathology in this model.

E. Similar level of functional avidity between the cross-reactive NP205 and PV NP38-specific CD8 T cells in the (PV+LCMV WT) double immune mice

The correlation data suggest that the cross-reactive NP205-specific CD8 T cells might interfere with PV NP38-specific CD8 T cells. For example, I showed that there was a significant delay in PV clearance comparing to homologous challenge (Fig 5.1). This might reflect an intrinsic property of the cross-reactive NP205-specific CD8 T cells or a different level of epitope expression on the APC. Therefore, I asked if there were any fundamental differences between these two CD8 T cell populations. Functional avidity studies employing different concentrations of peptide for stimulation of effector cells are often used to estimate the affinity of the TCR:pMHC interaction. High avidity antigen-specific T cell clones are usually regarded as the elite controllers of viral infections (Neveu et al., 2008). On the other hand, antigen-specific T cells that have a low affinity
TCR are implicated in many autoimmunity models and are associated with immune pathology (Harkiolaki et al., 2009; Zehn and Bevan, 2006). Hence, I was interested in finding out if there were functional avidity differences between the cross-reactive NP205-specific CD8 T cells and the PV NP38-specific CD8 T cells in the (PV+WT) double immune mice. Therefore, I isolated splenocytes from the (PV+WT) double immune mice and stimulated them separately with gradients of the LCMV NP205, PV NP205, and PV NP38 peptides. The difference in functional avidity was accessed by comparing the peptide concentration required to elicit 50% of the maximum IFNγ response in each of the antigen-specific CD8 T cells. I found that the functional avidities of the cross-reactive NP205-specific CD8 T cells and the PV NP38-specific CD8 T cells are quite comparable in 3 out of 4 (PV+WT) double immune mice (Figure 5.9).
Figure 5.9 Similar level of functional avidity between the cross-reactive NP205 and PV NP38-specific CD8 T cells in the (PV+LCMV WT) double immune Mice. Splenocytes from four double immune mice were harvested and stimulated \textit{ex vivo} with indicated peptide gradient. Standard ICS and FACs were performed. The \% IFN\gamma max was calculated by designating the \% of IFN\gamma response at 1,000nM as 100\%.
However, it is interesting to note that the functional avidity for PV NP205-specific CD8 T cells was lowest among the 3 responses. Therefore, it is possible that the sequence of immunization (PV+LCMV WT) generates a cross-reactive NP205-specific CD8 T cell population more sensitive to the LCMV NP205 epitope. However, a more precise analysis is needed to conclude that this finding is significant. Based on this experiment, both cross-reactive NP205-specific CD8 T cells and the PV NP38-specific CD8 T cells are high avidity T cells that display very little difference in their respective peptide sensitivity (Figure 5.9). Occasionally, there could be a situation where the cross-reactive NP205-specific CD8 T cell populations have a significantly lower functional avidity than the PV NP38-specific CD8 T cell populations (Figure 5.9). Again, this observation highlights the private specificity of the cross-reactive NP205-specific CD8 T cell population in each individual mouse. This observation could provide one of the mechanistic explanations on how the cross-reactive NP205-specific CD8 T cells are associated with immune pathology. However, the difference in the overall level of antigenic epitope expression on the cell surface could still be a major
reason why there is a significant delay in cross-reactive NP205-specific CD8 T cell-mediated PV clearance.

**F. Kb stabilization assay of LCMV NP205 peptides containing alanine mutated anchoring residues**

In addition to the r/V207A variant virus that contains the original point mutation from the LCMV NP-V207A variant virus, I also experimented with the possibility of generating a NP205 epitope variant that contains mutation(s) at the anchoring residue positions (Figure 4.6). Despite the possibility that the LCMV NP205 (V-A) peptide can still be presented *in vivo* (Figure 3.5), both ICS and MHC-Ig dimer experiments suggested that the point mutation resulted in a significant but not complete loss in immunogenicity (Figure 4.10 & 4.11). The low incidence of AFN seen in the (PV+LCMV NP-V207A) double immune mice following PV challenge could still be attributed to this small but “altered” NP205 response. Therefore we wanted to construct a LCMV NP205 variant virus that lacks the ability to present the cross-reactive NP205 epitope on the cell surface. Different versions of the LCMV NP205 peptides containing either single alanine
substitution at one of the two K^b anchoring residues (positions six and eight) or alanine residues at both anchoring positions were synthesized (Figure 5.10).
Figure 5.10  K\(^b\) stabilization assay of LCMV NP205 peptides containing alanine mutated anchoring residues. Different variant LCMV NP205-212 peptides were incubated with RMA-S cells with graded concentrations. Following overnight incubation, surface staining with a monoclonal antibody against K\(^b\) was performed. The K\(^b\) MFI of the YTAKYPNL at 100μM was used as the 100% max. The data represent the average of duplicate samples ± SD.
Subsequently, a MHC stabilization assay was performed on each peptide to estimate its ability to bind to $K^b$. The 50% maximum binding curve indicates that there was a significant reduction among these alanine switched peptides in their ability to stabilize $K^b$ (Figure 5.10). The original LCMV NP205 (V-A) peptide has a 50% maximum binding capacity at approximately 0.05μM. On the other hand, the 50% maximum binding capacity for the LCMV NP205 peptides with an alanine at either anchoring residue was at the 5μM range, correlating to a hundred-fold reduction in the ability to stabilize $K^b$ (Figure 5.10). As predicted, the LCMV NP205 peptide that contained alanine in both anchoring residues displayed the most dramatic reduction in $K^b$ binding ability, as its $K^b$ MFI was only slightly above the un-pulsed control (720 vs. 500) at a 100μM concentration (Figure 5.10). Therefore, these data provide first hand evidence that some of the alanine switch mutations in the LCMV NP205 epitope could result in a LCMV variant with a “complete knockout” version of the NP205 epitope. So far, I have been able to generate two rescued variant viral stocks that contain a single alanine mutation in either one of the anchoring residues of $K^b$ (r/Y209A and r/L212A). My preliminary data show that the antigen-specific CD8 response in
mice immunized with r/L212A variant did not respond to any of the LCMV NP205 variant peptides by standard ICS assay (LCMV NP205, PV NP205, LCMV NP-Y209A, and LCMV NP-L212A). The “complete knockout” of the LCMV NP205 CD8 epitope would enable us to further clarify the nature of the diminished NP205 responses elicited by the LCMV NP-V207A variant.

**Chapter Summary**

Much evidence suggests that cross-reactive T cells are associated with immune pathology. In many of the autoimmune models, cross-reactive memory T cells previously primed by pathogens expressing epitopes that closely resemble self-antigen are the major mediators of autoimmune tissue destruction (Lünemann et al., 2008). Our data from chapter four suggest that the cross-reactive NP205-specific CD8 T cell population mediates the majority of the heterologous immunity in this system without any apparent immune pathology. However, a significantly delayed PV clearance kinetics by the cross-reactive
NP205-mediated heterologous immunity can be seen compared to the homologous challenge control (PV+PV) (Figure 5.1).

In (PV+LCMV) double immune hosts re-challenged with PV, a high incidence of AFN was observed. Therefore, I questioned whether the presence of a large number of subdominant cross-reactive NP205-specific CD8 T cells in the (PV+LCMV) double immune mouse might be responsible for development of AFN. Overall, my data suggest that the cross-reactive NP205-specific CD8 T cells in the (LCMV+PV) double immune mice are significant contributors to immune pathogenesis following PV challenge. First of all, I found that the incidence and magnitude of AFN is significantly higher in the (PV+LCMV) than the (PV+LCMV NP-V207A) double immune mice (Figure 5.3). In addition, the cross-reactive NP205-specific CD8 T cells are the dominant responders in the primary site of infection in the (PV+LCMV) double immune mice upon a PV challenge (Figure 5.4). Interestingly, my correlation studies reveal that two important antigen-specific CD8 T cell populations (one cross-reactive, one non cross-reactive) in the (PV+LCMV) double immune mice might play opposing roles upon PV challenge. PV NP38-specific CD8 T cells are the
immunodominant population in the PV-immune animals. Upon heterologous challenge, the immunodominance hierarchy is reversed by 1) a comprehensive attrition of the memory lymphocyte populations and 2) active expansion of the cross-reactive NP205-specific CD8 T cell populations that survive the attrition. Since homologous challenge appears to clear the virus much quicker than heterologous challenge, this would suggest that PV NP38-specific T cells are the default CTL response for PV viral clearance (Figure 5.1). On the other hand, the cross-reactive NP205-specific CD8 T cell populations can mediate a different level of protection, albeit with a much slower kinetics. Significantly delayed PV clearance kinetics might play an important role in the induction of immune pathology. One could imagine that prolonged activation might lead to direct tissue pathology and possibly secondary recruitment of non antigen-specific cells into the pathological sites. The exact mechanistic explanation of how the cross-reactive T cells might contribute to immune pathology is still under investigation. However, we did not find a significant difference in terms of the functional avidity between the cross-reactive NP205-specific CD8 T cell and the PV NP38-specific CD8 T cells in the (PV+LCMV) double immune animals (Figure 5.9). Therefore,
these data suggest that the difference in clearance could be due to a difference in antigenic epitope expression. On the other hand, whether there is a difference in cytokine expression profiles between these two T cell populations remains to be determined. It has been shown that activation by an APL could result in alteration in cytokine profile in antigen-specific T cells (Bashyam et al., 2006; Clute et al., 2005). Finally, these data support our hypothesis that the cross-reactive NP205-specific CD8 T cells play an important role in immune pathogenesis in the LCMV and PV heterologous challenge system.

The exact mechanism of AFN is still unknown in this system. However, in the LCMV and VV system, TNF is an important cytokine for the induction of AFN. Work done by Siwei Nie, a former graduate student in Dr. Liisa Selin’s lab has shed light into some possible mechanisms. A potential scenario has been postulated in this model, in which cross-reactive CD8 T cells would first recognize the VV-specific antigens on an APC. This would lead to cytokine production (IFNγ and TNF) and FASL expression by the cross-reactive CD8 T cells and further TNF production by the activated APCs. TNF would then signal through TNFR2 on the surface of adipocytes, which would lead to an increase in
FAS expression. When the activated CD8 T cells engage the adipocytes through the FAS/FASL pathways, this would induce death of adipocytes. Therefore, it is possible that TNF could play a similar role in immune-pathogenesis of the fat pad. In the LCMV system, IFNγ+/TNF+ (double) producers normally account for approximately 70 to 90% of the total IFNγ+ producers within a given antigen-specific CD8 T cell population. Although the role of IFNγ has not been addressed in this system, frequency of IFNγ-producing antigen-specific CD8 T cell might provide an accurate estimation of the frequency of antigen-specific CD8 T cells in this system. However, the use of MHC tetramers or dimers would be more useful in this regard.

The use of the ratio between the cross-reactive NP205 and PV NP38-specific CD8 T cell populations suggested that the induction of immune-pathogenesis by the cross-reactive NP205-specific CD8 T cell populations is highly dependent on the presence of PV NP38-specific CD8 T cell. First of all, the significant difference in AFN occurrence between the (PV+ LCMV WT) and (PV+ LCMV NP-V207A) double immune animals does suggest role of cross-reactive NP205-specific CD8 T cells play in immune-pathogenesis. Second of
all, the correlation data presented in Fig 5.6 suggest that PV NP38 is a protective epitope. Therefore, even a small percentage of PV NP38 can dramatically increase the kinetics of PV clearance. Therefore, the use of the ratio between the frequency of cross-reactive NP205 and PV NP38-specific CD8 T cells suggest a threshold balance in terms of pathology and protection. The data suggest that as the ratio between the cross-reactive NP205 and PV NP38-specific CD8 T cells reach a point, the cross-reactive NP205-specific CD8 T cells could dominate the protective PV NP38-specific CD8 T cells response through a mechanism that is yet unknown.

One important question about this system is the possibility of T cell clonal exhaustion. As our data suggest, the LCMV NP-V207A variant is able to induce T cell clonal exhaustion in a similar manner as LCMV clone 13 (Fig 3.11). We chose the high challenge dose in order to drive the expansion of cross-reactive NP205-specific CD8 T cell response. Although original reports showed the induction of T cell clonal exhaustion in animals that received high dose i.v. (2x10^6 pfu) (Ahmed and Oldstone, 1988; Ahmed et al., 1984), the dose (1x10^6 pfu i.p.) we challenged the PV-immune mice could potentially result in partial clonal
exhaustion. In fact, 2 animals in the PV+LCMV WT group and 1 animal in the PV+LCMV NP-V207A group had to be excluded from the overall data pool because T cell clonal exhaustion was observed in GP33 and NP396-specific CD8 T cell populations before PV challenge. However, the majority of ICS data from both (PV+LCMV WT) and (PV+LCMV NP-V207A) double immune animals showed that the dose of 1×10^6 i.p. Induced a optimal TNF and IFNγ by the antigen-specific CD8 T cells (Fig 5.1 and 5.2). However, experiments looking at surface expression of PD-1 on those antigen-specific CD8 T cells have not been performed. Some reports have shown that IL-10 play a crucial role in controlling persistent virus infections, possibly through suppression of antigen-specific T cell responses (Brooks et al., 2008; Brooks et al., 2006). However, there has not been any report on whether IL-10 might be playing a role in immune-pathogenesis in the (PV+LCMV WT) double immune mice at the dose we administered i.p. Finally, the development of AFN (pathology) in this model strongly argue against T cell clonal exhaustion since the pathology are usually dependent on T cell responses. Most importantly, the original observation that
cross-reactive NP205-specific CD8 T cells might contribute to immune pathology
was conducted in the LCMV Armstrong background.
Chapter VI
Discussion

Heterologous immunity typically displays either a delayed viral clearance or a change in the pattern of immune pathology due to the activation of cross-reactive memory lymphocytes. Although the protective side of heterologous immunity is often overlooked, it can be beneficial to the host. For example, LCMV-immune mice can withstand a higher dose of VV challenge compared to naïve mice. However, these immune mice develop immune pathology in the abdominal fat pad known as acute fatty necrosis (Selin et al., 1998). This illustrates a compromise between partial protective immunity conferred by these cross-reactive memory T cell populations verses the development of immune pathology. In fact, some evidence from studies in humans suggested that T cell-mediated heterologous immunity is often associated with immune pathologies (Clute et al., 2005; Lünemann et al., 2008; Urbani et al., 2005).

One of the key features of mammalian adaptive immunity is its antigen specificity and quick recall response when it detects an antigen for the second time. A fully differentiated memory T cell is capable of multiple effector functions
and resembles a double-edged sword as it can either lead to clearance of the pathogen or immune pathology if not regulated properly. The LCMV+PV heterologous immunity model represents one of the prototypical experimental systems to examine the nature of T cell cross-reactivity and heterologous immunity. First, this system contains well-defined cross-reactive NP205 CD8 epitopes between LCMV and PV. The high degree in aa similarity between the two K\(^b\)-restricted CD8 epitopes results in a cross-reactive NP205-specific CD8 T cell response that is bi-directional and predictable. Using this system, our lab has made fundamental observations with regard to T cell cross-reactivity and heterologous immunity. First, T cell cross-reactivity between LCMV and PV leads to a dramatic alteration in the immunodominance hierarchy of the host (Brehm et al., 2002). Second, heterologous immunity between LCMV and PV results in a partial protective immunity without any apparent immune pathology. Third, the overall cross-reactive NP205-specific CD8 TCR repertoire becomes extremely narrowed following a heterologous infection. The overall aims of my thesis were 1) to establish a direct link between the cross-reactive NP205-specific CD8 T cells and heterologous immunity between LCMV and PV, and 2)
to investigate the role of cross-reactive NP205-specific CD8 T cells in immune pathogenesis in this heterologous immunity model.

**Narrowing of the TCR repertoire and generation of CTL escape variants**

Why certain antigen-specific TCR repertoires are narrower than others remains unclear. As suggested by Turner et al, it is possible that inherent features in a given antigenic epitope can select only an antigen-specific response that constitutes a “narrow” TCR repertoire (Turner et al., 2005). In addition, a difference in MHC polymorphism can also affect TCR repertoire selection (Messaoudi et al., 2002). Messaoudi et al showed that mice expressing the $K^{bm8}$ haplo-type are more resistant to a lethal dose of herpes simplex virus type one (HSV-1) challenge as compared to normal B6 mice. This might be due to the fact that HSV-8p-specific CD8 T cells generated from the $K^{bm8}$ mice displayed a broader TCR repertoire and higher functional avidity. On the other hand, it has been shown that the overall breadth of antigen-specific TCR repertoire can be subjected to skewing under constant activation and selection such as chronic viral infections or homeostatic proliferation (Lin et al., 2008). Finally, significant skewing of the cross-reactive NP205-specific CD8 TCR repertoires was
observed in the LCMV+PV heterologous immunity model (Cornberg et al., 2006). Therefore, we predicted that under a dominant but narrowed cross-reactive NP205-specific CD8 T cell response, one of the immunological consequences would be the generation of NP205 CTL variants.

An LCMV NP205 CTL variant (LCMV NP-V207A) was isolated in a PV-immune mice challenged with LCMV clone 13. The mutation reflects both the specificity and a high degree of conservation of the NP205 epitopes between different arenaviruses. The point mutation within the LCMV NP-V207A variant results in switching of valine to alanine at the third non-anchoring aa position of the NP205 epitope. Since all the NP205 epitope aa sequences so far indicate that the third aa position can only be either a valine or an alanine, the mutated NP205 CD8 epitope now resembles the Lassa virus NP205 sequence. Most importantly, immunization of B6 mice with the LCMV NP-V207A variant results in a greatly diminished cross-reactive NP205-specific CD8 T cell response. This observation strongly suggests that this point mutation results in a dramatic loss of immunogenicity of the NP205 epitope.
To further ascertain that this mutation is responsible for the greatly diminished cross-reactive NP205-specific CD8 response in B6 mice, rescued LCMV were generated in vitro by our collaborators at the Scripps Research Institute using molecular biology techniques. Again, the rescued LCMV virus (r/V207A) that contains the original point mutation also results in a significantly diminished cross-reactive NP205-specific CD8 response. In addition, studies using MHC-Ig dimers loaded with either wild type LCMV NP205 or LCMV NP205 (V-A) peptides for detection of antigen-specific CD8 T cell responses support our original conclusion that this mutation results in a significant reduction in LCMV NP205 immunogenicity.

Factors such as CTL strength, viral fitness, epitope structure, and the nature of virus all play a role in determining the emergence of a CTL escape variant. Narrowing of the TCR repertoire has been associated with an increased frequency of viral escape variants (Charini et al., 2001; Meyer-Olson et al., 2004). Therefore, a dominant cross-reactive NP205-specific CD8 T cell response with a narrowed TCR repertoire should increase the odds of NP205 cross-reactive CTL escape due to the reduction in the ability to recognize
potential epitope variants. Here, we demonstrated in this system that one of the immunological consequences of T cell mediated heterologous immunity is the generation of viral CTL escape variants \textit{in vivo}.

**Cross-reactive NP205-specific CD8 T cells are mediators of heterologous immunity between LCMV and PV**

Previously, our lab showed that heterologous challenge between LCMV and PV resulted in partial protective heterologous immunity. However, a direct link between the cross-reactive NP205-specific CD8 T cells and heterologous immunity still remained to be established. For instance, bystander T cell activation has been suggested to play a role in some heterologous immunity models (Gilbertson et al., 2004; Polley et al., 2005). Heterologous challenge experiments were carried out to test whether there was a direct link between the cross-reactive NP205-specific CD8 T cells and heterologous immunity in the LCMV+PV model. Specifically, we reasoned that if the cross-reactive NP205-specific CD8 T cells were directly mediating heterologous immunity between LCMV and PV, a significant loss in heterologous immunity should be observed in the LCMV NP-V207A-immune mice challenged with PV. However, it is
conceivable that additional cross-reactive epitopes could take a dominant role in
the LCMV NP-V207A-immune mice challenged with PV. Therefore, a series of
adoptive transfer studies and peptide screening experiments were conducted to
screen for additional cross-reactive epitopes. Our preliminary data suggested
that there was very little indication of additional cross-reactive CD8 epitopes in
LCMV NP-V207A-immune mice challenged with PV. Finally, the protection data
derived from both systems (LCMV NP-V207A and r/V207A) suggested that the
cross-reactive NP205-specific CD8 T cells are mediators of the majority of
heterologous immunity between LCMV and PV. This is the first evidence that
cross-reactive T cells are mediators of heterologous immunity between LCMV
and PV in vivo using a natural infection model. In addition, it also demonstrates
that bystander activation plays very little role, if any, in the LCMV+PV
heterologous immunity model.

**Contribution of the cross-reactive NP205-specific CD8 T cells to immune pathogenesis**

Cross-reactive T cells are often associated with various types of tissue
pathologies in both murine models and humans. The central focus of my thesis
was to address the question of how cross-reactive T cells contribute to immune pathogenesis. Experiments from our lab showed that (PV+LCMV WT) double immune mice developed a high incidence as well as high level of AFN after a final challenge with PV. These data suggest that cross-reactive NP205-specific CD8 T cells could play an important role in immune pathogenesis.

The fact that we found a significantly higher incidence of AFN in the (PV+LCMV WT) than the (PV+LCMV NP-V207A) double immune groups support our hypothesis that the cross-reactive NP205-specific CD8 T cells populations play an important role in the development of AFN. In addition, since a small percentage of (PV+LCMV NP-V207A) double immune animals still developed AFN following PV challenge, we question whether a small but altered cross-reactive NP205-specific CD8 T cell response still exists within the animals. Therefore, generation of the rescued LCMV variants that contain the mutation(s) at the anchoring residues of the LCMV NP205 epitope is underway to address this particular question.

There is additional evidence to support our hypothesis. First of all, cross-reactive NP205-specific CD8 T cells dominate the memory pool of the
(PV+LCMV WT) double immune mice. In addition, these cross-reactive NP205-specific CD8 T cells are also present at a high proportion in the pathological site following infection. Normally, PV NP38 stimulates the dominant CD8 response in mice immunized with PV whereas PV NP205 stimulates a subdominant CD8 T cell response. However, PV-immune animals challenged with LCMV resulted in reversal of the immunodominance hierarchy. In addition, type I IFN-induced attrition drives the non cross-reactive NP38-specific CD8 T cell frequency further down.

Second, the correlation studies looking at linear relationships between the frequency of the cross-reactive NP205-specific CD8 T cells in the (PV+LCMV WT) double immune mice and the severity of AFN suggest that two antigen-specific CD8 T cells might be playing opposite roles in this model. Therefore the outcome of immune pathology could be decided by the interplay between these two CD8 T cell populations. For example, when we looked at linear correlation between the PV NP38-specific CD8 T cell responses in the (PV+LCMV WT) double immune mice and the severity of AFN following PV challenge, we observed a significant negative linear correlation. This suggests that PV NP38-
specific CD8 T cells appear to confer a protective response from immune pathology. On the other hand, the linear correlation between the ratio of the NP205/NP38 in the (PV+LCMV WT) double immune mice and the severity of AFN following PV re-challenge suggests that the cross-reactive NP205-specific CD8 T cells play an important role in immune pathology.

Third, the viral protection data provide a possible mechanistic explanation for how the cross-reactive NP205-specific CD8 T cells might contribute to immune pathogenesis. A comparison of PV clearance kinetics between homologous (PV+PV) and heterologous (LCMV+PV) challenge suggests that different antigen-specific populations might mediate different levels of protection. Since a homologous challenge (PV+PV) sequence clears the virus much faster compared to a heterologous challenge sequence (LCMV+PV), this implies that PV NP38-specific CD8 T cells play a more important role than the cross-reactive NP205-specific CD8 T cells in mediating protective immunity to PV. Therefore, a significant difference in PV clearance kinetics could be the reason why it is possible to observe a loss in protective immunity to PV in the (PV+LCMV WT) double immune mice under certain conditions.
One of the reasons why cross-reactive T cells are sometimes associated with immune pathology is low affinity/avidity ligand interaction. First of all, high avidity TCR/pMHC interactions are more specific and are usually correlated with viral control (Neveu et al., 2008). On the other hand, low affinity cross-reactive T cells have been implicated to play a role in dengue hemorrhagic fever, a severe clinical manifestation that occurs at higher frequency in individuals sequentially infected with two different serotypes of dengue virus (Mongkolsapaya et al., 2003). Studies also suggest that different autoimmune diseases are caused by low affinity cross-reactive T cells (Harkiolaki et al., 2009; Zehn and Bevan, 2006). I showed in chapter V that the overall functional avidities (cross-reactive NP205 and PV NP38) within each (PV+LCMV WT) double immune mouse are similar and are considered high avidity interactions. However, splenocytes isolated from one of the double immune mice displayed a very low functional avidity to PV NP205 peptide. This is reminiscent of one report showing that boosting with a high dose of a heterologous peptide in a conventional prime-boost regimen can lead to CD8 T cells with low functional avidity (Narayan et al., 2007). A low functional avidity to the PV NP205 peptide can also be explained by the
sequence of immunization in these double immune mice. Therefore, these low avidity cross-reactive T cells might not be able to control the virus efficiently. However, because the functional avidities of the cross-reactive NP205-specific CD8 response and the PV NP38-specific CD8 response are comparable in the majority of the (PV+LCMV WT) double immune mice, other factors could also play a role. For example, a difference in the relative surface expression of each epitope represents an interesting possibility that could lead to a difference in PV clearance.

The other possible mechanism is a change in cytokine profiles following stimulation by a heterologous ligand. There is evidence suggesting that different levels of T cell activation can be modulated via APLs that display different characteristics. Depending on its affinity to the TCR and overall aa sequence, an APL can be characterized as a full agonist, a partial agonist, and an antagonist. For example, different APLs of the LCMV GP33 epitope have been shown to elicit different levels of calcium flux in naïve GP33-specific P14 transgenic T cells, indicating that these naïve transgenic TCR populations could respond to each APL in a slightly different manner and lead to differential downstream
effector functions (Bachmann et al., 1997). Other reports showed that APLs could lead to T cell anergy through alteration of TCR signal transduction patterns (Sloan-Lancaster et al., 1994; Sloan-Lancaster et al., 1996). In the LCMV system, there are reports showing that different variants of the GP33 epitope could elicit different functional responses in the antigen-specific CD8 T cells both in vitro and in vivo (Martin et al., 1996). For example, different variants of the GP33 epitope display limited abilities to induce GP33-specific CD8 T cell killing in in vivo CTL assays (Bättig et al., 2005).

APLs can play an important role in T cell mediated cross-reactivity and heterologous immunity. Cross-reactive epitopes can potentially deliver partial TCR signals and result in alteration in T cell functions. For example, the cross-reactive epitope D2 variant 71-79 from type 2 dengue that acts as a partial agonist to DV3 immune T cells induced very little T cell proliferation and IFNγ production in a heterologous dengue virus infection (Zivny et al., 1999). In addition, the same group also showed that there is an alteration in T cell cytokine profile following a heterologous peptide stimulation (Bashyam et al., 2006). Finally, ex vivo stimulation of auto-reactive T cells clones specific to myelin basic
protein by an APL led to differential secretion of cytokines (Ausubel et al., 1999; Ausubel et al., 1997). Similar observations were made in our lab where we found that a specific heterologous challenge sequence with an unrelated organism led to a change in cytokine profile of the host (Chen et al., 2003; Clute et al., 2005).

In conclusion, we have made the following observations in regard to the LCMV+PV heterologous challenge system. First of all, the cross-reactive NP205-specific CD8 T cells result in the dramatic alteration in the immunodominance hierarchy of the second incoming virus, possibly through immunodomination. Second of all, the expansion of the cross-reactive NP205-specific CD8 T cells leads to an extremely narrowed TCR repertoire, and could result in generation of a viral CTL escape variant. Third, the cross-reactive NP205-specific CD8 T cells are the mediators of heterologous immunity, associated with a significantly delayed PV clearance kinetics when compared to homologous challenge. The induction of immune pathology in this system possibly reflects the interplay between two different antigen-specific CTL populations through mechanisms that are not yet described. Finally, all the evidence suggests that heterologous immunity comes at a greater cost to a host
than homologous immunity due to its association with immune pathology. Therefore, the caveat of heterologous immunity really falls between the extent of T cell-mediated immune pathology versus host survival.

Finally, the data in this study bear important implications on the design of T cell vaccines in the future. Specifically, removal of potential cross-reactive CD8 T cell epitopes might prove to be beneficial in certain situations. First of all, T cell-mediated cross-reactive responses have the potential to bias the establishment of the subsequent immune response, as evidenced by the alteration of immunodominance hierarchy patterns (Brehm et al., 2002). Second of all, a single dominant cross-reactive T cell response could lead to either viral escape (chapter III) or competition against a more protective response (chapter V). This sub-optimal response could lead to delay in viral clearance and prolonged cytokine production and bystander recruitment, which together could contribute to induction of immune pathology (Fig 5.1).

The difficulty in providing an accurate prediction in highly diversified populations such as humans lies in the fact that all the individuals have a unique history of vaccination and infections. However, it might still be possible to
delineate a common denominator that could provide a useful prediction in a given population. For example, removal of the HCV NS3 1073-1081 cross-reactive epitope, which cross-reacts with IAV NA 231-239, might prove to be beneficial to the majority of HLA-A2-restricted individuals receiving HCV vaccine in the future since most of them will contain potential cross-reactive memory T cell populations from either IAV immunization or infections. Furthermore, therapeutic interventions that aim at elimination of the cross-reactive memory T cell populations could also be utilized once potential cross-reactive epitopes are identified.
Chapter VII

Reference


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