CHARACTERIZATION OF $CD8^+$ T CELLS FOLLOWING VACCINATION WITH AN INACTIVATED WHOLE VIRUS VACCINE

by

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Abstract

Lymphocytic choriomeningitis virus (LCMV) is a natural mouse pathogen, and acute infection of adult mice induces a robust and protective $CD8^+$ T cell response that persists for the life of the animal. These attributes make murine LCMV infection an ideal model in which to develop and test vaccine strategies designed to elicit $CD8^+$ T cell immunity. In these studies, vaccination of adult C57BL/6 mice with hydrogen peroxide (H_2O_2)-inactivated whole LCMV virions results in the production of $CD8^+$ T cell immunity with an altered immunodominance hierarchy relative to infection with acute or chronic LCMV strains. Higher percentages of vaccine-induced $CD8^+$ T cells express multiple effector cytokines relative to infection-induced $CD8^+$ T cells, and this enhanced memory-type cytokine expression profile is present as early as 8 days following vaccination. H_2O_2-LCMV vaccination was sufficient to protect mice from chronic LCMV infection following challenge with the chronic viral variant, LCMV-Clone 13, and this protection was mediated directly by $CD8^+$ T cells. Further investigations, including vaccinations with individual LCMV peptides, revealed a high degree of cross-reactivity between LCMV peptides that had little or no sequence homology. This level of cross-reactivity among peptides derived from a single virus has not been previously observed. This work demonstrates that it is possible to achieve durable and protective $CD8^+$ T cell-based immunity using an H_2O_2-inactivated whole virus vaccine, and reveals previously undescribed cross-reactivity among multiple LCMV-specific $CD8^+$ T cell populations.

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List of Abbreviations

LCMV – Lymphocytic choriomeningitis virus

H_2O_2 – hydrogen peroxide

GP-C – LCMV glycoprotein precursor

GP1 – LCMV glycoprotein 1

GP2 – LCMV glycoprotein 2

NP – LCMV nucleoprotein

L – LCMV L polymerase

Z – LCMV Z protein

kDa – kiloDalton

RdRp – RNA-dependent RNA polymerase

NK – Natural killer cell

MCMV – Murine cytomegalovirus

WNV – West Nile virus

IAV – Influenza A virus

PAMP – Pathogen associated molecular pattern

RLH – retinoic acid inducible gene I-like helicase

TLR – Toll-like receptor

IC – Intra cerebral

PFU – Plaque forming unit

β_2m – Beta 2 microglobulin

YFV – Yellow fever virus

VV – Vaccinia virus

MV – Measles virus

CMV – Human cytomegalovirus

ICCS – Intracellular cytokine staining

ELISPOT – Enzyme-linked immunospot

H_2O_2-LCMV – Peroxide inactivated LCMV

LCMV-Arm – LCMV Armstrong strain

LCMV-Clone 13 – Chronic LCMV Clone 13 strain

HCV – Hepatitis C virus

HIV – Human immunodeficiency virus

VZV – Varicella zoster virus

PV – Pichinde virus

MHC-I – Major histocompatibility complex I

APC – Antigen presenting cell

pAPC – Professional antigen presenting cell

DC – Dendritic cell

TAP – Transporter associated with antigen processing

HBC – Hepatitis B core antigen

APL – Altered peptide ligand

TCR – T cell receptor

MPL – monophosphoryl lipid A

IFNγ – Interferon gamma

TNFα – Tumor necrosis factor alpha

VLP – Virus-like particle

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Chapter 1 - Introduction

Overview

The focus of this thesis is to analyze $CD8^+$ T cell immunity to lymphocytic choriomeningitis virus (LCMV) subsequent to vaccination with either inactivated whole virus or peptide epitopes. Our first goal was to understand the similarities and differences between $CD8^+$ T cell immunity derived in response to either infection with live virus or vaccination with killed whole virions. To accomplish this, we used a novel vaccination strategy, in which whole LCMV was killed using hydrogen peroxide (H_2O_2) as a vaccine preparation method. Our analysis focused on the immunodominance profiles and cytokine production profiles of $CD8^+$ T cells produced in response to infection or vaccination. Our work revealed that the $CD8^+$ T cell response to H_2O_2-LCMV vaccination has a different immunodominance profile than the T cell response to live LCMV infection. The second focus of this work was the analysis of cross-reactivity among LCMV-specific $CD8^+$ T cell responses. To investigate this, we vaccinated mice with immunodominant peptides from LCMV to generate animals with a single LCMV-specific $CD8^+$ T cell population. We then screened these T cell populations against other known LCMV peptide epitopes. Surprisingly, these results demonstrated that certain LCMV-derived peptide epitopes are able to stimulate more than one $CD8^+$ T cell populations, and these peptides are cross-reactive despite having little or no sequence homology.

Chapter 1 of this thesis is a general background on the LCMV model system and the vaccine strategy used in this work. Chapters 2 and 3 are

modifications of review articles written during my thesis work, and serve as an extended introduction. Chapters 4 and 5 contain the results of the work just described, and Chapter 6 is an extended discussion of our results.

LCMV

Discovery and Classification

LCMV was the first member of the virus family, Arenaviridae, to be discovered. Charles Armstrong first isolated the virus from a fatal case of aseptic meningitis during a 1933 outbreak of St. Louis encephalitis virus (Murphy 1975, Buchmeier et al. 2006). Shortly thereafter, LCMV was discovered to be an endemic pathogen in laboratory rodent colonies. In the 35 years after the discovery of LCMV, several other morphologically and serologically linked viruses, including Machupo and Tacaribe viruses, were discovered (Murphy 1975, Buchmeier et al. 2006). In 1970, after the discovery of Lassa fever virus, the Arenaviridae family classification was established (Murphy 1975). The family is named after the sandy appearance of virions when viewed using electron microscopy. The two major classes in the family are Old and New world arenaviruses, and this distinction is made based on the range of the viruses' zoonotic reservoir species (Charrel et al. 2008). LCMV is unique among the arenaviruses in that it is the only member of the family present worldwide, due to the distribution of its native host, *Mus musculus*. Despite its global distribution, LCMV is most genetically homologous with the Old world arenaviruses, and it is classified as such (Charrel et al. 2008, Albariño et al. 2010).

Genome

Arenaviruses are enveloped, bisegmented, negative sense, single-stranded RNA viruses (Buchmeier et al. 2006). The genome is made up of two RNA strands, a 7.2 kilobase L strand and a 3.5 kilobase S strand. Each of the strands encodes two viral proteins separated by a stem-loop structure. While the virus is technically classified as a negative sense RNA virus, it actually employs a unique ambisense coding strategy. On each of the two strands, the viral gene encoded on the 5' side of the stem-loop structure is pseudo-positive sense RNA, while the viral gene encoded on the 3' side of the stem loop structure is negative sense RNA (Fig. 1.1) (Sánchez and la Torre 2006, Buchmeier et al. 2006, Emonet et al. 2009).

The viral genome encodes 4 proteins, all of which are present in infectious virions. LCMV nucleoprotein (NP) protein is the most abundant protein in infected cells as well as the virions themselves. The viral genome complexes with NP to form helical structures that make up the bulk of the virions(Buchmeier et al. 2006). LCMV glycoproteins 1 and 2 (GP-1 and GP-2) are derived from a common glycoprotein precursor (GP-C) that is posttranslationally cleaved by the cellular site 1 protease (Buchmeier et al. 2006, Emonet et al. 2009). GP-1 and GP-2 combine to form spiked structures on the surface of the virions involved with virus entry and replication. GP-1 is known to interact with α-dystroglycan on the cell surface to facilitate adhesion of virions to the cellular membrane (Kunz et al. 2002, Kunz 2009). GP-2 mediates the pH-dependent fusion of virion and endosomal membranes required for virus entry into the cytosol. LCMV L

polymerase (L) is an RNA-dependent RNA polymerase (RdRp). The L protein forms oligomers during the viral replication cycle, and it is required for viral replication (Sánchez and la Torre 2006, Buchmeier et al. 2006). LCMV Z protein (Z) is a RING zinc finger protein that is believed to have at least two roles. Buildup of Z in the late phase of the replication cycle is known to inhibit new RNA synthesis and promote virus assembly and budding (Sánchez and la Torre 2006, Buchmeier et al. 2006). Z is also thought to be analogous to matrix protein in other negative strand RNA viruses and play a role in linking nucleoprotein complexes to the viral membrane (Sánchez and la Torre 2006).

The NP and L genes are present on the negative sense segments of S and L genes respectively (Fig. 1.1). The RdRp L protein transcribes these genes into positive sense mRNA for use during replication. The GPC and Z are encoded on the pseudo-positive sense segments of the S and L genomic segments respectively (Fig. 1.1). During viral replication, the RdRp L protein mediates the production of a negative sense anti-genome for the pseudo-positive portions of both the S and L strands. Transcription and translation then proceed as described for the genes present on the negative sense segments, using this negative sense anti-genome as template (Buchmeier et al. 2006). It is important to note that none of the four LCMV genes can be translated directly from genomic RNA, including the GPC and Z genes on the pseudo-positive sense RNA segments, and naked LCMV genomic RNA is not infectious (Sánchez and la Torre 2006, Buchmeier et al. 2006).

Infection / Pathogenesis

LCMV is endemic in its zoonotic reservoir species, *Mus musculus.* Primary infection of adult animals most commonly begins with inhalation of an infected aerosol, typically urine (Buchmeier et al. 2006). Using α-dystroglycan as its receptor, LCMV is capable of infecting most cell types in the mice, and lung, liver, spleen, kidney, and brain are all sites of viral replication (Buchmeier et al. 2006). Acute infection in adult mice is largely asymptomatic and well controlled by the adaptive immune response, particularly the $CD8^+$ T cell response (Oldstone 2002), and virus is cleared from all organs within 15 days of infection (Wherry et al. 2003b). The virus is non-cytolytic, and congenital or neonatal infection of mice leads to systemic virus replication and central tolerance of LCMV (Oldstone 2002). These animals become persistently viremic and shed virus in their urine for the remainder of their lives (Oldstone 2002, Buchmeier et al. 2006).

Human infection most commonly occurs because of exposure to infected rodent excrement or contaminated fomites. LCMV infection of immunocompetent humans is rarely fatal, and most human infections result in a brief febrile illness or aseptic meningitis with no known long-term sequelae (Buchmeier et al. 2006, Bonthius et al. 2007a, 2007b). However, prenatal LCMV infection results in far more severe consequences and may lead to mental retardation, epilepsy, cerebral palsy, ataxia, and blindness (Bonthius et al. 2007a, 2007b). LCMV infection has also been shown to cause fatal disease in immunosuppressed transplant recipients (Gautam et al. 2007, Araki et al. 2010). The prevalence and

incidence of LCMV infection in humans is not precisely known; however, studies have shown rates of seroprevalence ranging between 1.2% and 11% of the population (Buchmeier et al. 2006, Bonthius 2009).

The Immune Response to LCMV

Innate Immunity

Following viral infection, the innate immune system is the first line of defense that pathogens encounter. The innate immune response can be directly antiviral and at the same time support the development of adaptive immune responses (Akira et al. 2006; Kumar et al. 2011). The two most well understood aspects of the innate immune response to LCMV are the natural killer (NK) cell response and the type I interferon response to infection. LCMV infection induces NK cell proliferation and activation, with the peak of the response observed at around 3 days post infection (Orange and Biron 1996, Oldstone 2002). However, NK cells appear to have little ability to kill virally infected cells or to produce IFNγ in response to LCMV infection (Orange and Biron 1996, Oldstone 2002). This is in sharp contrast to the NK cell response to another natural mouse pathogen, murine cytomegalovirus (MCMV). During early MCMV infection, the NK cell response is highly antiviral, killing MCMV infected cells and producing IFNγ (Orange and Biron 1996, Oldstone 2002). This robust NK cell response contributes to early viral control in the case of MCMV infection. The inability of NK cells to kill LCMV infected targets is most likely because LCMV infection does

not result in the down regulation of major histocompatibility complex (MHC)-I, a primary mechanism NK cells use to identify infected targets (Oldstone 2002).

Unlike the NK cell response, the innate type I interferon response to LCMV infection plays a significant role in establishing control of infection. Following LCMV infection, the phagocytic cells of the innate immune system (monocytes, macrophages, and dendritic cells) are exposed to viral components because they either phagocytize virions and virus-infected cells or because they are infected directly. This causes activation of pathogen associated molecular pattern (PAMP) receptors such as toll-like receptors (TLRs) and retinoic acid inducible gene I-like helicases (RLHs). Activation of these PAMP receptors causes production of type I interferons (IFNα/β) via a variety of second messengers (Akira et al. 2006, Kumar et al. 2011). This type I interferon response has been shown to increase antigen presentation in dendritic cells as well as increase the proliferation, survival, and cytokine production ability of $CD8^+$ T cells (Zhou et al. 2005, Thompson et al. 2006, Jung et al. 2008, Zhou et al. 2008). MyD88 is a universal adapter protein required for the majority of TLRs to function. When MyD88 knockout mice are infected, the type I interferon response to LCMV is blunted. Unlike wild-type mice, these animals remain viremic throughout the course of infection, and they ultimately die between 30 and 40 days post-infection (Jung et al. 2008). It has also been shown that MyD88 deficient animals are unable to control primary West Nile virus (WNV) and influenza A virus (IAV) infection (Seo et al. 2010, Szretter et al. 2010).

Therefore, the innate immune system is essential to the control of LCMV infection, but its role is largely in support of the adaptive immune response.

Antibodies

Humoral immunity has been shown to provide some level of protection against LCMV; however, it has a limited effect on viral clearance or pathogenesis during primary LCMV infection (Cerny et al. 1988, Wright and Buchmeier 1991, Baldridge and Buchmeier 1992, Buchmeier et al. 2006). Passive transfer of neutralizing anti-LCMV antibodies into naïve mice was shown to protect the animals from lethal intra-cerebral (IC) LCMV challenge by suppressing viral replication until a $CD8^+$ T cell response was mounted (Wright and Buchmeier 1991). Transfer of humoral immunity from mother to pup through nursing has also been shown to protect animals against chronic perinatal infection as well as lethal IC LCMV challenge (Baldridge and Buchmeier 1992). B cell-depleted animals show an increase in the magnitude and duration of serum viremia following primary LCMV infection, but they do not demonstrate any increased pathology compared to control animals (Cerny et al. 1988). Humoral immunity is not required for clearance of LCMV or the generation of a memory $CD8^+$ T cell population curing primary infection (Cerny et al. 1988). Therefore, while it is capable of providing some level of protection, humoral immunity is not a required component of the anti-LCMV immune response in adult animals.

$CD4^+$ T Lymphocytes

$CD4^+$ T cells are not required for clearance of LCMV under all circumstances; however, an absence of $CD4^+$ T cells makes infected animals more susceptible to chronic viral infection. Several groups have demonstrated that animals that are either $CD4^+$ T cell-depleted or genetically deficient in $CD4^+$ T cell expression are able to control primary infection with LCMV (Ahmed et al. 1988, Kasaian et al. 1991, Herrath et al. 1996). Interestingly, animals infected with chronic LCMV strains or high doses of acute strains demonstrated a requirement for $CD4^+$ T cell help to clear infection (Battegay et al. 1994, Matloubian et al. 1994). The chronic LCMV variants used by many labs, such as LCMV-Clone 13 and LCMV-28b, exhibit delayed viral clearance compared to acute LCMV strains, such as LCMV-Arm and LCMV-WE, but ultimately infection is controlled in wild-type mice and viremia subsides between 30 and 90 days post-infection (Matloubian et al. 1994). However, when $CD4^+$ T cells are depleted, mice infected with the chronic LCMV variants, LCMV-Clone 13 and LCMV-28b, are unable to control the infection, and they become lifelong carriers of the virus (Matloubian et al. 1994). Additionally, the ability of $CD4^+$ T cell knockout mice to control infection is dependent on the dose of virus inoculum. $CD4^{-/-}$ animals infected with 2×10^2 plaque forming units (PFU) of the acute LCMV-WE strain were able to control viremia and clear infection, while animals infected with 2×10^6 PFU (a dose controlled by $CD4^+$ T cell replete mice) of this same strain became chronically infected (Battegay et al. 1994). It is important to note that the contribution of $CD4^+$ T cells to the adaptive immune response

against LCMV infection is dependent upon the presence of and antiviral $CD8^+$ T cell response. Beta 2 microglobulin (β_2m) knockout mice, which cannot mount $CD8^+$ T cell responses, are unable to control infection with acute or chronic LCMV variants (Matloubian et al. 1994). $\beta_2m^{-/-}$ animals experience life long viremia following infection with acute or chronic LCMV strains, despite having an intact antiviral $CD4^+$ T cell and antibody response. The body of work discussed here indicates that $CD4^+$ T cell immunity is a requirement for a fully functional antiviral $CD8^+$ T cell response. Additionally, a requirement for $CD4^+$ T cell help during the priming of $CD8^+$ T cell responses has been demonstrated in vaccinia virus and *Listeria* infection models (Shedlock and Shen 2003, Sun and Bevan 2003). The ability of animals to mount an effective immune response against high dose challenge and chronic LCMV variants is impaired in the absence of an antiviral $CD4^+$ T cell response.

$CD8^+$ T Lymphocytes

Since it's discovery, LCMV has become an indispensable tool in immunological research. This is particularly true of the relationship between LCMV and $CD8^+$ T cell research. Murine LCMV infection has been the model system used in a number of seminal discoveries regarding $CD8^+$ T cells. LCMV was critical to such breakthroughs as the discovery of MHC restriction (Zinkernagel and Doherty 1974) and the discovery of $CD8^+$ T cell memory maintenance in the absence of antigen (Murali-Krishna et al. 1999). In previous sections, I have reviewed the role that innate immunity, antibodies, and $CD4^+$ T cells play in the immune response to LCMV. The over arching theme to each of

these sections has been the idea that innate immunity, antibodies, and $CD4^+$ T cells work in support of the $CD8^+$ T cells response, but without $CD8^+$ T cell immunity LCMV cannot be cleared. Here, I will describe the role that $CD8^+$ T cells play in acute and chronic LCMV infection.

Primary murine infection with acute LCMV strains results in a $CD8^+$ T cell response that is detectable by day 5 post-infection and reaches peak magnitude around day 8 post-infection (Oldstone 2002, Masopust et al. 2006, Raué and Slifka 2009). The primary $CD8^+$ T cell response to LCMV infection can constitute as much as 50-80% of the $CD8^+$ T cell compartment at the peak of the response (Butz and Bevan 1998a, Murali-Krishna et al. 1998). This $CD8^+$ T cell response is cytotoxic directly ex vivo and kills LCMV-infected cells in a perforin dependent manner (Walsh et al. 1994, Kägi et al. 1994). Since the virus is non-cytolytic, the $CD8^+$ T cell response is responsible for the bulk of cell death that occurs during infection (Oldstone 2002). Animals experience brief serum viremia around 5 days after infection, and the virus is cleared form all tissues by around day 15 after infection (Wherry et al. 2003b). Animals that are $CD8^+$ T cell deficient, or have defects in their antigen processing and presentation pathway that make them unable to present antigen to T cells via MHC-I, are unable to clear LCMV and become lifelong carriers of the virus (Fung-Leung et al. 1991, Matloubian et al. 1994).

However, the robust $CD8^+$ T cell response induced by LCMV infection can actually be a two-edged sword. The same T cell response that is responsible for clearance of the virus can result in severe immunopathology and death under

certain circumstances. Intra-cerebral infection is a frequently used measure of protective LCMV-specific $CD8^+$ T cell responses in mice. In this model, animals are injected IC with 1-10 PFU of an acute LCMV strain. In LCMV naïve, animals the virus replicates within the meninges and choroid plexus (McGavern et al. 2002). After the adaptive immune response is initiated, LCMV-specific $CD8^+$ T cells infiltrate the central nervous system and exert their cytotoxic effects on the infected tissues (McGavern et al. 2002). This results in damage to the blood brain barrier and uncontrolled edema, which ultimately leads to herniation of the brain stem and death of the animal within 6-8 days after infection (Matullo et al. 2010). This pathology does not occur if a pre-existing $CD8^+$ T cell response to LCMV exists, and animals with pre-existing anti-LCMV $CD8^+$ T cell responses are able to clear IC infection without long-term sequelae (Klavinskis et al. 1989, Hassett et al. 2000).

Another model of LCMV infection that is frequently used is the chronic infection model. This group can be further subdivided into the perinatal infection model and the immunosuppressive LCMV variants model. In the perinatal infection model, LCMV infects the thymus of the developing mouse, causing in negative selection of LCMV-specific T cells (Oldstone 2002, Buchmeier et al. 2006). This results in development of a lifelong LCMV carrier state in these animals. Interestingly, it is possible to clear these carrier animals of LCMV infection by adoptive transfer of T cells from LCMV-immune animals (Jamieson et al. 1987). In the immunosuppressive LCMV variant model, animals are infected with LCMV strains that preferentially infect $CD11c^+$ dendritic cells

(Sevilla et al. 2000). These viruses, such as LCMV-Clone 13 and LCMV-28b, have higher affinity for α-dystroglycan than wild type viruses, and exhibit increased dendritic cell tropism (Sevilla et al. 2000). These immunosuppressive chronic LCMV strains cause chronic activation of antigen-specific T cells and exhibit delayed viral clearance compared to acute LCMV variants, such as LCMV-Arm and LCMV-WE. However, chronic viral infection is ultimately controlled in wild type mice and viremia subsides between 30 and 90 days post infection (Matloubian et al. 1994). If $CD8^+$ or $CD4^+$ T cell mediated immunity is abrogated at the time of infection with immunosuppressive LCMV variants, the infected animals lose the ability to clear the virus and become lifelong carriers (Matloubian et al. 1994).

The LCMV acute and chronic infection models exhibit dependence on $CD8^+$ T cell immunity for control and clearance of viral infection. For this reason, LCMV has been frequently used as a model system to test vaccination strategies and gain insight into the fundamental biology of $CD8^+$ T cell immunity (Slifka et al. 1996, Murali-Krishna et al. 1998, Badovinac et al. 2003, Kotturi et al. 2007).

Immunodominance

The constellation of possible antigenic determinants for $CD8^+$ T cells is enormous. Even a small virus, such as LCMV, contains more than 1,200 peptide epitopes predicted to bind MHC-I by computer modeling (Kotturi et al. 2007). However, the antiviral T cell response to LCMV infection is directed against a small subset of the total possible stimulating peptides, (Yewdell and Bennink 1999, Chen and McCluskey 2006). Among this small group of stimulatory

peptides, some induce a higher degree of clonal expansion and survival than others. This phenomenon generates specific immunodominance patterns for each combination of pathogen and MHC-I allele. If we are able to understand precisely the factors that govern immunodominance then it may enable vaccinologists to exploit this knowledge in vaccine development. For this reason, an understanding of immunodominance has long been a goal of scientists interested in rational vaccine design (Yewdell 2010). Some of the factors that may play a role in determining immunodominance include the ability of $CD8^+$ T cell populations to produce and respond to IFNγ (Liu et al. 2004, Whitmire et al. 2005), competition between T cells for APCs (Kastenmuller et al. 2007), the duration of antigen exposure (Yoshimura et al. 2004), the magnitude of antigen load (La Gruta et al. 2006), the affinity of peptide for MHC-I (Kotturi et al. 2008), efficiency of antigen processing and presentation (Yewdell and Bennink 1999), and frequency of naïve T cell precursors (Kotturi et al. 2008).

Following acute LCMV infection of C57BL/6 mice, the $CD8^+$ T cell responses to NP396 and GP33 peptide epitopes are known to dominate the immune response (Wherry et al. 2003b, Kotturi et al. 2007). It has also been shown that this immunodominance profile evolves after infection (NP396 becoming more dominant), independently of functional avidity maturation and likely in the absence of antigen (Raué and Slifka 2009). Immunodominance has also been shown to shift following re-infection with the same virus. In the case of LCMV, the $CD8^+$ T cell response to GP276 emerges as a co-dominant response during re-infection (Tebo et al. 2005). The largest changes in immunodominance

are observed following infection with chronic LCMV variants, such as LCMV-Clone 13. In the case of LCMV-Clone 13 infection, the $CD8^+$ T cell response is initially dominated by the NP396 and GP33. However, persistent high antigen levels cause exhaustion of the $CD8^+$ T cell responses, and lead to the clonal deletion of the NP396-specific T cell response and cause the GP33-specific T cell response to become functionally anergic (unable to produce cytokine in response to stimulation) (Zajac et al. 1998, Wherry et al. 2003b, Mueller and Ahmed 2009). This $CD8^+$ T cell dysfunction is prevented if an LCMV-specific $CD8^+$ T cell response is present prior to LCMV-Clone 13 challenge (Oldstone et al. 1993, Shen et al. 1995, Slifka et al. 1996, Lanier et al. 1999, Takagi et al. 2009). A pre-existing LCMV-specific $CD8^+$ T cell response blunts viral replication, and prevents the chronic high viral loads associated with immune dysfunction in chronic LCMV infection. These factors make murine LCMV infection an ideal model in which to study vaccines that stimulate $CD8^+$ T cell immunity.

MHC-I-Restricted Antigen Presentation

The generation of anti-LCMV $CD8^+$ T cells requires the stimulation of antigen-specific naïve T cells by antigen presenting cells (APC) loaded with LCMV peptides. There are two primary mechanisms by which viral peptides may become bound to MHC-I and presented on the cell surface. The first of these mechanisms is referred to as direct presentation. In the case of direct presentation, nascent viral peptides, within the cytoplasm of a virally infected APC, are degraded by the proteasome (Eisenlohr et al. 2007, Vyas et al. 2008).

The polypeptide fragments produced are transported across the membrane of the endoplasmic reticulum by the transporter associated with antigen processing (TAP), where they undergo further digestion by amino-peptidases, and a small percentage of these resulting polypeptides become bound to MHC-I (Eisenlohr et al. 2007, Vyas et al. 2008). The peptide loaded MHC-I molecules then undergo vesicular transport to the cell surface, where they are able to interact with T cell receptors (TCR) and stimulate an anti-viral $CD8^+$ T cell response (Vyas et al. 2008). The other way in which viral antigen may become bound to surface MHC-I molecules is referred to as cross-presentation. Cross-presentation is known to occur by at least two mechanisms. The first of these is a TAP-dependent mechanism in which exogenous antigen is phagocytosed and is then transported from the phagocytic vesicle into the cytosol to be processed via the direct presentation pathway (Rock and Shen 2005, Raghavan et al. 2008). The second pathway is a TAP-independent mechanism in which antigen is phagocytosed, followed by cathepsin S-mediated degradation of the antigen within the phagocytic vesicle. The polypeptide fragments then bind to MHC-I within the vesicle, and are transported to the cell surface for presentation (Rock and Shen 2005, Raghavan et al. 2008). It has proven difficult to compare the efficiency of direct presentation and cross-presentation quantitatively; however, direct presentation of antigen is believed to be a more efficient process than cross-presentation (Storni and Bachmann 2004). The distinction between direct presentation and cross-presentation becomes important in the context of killed virus vaccines because fully inactivated virions are unable to induce direct

presentation of viral antigen, and are able to produce $CD8^+$ T cell immunity less efficiently than many live-attenuated vaccines.

Vaccination

Antiviral Vaccination

The discovery and development of vaccination over the last 200 years is one of the great success stories in medicine. Vaccination originated with Edward Jenner in the late 18th century, who discovered that people he deliberately infected with cowpox (though it is now believed this may have actually been horsepox (Dasgupta et al. 2007)) were protected from infection with smallpox (Jenner 1798, 1800). The worldwide acceptance and implementation of smallpox vaccination ultimately led to the elimination of *Variola major* and *Variola minor* as pathogens, and in 1980 the World Health Organization certified that smallpox had been eradicated (Bhattacharya 2008). Prior to vaccination, smallpox infection was the cause of an estimated 400,000 deaths per year in Europe (Hays 2005). Since Jenner's time, vaccines have been developed to combat more than a dozen viral infections, and there are currently 14 antiviral vaccines approved for use by the Food and Drug Administration in the United States (Amanna and Slifka 2011).

The two main varieties of vaccines currently used are live-attenuated vaccines and inactivated, whole virus or subunit vaccines. Of the antiviral vaccines licensed for use in the United States, roughly 50% are live-attenuated vaccines and 50% are whole virus or subunit vaccines. For some viruses, such

as influenza and polio, both inactivated as well as alive-attenuated vaccines have been developed. However, a major focus of vaccine development is improvement of product safety, and live-attenuated vaccines occasionally have higher rates of morbidity and mortality than their inactivated counterparts. For instance, smallpox vaccination is known to result in between 1-8 deaths per million doses administered (Kretzschmar et al. 2006). Current yellow fever vaccines are known to cause 1-2 deaths per million vaccinees, and the rate of adverse events is nearly 10-fold higher among elderly populations (Kitchener 2004, Lindsey et al. 2008). Finally, live-attenuated oral polio vaccine, which is still used in developing countries, continues to be responsible for new outbreaks of vaccine-associated paralytic poliomyelitis each year (Centers for Disease Control and Prevention (CDC) 2009).

The two chemical methods currently used for inactivation of whole virus vaccines are formaldehyde inactivation and β-propiolactone inactivation (Stauffer et al. 2006). However, chemical inactivation can lead to degradation of the antigenic properties of the inactivated virions (Brown 1993). In one well known example, a formaldehyde-inactivated respiratory syncytial virus vaccine caused exacerbated disease in 16 and the death of 2 children who received the vaccine and were later infected with respiratory syncytial virus (Kim et al. 1969). In another case, a formaldehyde-inactivated measles vaccine failed to generate a protective immune response, and led to an atypical measles syndrome in vaccinees (Annunziato et al. 1982). This has led our lab to develop a new viral inactivation strategy, designed to preserve the antigenicity of whole virions.

H_2O_2 Inactivation

Oxidation with H_2O_2 is a critical mechanism of pathogen inactivation in innate immune cells, such as macrophage and dendritic cells (Valko et al. 2007). Hydrogen peroxide has also been used as an antiseptic since its discovery the early 19th century (Robertson 1975). Our lab has recently developed a series of H_2O_2-inactivated whole virus vaccines using LCMV, West Nile virus, yellow fever virus, and others that are currently being tested in mice and non-human primates. This has proven to be a remarkably effective strategy for inactivating whole virus preparations. We are able to achieve at least a 6 $\log_{10}$ reduction in LCMV virus titers by treating the virions with 3% H_2O_2 for 2 hours (Amanna, Raué, and Slifka. manuscript submitted). We are able to completely inactivate LCMV while maintaining the presence of intact virus particles (Fig 1.2). At the same time, whole West Nile virus and vaccinia virus virions inactivated with H_2O_2 are able to induce significantly higher levels of humoral immunity than formaldehyde inactivated virus (Amanna, Raué, and Slifka. manuscript submitted). These results encouraged us to move forward and determine the ability of H_2O_2-inactivated LCMV to induce $CD8^+$ T cell responses. We then went on to examine differences in immunodominance and cytokine production profiles between vaccine-induced and infection-induced $CD8^+$ T cells. The final data section of this thesis is an examination of cross-reactivity among LCMV-specific $CD8^+$ T cells.

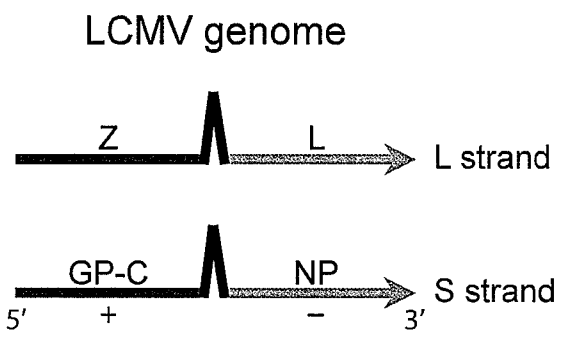

Figure 1.1: LCMV genome

The LCMV genome consists of two ambi-sense RNA strands. Each of the two strands encodes two genes separated by a stem-loop structure. The RNA on the 5' end of the stem loop is pseudo-positive sense, while the RNA on the 3' end of the stem-loop is negative sense. The L strand is 7.3 kilobases in size and contains the 11 kDa Z protein as well as the ~200 kDa L polymerase protein. The S strand is 3.4 kilobases and contains the 75 kDa GP-C protein as well as the ~63 kDa NP protein.

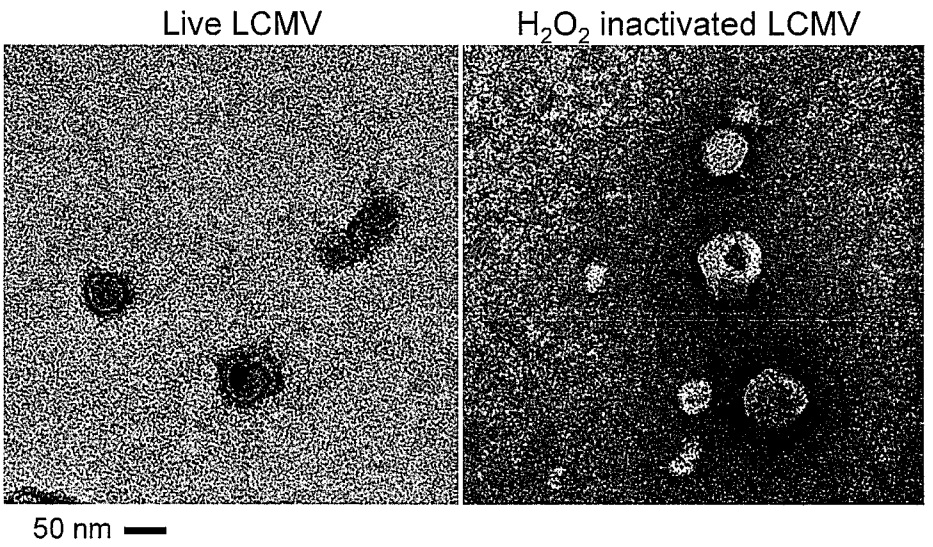

Figure 1.2: Intact LCMV virions are present following H_2O_2-inactivation

High titer LCMV was inactivated using 3% H_2O_2 for 2 hours at room temperature. Following inactivation, electron microscopy grids were prepared using untreated LCMV and H_2O_2-inactivated LCMV. The grids were coated with uranyl acetate, dried, and analyzed. The images shown were captured at 37,000 X magnification.

Chapter 2 - The Immunostimulatory Power of Acute Viral Infection

Several groups have demonstrated that antiviral CD8+ T cell responses in mice can be enormous-with virus-specific T cells representing up to 50%-80% of the total $CD8^+$ T cell population at the peak of the immune response and/or the anatomical site of infection (Murali-Krishna et al. 1998). Such dramatic T cell responses were thought to be associated with only a few select pathogens (e.g., lymphocytic choriomeningitis virus; LCMV) or perhaps related to the large virus doses and invasive routes of infection (e.g., intra- peritoneal administration) that are often used during experimental infection of animals. Acute viral infection of humans, on the other hand, was thought to result in a much smaller $CD8^+$ T cell response. However, Miller et al. (2008) provide compelling evidence indicating that the magnitude of $CD8^+$ T cell responses identified in humans after acute viral infection might indeed rival the magnitude of antiviral T cell responses observed in experimental animal models.

Miller et al. (2008) analyzed virus-specific $CD8^+$ T cell responses against two unrelated viruses: yellow fever virus (YFV) and vaccinia virus (VV). Although both YFV and VV represent acute viral infections, they differ in many ways. YFV is a small RNA virus that encodes just ten genes, and following subcutaneous inoculation, it spreads systemically, resulting in a transient viremia in the infected host. In contrast, VV is a large DNA virus encoding ~200 genes, and after transcutaneous inoculation, it rarely spreads systemically and the viral infection is

localized mainly to the inoculation site on the skin. If the authors had studied only one virus, then one might have argued that the ensuing results could be specific to just that one model. However, similar results were observed with two different viruses, lending credence to their assertion that the magnitude of the virus-specific T cell response might be larger than previously thought. Comparison of the antiviral T cell responses in both model systems side-by-side and the finding of similar outcomes within both systems (antiviral T cell responses potentially as high as 12%–40%) suggest that strong T cell activation following acute viral infection might be the rule instead of the exception.

Miller et al. (2008) used a combination of three main approaches to T cell quantitation: peptide-MHC tetramer staining, intracellular cytokine staining after *in vitro* stimulation, and phenotypic analysis of activation markers (Fig. 2.1). Each of these approaches has advantages and drawbacks. Staining of $CD8^+$ T cells with their cognate peptide antigen in the context of fluorochrome-labeled MHC Class I molecules provides direct evidence of antigen specificity but does not necessarily demonstrate function. On the other hand, phenotypic analysis of peptide-MHC tetramer-positive T cells does provide information about the native and unmanipulated in vivo phenotype of the cells. Moreover, some functions, such as granzyme and perforin expression, are probably best measured by staining of tetramer-positive T cells, but this still represents only indirect evidence of potential lytic activity. Other drawbacks associated with peptide-MHC tetramer staining are that it requires *a priori* knowledge of peptide specificity and that it only measures one peptide-specific T cell population at a time. If the peptide-

MHC combination of interest happens to be a subdominant epitope, then the majority of the T cell response could go unmeasured (and, unfortunately, unnoticed) by this approach. This might explain why tetramer staining, although highly specific, provided the lowest sensitivity in terms of detection of the total number of VV-specific T cells (0.2%-0.5% MHC Class I tetramer+ at the peak of the immune response) in the study by Miller et al. (2008). Even so, it provided kinetic data that was strikingly similar to a previous study showing that the peak VV-specific $CD8^+$ T cell response occurred at 14 days postinfection and then declined rapidly thereafter (Terajima et al. 2003).

Intracellular cytokine staining (ICCS) provides a direct functional readout via the production and detection of antiviral cytokines. If the virus of interest can be used for direct stimulation, then one may theoretically stimulate the total peptide-specific response - as long as both dominant and subdominant peptides are similarly processed and presented by the infected APC used in the assay. This is the method of choice for large viruses such as VV, because this virus preferentially infects human monocytes and presents peptides to both $CD4^+$ and $CD8^+$ T cell populations (Dasgupta et al. 2007). Alternatively, pools of overlapping peptides can be used to stimulate antiviral T cell responses directly ex vivo, and with small viruses such as YFV, this may be the preferred approach for measurement of the total virus-specific T cell response. Using direct ex vivo ICCS assays, Miller et al. (2008) show that antiviral CD8+ T cell responses against both YFV and VV appear to peak around 14 days after infection, with

virus-specific IFNγ^+ T cell frequencies reaching approximately 2% and 3%–14% of the total CD8+ T cell response for YFV and VV, respectively.

An intriguing development noted in the study by Miller et al. (2008) is that it is possible that MHC Class I tetramer staining and ICCS might both be measuring only a fraction of the total virus-specific T cell response. The authors examined the expression of phenotypic markers of T cell activation, CD38 and HLA-DR, the proliferation marker, Ki-67, as well as the anti-apoptotic protein, Bcl-2. They concluded that the total activated T cell response induced by acute viral infection might be up to 3-fold higher than the total T cell response that is measured by ICCS. The basis for this assumption is provided by the observation that MHC Class I tetramer+ VV-specific T cells are uniformly CD38$^+$HLA-DR$^+$ and Ki-67$^+$Bcl-2$^-$ at the peak of the T cell response. In contrast, MHC Class I tetramer+ T cells specific for Epstein-Barr virus or influenza virus remained negative for these phenotypic marker combinations during the effector phase that follows either YFV or VV infection - indicating that bystander activation of preexisting memory T cells of other specificities was not occurring. The kinetics of T cells expressing this activated phenotype also closely match those of the antigen-specific T cells measured by ICCS as well as by MHC Class I tetramer staining. However, YFV-specific and VV-specific memory T cells soon lose the CD38$^+$HLA-DR$^+$ and the Ki-67$^+$Bcl-2$^-$ phenotype, and therefore these markers are only useful for approximating the total T cell response elicited during the acute phase of infection. Nonetheless, this provides food for thought in terms of

the realization that the antiviral T cell response might be larger than what we can measure through current functional assays such as IFNγ-based ICCS.

There are several questions that remain unanswered with regard to the Miller study (Seder et al. 2008). For instance, if intracellular IFNγ production "tags" only one- third of the total virus-specific T cell response (as measured by an activated $CD38^{+}HLA\text{-}DR^{+}$ or $Ki\text{-}67^{+}Bcl\text{-}2^{-}$ T cell phenotype), then what cytokines, if any, do the other two-thirds of the $CD8^{+}$ T cells make following stimulation with antigen? Does this mean that the majority of the T cell response represents non-IFNγ producers in humans, or is it possible that our approaches to *in vitro* stimulation require further optimization? Others have commented that because of the multifunctional nature of heterologous T cell populations, measurement of any one cytokine alone could lead to a lower, more conservative estimate of the total antigen-specific T cell response (Seder et al. 2008). With this in mind, it will be interesting to learn the full spectrum of human cytokine production profiles that are elicited following various acute viral infections in both the effector and the memory phases of the immune response.

On the basis of the results of the study by Miller et al. (2008), it appears clear that virus-specific T cell responses following acute infection in humans are larger than expected and vary substantially depending on the efficiency of the technique used to measure them. Importantly, both YFV and VV represent viruses that infect the host through a peripheral route. It will be interesting to learn whether respiratory viral infections such as influenza virus or respiratory syncytial virus are also capable of eliciting large frequencies of virus-specific

$CD8^+$ T cells in the bloodstream. Further in-depth analysis of virus-specific T cell kinetics will be important, given that it appears that $CD8^+$ T cell responses decay very rapidly in the short term (i.e., the first weeks after infection) compared to the long term (i.e., months or years after vaccination (Co et al. 2002, Hammarlund et al. 2003, Crotty et al. 2003). Analysis of T cell responses following booster vaccination or re-infection will be another interesting avenue of investigation. Together, these studies will lead to a better understanding of antiviral T cell responses and maintenance of immunological memory in humans.

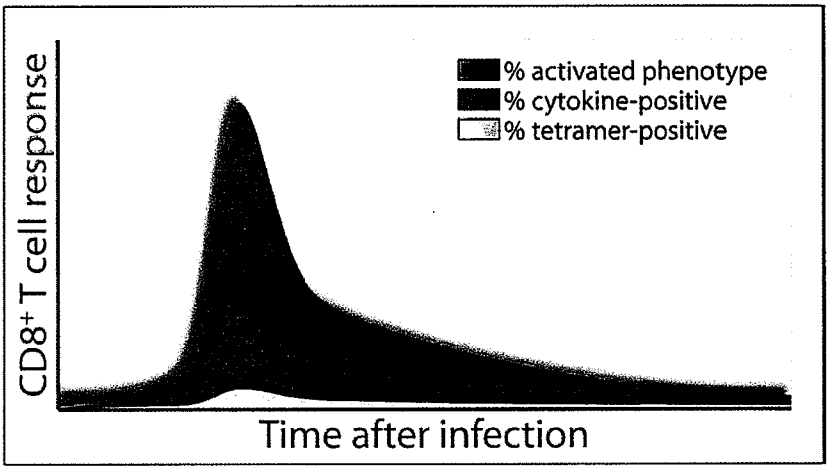

Figure 2.1: Virus-Induced T Cell Responses During Acute Infection

This figure illustrates the concept that current methods for the detection of virus-specific T cells might represent a substantial underestimate of their true frequency during the course of an acute viral infection. The MHC Class I tetramer-positive population represents T cells that bind a single peptide-specific MHC Class I tetramer, such as the HLA-A2-VV^{CLT} tetramer used in the study by Miller et al. (2008). The cytokine-positive population represents cells that produce cytokines such as IFNγ after stimulation with live virus or overlapping 15-mer peptide pools followed by intracellular cytokine staining (ICCS) directly ex vivo. The activated phenotype population represents either the $CD38^{+}HL$ -DR^{+} subset or the Ki-$67^{+}Bcl\text{-}2^{-}$ subset, which are shown by Miller et al. (2008) to be roughly equivalent in magnitude. The data presented in this study indicate that methods such as ICCS might identify only about one-third of the total human $CD8^{+}$ T cell response elicited by an acute viral infection.

Chapter 3 - Longevity of T-Cell Memory Following Acute Viral Infection

Abstract

Investigation of T cell-mediated immunity following acute viral infection represents an area of research with broad implications for both fundamental immunology research as well as vaccine development. Here, we review techniques that are used to assess T cell memory including limiting dilution analysis, enzyme-linked immunospot (ELISPOT) assays, intracellular cytokine staining (ICCS), and peptide-MHC Class I tetramer staining. The durability of T cell memory is explored in the context of several acute viral infections including vaccinia virus (VV), measles virus (MV), and yellow fever virus (YFV). Following acute infection, different virus-specific T cell subpopulations exhibit distinct cytokine profiles, and these profiles change over the course of infection. Differential regulation of the cytotoxic proteins, granzyme A, granzyme B, and perforin are also observed in virus-specific T cells following infection. As a result of this work, we have gained a broader understanding of the kinetics and magnitude of antiviral T cell immunity as well as new insight into the patterns of immunodominance and differential regulation of cytokines and cytotoxicity-associated molecules. This information may eventually lead to the generation of more effective vaccines that elicit T cell memory with the optimal combination of functional characteristics required for providing protective immunity against infectious disease.

Introduction

The concept of immunological memory is well established, but it was not until the twentieth century that the cellular origins of antimicrobial immunity and the basis of immunological memory first began to be elucidated (Masopust et al. 2007). It is remarkable how the field of immunology has changed in the four decades that have passed since Mitchell & Miller postulated the existence of T cells as a distinct subset of small lymphocytes (Mitchell and Miller 1968) (Fig. 3.1). During this time, several of the initial questions regarding T cell specificity and function have been answered and yet many new questions regarding the dynamics and functional attributes of the memory T cell compartment have been raised. To answer these questions, a multitude of quantitative techniques have been developed and optimized to assess T cell memory. In the early days of immunology, these techniques often consisted of bulk analysis of broadly defined cell populations with little understanding of the mechanisms employed in their function. Today, it is possible to analyze cytokine production, cytolytic potential and the phenotype of highly defined subsets of even rare T cell populations directly ex vivo. Thus, the evolution of T cell analysis (Fig. 3.1) is characterized by a trend toward measuring more precisely defined T cell subsets and developing a progressively more refined ability to determine function at the single cell level.

Cytotoxic activity has been a mainstay of T cell analysis since its development in the 1970s. Through the use of Cr^{51}-release assays, Cerottini *et al.* demonstrated that the cytotoxic T cells present in a mixed population of

lymphocytes could be depleted with anti-θ antibodies (Cerottini et al. 1970). Shortly thereafter, others proved that depletion of B-cells had no effect on the cytotoxic properties of mixed lymphocyte populations (Golstein et al. 1972), proving that it was indeed the thymus-derived lymphocyte population that was responsible for cell-mediated destruction of allogenic targets. This discovery was followed by the work of two independent groups who demonstrated that cells bearing the CD8 antigen were responsible for the cytolytic activity of T cells (Shiku et al. 1975, Kisielow et al. 1975, Cantor and Boyse 1975), further defining this population. During this time, Taswell and colleagues developed limiting dilution assays which allowed the frequency of antigen-specific T cells to be quantitatively determined (Taswell et al. 1979, 1980, Taswell 1981). Limiting dilution assays remained the cornerstone of T cell quantitation until the late 1980s and early 1990s when the development of cytokine ELISPOT assays (Czerkinsky et al. 1988), intracellular cytokine staining (ICCS) (Jung et al. 1993, Maino et al. 1995, Picker et al. 1995) and peptide-MHC Class I tetramers (Altman et al. 1996) greatly expanded the number of techniques that could be used for quantitatively measuring T cell responses directly ex vivo. In a landmark study published in 1998, Murali-Krishna *et al.* (Murali-Krishna et al. 1998) demonstrated that IFNγ ELISPOT assays, IFNγ ICCS, and peptide-MHC Class I tetramers all identified the same approximate frequency of peptide-specific $CD8^+$ T cells following infection of mice with lymphocytic choriomeningitis virus (LCMV). Moreover, they compared these new techniques to the standard approach of limiting dilution analysis and demonstrated that the number of

antigen-specific T cells determined by the older technique was off by 10-fold or more. In other words, limiting dilution analysis, the best technique available up until the 1990's, was detecting <10% of the total virus-specific T cell response identified by ELISPOT, ICCS, or peptide-MHC Class I tetramers. By performing T cell quantitation by each of these approaches in one comprehensive study, this work provided the first "Rosetta stone" for understanding how these different approaches to T cell quantitation compared with each other in direct side-by-side analysis. Remarkably, peak antiviral T cell responses to LCMV reach about 50-80% of the total $CD8^+$ T cells in the spleen (Butz and Bevan 1998a, Murali-Krishna et al. 1998). This is likely due to the tropism of the virus; LCMV infects lymphoid tissues such as the spleen and virus-specific T cells preferentially home to, and proliferate at, sites of infection. Peak $CD8^+$ T cell responses against vaccinia also reach ~25% of total splenic T cells (Harrington et al. 2002) and frequencies of virus-specific T cells in nonlymphoid organs can also be quite high (Masopust et al. 2001). For instance, the frequency of virus-specific T cells may reach 25-80% of the total T cell population in the lungs following acute respiratory infection (Hogan et al. 2001, Belz et al. 2001, Chang and Braciale 2002) or in the brain following infection with neurotropic viruses (Johnson et al. 1999, Marten et al. 2003). The high frequency of virus-specific T cells observed in these murine studies was thought to be restricted to rodent models of acute viral infection. However, as discussed later in this chapter, high frequencies of virus-specific T cells are now being identified during acute human infection as well. With the advent of polychromatic flow cytometry, in the last decade it has

become possible to analyze ten or more fluorescence parameters (Perfetto et al. 2004) and this now makes it feasible to simultaneously analyze phenotype, cytokine production, cytolytic potential, proliferative status, and viability of T cells identified by peptide-MHC tetramers or by antigenic stimulation. These advances in technology have lead to exciting new developments in our understanding of human T cell memory.

Memory T cell responses following acute viral infection

T cell memory can be surprisingly long-lived and studies examining the duration of cellular immunity following smallpox vaccination have demonstrated that antiviral $CD4^+$ and $CD8^+$ T cell responses can be identified for up to 75 years after a single acute viral infection (Hammarlund et al. 2003, Slifka 2004). Measurement of virus-specific T cell frequency however, represents only one dimension of T cell memory, and as our ability to quantitate and functionally assess T cells has evolved, our understanding of the dynamics and duration of T cell memory to acute viral pathogens has continued to grow. There are no cross-reactive orthopoxviruses endemic to the US and so analysis of immunity following smallpox vaccination with vaccinia virus (VV) provides an opportunity to measure T cell memory in the absence of environmental re-exposure (Slifka 2004). Likewise, analysis of T cell memory following childhood measles virus (MV) infection or vaccination (e.g., MMR; measles, mumps, rubella vaccination) or yellow fever virus (YFV) vaccination also provide important information regarding T cell memory to viruses that cause only rare outbreaks (MV) or are no longer endemic in the US (YFV).

Edward Jenner was the first to formally demonstrate long-term protective immunity against orthopoxviruses (Jenner 1798) and in 1800, he published a report demonstrating that immunity following cowpox infection (the basis of contemporary smallpox vaccination) could be maintained for >50 years after infection (Jenner 1800). Over 200 years passed before the technology was available to quantitatively measure the duration/half-life of human T cell responses following smallpox vaccination. In one study, VV-specific T cell responses measured mainly by IFNγ ELISPOT analysis showed that T cell memory was detectable for >50 years while declining slowly with a half-life of ~14 years (Crotty et al. 2003). Likewise, we measured the frequency of $IFN\gamma^{+}TNF\alpha^{+}$ VV-specific memory T cells by ICCS and found that memory could be maintained for up to 75 years and that virus-specific $CD4^{+}$ and $CD8^{+}$ T cell responses declined with a half life of approximately 8-15 years (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008) (Fig. 3.2). Despite using different T cell quantitation techniques, these independent studies were in close agreement in terms of the estimated half-life of long-term T cell memory. However, these studies were focused primarily on memory T cell responses analyzed several years after vaccination and further studies have now examined the earlier kinetics of VV-specific T cell responses (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008). Interestingly, primary antiviral T cell responses in most human subjects peak between 14-21 days after VV infection (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008), which is a

substantial delay compared to VV-specific T cell responses in mice, which peak within the first 7 days after infection (Harrington et al. 2002). Another interesting observation revealed by these studies (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008) is that antiviral $CD8^+$ T cell responses decline dramatically over the course of the first few weeks/months after the infection has cleared before reaching a more stable, albeit slowly declining plateau phase of immunological memory. Comparing studies that examined the early VV-specific T cell response (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008) to the studies that focused on long-term T cell memory (Hammarlund et al. 2003, Crotty et al. 2003), it appears that antiviral T cell responses may decline in a biphasic manner; a rapid initial decline in virus-specific T cell numbers followed by a slower decline in T cell memory at later time points. With this in mind, it is possible that the 8-15 year half life of T cell memory following VV infection (Hammarlund et al. 2003, Crotty et al. 2003) may be an overly conservative estimate and once these different decay rates are separated, the duration of late-stage T cell memory at >1 year post-infection may be longer than currently estimated.

Analysis of virus-specific $CD4^+$ T cell memory versus $CD8^+$ T cell memory following VV infection has also revealed some interesting differences between these two T cell subsets. Although the long term T cell half life estimates are similar ($T_{1/2}$ = 8-12 years and $T_{1/2}$ = 8-15 years for $CD4^+$ and $CD8^+$ T cells, respectively), $CD4^+$ T cell memory appeared to be maintained more efficiently

than $CD8^+$ T cell memory since nearly half of VV-immune subjects lost detectable $CD8^+$ T cell responses within 20 years after vaccination whereas $CD4^+$ T cell responses declined but remained readily detectable in most individuals during the same observation period (Hammarlund et al. 2003). Similar results were found by an independent group (Amara et al. 2004) and this observation may be due, at least in part, to strikingly different dynamics in the early kinetics of the T cell response following VV infection. When compared side-by-side, VV-specific $CD4^+$ T cell numbers do not reach the same peak levels as the coinciding $CD8^+$ T cell response, but they also do not decline as sharply as their $CD8^+$ T cell counterparts during the first weeks/months after infection (Amara et al. 2004, Miller et al. 2008). This indicates that the kinetics and relative magnitude of human $CD8^+$ and $CD4^+$ T cell subpopulations differ substantially following this acute viral infection and a better understanding of why these differences exist will be an important area of future investigation.

Lifelong immunity occurs following childhood infection with MV and this was perhaps most clearly demonstrated by Panum (Panum 1847) who showed that following a MV epidemic in 1781, isolated inhabitants on the remote Faroe Islands were protected against reinfection during a second outbreak that occurred 65 years later in 1846. Since the island was not visited during the intervening years between these two outbreaks, it appears that antiviral immunity persisted in the absence of environmental re-exposure. It is believed that strong T cell responses are important for protection against MV (van Els and Nanan 2002) and several techniques have been used to measure MV-specific T cell

responses including direct *ex vivo* CTL assays, ELISPOT, ICCS and peptide-MHC Class I tetramer staining (Jaye et al. 1998, Nanan et al. 2000, van Els and Nanan 2002, Ota et al. 2007). One study (Naniche et al. 2004) examined the relative levels and duration of MV-specific T cell memory following 10 days of *in vitro* expansion of carboxyfluorescein succinimidyl ester (CFSE)-labeled lymphocytes. Following this period of expansion, MV-reactive $CD4^+$ and $CD8^+$ T cell responses could be detected for up to 34 years after vaccination and interestingly, the study suggests that $CD8^+$ T cell memory remained largely intact whereas $CD4^+$ T cell responses appeared to decline over time. This appears to be different from the results observed following VV infection wherein $CD8^+$ T cells initially decline more rapidly than $CD4^+$ T cells and then at later time points both populations decline slowly with similar decay rates. Further studies will be needed to determine if $CD4^+$ and $CD8^+$ T cell memory responses following VV infection represent a paradigm for T cell responses to other acute viral infections or whether each viral infection instead triggers T cell memory with different long-term kinetic patterns for $CD4^+$ and $CD8^+$ T cell subsets.

Humoral immunity against YFV can be maintained for up to 75 years after infection (Sawyer 1931) but there is relatively little known about the overall duration of YFV-specific T cell responses. One longitudinal study identified stable T cell memory by IFNγ ELISPOT analysis for up to 18 months after YFV infection (Co et al. 2002) but another more recent study by Miller *et al.* (Miller et al. 2008)(Miller et al. 2008) has provided further insight into the kinetics and magnitude of the early phases of the antiviral T cell response to YFV. In this

study, antiviral T cell responses against YFV and VV were compared using multiple quantitation techniques including peptide-MHC Class I tetramer staining, ICCS and two sets of phenotypic markers that identified activated T cell populations. As indicated in Figure 3.3, peptide-MHC Class I tetramer staining identifies a small defined T cell population with specificity to only a single peptide epitope bound to one MHC haplotype. Use of IFNγ ICCS (or ELISPOT) allows T cells of multiple antigenic specificities to be identified by using peptide pools or infected APC for stimulation. If more than one cytokine is used for T cell quantitation, then an even larger frequency of virus-specific T cells can be identified because it will include T cell populations that do not produce IFNγ directly *ex vivo*. In addition to these antigen-specific assays (peptide-MHC Class I tetramer binding or cytokine production), analysis of virus-induced T cell populations can also be indirectly estimated by measuring the frequency of T cells bearing an activated $CD38^{+}HLA\text{-}DR^{+}$ or $Ki\text{-}67^{+}Bcl\text{-}2^{-}$ phenotype (Zaunders et al. 2006, Miller et al. 2008). Using either of these phenotypic marker combinations, it appears that there are ~3-fold more T cells elicited by VV and YFV infection than what are measured using other current approaches to T cell quantitation. Based on the Miller study (Miller et al. 2008), the frequency of virus-specific T cells at the peak of the antiviral immune response could reach as high as 12% to 40% of total peripheral T cells following YFV or VV infection, respectively. This is intriguing because it indicates that human T cell responses to acute viral infection may be far more robust than previously realized (Walker and Slifka 2008, Miller et al. 2008). These studies also showed that YFV-specific

T cell kinetics mimic the responses observed following VV infection, with antiviral T cell responses peaking by ~14 days after infection and then dropping rapidly before reaching the memory phase of the immune response within 1-6 months after infection.

Functional attributes of human memory T cells

Concerns with bioterrorism and the threat of natural emerging/re-emerging infectious disease has greatly accelerated our understanding of human T cell immunology. As noted above, we have gained considerable information in terms of the induction and maintenance of immunological memory. We have also made substantial progress in understanding the basic antiviral functions of human $CD4^+$ and $CD8^+$ T cells and their role in antiviral immunity. The NIH funded a program entitled, "Large Scale B- and T-cell Epitope Discovery" and this has led to an explosion of research dedicated to identifying $CD4^+$ and $CD8^+$ T cell epitopes in a variety of human pathogens (see http://www.immuneepitope.org). For orthopoxvirus research in particular, this has revolutionized the field. Over 170 human and murine T cell epitopes have been mapped in VV (Kennedy and Poland 2007) and this has led to the opportunity to ask immunological questions that would have otherwise been unfeasible. Is T cell immunodominance a factor in shaping the immune response to a complex virus in outbred human populations? What are the attributes of highly immunogenic viral proteins? Are early gene products targeted more often than late genes? With a toolbox of mapped $CD4^+$ and $CD8^+$ T cell epitopes in hand, we are beginning to find answers to these fundamental questions.

T cell immunodominance occurs when the majority of the T cell response is directed to only a small number of potential peptide epitopes. VV represents a large DNA virus with approximately 180 predicted open reading frames (ORFs) and the potential to harbor many peptide epitopes. Close to 120 human $CD8^+$ T cell epitopes have been mapped across 103 VV ORFs, making it clear that the antiviral $CD8^+$ T cell response following this acute viral infection is remarkably broad (Kennedy and Poland 2007). This is not just a characteristic of VV since $CD8^+$ T cell responses to MV are also diverse and one study mapping HLA-A2-restricted T cells found that no single peptide dominated the T cell response (Ota et al. 2007). Similar to VV-specific $CD8^+$ T cell responses, VV-specific $CD4^+$ T cell responses are surprisingly diverse with $CD4^+$ T cells recognizing 122 different VV ORFs (Jing et al. 2008). On average, each subject developed $CD4^+$ T cell responses against 39 VV ORFs with a range of 13 to 63 VV ORFs. In comparison, a proteome-wide analysis of antiviral $CD4^+$ T cell responses to human cytomegalovirus (CMV) revealed a median of 12 CMV ORFs with as many as 39 CMV ORFs being recognized by $CD4^+$ T cells (Sylwester et al. 2005). Although these viruses are similar in genome size and number of ORFs, they differ significantly in the sense that VV induces only an acute infection that is rapidly cleared whereas CMV induces a chronic and lifelong infection.

A meta-analysis of viral protein immunogenicity was performed based on 8 studies that together included 151 human and murine $CD8^+$ T cell epitopes spanning 62 VV ORFs (Kennedy and Poland 2007). Based on VV protein expression kinetics, 47% of the $CD8^+$ T cell response targeted early genes, 19%

targeted late genes and the remaining 34% of the response targeted genes with unknown kinetics or both early and late kinetics. Although not absolute, these results indicate that $CD8^+$ T cells show a trend toward preferentially targeting early gene products. Comparisons between viral proteins divided according to functional attributes (e.g., replication/viral regulation, virulence/host range or structural) did not reveal a clear preference in $CD8^+$ T cell recognition in this meta-analysis. Comparison of protein localization indicated that nearly half (48%) of the $CD8^+$ T cell response targeted intracellular proteins with 12% of the T cells targeting membrane proteins, 8% targeting secreted proteins and 32% of the response directed towards proteins of unknown localization. In a large proteomic analysis of $CD4^+$ T cell responses to VV, the most commonly targeted ORFs included structural proteins and proteins with late expression kinetics (Jing et al. 2008). There was also a trend towards higher recognition of larger vs. smaller virus proteins (Jing et al. 2008). Similar results were observed after mapping murine T cell epitopes in VV-infected mice showing that $CD8^+$ T cell responses tended to target early gene products whereas $CD4^+$ T cell epitopes showed a modest trend towards recognition of late gene products (39% early, 61% late gene recognition by $CD4^+$ T cells, respectively) (Moutaftsi et al. 2007). Together, this indicates that T cell responses to a complex virus such as VV is broad and targets a wide variety of proteins based on localization, structure/size and time of gene expression, which together is likely to provide efficient recognition of infected cells during the course of acute viral infection.

Perhaps the most important aspect of T cell memory is the ability to express a variety of antiviral effector molecules upon cognate interactions with their specific peptide antigen. VV-specific T cells produce a number of different cytokines including IFNγ, TNFα, IL-2, IL-4, IL-13, and MIP1β (Amara et al. 2004, Precopio et al. 2007, Kannanganat et al. 2007), costimulatory adhesion molecules such as CD40L (Kannanganat et al. 2007, Sette et al. 2008) and cytolytic molecules such as granzyme A, granzyme B, and perforin (Rock et al. 2005, Nowacki et al. 2007, Precopio et al. 2007, Miller et al. 2008). The expression pattern of different effector molecules depends on the T cell subpopulation (CD4 vs. CD8) as well as the time point examined after infection, since expression profiles often change dramatically between the peak of the antiviral T cell response and the resting memory stages of the immune response. Although both $CD4^+$ and $CD8^+$ T cells have the ability to express IFNγ, TNFα, and IL-2, only VV-specific $CD4^+$ T cells express the Th2 cytokine, IL-13 (Amara et al. 2004). IFNγ is the most common cytokine used to measure T cell responses and in murine models there is essentially a 1:1 ratio between $IFN\gamma^+$ $CD8^+$ T cells and peptide-MHC Class I $tetramer^+$ T cells (Murali-Krishna et al. 1998). With human T cells (especially human $CD4^+$ T cells), it is becoming clear that IFNγ production may identify only a subpopulation of the total virus-specific T cell response. Some studies have identified VV-specific $CD4^+$ T cell clones that proliferate against VV antigens, but fail to produce IFNγ (Jing et al. 2007). Likewise, other studies have identified primary VV-specific $CD4^+$ T cells that were IFNγ-negative but still produced other cytokines including TNFα, IL-2, or IL-13 in

response to VV stimulation (Amara et al. 2004, Zaunders et al. 2006). Indeed, analysis of IFNγ, TNFα, and IL-2 production by VV-specific $CD4^+$ T cells revealed T cell subpopulations producing each of the 7 possible combinations of these 3 cytokines (Kannanganat et al. 2007). Compared to $CD4^+$ T cells, VV-specific $CD8^+$ T cells are more likely to express IFNγ. However, detailed analysis of cytokine profiles including IFNγ, TNFα, IL-2 and MIP1β also demonstrate the existence of VV-specific $CD8^+$ T cell subpopulations that express a variety of cytokines in the absence of IFNγ production (Precopio et al. 2007). These variations in cytokine expression patterns may explain the dichotomy observed in long-term $CD8^+$ T cell responses measured by Hammarlund *et al.* (Hammarlund et al. 2003). In those studies, polyfunctional $CD4^+$ and $CD8^+$ T cell responses were measured on the basis of dual production of two cytokines, IFNγ and TNFα. Although $CD4^+$ T cell responses appeared fairly uniform in their decay rates, $CD8^+$ T cell responses split into two groups by 20 years post-vaccination wherein about half of VV-immune individuals maintained detectable $CD8^+$ T cell memory and the other half of the sample population appeared to lose detectable VV-specific $CD8^+$ T cell responses (Fig. 3.2). Retesting of a subset of these samples has revealed that many of the VV-specific $CD8^+$ T cells examined at >20 years after infection had not actually disappeared, but instead appear to make other cytokine combinations besides IFNγ and TNFα (Slifka and Hammarlund, unpublished results) and were not previously identified because they did not produce both IFNγ and TNFα. This emphasizes the point illustrated in Figure 3.3 indicating that it is important to measure as many cytokine combinations as

possible when quantitating T cell memory because measuring any cytokine alone is likely to lead to a conservatively lower estimate of the total antigen-specific T cell response.

Perforin and granzyme B are expressed by nearly all VV-specific MHC Class I tetramer-positive $CD8^+$ T cells at early time points after infection (Miller et al. 2008). Interestingly, <8% of VV-specific $CD8^+$ T cells express perforin directly *ex vivo* by one month after infection (Rock et al. 2005). However, the memory T cells are able to re-express perforin following 7 days of *in vitro* restimulation, indicating that although this important cytolytic molecule is rapidly downregulated *in vivo* after VV infection has cleared, it can be quickly upregulated after re-exposure to specific viral antigens (Miller et al. 2008). In contrast to perforin, other cytolytic molecules such as granzyme A and granzyme B continue to be expressed in a sizeable subpopulation of MHC Class I tetramer-positive or restimulated $IFN\gamma^+$ $CD8^+$ T cells at 1 month post-infection (Rock et al. 2005, Precopio et al. 2007, Miller et al. 2008). The proportion of $CD8^+$ T cells expressing both granzyme A and granzyme B declines from 60% at one month to 33% of the virus-specific T cell response at one year post-infection (Rock et al. 2005). This is still a relatively high percentage of memory cells expressing granzymes when compared to perforin expression, which has dropped to nearly baseline levels within the first month after infection. This indicates that, similar to shifting virus-specific cytokine expression profiles, cytolytic proteins such as perforin and granzymes A and B are differentially regulated after acute viral infection.

In contrast to $CD8^+$ T cells, there is much less known about the kinetics and expression levels of cytolytic proteins in cytotoxic $CD4^+$ T cells. Although most cytotoxic T lymphocytes are $CD8^+$ T cells, it is important to note that cytotoxic $CD4^+$ T cells have been identified directly *ex vivo* following human MV infection (Jaye et al. 1998) and the development of cytolytic $CD4^+$ T cell responses against acute and chronic viral infections are far more common than one might expect. Virus-specific $CD4^+$ CTL are MHC Class II-restricted (Schmid 1988, Bourgault et al. 1989, Penna et al. 1992, Littaua et al. 1992, Erickson and Walker 1993, Demkowicz et al. 1996, Mitra-Kaushik et al. 2007) and have been identified following infection with MV (Jaye et al. 1998), VV(Littaua et al. 1992, Erickson and Walker 1993, Demkowicz et al. 1996, Mitra-Kaushik et al. 2007), polio (Wahid et al. 2005), dengue (Green et al. 1997), influenza (Bourgault et al. 1989), hepatitis B virus (Penna et al. 1992), varicella zoster virus (Arvin et al. 1991), Epstein Barr virus (Bourgault et al. 1989, Münz et al. 2000), herpes simplex virus (Schmid 1988) and CMV (Gyulai et al. 2000, Appay et al. 2002). Although MHC Class II (e.g., HLA-DR) is typically expressed on professional APC, following infection and the resulting inflammatory cytokine response, MHC Class II is upregulated on non-professional APC including human epithelial cells (Rossi et al. 1990, Wang et al. 1997, Stríz et al. 2000, Papon et al. 2002, Hegde and Johnson 2003, Rees et al. 2003) as well as highly activated virus-specific T cells (Miller et al. 2008) and potentially other cell types as well. This indicates that during acute infection, cytolytic $CD4^+$ T cells may be capable of enhanced

immune surveillance due to the transient upregulation of MHC Class II on a broader array of host cells.

Conclusions

Over the last several decades a number of different techniques have been developed to assess the duration and functional characteristics of T cell-mediated immunity following acute viral infection. These techniques have been refined to permit the detection of precisely defined, low frequency antigen-specific T cell subsets directly ex vivo. Analysis of T cell memory following infection with VV, MV or YFV has provided valuable insight into the kinetics, magnitude and duration of virus-specific T cell responses. $CD4^+$ and $CD8^+$ T cell memory has been demonstrated up to 75 years after VV infection and 34 years following MV infection. Although the half-life of VV-specific T cells has been calculated at 8-15 years following VV infection, it is unclear if this degree of immunological memory is representative of other acute viral infections or if this is specific only to VV infection. Likewise, more information on the relative duration of $CD4^+$ versus $CD8^+$ T cell memory following acute viral infection is needed in order to determine if there are virus-specific patterns of T cell memory or if the immune response to a variety of viruses is similar. The expression of effector cytokines and cytotoxic proteins has been shown to evolve throughout the course of primary viral infection and memory T cell generation. This effect can be observed in shifting cytokine production profiles of virus-specific T cells as well as the differential regulation of perforin compared to granzyme A and granzyme B. The biological relevance of these various patterns of effector molecule

expression in the context of acute human infection have yet to be fully understood.

Analysis of T cell memory following acute infection by viruses such as VV, MV and YFV illustrate both the great strides that have been made in our knowledge of T cell-mediated immunity as well as the sizeable gaps that remain in our understanding of human T cell immunobiology. It will be exciting to learn the mechanisms that govern the longevity of memory T cells induced by acute viral infection and learn how to best mimic these forms of immunological memory by developing improved vaccines that elicit effective and long-lived T cell responses.

Acknowledgements

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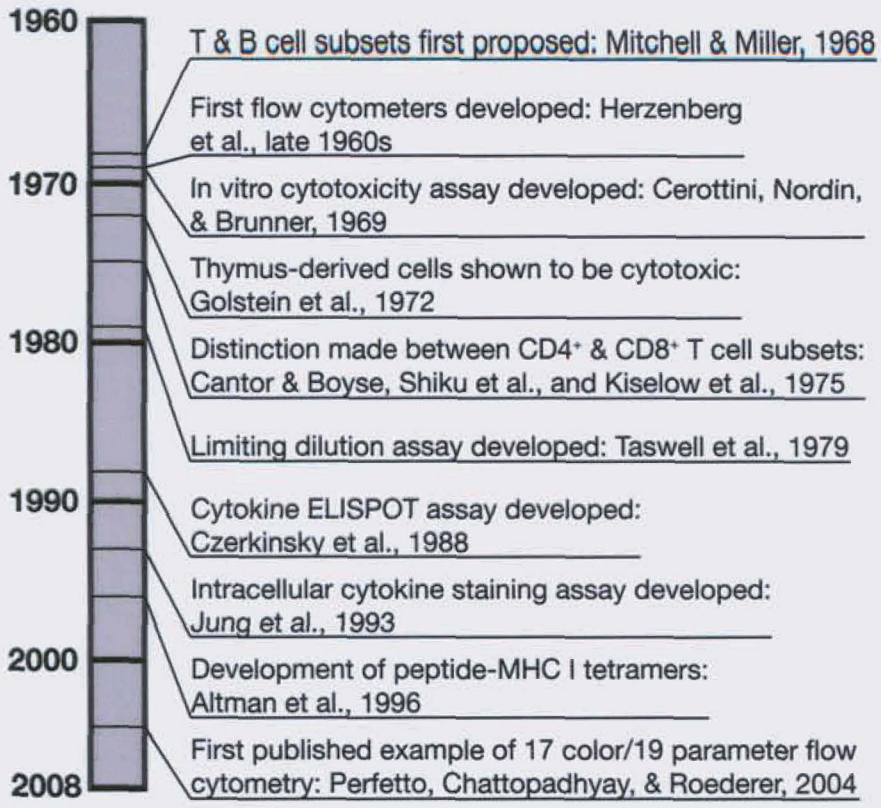

Figure 3.1: Historical advances involved with analysis of T cell memory

In 1968, Mitchell & Miller proposed the existence of a subset of lymphocytes that were thymus-derived and distinct from bone marrow-derived B cells (Mitchell and Miller 1968), setting the next four decades of T cell research in motion. The development of the *in vitro* cytotoxicity assay in the late 1960s (Golstein et al. 1972) made it possible to determine that T cells, and not B cells, were the component of small lymphocytes that were directly cytotoxic (Golstein et al. 1972). In the mid 1970s, the concept of $CD4^+$ and $CD8^+$ T cell subsets arose when it was determined that helper activity and cytotoxic activity were restricted to distinct subpopulations of T cells (Shiku et al. 1975, Kisielow et al. 1975). In the late 1960s, the first flow cytometers were developed (Herzenberg et al. 2002) and this technology would later come to play an integral role in T cell analysis. In the late 1970s, the limiting dilution assay was developed (Taswell et al. 1979, 1980, Taswell 1981). This is a significant landmark in T cell analysis because the limiting dilution assay was the first technique to quantitatively assess T cell responses to antigen. In the late 1980s, the cytokine ELISPOT (enzyme-linked immunospot) assay was developed, allowing T cell responses to be quantitatively measured without requiring ex vivo T cell expansion (Czerkinsky et al. 1988). In the early 1990s, intracellular cytokine staining assays were developed, making it possible to quantitate the expression of multiple cytokines directly ex vivo (Jung et al. 1993, Maino et al. 1995, Picker et al. 1995). In 1996, peptide-MHC Class I tetramers were developed (Altman et al. 1996). The introduction of tetramer reagents made it possible to quantitatively measure the frequency of peptide-specific T cell populations regardless of their function/cytokine profiles and without performing ex vivo restimulation. Since the antigen-specific T cells do not require restimulation to be visualized with peptide-MHC Class I tetramers, the native *in vivo* phenotype of the cells is also preserved. By the late 1990s and through today, polychromatic flow cytometry (Herzenberg et al. 2002, Perfetto et al. 2004) has revolutionized the study of T cell function and phenotype by making it possible to simultaneously analyze T cell lineage markers, phenotype, cytokine profiles, and cytotolytic protein expression of even rare T cell populations directly ex vivo.

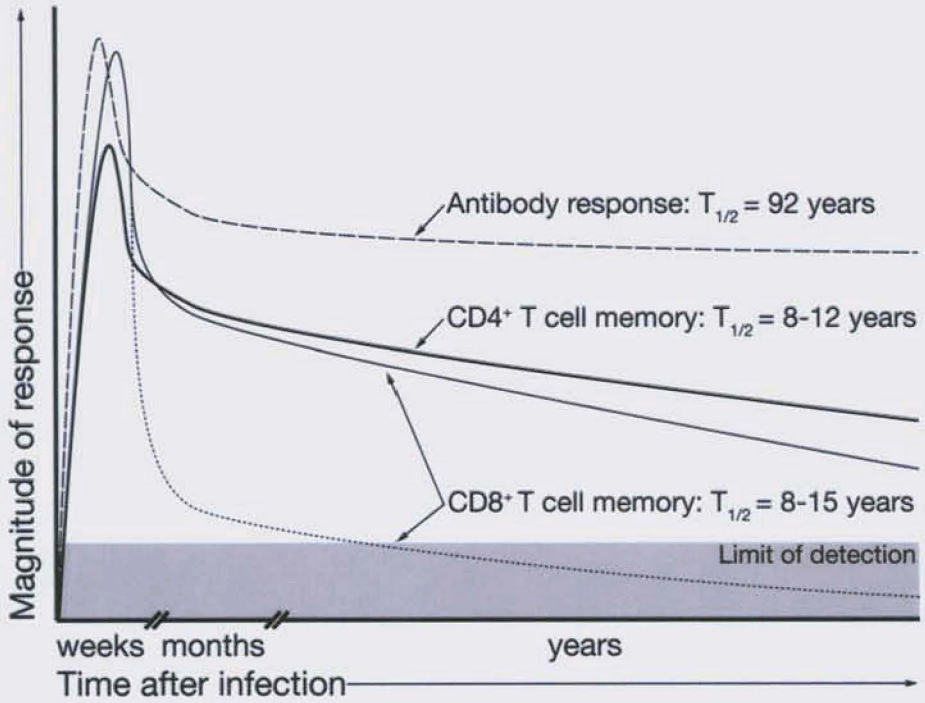

Figure 3.2: Immunological memory following vaccinia virus (VV) infection.

Vaccinia virus infection provides a prototype for understanding the kinetics and duration of antiviral immunity following acute viral infection. Antiviral antibody responses peak within a few weeks after infection and after passing through a short period of decline, the humoral immune response remains quite stable, declining with a half-life of approximately 92 years (Amanna et al. 2007). Virus-specific $CD4^+$ and $CD8^+$ T cell responses peak within 2-3 weeks after infection (Terajima et al. 2003, Amara et al. 2004, Kennedy et al. 2004, Treanor et al. 2006, Miller et al. 2008) and then following an early rapid decay rate, the estimated half-life of T cell memory is approximately 8-15 years (Hammarlund et al. 2003, Crotty et al. 2003). Unlike the antiviral $CD4^+$ T cell response that is relatively uniform in terms of decay kinetics, antiviral $CD8^+$ T cell responses appeared to split, with approximately half of the VV-immune population losing detectable $CD8^+$ T cell memory at some time in the 20 years post-vaccination. Further studies are needed, but it is possible that by using multiple cytokine combinations for estimating memory T cell frequencies, we may find $CD8^+$ T cell memory maintained in a larger proportion of subjects than the number determined based only the frequency of $IFN\gamma^+TNF\alpha^+$ T cells (Hammarlund et al. 2003).

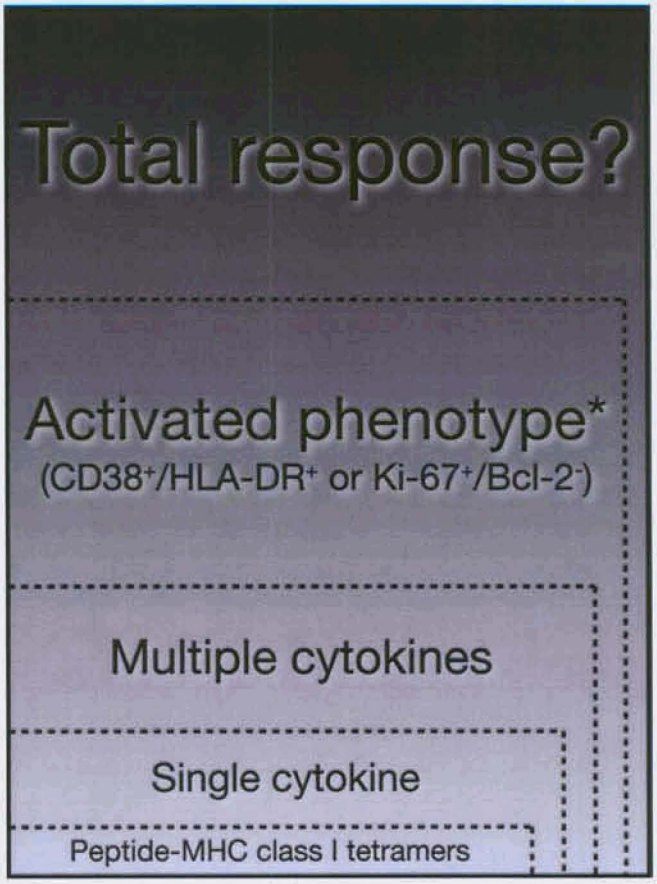

Figure 3.3: Comparison between different quantitation approaches used to measure T cell memory.

The measured frequency of antigen-specific T cells depends to a great extent on which techniques are used for their quantitation. This figure shows an illustration of the proportion of a virus-specific T cell response that can be detected using currently available techniques. Peptide-MHC Class I tetramer analysis is highly specific, but identifies the lowest percentage of the total virus-specific T cell response due to measuring only T cells with a single peptide specificity. Measuring a single cytokine such as IFNγ by ICCS allows identification of a potentially broader subset of T cells than using a peptide-MHC Class I tetramer due to the ability to use pools of peptides, entire viral antigens or virus-infected APC. Performing ICCS that is based on the production of multiple cytokines may further increase the number of virus-specific T cells that are measured due to identification T cell populations that may not produce any one cytokine of interest. For instance, several studies have found virus-specific T cell populations that fail to produce IFNγ, the most common cytokine used for measuring T cell memory. T cell quantitation based on phenotypic analysis using markers such as $CD38^+HLA\text{-}DR^+$ or $Ki\text{-}67^+Bcl\text{-}2^-$, is capable of detecting the highest proportion of activated T cells during the early stages of infection. Although we may never know the "total" T cell response mounted during acute human infection, the various assays described here provide the initial steps towards achieving this goal.

*Note: Estimating an antiviral T cell response based on phenotypic markers ($CD38^+HLA\text{-}DR^+$ or $Ki\text{-}67^+Bcl\text{-}2^-$) is only valid during the first few weeks after infection since these activation markers are rapidly down-regulated on virus-specific T cells at later time points.

Chapter 4 - Characterization of $CD8^+$ T cells generated with an H_2O_2-inactivated whole virus vaccine

Abstract

$CD8^+$ T cells play an important role in protection against both acute and persistent viral infections, and new vaccines that induce $CD8^+$ T cell immunity are currently needed. Here, we show that lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells can be generated in response to a non-replicating H_2O_2-inactivated whole virus vaccine (H_2O_2-LCMV). Twenty H-2^b-restricted peptide epitopes were used to stimulate virus-specific $CD8^+$ T cells after LCMV-Armstrong (LCMV-Arm) infection or following immunization with H_2O_2-LCMV. Vaccine-induced $CD8^+$ T cell responses exhibited an increased ability to produce multiple cytokines at early time points following immunization compared to infection-induced responses. Vaccination with H_2O_2-LCMV induced the expansion of a narrow subset of the virus-specific $CD8^+$ T cells induced by LCMV-Arm infection, resulting in a distinct immunodominance hierarchy. Acute LCMV infection stimulated immunodominance patterns that shifted over time or after secondary infection, whereas vaccine-generated immunodominance profiles remained stable even following subsequent infection. Vaccine-induced $CD8^+$ T cell populations expanded sharply in response to challenge and were then maintained at high levels, with responses to individual epitopes occupying up to 40% of the $CD8^+$ T cell compartment at 35 days after challenge. H_2O_2-LCMV vaccination protected animals from LCMV-Clone 13 challenge, and CD8+ T cells

mediated this protection. These results indicate that vaccination with inactivated whole LCMV virions results in a protective $CD8^+$ T cell response, and that vaccine-induced T cells may have improved development kinetics and functional characteristics compared to those generated in response to infection.

Introduction

CD8+ T cells play a critical role in the defense against intracellular pathogens by eliminating virus-infected cells (Slifka et al. 1996, Brien et al. 2007). However, the overwhelming majority of current antiviral vaccines mediate their protective effects through induction of antibody responses, and frequently elicit little or no $CD8^+$ T cell immunity (Plotkin 2010). Many of the current targets of vaccine development are viral infections in which a strong $CD8^+$ T cell response will be important or required for protection. In the case of hepatitis C virus (HCV), it is believed that sterilizing immunity can be achieved if a sufficient cellular immune response is present (Thimme et al. 2002). In the case of other vaccine targets, such as human immunodeficiency virus (HIV), human cytomegalovirus (CMV), and varicella zoster virus (VZV), control of viral replication and pathogenesis appear to be profoundly dependent on the presence of $CD8^+$ T cell responses (Okoye et al. 2009, Hansen et al. 2010, Plotkin 2010, Amanna and Slifka 2011). New vaccines that are able to stimulate robust $CD8^+$ T cell based immunity are needed, and their development has the potential to significantly reduce morbidity and mortality in human populations.

$CD8^+$ T cell responses are often directed against a small subset of the total possible epitopes in any given pathogen (Chen and McCluskey 2006).

Within the responding T cell populations, some epitopes induce a higher degree of clonal expansion and/or survival than others, resulting in predictable immunodominance patterns for each combination of pathogen and major histocompatibility complex-I (MHC-I) (Yewdell and Bennink 1999). The identification of the factors that determine which epitopes in a pathogen will be both immunogenic and immunodominant has long been a goal of rational vaccine design (Yewdell and Haeryfar 2005). Some mechanisms believed to play a role in determining immunodominance include antigen load (La Gruta et al. 2006), duration of antigen exposure (Yoshimura et al. 2004), efficiency of antigen processing and presentation (Yewdell and Bennink 1999), frequency of naïve T cell precursors (Kotturi et al. 2008), competition between T cells for APCs (Kastenmuller et al. 2007), and the ability of $CD8^+$ T cell populations to produce and respond to IFNγ (Liu et al. 2004, Whitmire et al. 2005).

In this study, we investigate how vaccination with H_2O_2-inactivated LCMV influences the immunodominance of the resulting MHC-I restricted $CD8^+$ T cell responses in C57BL/6 mice, and compared these responses to those elicited by acute and chronic LCMV infection. Reactive oxygen species (ROS), such as H_2O_2, are utilized by innate immune cells to inactivate viral and bacterial pathogens (Miller and Britigan 1997). We chose to adopt this same strategy, and we produced our vaccine candidate by inactivating purified LCMV virions with H_2O_2. This novel vaccine approach has been found to preserve the antigenic structure of virions more readily than other inactivation methods, such as heat or formalin inactivation (Amanna, Raué, and Slifka, manuscript submitted). The

immune response to LCMV infection in mice has been extensively characterized, making it an ideal system in which to test the ability of our vaccine to elicit $CD8^+$ T cell immunity. LCMV is a natural mouse pathogen (Buchmeier et al. 2006, Charrel et al. 2008), and many virus-specific MHC-I restricted peptide epitopes have been previously mapped (Gairin et al. 1995, Oxenius et al. 1995, van der Most et al. 1998, Kotturi et al. 2007, Dow et al. 2008). The kinetics of viral infection have been described for both acute and chronic strains of the virus (Matloubian et al. 1994, Wherry et al. 2003b), and LCMV-specific $CD8^+$ T cells are required to clear acute infection as well as to protect from chronic or lethal challenge (Fung-Leung et al. 1991, Matloubian et al. 1994, Shen et al. 1995, Slifka et al. 1996).

Here, we demonstrate that virus-specific CD8+ T cells can be elicited in response to an H_2O_2-inactivated whole-virus vaccine. We found that vaccination with H_2O_2-LCMV stimulates a subset of the 20 different MHC-I restricted $CD8^+$ T cell responses observed during acute or chronic LCMV infection. Interestingly, vaccine-induced $CD8^+$ T cell responses are highly polyfunctional, expressing a cytokine production profile associated with highly active antiviral T cell populations (Betts et al. 2006); and these cells were present at time points as early as 8 days post vaccination. In contrast, $CD8^+$ T cells induced by acute LCMV-Arm infection require several weeks to achieve a similar cytokine production profile. Once immunodominance is established by H_2O_2-LCMV vaccination, it does not change following booster vaccination or subsequent viral infection. This is unlike the shifting immunodominance pattern observed

following acute LCMV-Arm infection. The T cell responses to individual peptide epitopes in animals that were vaccinated with H_2O_2-LCMV prior to challenge with live virus were robust, and constitute 25-50% of the $CD8^+$ T cell compartment during the memory phase post-challenge. Most importantly, animals vaccinated with H_2O_2-LCMV were protected from chronic LCMV-Clone 13 challenge in a $CD8^+$ T cell-dependent manner. These results show that the $CD8^+$ T cell response to H_2O_2-inactivated LCMV vaccination differs sharply from the response to LCMV infection in both immunodominance and functional attributes, while providing effective $CD8^+$ T cell-mediated protection against chronic infection.

Results

Functional avidity response curves of H-2^b-restricted LCMV-specific peptide epitopes

In this work, we used a group of 20 peptide epitopes to stimulate LCMV-specific $CD8^+$ T cell responses. These peptide epitopes were originally discovered by a number of different laboratories, and are believed to stimulate the bulk, if not all, of LCMV-specific $CD8^+$ T cells (Gairin et al. 1995, van der Most et al. 1998, Kotturi et al. 2007). In order to determine the optimal concentration to use in our *in vitro* culture assays, we performed peptide dose titrations in which T cells from LCMV-immune C57BL/6 mice were stimulated with concentrations of peptide ranging from 10^{-4}-10^{11} molar for 6 hours in the presence of brefeldin A. Following stimulation, intracellular cytokine staining

(ICCS) was performed, and the percentage of $CD8^+$ T cells producing IFNγ in response to stimulation was determined by flow cytometry. The results of this work were used to generate functional avidity response curves for each of the 20 peptide epitopes we chose to use (Fig. 4.1). It should be noted that 27 potential peptide epitopes were originally tested based on work by Kotturi et al., 2007, but we were unable to stimulate $CD8^+$ T cell responses at peptide dose tested between 10^{-4}-10^{11} molar using 7 of the peptides (Kotturi et al. 2007). Responses are shown at each peptide concentration as a percentage of the maximum $CD8^+$ T cell response achieved for that peptide. The functional avidity curves are grouped according to the LCMV protein that the peptide epitopes are derived from; NP (Fig. 4.1A), GP (Fig 4.1B), or L (Fig. 4.1C). A dashed line on each plot shows the peptide concentration required to achieve 50% of the maximum response for each peptide. Peptide concentrations able to stimulate the maximum $CD8^+$ T cell response for each peptide were also determined using these functional avidity curves. These peptide concentrations ranged from 1 X 10^{-4} – 1 X 10^{-6} molar and were used for all in vitro stimulation throughout the rest of the experiments in this thesis. The peptide concentrations at which each of the peptide epitopes induce ½ maximum and maximum possible stimulation in reactive $CD8^+$ T cells are listed in Table 4.1.

H_2O_2 inactivation of LCMV

In this paper, we use a novel virus inactivation technique, H_2O_2 inactivation of whole LCMV virions. This method preserves the antigenicity of inactivated virions more readily than heat or formaldehyde inactivation (Amanna,

Raué, and Slifka, manuscript submitted). Using 3% H_2O_2 to inactivate purified LCMV, it is possible to get at least a million-fold reduction in infectious virus titer within 2 hours. Complete LCMV inactivation was confirmed by directly performing plaque assays, or by co-culturing up to 200 µg of inactivated material (equivalent to 4 vaccine doses) on BHK cells followed by plaque assay. As an additional test for live virus, $RAG^{-/-}$ animals were injected intraperitoneally with up to 200 µg of inactivated material and serum viremia was subsequently measured using plaque assay. Co-culture and $RAG^{-/-}$ infection assays are capable of detecting 1-2 infectious particles in 200 µg (~2 X 10^9 virus-equivalents) of inactivated material, and confirmed that our inactivated material was devoid of live LCMV virions. The LCMV vaccine preparation used in these experiments contained 50 µg H_2O_2-inactivated LCMV with 5 µg MPL adjuvant, delivered subcutaneously.

Peptide stimulation and gating strategy in H_2O_2-LCMV vaccinated animals

The primary assay used to determine the presence of LCMV-specific $CD8^+$ T cells in these experiments was peptide stimulation followed by ICCS and flow cytometry analysis. In order to achieve high sensitivity and specificity, we used a gating process following flow cytometry analysis that eliminated aggregates as well as dead cells prior to scatter and lineage gating. The gating strategy used is outlined in Figure 4.2. A forward scatter-area versus forward scatter-height gate was used to perform doublet discrimination, so that only single cells were analyzed. Cells that were dead immediately following

stimulation were identified and removed using the amino vital dye Aqua. This dye functions by binding free amines within the cytoplasm of cells with compromised lipid bilayers. Light scatter gating and lineage gating were then performed. $CD8^+$ T lymphocytes were analyzed for IFNγ, TNFα, and IL-2 production. To increase the sensitivity of the assay, between 400,000 and 800,000 events were typically collected for each sample.

H_2O_2-LCMV vaccination stimulates a long-lasting, highly polyfunctional, NP396-specific $CD8^+$ T cell response

Primary infection of C57BL/6 mice with LCMV-Arm induces a protective effector $CD8^+$ T cell response that peaks in magnitude around 8 days post infection, becoming a stable memory response between 30 and 70 days post infection (Murali-Krishna et al. 1998, Whitmire et al. 2000, Wherry et al. 2003a). To compare the T cell immunity generated by our H_2O_2-LCMV vaccine to the responses induced by LCMV-Arm infection, we measured the $CD8^+$ T cell responses to the immunodominant epitope, LCMV NP396, from 8 to 70 days post infection or vaccination (Fig. 4.3A). At day 8, the average number of NP396-specific $CD8^+$ T cells per spleen is about 150 fold lower in the vaccinated group than observed in the LCMV infected group. However, the number of virus-specific $CD8^+$ T cells in vaccinated animals only declined 30-40% between day 8 and day 35 after vaccination, whereas the LCMV-Arm infected animals declined 80-90% during the same time period. Although H_2O_2-LCMV vaccination induces an NP396-specific T cell response that is lower in magnitude than LCMV-Arm infection, the vaccine-induced response is stable over the study period and

experiences less contraction than the T cell response induced by acute viral infection.

The ultimate goal of vaccine-induced $CD8^+$ T cell memory is the generation of a stable antigen-specific T cell population that is able to rapidly respond to rechallenge. Polyfunctional T cells, capable of producing multiple cytokines, have been associated is associated with more effective control of chronic viral infections such as CMV and HIV (Betts et al. 2006, Seder et al. 2008, Sun et al. 2008). The $CD8^+$ T cell response to LCMV-Arm infection becomes more polyfunctional as it transitions from an effector to a memory response (Slifka and Whitton 2000, Whitton et al. 2004, Whitmire et al. 2007). Here, we demonstrate that H_2O_2-LCMV vaccine-induced $CD8^+$ T cells are capable of producing multiple cytokines as early as 8 days after vaccination (Fig. 4.3B & 4.3C). On average, 98±19% of NP396-specific $CD8^+$ T cells produce both IFNγ and TNFα in response to direct ex vivo peptide stimulation at day 8 post-vaccination. In contrast, only 55±10% of NP396-specific $CD8^+$ T cells at day 8 post-infection produce both IFNγ and TNFα. The disparity in IL-2 production between virus-specific $CD8^+$ T cells from vaccinated and infected animals 8 days after infection is even greater. 50±13% of the vaccine-induced $CD8^+$ T cells are able to produce IL-2 following NP396 peptide stimulation, compared to 1±0.3% of the T cells induced by LCMV-Arm infection. However, LCMV-Arm infected animals have an evolving response as the $CD8^+$ T cells progress from the effector to the memory phase. This means, as the virus-specific T cells mature they produce a greater variety of cytokines. By day 70

post infection, the percentage of NP396-specific T cells that were polyfunctional, producing IFNγ, TNFα, and IL-2 in response to peptide stimulation, increased from 1±0.3% to 17±8%. However, even with this increase, the LCMV-Arm infected animals did not achieve the same percentage of polyfunctional $CD8^+$ T cells as the H_2O_2-LCMV vaccinated animals. H_2O_2-LCMV vaccination consistently induced a higher percentage of $IFN\gamma^+$, $TNF\alpha^+$, $IL\text{-}2^+$ $CD8^+$ T cells, with 44%-55% of the NP396-specific population being triple cytokine producers at each time point examined. These data indicate that our H_2O_2-LCMV vaccine strategy is capable of inducing $CD8^+$ T cell responses that are durable and have a higher percentage of polyfunctional T cells compared to immune responses elicited by LCMV-Arm infection.

Immunodominance following acute LCMV-Arm infection or H_2O_2-LCMV vaccination

The $CD8^+$ T cell response to LCMV in C57BL/6 mice is a well characterized model, and a number MHC-I-restricted T cell epitopes have been identified (Gairin et al. 1995, Oxenius et al. 1995, van der Most et al. 1998, Kotturi et al. 2007, Dow et al. 2008). This provides the opportunity to investigate potential differences in the immunodominance hierarchies between H_2O_2-LCMV vaccinated animals and LCMV-Arm infected animals. We selected a panel of the 20 most immunogenic LCMV peptides, consisting of 4 nucleoprotein epitopes (NP165, NP205, NP238 & NP396), 7 glycoprotein epitopes (GP33, GP61, GP92, GP118, GP221, GP276, & GP365), and 9 polymerase epitopes (L156, L313, L338, L349, L455, L663, L775, L1428, & L2062). The

immunodominance hierarchies at 8 days following LCMV-Arm infection or H_2O_2-LCMV vaccination were determined using this 20 epitope panel (Fig. 4.4). Following LCMV-Arm infection, $CD8^+$ T cell responses to all 20 peptide epitopes were observed. Responses to NP396, GP33, and L156 were co-dominant, and intermediate responses, consisting 5% of the $CD8^+$ T cell population or greater, were observed when we stimulated with L338, L455, L2062, NP165, NP205, GP61, GP118, and GP276 peptides. Low frequency, but reproducible, T cell responses to L313, L349, L663, L775, L1428, NP238, GP92, GP221, and GP365 peptides were also detected. Following H_2O_2-LCMV vaccination, the immunodominance pattern was restricted to 3 main peptide epitopes, NP396, L156, and L455. We also identified what appears to be a weak response to GP33, though the average GP33 response was less than 2 fold over background and was not detected in all vaccinated animals. The remainder of the $CD8^+$ T cell responses induced by LCMV-Arm infection were not detectable following vaccination. Similar immunodominance patterns were observed if mice were vaccinated with LCMV antigen that was inactivated using H_2O_2, formaldehyde, or heat (56° C) (data not shown). This suggests that the immunodominance pattern observed following H_2O_2-LCMV vaccination is not necessarily an artifact of H_2O_2 inactivation of the virus since similar results were observed when other inactivation approaches were tested.

Direct *ex vivo* peptide stimulation and ICCS assay is sensitive and allows detection of responses as low as 0.04% of the $CD8^+$ T cell population. However, the overall magnitude of T cell responses elicited by H_2O_2-LCMV vaccination was

low, and we wanted to determine if a booster vaccination would or make it possible to detect low frequency responses that were present, but not detectable, after a single vaccination. Additionally, we were interested to determine if changes in immunodominance would be observed after booster vaccination. To accomplish this, we conducted a series of prime followed by boost and prime followed by challenge experiments (Fig. 4.5). In these experiments, animals were vaccinated using H_2O_2-LCMV or infected with LCMV-Arm at day 0. The animals were then boosted by H_2O_2-LCMV vaccination or challenged with LCMV-Arm infection 28 days after their primary infection or vaccination. The $CD8^+$ T cell responses were then analyzed at 5 days (Fig. 4.5A-C) or 42 days (Fig. 4.5D-F) post secondary vaccination or infection. When animals were infected at day 0 and challenged at day 28 with LCMV-Arm, we observed evolution of the immunodominance profile during the study period, compared to the immunodominance profile of animals that were not challenged. At day 5 following LCMV-Arm challenge, GP276 emerged as a co-dominant epitope with NP396, GP33, L156, and L455 (Fig. 4.5A). By day 42, the response to the GP61 peptide had also joined the other 5 co-dominant responses (Fig. 4.5D). $CD8^+$ T cell responses to the other individual peptide epitopes represented less than 1% of the $CD8^+$ T cell response, and some previously observed subdominant responses became undetectable at this point. These results are supported by previously published work, showing evolution of immunodominance coincident with LCMV re-infection (Tebo et al. 2005). It has been shown, for example, that GP276 emerges as a co-dominant epitope following secondary LCMV infection

(Tebo et al. 2005). However, the immunodominance hierarchy of this broad set of recently described peptides has not been determined following secondary infection. LCMV-Arm immune animals had modest $CD8^+$ T cell responses after challenge compared to animals undergoing primary LCMV-Arm infection. This is likely due to the ability of the pre-existing LCMV-specific T cell response in these animals to rapidly control viral infection. When animals were vaccinated with H_2O_2-LCMV at day 0 and boosted with H_2O_2-LCMV at day 28, the result was strikingly different in that the immunodominance profile did not evolve. There was no change in immunodominance between primary H_2O_2-LCMV vaccination (Fig. 4.4B), 5 days post H_2O_2-LCMV boost (Fig. 4.5B), or 42 days post H_2O_2-LCMV boost (Fig. 4.5E). There was, on average, a 3-fold increase in the magnitude of the LCMV-specific $CD8^+$ T cell responses following booster vaccination. However, no epitope spreading to new specificities was detected following booster vaccination. This phenomenon of "fixed" immunodominance was also observed when animals were vaccinated with H_2O_2-LCMV at day 0 and infected with LCMV-Arm at day 28. These animals displayed the same immunodominance profile as the other vaccinated animals, generating a dominant response to NP396 and two subdominant responses to L156 and L455. However, the responses to these 3 peptide epitopes were large, comprising 81% of the $CD8^+$ T cell compartment at day 5 after challenge (Fig. 4.5C) and 59% of the total $CD8^+$ T cell compartment at day 42 following challenge (Fig. 4.5F). These data indicate that while H_2O_2-LCMV vaccine-induced responses constitute a small percentage of the $CD8^+$ T cell repertoire after vaccination, these

responses exhibit stable immunodominance patterns and are capable of rapidly expanding to high frequencies that are maintained at elevated levels after challenge.

H_2O_2-LCMV vaccine provides protection from chronic LCMV-Clone 13 challenge

We next determined whether the $CD8^+$ T cell immunity induced by our H_2O_2-LCMV vaccination was protective against chronic LCMV challenge. Protection from chronic LCMV challenge is an established tool used to measure the efficacy to T cell immunity to LCMV (Oldstone et al. 1993, Shen et al. 1995, Slifka et al. 1996, Lanier et al. 1999, Takagi et al. 2009). LCMV-Clone 13 infection of naïve C57BL/6 mice causes persistent viremia as well as predictable T cell dysfunction, both of which are preventable if infected animals have a pre-existing LCMV-specific $CD8^+$ T cell response (Wherry et al. 2003b, Shin and Wherry 2007). Animals were vaccinated with H_2O_2-LCMV and either 8 or 28 days following vaccination they were challenged with LCMV-Clone 13. These post-vaccination time points were selected to determine if both early and memory phase $CD8^+$ T cell responses were protective. Naïve and LCMV-Arm immune animals were also challenged as controls, and to determine the effects of LCMV-Clone 13 infection on immunodominance using the extended peptide array. Following challenge, viremia was determined weekly by plaque assay (Fig. 4.6A). The group vaccinated with H_2O_2-LCMV at 8 days prior to challenge had no detectable viremia during the study period, while the group vaccinated with H_2O_2-LCMV at 28 days prior to challenge had low-level viremia in 2 of 7 animals tested

at day 7 post challenge. This viremia was transient, and resolved by day 14 post challenge. Interestingly, this level of protection is similar to that achieved in previous work using a recombinant *Listeria* vaccine expressing LCMV-nucleoprotein (Slifka et al. 1996). When this live listeria vector was used to induce immunity animals were protected, as in our system, but they also experienced breakthrough viremia during the first week after challenge. None of the LCMV-Arm immune animals demonstrated viremia following challenge, while all naïve animals had persistent viremia following challenge. To determine if $CD8^+$ T cells directly mediated protection, we vaccinated animals with H_2O_2-LCMV followed 8 days later by $CD8^+$ T cell depletion and LCMV-Clone 13 challenge (Fig. 4.6B). In this experiment, the $CD8^+$ T cell-depleted animals failed to control LCMV-Clone 13 challenge and experienced viremia indistinguishable from naïve mice. This suggests that the $CD8^+$ T cell responses generated through H_2O_2-LCMV vaccination are functional and protective against chronic LCMV challenge.

Thirty-five days after challenge, immunodominance was determined using our panel of 20 LCMV peptide epitopes. Immunodominance profiles are presented as a percentage of the $CD8^+$ T cell compartment to illustrate the large differences in magnitude of the T cell responses between the vaccinated and control groups (Fig. 4.6C, E, G, & I). The immunodominance profiles are also shown with individual responses normalized to the total LCMV-specific response to highlight the differences in hierarchy between the vaccinated and control groups (Fig 4.6D, F, H, & J). Naïve animals developed significant antiviral T cell

dysfunction following challenge. In these animals, $CD8^+$ T cell responses to NP396 and L455 underwent clonal deletion and responses to GP33 became functionally anergic (unable to produce IFNγ in response to peptide stimulation) (Fig. 4.6C-D and 4.7A-B). Functional anergy was determined for the GP33 epitope by comparing percentage peptide-MHC tetramer positive cells to the percentage of cells able to make IFNγ in response to GP33 peptide stimulation (Fig. 4.7B). Responses specific for GP276 and L156 emerged as the immunodominant T cells in these animals. Mice infected with LCMV-Arm 28 days prior to challenge (Fig. 4.6E-F) suffered no T cell dysfunction and had immunodominance profiles similar to animals examined six weeks following LCMV-Arm challenge (Fig. 4.5D). Animals challenged either 8 days (Fig. 4.6G-H) or 28 days (Fig. 4.6I-F) following H_2O_2-LCMV vaccination did not display functional anergy and had intact $CD8^+$ T cell responses to NP396, GP33, L156 and L455 peptides. The vaccinated animals had immunodominance profiles similar to other animals receiving H_2O_2-LCMV vaccination. In addition, the vaccinated animals maintained large responses to the immunodominant epitopes. The average response to the immunodominant NP396 epitope in the group vaccinated 8 days prior to LCMV-Clone 13 challenge was 46±3% of the total $CD8^+$ T cell compartment at day 35 post challenge. The data in these experiments indicate that our H_2O_2-LCMV vaccine strategy protects animals from both the serum viremia and T cell dysfunction associated with chronic LCMV infection.

In these experiments, we were also able to test the ability of vaccination to protect animals from $CD8^+$ T cell dysfunction. LCMV-Clone 13 infection is known to induce both functional exhaustion and clonal deletion of LCMV-specific $CD8^+$ T cells by persistently expressing high levels of viral antigen (Wherry et al. 2003b, Kotturi et al. 2007, Mueller and Ahmed 2009). Here, we define functional exhaustion as the presence of antigen-specific $CD8^+$ T cells, as determined by peptide-MHC tetramer staining, that are unable to make cytokine in response to stimulation with their cognate antigen. Clonal deletion is defined the absence of a $CD8^+$ T cell population, as determined by peptide-MHC tetramer staining. In LCMV-Clone 13 infection of naïve C57BL/6 mice, $CD8^+$ T cell responses to NP396 undergo clonal deletion, while $CD8^+$ T cell responses to GP33 experience functional exhaustion (Fig. 4.7). Both of these effects can be abrogated during LCMV-Clone 13 infection if a pre-existing LCMV-specific $CD8^+$ T cell response present (Fig. 4.7). These vaccinated animals mounted $CD8^+$ T cell responses of similar or larger magnitude than LCMV immune control animals, and they displayed a one-to-one ratio of peptide-MHC tetramer positive to cytokine producing T cells. These results indicate that H_2O_2-LCMV vaccination is protective against LCMV-Clone 13 induced functional exhaustion and clonal deletion.

H_2O_2 vaccination primes a GP34, but not a GP33-specific $CD8^+$ T cell response

One of the more interesting findings in this work came when we analyzed the frequencies of the GP33 epitope after H_2O_2-LCMV vaccination and LCMV-

Clone 13 challenge. The immunodominant "GP33" peptide epitope is actually two distinct overlapping epitopes, the D^b-restricted GP33-41 and the K^b-restricted GP34-41 epitopes. For the purposes of peptide stimulation directly *ex vivo*, the GP33-41 9mer peptide is capable of stimulating both populations of $CD8^+$ T cells. We obtained peptide-MHC tetramers for both epitopes, which allowed us to monitor the levels of each population independently. When H_2O_2-LCMV vaccinated animals were challenged with LCMV-Clone 13, we found a skewing of the immune response toward GP34-specific T cells (Fig. 4.8). Animals challenged 28 days after vaccination showed a 5:1 ratio of GP34-specific T cells to GP33-specific T cells, while animals challenged 8 days after vaccination showed a 10:1 ratio of GP34- to GP33-specific T cells (Fig. 4.8). This is in stark contrast to the essentially 1:1 ratio of GP34- to GP33-specific T cells that is present in naïve or LCMV-immune animals that are challenged with LCMV clone 13. These results indicate that H_2O_2-LCMV vaccination is priming a GP34-specific $CD8^+$ T cell response, but not a GP33-specific T cell response. These results also shed some light on the low-level "GP33" responses we observed during our immunodominance experiments. In our initial vaccination experiments we observed clear $CD8^+$ T cell responses to the NP396, L156, and L455 peptides (Fig. 4.4B). We also observed what appeared to be very low-level induction of a GP33-specific response in some animals, but it was not clearly discernable above background. The data from these experiments suggest what we observed earlier was most likely a low frequency GP34-specific response,

meaning the list of peptide epitopes that is able to stimulate a $CD8^+$ T cell response during H_2O_2-LCMV vaccination must also include GP34.

Discussion

In these experiments, we utilized a novel H_2O_2-inactivation strategy to generate an inactivated whole-virus vaccine. Our results demonstrate that H_2O_2-LCMV vaccination generates a durable LCMV-specific memory $CD8^+$ T cell response. We were able to show that H_2O_2-LCMV induced $CD8^+$ T cell responses are persistent for at least 10 weeks post vaccination. These vaccine-induced $CD8^+$ T cells have a highly polyfunctional cytokine profile, with around 50% of the responding cells being triple cytokine producing ($IFN\gamma^+$, $TNF\alpha^+$, and $IL\text{-}2^+$) as early as 8 days after vaccination. Our results show that H_2O_2-LCMV vaccination induces a subset of the $CD8^+$ T cell responses observed following LCMV-Arm infection. Once established, this vaccine-induced immunodominance profile did not change over time or following subsequent LCMV infection, in contrast to the shifting immunodominance profile observed after acute LCMV-Arm infection. The $CD8^+$ T cell immunity induced by this H_2O_2-inactivated vaccine was also capable of protecting animals from chronic LCMV-Clone 13 challenge. These results indicate that the H_2O_2-inactivated vaccine platform is capable of generating biologically relevant T cell immunity.

It is clear that different immunodominance patterns emerge depending on whether viral antigen is presented in the context of acute infection, chronic infection, or vaccination in C57BL/6 mice. Acute LCMV-Arm infection generates a $CD8^+$ T cell response against at least 20 peptide epitopes (Kotturi et al. 2007).

This response to infection has an evolving immunodominance profile as the T cells progress from the effector phase to the memory phase of T cell maturation, and it changes further in response to LCMV re-challenge. The most immunodominant peptide epitopes following primary LCMV-Arm infection are NP396, GP33/34, and L156. This repertoire of immunodominant epitopes expands during re-infection with LCMV-Arm to include NP396, GP33, GP61, GP276, and L455. In the case of primary LCMV-Clone 13 infection, GP276 and L156 are the immunodominant epitopes and the responses to previously co-dominant epitopes are diminished (NP396, GP61, and L455) or become functionally anergic (GP33). The immunodominance pattern following H_2O_2-LCMV vaccination is different from the pattern observed during either acute or chronic LCMV infection. In this case, the most dominant responses are directed against NP396, L156, and L455; and there is a weak subdominant response against GP34 observed in some animals. The responses to all other antigenic determinants are below the limit of detection in the case of H_2O_2-LCMV vaccination. These results raise the question of what features of the individual peptide epitopes, or the $CD8^+$ T cell responses to those epitopes, allow one response to be primed by vaccination while others are not.

A potential difference between LCMV-Arm infection and H_2O_2-LCMV vaccination is the abundance of viral protein within professional antigen presenting cells (pAPC) involved with the priming of the T cell responses. LCMV viral nucleoprotein is believed to be the most abundant protein in both LCMV-infected cells as well as in LCMV virions (Buchmeier et al. 2006). However,

differences in viral protein abundance alone cannot account for the differences in immunodominance observed between LCMV-Arm infected animals and H_2O_2-LCMV vaccinated animals. If vaccine-induced immunodominance patterns were due to antigen abundance in the vaccine, we might expect to see responses to a single protein dominate the response; however, immunodominance patterns to that protein would likely be preserved. Instead, we observed that responses to LCMV epitopes within the same protein, such as NP165 and NP396, vary greatly in their ability to be elicited by our whole virus vaccine. In LCMV-Arm infected C57BL/6 mice, $CD8^+$ T cell responses to NP165 and NP396 are present following infection (Fig. 4.4A) whereas H_2O_2-LCMV vaccination yields a response to only NP396 (Fig. 4.4B). Additionally, differences in immunodominance also cannot be attributed to the absence of any of the 3 main viral proteins in the vaccine. LCMV virions contain all proteins encoded in the viral genome (Buchmeier et al. 2006), so professional antigen presenting cells (pAPC) should ideally be capable of presenting any antigenic determinant within the virus. Indeed, our results also show evidence of vaccine-induced responses to antigenic determinants from each of the 3 major viral proteins NP, GP, and L. In addition we observe intra-protein variability in the immunodominance patterns of H_2O_2-LCMV vaccinated animals compared to LCMV-Arm infected animals.

Another possible explanation for the differences in immunodominance we observed between vaccinated and infected animals are differences in peptide-MHC affinity. Among the identified LCMV epitopes, there is significant variability in peptide affinity for MHC-I. The affinity of MHC-I for the majority of LCMV-

specific peptide epitopes used in these experiments has been previously elucidated (Kotturi et al. 2007), and it has been suggested that peptide-MHC-I affinity may play a role in determination of immunodominance (Kotturi et al. 2008). We did not observe any correlation between peptide-MHC-I affinity and immunodominance hierarchies in either infected or vaccinated animals (Spearman correlation, 95% confidence interval, P=0.144). Additionally, MHC-I binding affinity did not predict which peptide epitopes would be antigenic following vaccination. Several peptide epitopes that did not stimulate $CD8^+$ T cell responses following vaccination have affinity for MHC-I that is equivalent to or greater than the immunodominant vaccine epitope, NP396. It does not appear that peptide affinity for MHC-I predicts immunodominance following H_2O_2-LCMV vaccination.

$CD8^+$ T cell immunodominance differences between LCMV vaccinated and infected animals are likely due to the functioning of cross-priming as the primary mechanism of antigen presentation following vaccination and direct-presentation dominating during live infection. We rigorously test our vaccine preparations to ensure all virus has been thoroughly killed. Additionally, LCMV virions contain an ambisense genome that is not infectious in the absence of functioning LCMV polymerase protein (Sánchez and la Torre 2006, Buchmeier et al. 2006). Our results suggest that some antigenic determinants present in LCMV are likely more capable of cross-priming $CD8^+$ T cell responses than others. Transporter associated with antigen processing (TAP)-dependent as well as TAP-independent antigen processing pathways play a role in both direct-

presentation as well as cross-presentation of antigen (Rock and Shen 2005, Raghavan et al. 2008). Interestingly, it has been shown during LCMV infection that NP396 processing can occur via TAP-independent as well as TAP-dependent pathways whereas GP33 processing occurs exclusively via a TAP-dependent mechanism in pAPCs (Hombach et al. 1995, Sigal and Rock 2000). It is not known at this time how the antigenic determinants that are capable of priming T cell responses in our H_2O_2-LCMV vaccine system are processed, but this suggests that NP396 could be immunodominant following vaccination because it is able to be cross-presented in via TAP-dependent as well as TAP-independent mechanisms. Whether a specific peptide is capable of being processed via a TAP-dependent or a TAP-independent pathway during infection does not appear to play a role in which antigenic determinants are immunodominant since NP396 and GP33 are co-dominant following LCMV-Arm infection. It will be interesting to determine whether one antigen processing pathway is favored over another in the case of our H_2O_2-LCMV vaccine. Based on the ability of NP396 to be processed in a TAP-independent manner, we predict that TAP-independent antigen processing will also dominate antigen processing of our inactivated vaccine.

Another factor that may play a role in the different immunodominance patterns observed after LCMV-Arm infection, LCMV-Clone 13 infection, and vaccination with H_2O_2-LCMV is APC usage. There is evidence that the constellation of peptide epitopes that are presented via MHC-I can vary depending on the type of APCs performing the antigen presentation (Butz and

Bevan 1998b). In the case of direct presentation of LCMV, it has been shown that a professional APCs (pAPC) line processes and presents the GP33 and NP396 peptides most efficiently following infection, while a fibroblast cell line processes and presents the GP276 peptide following infection (Butz and Bevan 1998b). At the same time, it is believed that $CD8^+$ dendritic cells (DC) are primarily responsible for cross-presentation in mice (Guermonprez et al. 2002). This means that during LCMV-Arm infection there are likely many professional and non-professional APC types functioning to stimulate the $CD8^+$ T cell response, while vaccination with H_2O_2-LCMV likely results in the cross-presentation of vial antigen via $CD8^+$ DCs. It is possible that differences in APC usage could account for some of the differences in immunodominance we observed between vaccinated and infected animals.

An interesting finding from these experiments was the large magnitude of LCMV-specific $CD8^+$ T cell responses in animals that were H_2O_2-LCMV vaccinated and subsequently challenged with LCMV-Arm. The immunodominant responses were 2-5 fold greater at 35-42 days post challenge in animals that received H_2O_2-LCMV vaccination than in animals that were LCMV-Arm immune prior to challenge. One possible explanation for this effect is the larger $CD8^+$ T cell response in previously infected animals efficiently blocks virus replication, resulting in less of a boosting effect in these mice. H_2O_2-LCMV vaccination induces $CD8^+$ T cell immunity with a central memory phenotype and cytokine profile early after vaccination, and these cells exhibit immunodomination over the T cells specific for other LCMV epitopes. A second possible explanation is that

vaccine-induced $CD8^+$ T cells are capable of producing large amounts of IL-2. This IL-2 could potentially function in an autocrine manner, promoting strong proliferation in response to challenge. Additionally, there is evidence that vaccination strategies that do not induce large inflammatory responses result in $CD8^+$ T cell immunity that is more easily restimulated. A study by Pham et al., 2009, demonstrated that the $CD8^+$ T cell response to primary vaccination was larger when high dose (100 µg CpG-ODN) was used as adjuvant; however, $CD8^+$ T cell responses to booster vaccination were larger in animals that did not receive adjuvant during their primary vaccination (Pham et al. 2009). This indicates that the cytokine environment $CD8^+$ T cell responses are primed in may play a role in their ability to respond to challenge. H_2O_2-LCMV vaccine-induced $CD8^+$ T cell responses in these experiments are stimulated in the absence of high dose adjuvant or the cytokine stress of LCMV-Arm infection. This may afford vaccine-induced $CD8^+$ T cells a selective advantage, enabling them to rapidly proliferate and be maintained at high concentrations during and after challenge.

In this work, we demonstrate that an H_2O_2-inactivated LCMV vaccine is capable of inducing an antigen-specific $CD8^+$ T cell response in C57BL/6 mice. Immunodominance was determined using H-2^b-restricted LCMV peptide epitopes following LCMV-Arm infection as well as immunization with purified H_2O_2-LCMV. We show that H_2O_2-LCMV vaccination stimulates a subset of the $CD8^+$ T cell specificities induced during acute LCMV-Arm infection, and that immunodominance differs substantially following H_2O_2-LCMV vaccination versus LCMV infection. Vaccine-induced $CD8^+$ T cell responses were found to be highly

polyfunctional, meaning a high percentage of these vaccine-induced T cells were able to produce IFNγ, TNFα, and IL-2 in response to stimulation with cognate antigen. This advantageous cytokine profile was present in vaccine-induced T cells as early as 8 days after vaccination, while T cells stimulated in response to infection were not able to achieve equivalent levels of polyfunctional cytokine production in these experiments. The presence of these polyfunctional T cells has been associated with increased viral control in other systems (Betts et al. 2006, Seder et al. 2008, Sun et al. 2008). Additionally, our results show that vaccination with a single dose of H_2O_2-inactivated LCMV is capable of inducing a protective $CD8^+$ T cell response. Our challenge model also demonstrates that H_2O_2-LCMV vaccinated animals were protected against functional exhaustion and clonal deletion of LCMV-specific $CD8^+$ T cells. Additionally, vaccinated animals were protected from LCMV-Clone 13-induced chronic viremia, and this protective effect was lost if $CD8^+$ T cells were depleted. These results provide an important proof-of-principle regarding the use of an H_2O_2-inactivated whole virus vaccine preparation. The ability of H_2O_2-LCMV-induced $CD8^+$ T cell responses to be boosted early after vaccination and then maintained at high levels following challenge indicate that H_2O_2 inactivation of live virus may be an effective vaccine strategy in circumstances wherein a $CD8^+$ T cell response is needed for protection.

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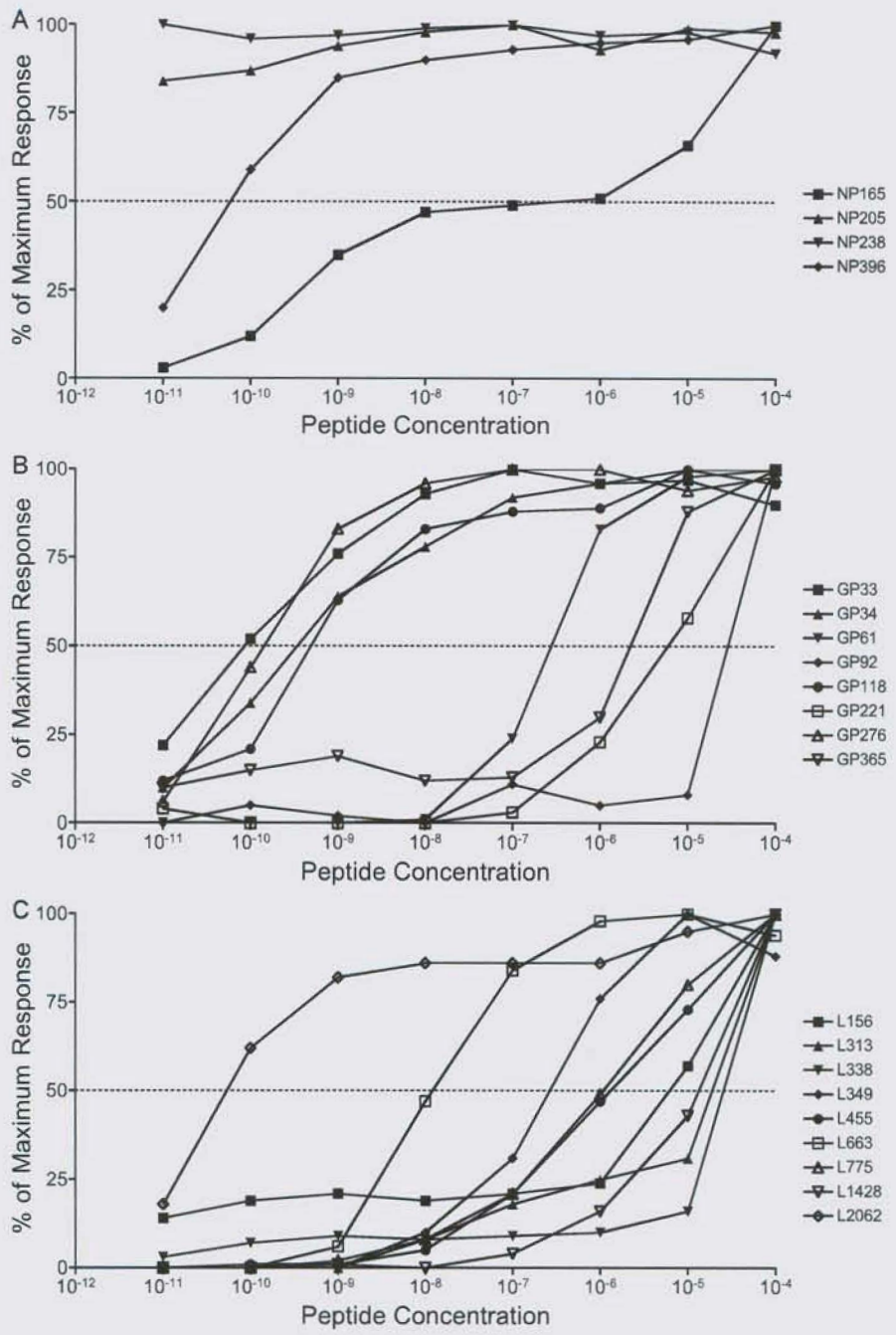

Figure 4.1: Functional avidity of LCMV-specific CD8$^+$ T cells

CD8$^+$ T cells from LCMV immune C57BL/6 mice were stimulated using 20 separate LCMV-derived peptide epitopes, followed by ICCS for IFNγ and flow cytometry analysis. Concentrations of peptide used ranged from 10^{-4} M to 10^{-11} M. These graphs show the percentage of the maximum antigen-specific CD8$^+$ T cell response achieved at each peptide concentration. The three graphs show the functional avidity curves for the LCMV NP (A), GP (B), and L (C) peptide epitopes. Results are the average of 2-4 LCMV-immune mice per group, over 1-2 experiments.

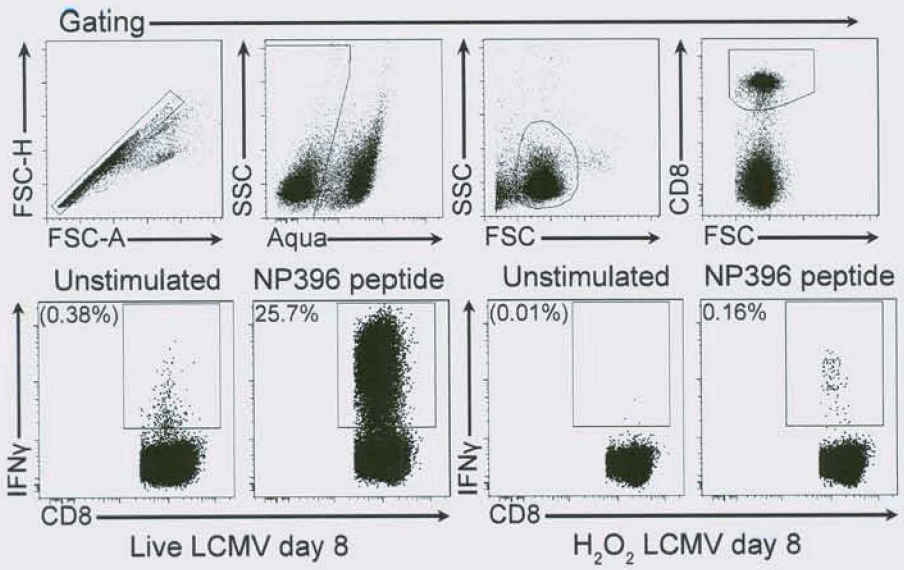

Figure 4.2: Determination of LCMV-specific $CD8^+$ T cell frequencies following vaccination

C57BL/6 mice were vaccinated with 50 μg H_2O_2-LCMV formulated with 5 μg MPL or infected with 2 X 10^5 PFU of LCMV-Arm. Eight days following infection or vaccination cells were stimulated with the immunodominant LCMV peptide epitope NP396, followed by intracellular cytokine staining and flow Cytometry analysis. The FSC-area vs. FSC-height gate was used as a singlet gate to remove clumped cells from analysis. Gating on the Aqua negative population identified the cells that were viable at the conclusion of the assay. Lymphocyte gating was performed based on FSC X SSC characteristics and $CD8^+$ gates were then applied before the percentage of cytokine-positive T cells was determined.

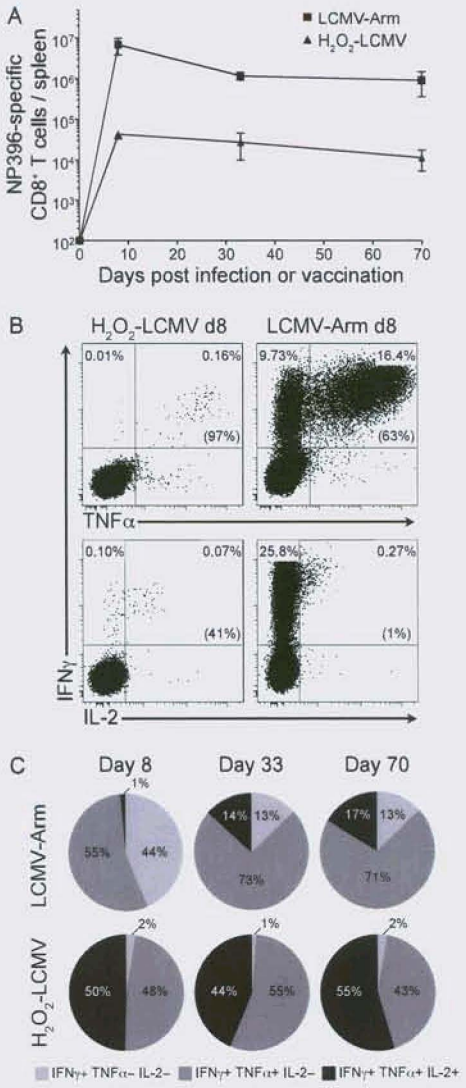

Figure 4.3: H_2O_2-LCMV vaccination induces long-lived polyfunctional $CD8^+$ T cell immunity

C57BL/6 mice were vaccinated with 50 μg H_2O_2-LCMV formulated with 5 μg MPL or infected with 2 X 10^5 PFU of LCMV-Arm. T Cells were stimulated with the immunodominant LCMV peptide epitope NP396, directly ex vivo, followed by ICCS. The total numbers of IFNγ producing $CD8^+$ T cells per spleen were calculated at days 8, 33, and 70 after H_2O_2-LCMV vaccination or LCMV-Arm infection (A). Representative cytokine production profiles at 8 days following vaccination or infection are shown (B). Data in parentheses indicates the percentage of IFNγ positive events that also produce either TNFα or IL-2. Average cytokine profiles following infection or vaccination were determined (C). The three cytokine production profiles shown in panel C are single positive ($IFN\gamma^+$ only), double positive ($IFN\gamma^+$ and $TNF\alpha^+$), and triple positive events ($IFN\gamma^+$, $TNF\alpha^+$, and $IL\text{-}2^+$), with triple positive events being the most polyfunctional. Data in panels A and C represent 3-4 mice per group from 2 independent experiments.

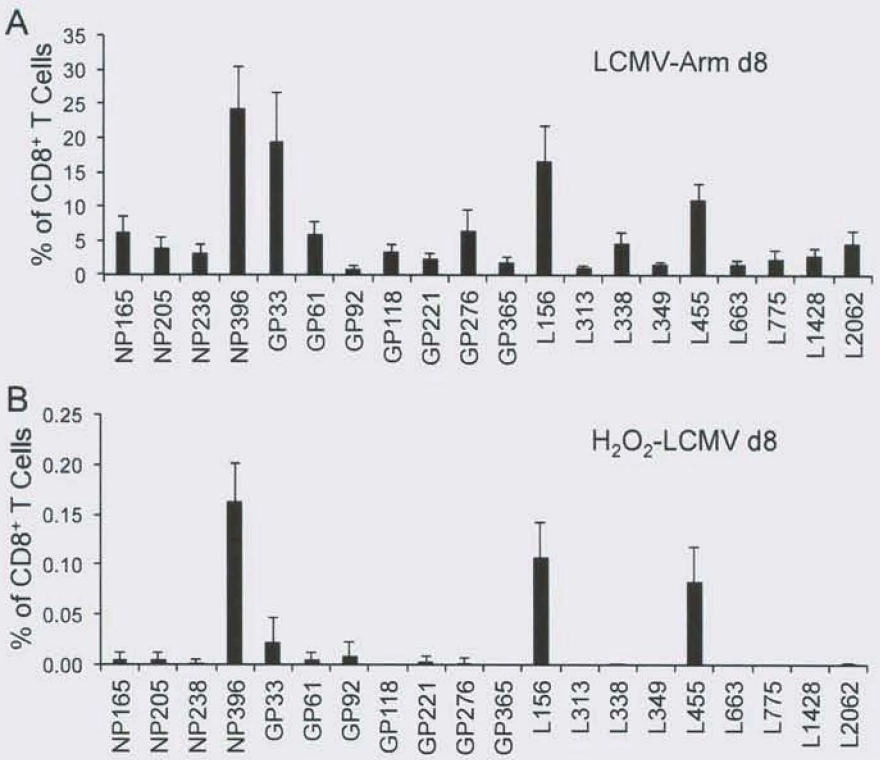

Figure 4.4: $CD8^+$ T cell immunodominance profiles differ following acute LCMV-Arm infection or H_2O_2-LCMV vaccination

C57BL/6 mice were infected with LCMV-Arm or vaccinated with H_2O_2-LCMV. Eight days later, T cell cultures were stimulated directly ex-vivo with 20 individual H-2^b-restricted LCMV epitopes, and IFNγ cytokine expression was determined using ICCS. Immunodominance profiles following primary LCMV-Arm infection (A) or H_2O_2-LCMV vaccination (B) were determined based on the percentage of $CD8^+$ T cells expressing IFNγ. H_2O_2-LCMV vaccinated animals (B) demonstrate a restricted immunodominance profile when compared to the LCMV-Arm infected animals (A). Graphs show the average of 3-4 mice per group from 2 independent experiments.

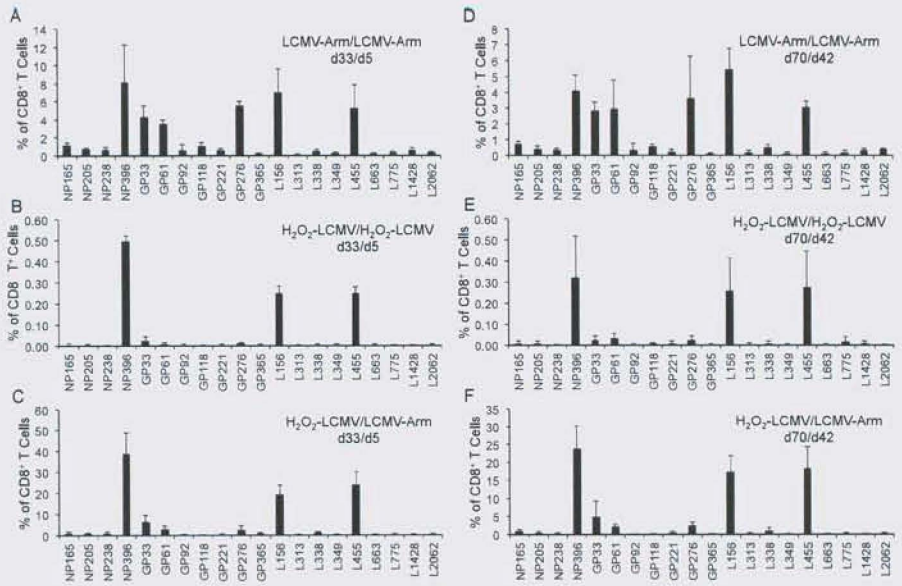

Figure 4.5: H_2O_2-LCMV vaccinated mice maintain stable $CD8^+$ T cell immunodominance patterns following subsequent infection or booster vaccination

C57BL/6 mice underwent primary infection with 2 X 10^5 PFU LCMV-Arm or vaccination with 50 μg H_2O_2-LCMV formulated with 5 μg MPL, and 28 days later received a secondary live-LCMV challenge or booster H_2O_2-LCMV vaccine. Primary and second ry vaccinations and infections were identical in dose. Combinations tested were LCMV-Arm infection followed by LCMV-Arm challenge (A & D), H_2O_2-LCMV vaccination followed by H_2O_2-LCMV boost (B & E), and H_2O_2-LCMV vaccination followed by LCMV-Arm challenge (C & F). Immunodominance profiles were determined by stimulating T cells with our panel of 20 LCMV peptide epitopes followed by ICCS for IFNγ at day 5 (A-C) and day 42 (D-F) post challenge or boost. The data represent the average of 3-4 mice per group from 2 independent experiments.

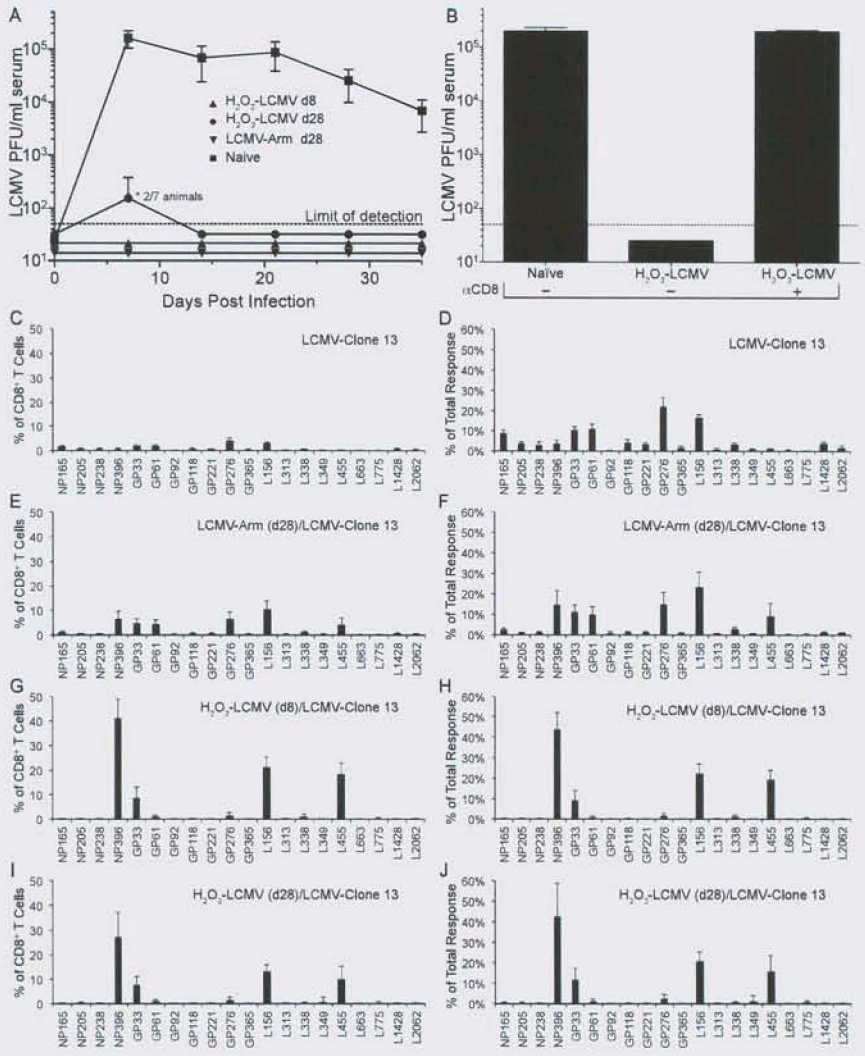

Figure 4.6: H_2O_2-LCMV vaccination protects mice against chronic LCMV-Clone 13 challenge in a $CD8^+$ T cell dependent manner

At 8 days or 28 days following H_2O_2-LCMV vaccination or at 28 days following LCMV-Arm infection C57BL/6 mice were challenged with 2 X 10^6 PFU LCMV-Clone 13 intravenously, and serum viremia was determined through plaque assay weekly for 35 days (A). To determine if vaccine-induced protection from challenge was $CD8^+$ T cell mediated, animals were H_2O_2-LCMV vaccinated followed by $CD8^+$ T cell depletion and LCMV-Clone 13 challenge (B). Data in panel B depicts average serum viremia 7 days following challenge. $CD8^+$ T cell immunodominance was determined at day 35 following LCMV-Clone 13 challenge by stimulating T cells with our panel of 20 LCMV peptide epitopes directly ex vivo followed by ICCS (C-J). The animals challenged were either naïve (C & D), infected with LCMV-Arm (E & F), or vaccinated with H_2O_2-LCMV (G & H, I & J). Challenge occurred 8 days (G & H) or 28 days (E & F, I & J) following infection or vaccination. Immunodominance profiles are displayed as the percentage of $CD8^+$ T cells producing IFNγ in response to each peptide epitope (C & E, G & I), or as the percentage of the total anti-LCMV response that each peptide epitope comprises (D & F, H & J). Graphs show the average of 3-4 mice per group over 2 independent experiments.

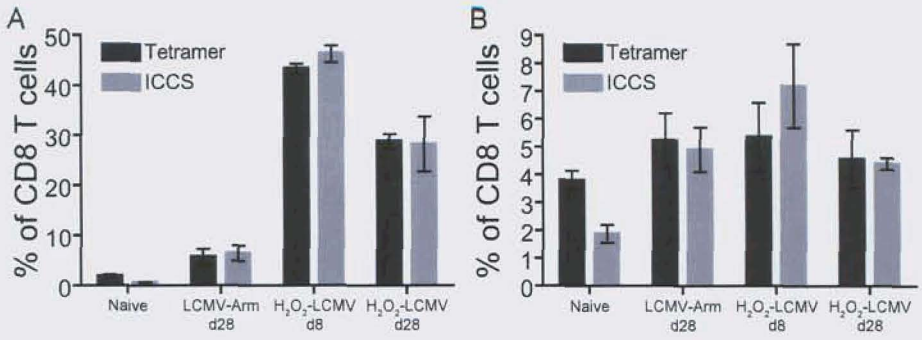

Figure 4.7: H_2O_2-LCMV vaccinated animals are protected from LCMV-Clone 13-induced T cell dysfunction

C57BL/6 mice were LCMV-Clone 13 challenged at either 8 days or 28 days following H_2O_2-LCMV vaccination or at 28 days following LCMV-Arm infection. 35 after LCMV-Clone 13 challenge the percentage of $CD8^+$ T cells that were NP396-specific (A) or GP33/34-specific (B) were determined using peptide stimulation followed by ICCS for IFNγ as well as peptide-MHC tetramer analysis. The results compare the percentage of antigen-specific $CD8^+$ T cells that were tetramer positive to the percentage of antigen-specific $CD8^+$ T cells that produce IFNγ in response to peptide stimulation. When GP33/34 analysis was done, peptide-MHC tetramers for both the GP33 and GP34 peptide epitopes were used to determine frequencies.

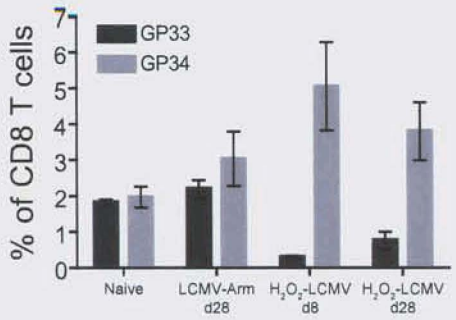

Figure 4.8: H_2O_2-LCMV vaccination induces a GP34-specific response but not a GP33-specific response

Animals were challenged with LCMV-Clone 13 intravenously at 8 days or 28 days following H_2O_2-LCMV vaccination or at 28 days following LCMV-Arm infection. On day 35 post LCMV-Clone 13 challenge the percentage of $CD8^+$ T cells that were GP33 or GP34-specific was determined using peptide-MHC tetramer analysis. H_2O_2-LCMV vaccinated animals developed a K^b-restricted GP34 response but not a D^b-restricted GP33 response following challenge, while control animals, previously infected with LCMV-Arm or LCMV-Clone 13, generate both GP33 and GP34 responses following challenge.

Peptide Epitope	Functional Avidity 1/2 Max (M)	*In Vitro* Stim. Concentrations (M)
NP165	1.5×10^{-7}	1×10^{-4}
NP205	$<1.0 \times 10^{-11}$	1×10^{-6}
NP238	$<1.0 \times 10^{-11}$	1×10^{-6}
NP396	6.0×10^{-11}	1×10^{-6}
GP33	9.0×10^{-11}	1×10^{-6}
GP34	4.0×10^{-10}	1×10^{-6}
GP61	3.0×10^{-7}	1×10^{-5}
GP92	3.0×10^{-5}	1×10^{-6}
GP118	5.0×10^{-10}	1×10^{-6}
GP221	$6.0E \times 10^{-6}$	1×10^{-4}
GP276	1.5×10^{-10}	1×10^{-6}
GP365	2.0×10^{-6}	1×10^{-5}
L156	$6.0E \times 10^{-6}$	1×10^{-4}
L313	$2.0E \times 10^{-5}$	1×10^{-4}
L338	2.5×10^{-5}	1×10^{-4}
L349	3.0×10^{-7}	1×10^{-5}
L455	1.5×10^{-6}	1×10^{-4}
L663	1.0×10^{-8}	1×10^{-6}
L775	1.0×10^{-6}	1×10^{-4}
L1428	1.5×10^{-5}	1×10^{-4}
L2062	5.5×10^{-11}	1×10^{-6}

Table 4.1: LCMV-specific $CD8^+$ T cell functional avidity

The values shown are peptide concentrations that stimulate 50% or of the maximum IFNγ response to each of the peptide epitopes as well as the peptide concentrations chosen for *in vitro* stimulation experiments. The values were determined using data from Figure 4.1.

Chapter 5 - Cross-reactivity of LCMV-specific $CD8^+$ T cell populations

Introduction

The $CD8^+$ T cell response to acute LCMV infection can be large. Other groups have estimated that the LCMV-specific response can constitute 50-80% of the $CD8^+$ T cell compartment at its peak (Murali-Krishna et al. 1998, Homann et al. 2001). It has also been shown that the T cell response to infection is antigen-specific, and bystander activation appears to play little or no role in the immune response to LCMV (Butz and Bevan 1998a, Murali-Krishna et al. 1998). In addition, many, if not all, of the H-2^b-restricted peptide epitopes have been elucidated (Gairin et al. 1995, van der Most et al. 1998, Kotturi et al. 2007). This has allowed us to determine the magnitude and immunodominance of the LCMV response, as described in Chapter 4. An interesting aspect of our results is that the total magnitude of LCMV-specific $CD8^+$ T cell responses can constitute greater than 100% of the $CD8^+$ T cell compartment at the peak of the response, if the responses to the 20 individual peptide epitopes are added together. Using the aggregate data from the individual $CD8^+$ T cell responses shown in Figure 4.4A, our results show that the average total magnitude of the $CD8^+$ T cell response 8 days following primary infection with LCMV-Arm is 125% of the compartment. The range for individual animals was 94% to 167% of all $CD8^+$ T cells. There are multiple possible explanations for this phenomenon. One explanation is that non-specific killing by highly activated cytotoxic T cells may

artificially inflate the frequency of antigen-specific $CD8^+$ T cells in culture. Another possible explanation is that LCMV-specific $CD8^+$ T cells were cross-reactive, and capable of being stimulated by more than one LCMV-derived peptide epitope. It was unclear if the magnitude of the total LCMV-specific $CD8^+$ T cell response was an experimentally induced artifact or the result of cross-reactive T cells. We carried out a series of peptide vaccination experiments in an attempt to determine if T cell cross-reactivity is, at least partially, responsible for the perceived magnitude of the $CD8^+$ T cell response to acute LCMV-Arm infection.

The murine T cell repertoire is highly diverse, and it is estimated that individual animals are capable of producing roughly 10^6 unique MHC-I restricted clonotypes (Casrouge et al. 2000, Nikolich-Zugich et al. 2004, Welsh et al. 2010). However, the potential constellation of unique peptides that can be generated in a 9 amino acid sequence is an even greater number of 5×10^{11}. If $CD8^+$ T cells were to be able to respond to every possible combination of 9mer amino acid sequence, there would have to be some level of cross-reactivity within $CD8^+$ T cell populations. Cross-reactivity also makes sense on a theoretical basis because of the multiple potential pathogens that will be encountered over the life of an animal. Even a small degree of cross-reactivity between viruses has the potential to enhance protection from a novel pathogen. In the early 1990s, several labs began to discover that multiple, seemingly unrelated, peptides from a homologous or heterologous virus were able to stimulate the same $CD8^+$ T cells (Kuwano et al. 1991, Anderson et al. 1992, Selin et al. 1994). More

recently, LCMV-specific $CD8^+$ T cells have been shown to be cross-reactive against antigen derived from Pichinde virus (PV) and vaccinia virus (VV) (Brehm et al. 2002, Kim et al. 2005, Cornberg et al. 2006). However, to our knowledge, an investigation of cross-reactivity among LCMV-specific $CD8^+$ T cell populations has not been conducted.

In this study, we performed peptide vaccinations in C57BL/6 mice, using 20 MHC-I-restricted LCMV peptide epitopes. We successfully generated vaccine-induced $CD8^+$ T cell responses to 12 of these peptide epitopes. These vaccine-induced $CD8^+$ T cell responses displayed a remarkable degree of cross-reactivity to non-homologous LCMV peptides. In some cases, vaccination with a single peptide induced $CD8^+$ T cells that could respond to 1-4 other LCMV peptides, having little or no sequence homology. These results suggest that LCMV-specific $CD8^+$ T cells are highly cross-reactive.

Results

Peptide vaccination to generate $CD8^+$ T cell responses

In order to determine if LCMV-specific T cells were cross-reactive with multiple peptide epitopes, we first generated $CD8^+$ T cell responses to individual peptides. It is possible to achieve relatively large $CD8^+$ T cell responses by serial vaccination with minimal peptide epitopes (Kochenderfer et al. 2007, Wick et al. 2011). We decided to use a similar peptide vaccination strategy to generate responses to individual LCMV peptide epitopes. A helper $CD4^+$ T cell response is required during priming to induce a functional memory $CD8^+$ T cell population

(Shedlock and Shen 2003, Sun and Bevan 2003), and other groups have included the Hepatitis B core antigen ($HBC_{128-140}$) in their vaccine preparations in order to generate a helper $CD4^+$ T cell response (Kochenderfer et al. 2007, Wick et al. 2011). Because the purpose of our experiments was to determine if cross-reactivity existed among $CD8^+$ T cell epitopes, we chose to eliminate this possible confounding factor in our cross-reactivity experiments. The vaccine preparation we used in these experiments was based on previously published formulas (Kochenderfer et al. 2007, Wick et al. 2011), and contained 50 µg of the LCMV peptide of interest, 10 µg CpG-ODN as adjuvant, and 100 ng IL-2 formulated in either PBS or incomplete Freund's adjuvant. Serial vaccinations were delivered subcutaneously on days 0, 3, and 6. Vaccinations delivered on days 0 and 3 were formulated in PBS, while the day 6 vaccination was formulated in incomplete Freund's adjuvant. The final vaccination was formulated in incomplete Freund's adjuvant in order to generate an antigen depot (Kündig et al. 1996). Using this technique, we were able to achieve peptide-specific T cell responses as large as 3.4% of the $CD8^+$ T cell compartment (Fig. 5.1). The average magnitude of responses in successfully vaccinated animals was 1.07% (range of 0.07% to 3.4%) of the $CD8^+$ T cell compartment (Figs. 5.1 & 5.3).

Vaccination with NP396 peptide induces a cross-reactive $CD8^+$ T cell response

In C57BL/6 mice, NP396 is among the most immunodominant peptide epitopes following primary infection with LCMV, with responding cells constituting

as much as 15-25% of the $CD8^+$ T cell compartment (Kotturi et al. 2007, Raué and Slifka 2009). Using our peptide vaccination strategy, we achieved frequencies of $CD8^+$ T cells that produced IFNγ in response to NP396 stimulation of up to 3.4% 7 days after the final vaccination (Fig. 5.1). The average magnitude of NP396-specific $CD8^+$ T cell responses that we obtained with this vaccine strategy over two separate experiments was 1.65% (Figs. 5.1 & 5.3). $CD8^+$ T cells produced in response to peptide vaccination demonstrated reduced polyfunctionality when compared to T cells produced in response to H_2O_2-LCMV vaccination. In a representative animal, the percentage NP396-specific $CD8^+$ T cells that were double positive for IFNγ and TNFα was 79% in response to peptide vaccination. In the same animal, the percentage of NP396-specific $CD8^+$ T cells that were triple positive for IFNγ, TNFα, and IL-2 was 2% (Fig. 5.2). This is in stark contrast to the 98% of double cytokine producing ($IFN\gamma^+$ and $TNF\alpha^+$) and 50% of triple cytokine producing ($IFN\gamma^+$, $TNF\alpha^+$, and $IL\text{-}2^+$) events found at a similar time point after H_2O_2-LCMV vaccination (Fig. 5.2). We observed similar cytokine production profiles when 50 µg $HBC_{128\text{-}140}$ was utilized as part of vaccine preparations, suggesting that a lack of $CD4^+$ T cell help was not responsible for this result. This data indicates that vaccination with H_2O_2-LCMV may elicit a $CD8^+$ T cell response with more advantageous cytokine production characteristics than vaccination with optimal peptide epitopes.

$CD8^+$ T cells from NP396 peptide vaccinated animals were screened against our panel of 20 LCMV-specific peptides. We found two other peptides that were able to stimulate NP396-specific T cells to varying degrees. The L156

peptide was able to stimulate an average of 67% of the NP396-specific T cells, while the L455 epitope was able to stimulate an average of 47% of the NP396-specific T cells (Figs. 5.1 & 5.3). Cross-reactivity of the NP396-specific T cells with L156 and L455 was consistent over more than 6 independent experiments, using multiple stocks of both the vaccinating and stimulating peptides. This indicates that cross-contamination of peptide stocks is not likely to be the source of these results. Non-cross reactive LCMV peptide epitopes showed no difference from unstimulated control samples (Figs. 5.1 & 5.3), indicating that the $CD8^+$ T cell stimulation observed in response to cross-reactive peptides was antigen-specific.

Multiple $CD8^+$ T cell responses to LCMV peptide epitopes cross-react

Following the discovery of cross-reactive NP396-specific $CD8^+$ T cells, we next determined the extent of cross-reactivity among other LCMV-specific $CD8^+$ T cells. To accomplish this task, we performed peptide vaccinations, as described in the previous section, using each of the 20 individual LCMV peptide epitopes. Seven days following the final vaccination, $CD8^+$ T cells from the animals were stimulated with the 20 individual LCMV peptide epitopes, followed by ICCS for IFNγ. The mice were vaccinated in groups of 2 animals each, and we detected $CD8^+$ T cell responses to the vaccinating antigen in at least one animal for 12 of the 20 tested peptide epitopes. Vaccinations using the other 8 peptide epitopes did not generate detectable T cell responses. In 9 of the 12 groups with $CD8^+$ T cell responses both animals responded to vaccination; however, 3 of the groups had a single animal that was a vaccine failure.

Remarkably, 11 of the 12 $CD8^+$ T cell populations that were stimulated demonstrated some level of cross-reactivity, and were capable of being stimulated by at least 2 peptides. $CD8^+$ T cells generated in response to NP165 were able to cross-react with GP221 and L349 (Fig. 5.3A). $CD8^+$ T cells generated in response to NP238 were able to cross-react with GP365, L338 and L1428 (Fig. 5.3B). $CD8^+$ T cells generated in response to NP396 were able to cross-react with L156 and L455, as previously described (Fig. 5.3C). $CD8^+$ T cells generated in response to GP33 were able to cross-react with L156 (Fig. 5.3D). $CD8^+$ T cells generated in response to GP118 were able to cross-react with L1428 (Fig. 5.3E). $CD8^+$ T cells generated in response to GP276 were able to cross-react with GP61 and L156 (Fig. 5.3F). $CD8^+$ T cells generated in response to L156 were able to cross-react with L455 (Fig. 5.3G). $CD8^+$ T cells generated in response to L338 were able to cross-react with L313, L663, and L1428 (Fig. 5.3H). $CD8^+$ T cells generated in response to L663 were able to cross-react with L313, L338, L349, and L1428 (Fig. 5.3I). $CD8^+$ T cells generated in response to L775 responded only to the vaccinating peptide, L775 (Fig. 5.3J). $CD8^+$ T cells generated in response to L1428 were able to cross-react with L313, L338, L349 and L663 (Fig. 5.3K). $CD8^+$ T cells generated in response to L2062 were able to cross-react with NP165 (Fig. 5.3L).

The ability of LCMV-specific $CD8^+$ T cells to be stimulated by multiple LCMV peptide epitopes is outlined in Tables 5.1 and 5.3. In Table 5.1 the interactions are sorted based the peptide used for vaccination, and all peptide epitopes capable of stimulating a given T cell population are listed. In Table 5.3

the interactions are sorted with regard to the peptide used for stimulation, and all T cell populations that are able to stimulated by that peptide epitope are listed. Table 5.3 illustrates that the ability of LCMV peptide epitopes to cross-react with $CD8^+$ T cells of different specificities is dominated by peptides derived from the L protein. In most cases, the cross-reacting peptide epitopes do not share sequence homology (Table 5.3). Both H-$2D^b$ and H-$2K^b$ MHC-I-restricted peptides were found to be cross-reactive, and these peptides have widely varying peptide-MHC binding affinities (Table 5.3). However, when we compared cross-reactivity with functional avidity a different story emerged. The LCMV peptide-specific $CD8^+$ T cell responses were divided into high and low functional avidity groups (Fig. 4.1 & Table 5.3). High functional avidity was defined as any peptide that was able to stimulate half of antigen-specific $CD8^+$ T cells at a peptide concentration of less than 1×10^9 molar. Low functional avidity peptides were defined as any peptide that required greater than 1×10^9 molar concentration to stimulate half of antigen-specific T cells. Interestingly, when functional avidity and cross-reactivity were compared (Table 5.3), no overlap existed between high functional avidity peptide epitopes and cross-reactive peptide epitopes. This means that peptides defined as low functional avidity were frequently capable of stimulating multiple $CD8^+$ T cell populations, while peptides defined as high functional avidity were capable of stimulating only a single T cell population.

The ability of LCMV-derived peptides to cross-react with each other is graphically represented in Figure 5.4. In this figure, each LCMV-derived peptide

capable of stimulating a $CD8^+$ T cell response of another specificity is shown. Arrows in the figure point from the peptide used for stimulation to the $CD8^+$ T cell population that is stimulated. When applicable, arrows run in both directions, indicating that cross-reactivity is bi-directional. Here, we observe that low functional avidity peptide epitopes are able to stimulate $CD8^+$ T cells of multiple specificities. These low functional avidity peptides are able to stimulate T cells that were primed using both high and low functional avidity peptides. However, high functional avidity peptide epitopes are only capable of stimulating $CD8^+$ T cells of a single specificity.

Discussion

The goal of this work was to determine whether LCMV-specific $CD8^+$ T cells are capable of being stimulated by more than one LCMV-derived peptide epitopes. Using peptide vaccination, we were able to generate measurable $CD8^+$ T cell populations against 12 of 20 individual antigenic determinants. T cells primed through peptide vaccination demonstrated a less polyfunctional production profile compared to T cells primed by vaccination with H_2O_2-inactivated LCMV (Fig. 5.2). This means there were fewer $CD8^+$ T cells elicited by peptide vaccination that were capable of making TNFα or IL-2 in addition to IFNγ. We were able to demonstrate that $CD8^+$ T cells generated in response to vaccination with individual LCMV peptides were highly cross-reactive, with some of these T cell populations able to respond to as many as 4 LCMV-derived peptides in addition to the original peptide used for vaccination. A recent publication demonstrated that arenavirus-specific $CD4^+$ T cell populations could

be stimulated by multiple, non-homologous, peptide epitopes (Kotturi et al. 2010). However, the level of cross-reactivity among peptide epitopes within the $CD8^+$ T cell response to LCMV is unprecedented. This data may indicate that a number of broadly specific low functional avidity $CD8^+$ T cell responses contribute to protection from acute or chronic viral infection.

It has been understood for some time that it is possible for $CD8^+$ T cells to cross react with multiple peptide epitopes, and the phenomenon has been demonstrated in both murine and human viral infections (Selin et al. 2006, Welsh et al. 2010). There are several antigenic determinant groups shared by LCMV, Pichinde virus (PV), and vaccinia virus (VV) that have been previously shown to be cross-reactive (Brehm et al. 2002, Kim et al. 2005, Cornberg et al. 2006). One of the best known examples of this is the NP205 epitope shared by the distantly related arenaviruses, LCMV, and PV. It has been shown that NP205-specific $CD8^+$ T cells generated in response to LCMV infection can be boosted by primary infection with PV (Selin et al. 1994, 1998, Brehm et al. 2002). The reverse is also true, and PV primed T cells are capable of responding to LCMV infection. It has also been shown that previous infection with LCMV heavily skews the $CD8^+$ T cell response to primary PV infection in favor of the NP205 peptide epitope, typically a subdominant antigenic determinant in PV infection (Brehm et al. 2002). This same group has also demonstrated that previous exposure to LCMV lowers peak PV viremia during primary PV infection 10-fold (Selin et al. 1998, Brehm et al. 2002). However, the NP205 peptide sequences derived from LCMV and PV are extremely homologous, and differ by only 2

amino acids (LCMV NP205: YTVKYPNL, PV NP205: YTVKFPNM) (Welsh et al. 2010). This essentially makes the NP205 epitopes derived from LCMV and PV altered peptide ligands (APL) of each other, making it difficult to compare this example to the more remarkable cross-reactivity observed across non-homologous LCMV peptides in our model system.

Cross-reactivity among $CD8^+$ T cells specific for LCMV and VV has also been demonstrated (Selin et al. 2006, Welsh et al. 2010). In those interactions, $CD8^+$ T cells from LCMV-immune mice were able to respond to heterologous infection with VV (Selin et al. 1994). However, the reverse is not true in this case, and LCMV infection is unable to stimulate a $CD8^+$ T cell response that can be boosted by VV infection (Selin et al. 1994, Clute et al. 2010). An interesting aspect of the interaction between LCMV and VV is that the cross-reacting $CD8^+$ T cell populations are inconsistent between animals. LCMV-primed $CD8^+$ T cell populations that are specific for GP34, GP118, and NP205 have all been observed to cross-react with the $A11R_{198}$ peptide epitope from VV (Welsh et al. 2010, Clute et al. 2010). However, the authors of that work were unable to predict from mouse-to-mouse which LCMV-primed $CD8^+$ T cell populations would be effectively stimulated by the VV-derived $A11R_{198}$ peptide epitope, and they believe that this effect was due to subtle differences in the private specificities of the T cell receptors (TCRs) expressed by the various animals (Welsh et al. 2010, Clute et al. 2010). In the case of this cross-reactivity network, the LCMV peptides displayed less homology with the $A11R_{198}$ peptide they were cross-reacting with (sharing 3-4 of 8 residues in common) than the LCMV interaction

with PV previously described. Interestingly, the $A11R_{198}$-specific $CD8^+$ T cells produced in response to VV infection are also capable of responding to stimulation with the VV peptide, $E7R_{130}$, meaning that they are cross reactive with both VV and LCMV peptide epitopes. The $A11R_{198}$ and $E7R_{130}$ peptides are homologous at 3 of 8 residues. Cross-reactivity between has actually been shown to be protective in the case of the interaction between LCMV and VV. Infection with VV subsequent to LCMV infection results in reduced peak viremia compared to infection with VV alone (Welsh et al. 2010). It should also be noted that $CD8^+$ T cells primed by vaccination the GP118 or GP33/34 peptides were among the T cell populations we found to be cross-reactive in our experiments (Fig. 5.4 & Table 5.1).

In the case of murine influenza A infection, both homologous and heterologous cross-reactivity are also observed. In the early 1990s a fascinating observation was made by using cytotoxic $CD8^+$ T cells primed in response to influenza nucleoprotein to kill target cells coated with influenza polymerase-derived peptide epitopes (Anderson et al. 1992). In this work, influenza polymerase-derived peptide epitopes, $PB2_{146}$ and $PB2_{187}$, were used to coat target cells and $CD8^+$ T cells primed by recombinant VV-NP infection were able to kill both sets of targets (Anderson et al. 1992). Oddly, $CD8^+$ T cells primed by VV-PB infection were unable to kill targets coated with the $PB2_{146}$ and $PB2_{187}$ peptides. The authors speculated that this was because the PB2-primed $CD8^+$ T cells were low functional avidity; however, it has been recently demonstrated that low functional avidity T cells are capable of efficiently killing infected targets

(Zehn et al. 2009). On the other hand, it has been shown that $CD8^+$ T cells specific for LCMV GP34 or GP276 are able to respond to stimulation with the influenza A peptides, $PB1_{703}$ and PA_{224}, respectively (Welsh et al. 2010). Similar to LCMV-primed $CD8^+$ T cells that cross-react with VV peptide epitopes, these GP34 and GP276-specific T cells are capable of cross-reacting both with other LCMV peptide epitopes as well as peptides from influenza A virus. These results indicate that while the level of cross-reactivity we observed among LCMV peptides is unprecedented, the concept of promiscuous $CD8^+$ T cells that interact with antigenic determinants from multiple viruses is established in the literature.

These results begin to depict a very intriguing picture of $CD8^+$ T cell cross-reactivity within and among different viruses. We know of at least 3 T cell populations primed by acute LCMV infection (GP34-, GP118-, and GP276-specific) that are capable of cross reacting with other LCMV peptides as well as peptides derived from either VV or influenza A virus. This may indicate that TCRs expressed by these cross-reactive $CD8^+$ T cells are broadly reactive, and able to interact with common motifs found within heterologous and homologous viral sequences. It is also possible that these T cell populations are able to provide protection from several viruses, other than the one that primed the original response

An interesting aspect of our findings was the relationship we were able to show between peptide-specific functional avidity and cross-reactivity. Our results demonstrate that peptide epitopes involved in high functional avidity interactions, such as NP396 and GP33 peptides, were unable to stimulate $CD8^+$ T cells of

other specificities. On the other hand, peptide epitopes involved with low functional avidity interactions, such as L156 and L663 were able to effectively stimulate multiple $CD8^+$ T cell populations of other primary peptide specificities. While the implications of these data are currently unknown, it has been demonstrated previously that high antigen load during T cell priming may promote the outgrowth of low functional avidity T cells (Alexander-Miller 2005, Kim et al. 2006, Kroger and Alexander-Miller 2007). There is data indicating that low functional avidity $CD8^+$ T cells are capable of killing in an antigen-specific manner, albeit at peptide concentrations that are 1-3 orders of magnitude higher than what is required for high functional avidity epitopes (Bennett et al. 2007, Zehn et al. 2009). It has also been shown that functional avidity can increase substantially for some, but not all, $CD8^+$ T cell populations as the responses mature (Slifka and Whitton 2001, Raué and Slifka 2009). It may be possible, that the ability of $CD8^+$ T cells to interact with multiple specificities of LCMV peptide on infected targets may augment the ability of $CD8^+$ T cells to effectively kill infected cells during acute viral infection, particularly before functional avidity has fully matured.

With these experiments, we were able to generate $CD8^+$ T cell populations through vaccination with optimal peptide epitopes. We show that these peptide vaccine-induced $CD8^+$ T cell responses have reduced polyfunctionality compared to T cells elicited by vaccination with H_2O_2-LCMV. We were able to demonstrate that several $CD8^+$ T cell populations, primed by a single synthetic peptide, were capable of cross-reacting with multiple antigenic determinants. We observed that

peptides capable of stimulating multiple $CD8^+$ T cell populations were derived from low functional avidity interactions, and that peptides derived from high functional avidity interactions were not capable of cross reactivity in our system. These results provide some insight into the level of broadly reactive $CD8^+$ T cell populations that exist within the immune response to LCMV, and raise many questions regarding the role of cross-reactive $CD8^+$ T cells.

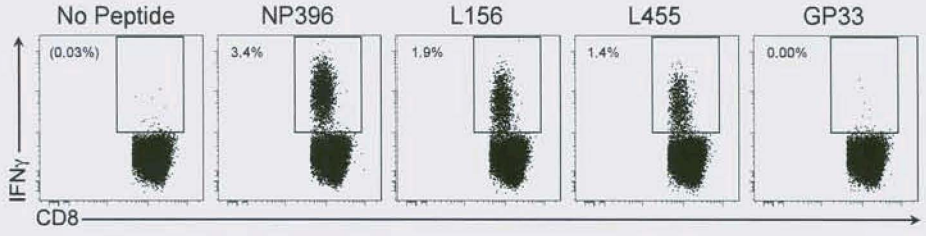

Figure 5.1: NP396-specific $CD8^+$ T cells cross-react with two other LCMV peptides

C57BL/6 mice were vaccinated using 50 µg of NP396 peptide formulated with 10 µg CpG-ODNs and 100 ng IL-2 in incomplete Freund's adjuvant subcutaneously. The animals were boosted on days 3 and 6 using the same vaccine formulation, and the experiment was performed on day 13. Cells from NP396 peptide-vaccinated animals were stimulated with LCMV peptide epitopes followed by ICCS. The dot plots show the response from a representative animal. $CD8^+$ T cells from NP396 peptide-vaccinated animals were able to produce cytokine in response to NP396, L156, and L455 peptide stimulation, but not GP33 peptide stimulation.

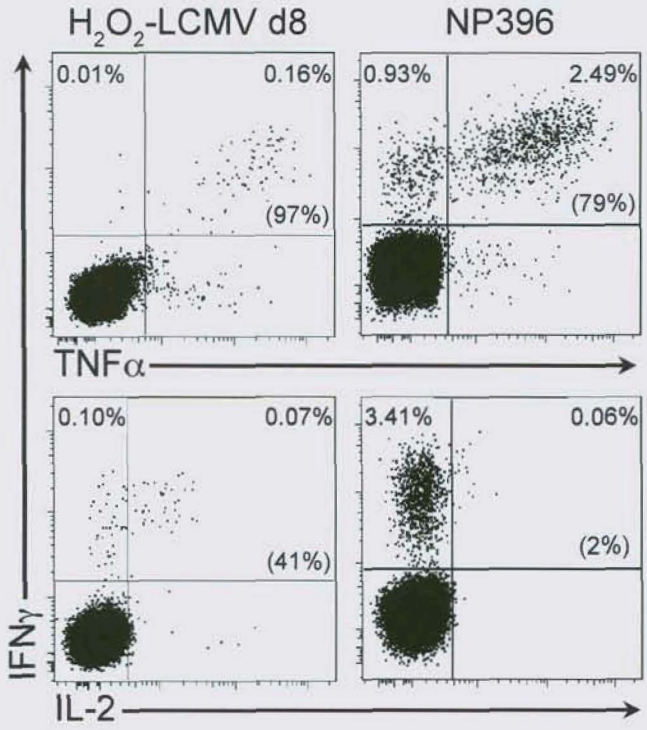

Figure 5.2: CD8$^+$ T cells primed by peptide vaccination exhibit low polyfunctionality

Cytokine production profile analysis was performed 8 days following H_2O_2-LCMV vaccination or 7 days final vaccine dose of NP396 peptide. The NP396-vaccinated animal was administered 3 doses of peptide vaccine, at days 0, 3, and 6. The cytokine production profiles shown are representative of other animals receiving the same vaccinations. Cytokine profiles from the H_2O_2-LCMV vaccinated animal are the same data shown in Figure 4.3, and are presented here for comparison purposes. Data in parentheses indicates the percentage of IFNγ positive events that also produce either TNFα or IL-2.

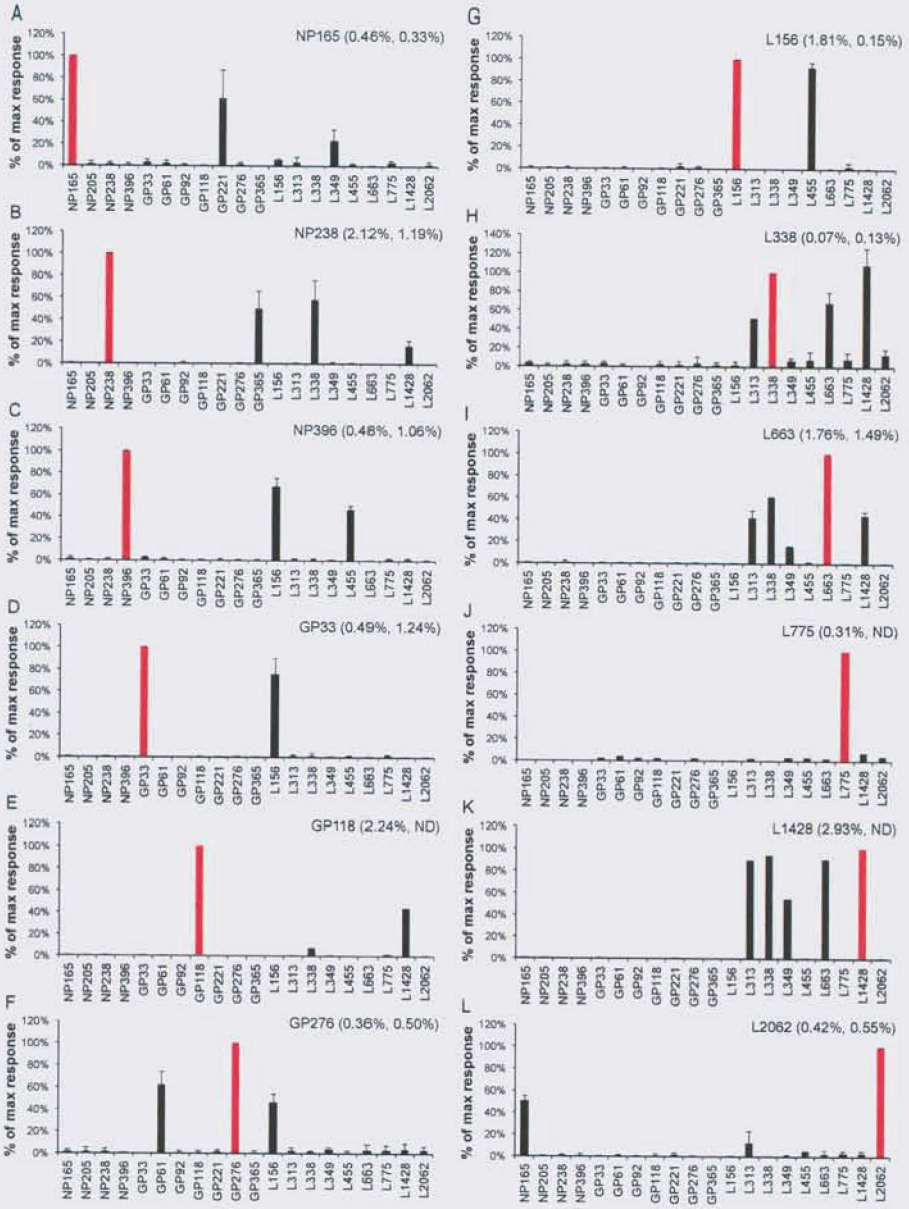

Figure 5.3: CD8$^+$ T cells stimulated by LCMV peptide vaccination are cross-reactive with other LCMV epitopes

Animals were vaccinated using 50 µg of LCMV peptide formulated with 10 µg CpG-ODNs and 100 ng IL-2 in incomplete Freund's adjuvant subcutaneously. Booster vaccinations of the same peptide were administered on days 3 and 6. On day 13 CD8$^+$ T cells were stimulated using our panel of 20 LCMV peptide epitopes followed by ICCS for IFNγ. Graphs show the response to the vaccinating peptide as 100% of the maximum response, and cross-reactivity with other peptide epitopes is shown as a percentage of the maximum response to the vaccinating peptide. Peptide vaccination resulted in responses to NP165 (A), NP238 (B), NP396 (C), GP33 (D), GP118 (E), GP276 (F), L156 (G), L338 (H), L663 (I), L775 (J), L1428 (K), and L2062 (L). Bars representing the vaccinating peptide are displayed in red. ND indicates a vaccine failure in one animal. Graphs represent 1-2 animals per group over a single experiment.

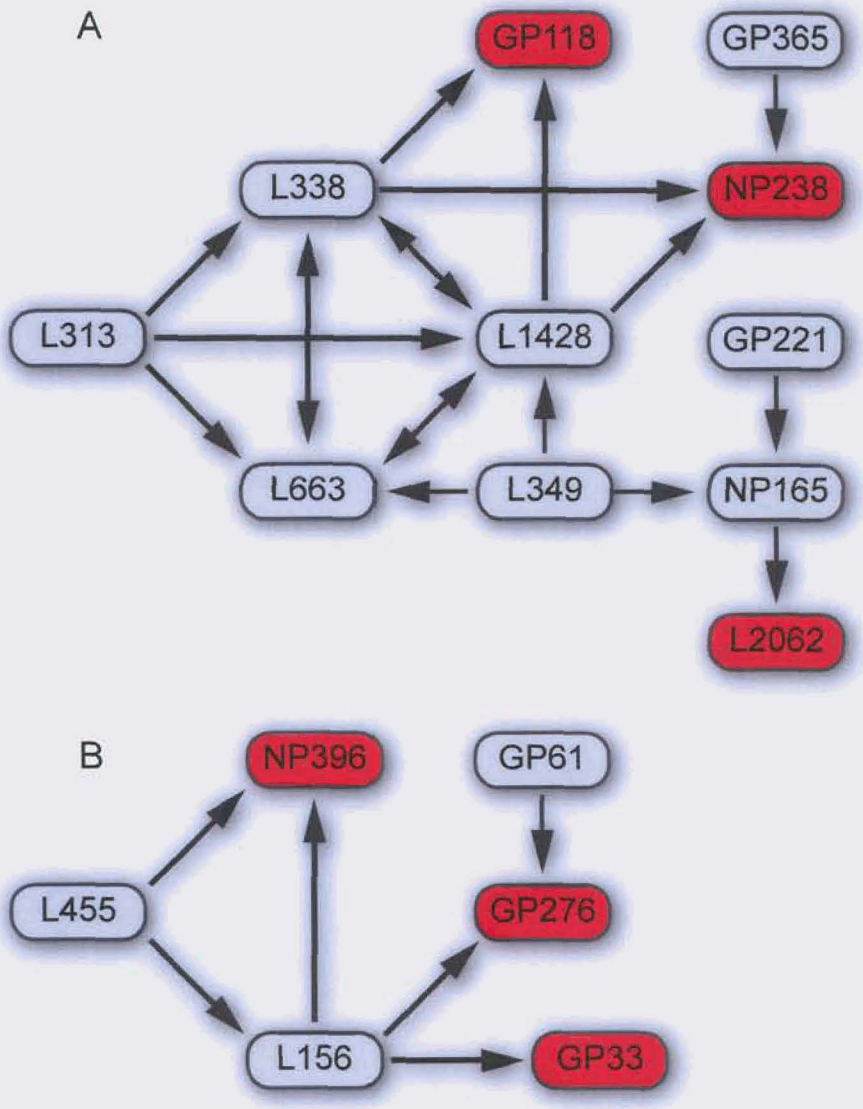

Figure 5.4: LCMV peptide epitope cross-reactivity map

This is a graphical representation of the cross-reactivity observed in our peptide vaccination experiments. Cross-reactivity was observed in two non-overlapping groups (A & B). The direction of the arrows indicates that a peptide indicated at the tail of the arrow was able to stimulate cells produced by vaccination with the peptide at the head of the arrow. For example, L156 is able to stimulate cells produced by NP396 peptide vaccination, but not vice versa. Double-headed arrows indicate that stimulation occurred in both directions. Peptide epitopes with functional avidity ½ max values of less than 1 X 10^9 molar are considered high functional avidity epitopes and are marked in red.

Peptide used for vaccination	Responding Peptide
NP165	NP165 GP221 L349
NP238	NP238 GP365 L338 L1428
NP396	NP396 L156 L455
GP33	GP33 L156
GP118	GP118 L338 L1428
GP276	GP276 GP61 L156
L156	L156 L455
L338	L338 L313 L663 L1428
L663	L663 L313 L338 L349 L1428
L775	L775
L1428	L1428 L313 L338 L349 L663
L2062	L2062 NP165

Table 5.1: LCMV-specific $CD8^+$ T cell responses sorted by vaccinating peptide

This table represents cross-reactivity observed in Figure 5.3. The column on the left is the peptide that was used to vaccinate animals. The column on the right represents all the LCMV peptide epitopes that were able to stimulate $CD8^+$ T cells from those animals. Peptides that failed to induce a $CD8^+$ T cell response after vaccination were NP205, GP61, GP92, GP221, GP365, L313, L349, and L455.

Peptide used for stimulation	Peptide used for vaccination
NP165	NP165 L2062
NP238	NP238
NP396	NP396
GP33	GP33
GP61	GP276
GP118	GP118
GP221	NP165
GP276	GP276
GP365	NP238
L156	L156 NP396 GP33 GP276
L313	L338 L663 L1428
L338	L338 NP238 GP118 L663 L1428
L349	NP165 L663 L1428
L455	NP396 L156
L663	L663 L338 L1428
L775	L775
L1428	L1428 NP238 GP118 L338 L663
L2062	L2062

Table 5.2: LCMV-specific $CD8^+$ T cell responses sorted by stimulating peptide

This table is a representation of cross-reactivity observed in Figure 5.3, sorted by stimulating peptide. LCMV peptide epitopes that were able to stimulate $CD8^+$ T cells from vaccinated animals are shown in the left column. The right column represents the peptide used to vaccinate the cross-reactive T cells.

Peptide Epitope	Sequence	Functional Avidity 1/2 Max (M)	Restriction Element	Binding Affinity (IC50 nM)
NP165	**SSLLNNQFGTM**	**1.5×10^{-7}**	**D^b**	**14000**
NP205	YTVKYPNL	$<1.0 \times 10^{-11}$	K^b	0.55
NP238	SGYNFSLGAAV	$<1.0 \times 10^{-11}$	K^b	0.38
NP396	FQPQNGQFI	6.0×10^{-11}	D^b	0.23
GP33	KAVYNFATC	9.0×10^{-11}	D^b	1439
GP34	AVYNFATC	4.0×10^{-10}	K^b	1.2
GP61	**GLKGPDIYKGVYQFKSVEFD**	**3.0×10^{-7}**	**D^b**	**ND**
GP92	CSANNSHHYI	3.0×10^{-5}	D^b	113
GP118	ISHNFCNL	5.0×10^{-10}	K^b	1.5
GP221	**SQTSYQYL**	**$6.0E \times 10^{-6}$**	**K^b**	**7.8**
GP276	SGVENPGGYCL	1.5×10^{-10}	D^b	414
GP365	**MGVPYCNY**	**2.0×10^{-6}**	**K^b**	**5740**
L156	**ANFKFRDL**	**$6.0E \times 10^{-6}$**	**K^b**	**4.6**
L313	**TSTEYERL**	**$2.0E \times 10^{-5}$**	**K^b**	**34**
L338	**RQLLNLDVL**	**2.5×10^{-5}**	**D^b**	**0.15**
L349	**SSLIKQSKF**	**3.0×10^{-7}**	**K^b**	**472**
L455	**FMKIGAHPI**	**1.5×10^{-6}**	**D^b**	**188**
L663	**VVYKLLRFL**	**1.0×10^{-8}**	**K^b**	**43**
L775	SSFNNGTL	1.0×10^{-6}	K^b	104
L1428	**NSIQRRTL**	**1.5×10^{-5}**	**K^b**	**58794**
L2062	RSIDFERV	5.5×10^{-11}	K^b	1.1

Table 5.3: LCMV-specific peptide epitope attributes

This table is a compilation of data on each of the 20 LCMV peptide-epitopes used in these experiments. Information regarding amino acid sequence, restriction element, and peptide-MHC binding affinity were taken from a publication by Alessandro Sette's group (Kotturi et al. 2007). Functional avidity ½ max values were calculated using peptide titration curves in Figure 4.1. Peptide epitopes with functional avidity ½ max values less than 1×10^9 molar are considered high functional avidity and are marked in red. Peptide epitopes that were able to cross-react with $CD8^+$ T cells primed by another epitope are shown in bold.

Chapter 6 - Discussion & Future Directions

The overall goal of this thesis was to compare the functional characteristics, protective ability, and specificity of $CD8^+$ T cells generated in response to acute LCMV infection, chronic LCMV infection, and LCMV vaccination. During the course of these experiments, we have been able to come to several interesting conclusions, which are listed below. However, many questions were raised during the course of this work that remain to be answered. The following section is a discussion of some of these questions in the context of the current immunological literature.

Summary of Results:

◆ It is possible to generate durable $CD8^+$ T cell responses to LCMV using an inactivated whole virus vaccine.

◆ $CD8^+$ T cell immunity generated in response to H_2O_2-LCMV vaccination has a more polyfunctional cytokine profile than T cell responses to live LCMV-Arm infection. Specifically, a higher percentage of vaccine-induced $CD8^+$ T cells are able to produce TNFα and/or IL-2 in response to stimulation,

◆ H_2O_2-LCMV-induced $CD8^+$ T cell responses underwent rapid expansion following challenge with LCMV-Arm, and were maintained at high levels following challenge.

◆ H_2O_2-LCMV vaccination generates a different immunodominance hierarchy than what is observed during either acute or chronic LCMV infection.

◆ Vaccine-induced immunodominance is "fixed," and does not shift upon booster vaccination or live LCMV challenge, while immunodominance hierarchies

following acute or chronic LCMV infection may change or evolve more dramatically.

◆ H_2O_2-LCMV vaccination protects against viremia and T cell dysfunction following LCMV-Clone 13 challenge.

◆ $CD8^+$ T cells primed using a single LCMV-derived peptide can respond to between 1-5 LCMV-derived peptide epitopes.

◆ Antigen-specific $CD8^+$ T cells primed through peptide vaccination have less polyfunctional cytokine profiles, with few IFNγ positive cells also able to produce IL-2, than T cells primed by vaccination with H_2O_2-LCMV.

◆ Low functional avidity LCMV peptides may be capable of stimulating more than a single $CD8^+$ T cell population, while high functional avidity peptides were only capable of stimulating a single $CD8^+$ T cell population.

Is it possible that cross-presentation of H_2O_2-LCMV antigen is responsible for the immunodominance hierarchy observed following vaccination?

The most likely mechanism by which H_2O_2-LCMV vaccination is able to stimulate an antigen-specific $CD8^+$ T cell response is through cross-presentation. Cross-presentation is the loading of exogenous antigen onto MHC-I. This is in contrast to direct-presentation in which endogenous antigen is used as a source for the peptides loaded onto MHC-I. A number of reasons contribute to the likelihood that cross-presentation is the mechanism at work following H_2O_2-LCMV vaccination. We are able to demonstrate killing of LCMV to a sensitivity of 1-2 infectious units per 200 µg (~2 X 10^9 PFU) of inactivated material, making it

unlikely that any infectious virus was present in vaccine preparations. Additionally, LCMV-Arm infection with very low doses (e.g., 10 PFU) of virus has been shown to result in immunodominance patterns similar to infection at high virus doses (personal communication, Dr. Hans-Peter Raué), suggesting that low-level LCMV-Arm infection is not responsible for the immunodominance pattern observed following H_2O_2-LCMV vaccination. It should also be noted that the LCMV genome is a mixture of negative-sense and pseudo positive-sense RNA that is not capable of being directly translated in the cell cytoplasm (Buchmeier et al. 2006). LCMV protein translation requires the presence of a functioning viral polymerase. The presence of LCMV polymerase activity is unlikely after the strong oxidizing treatment the virus receives during the H_2O_2 inactivation (Miller and Britigan 1997). Finally, reactive oxygen species, such as H_2O_2, have been shown to damage purine and pyrimidine bases (Valko et al. 2007). This makes it unlikely that any residual LCMV protein translation is occurring in animals that are vaccinated with H_2O_2-inactivated LCMV.

The immunodominance pattern of H_2O_2-LCMV-induced $CD8^+$ T cell immunity is fascinating if it is considered from the perspective of the known cross-presentation pathways. Cross-presentation is known to occur by at least two mechanisms TAP-dependent and TAP-independent. The TAP-dependent pathway is generally considered to be the more efficient cross-presentation mechanism, and is 1-2 orders of magnitude more efficient than the TAP-independent pathway (Sigal and Rock 2000). It is believed that the LCMV-NP396 peptide epitope can be processed and presented in a TAP-independent

as well as a TAP-dependent manner (Sigal and Rock 2000). With this in mind, it is interesting that the NP396 peptide epitope is immunodominant following H_2O_2-LCMV vaccination and responses to the GP33/34 epitopes (which are processed via a TAP-dependent mechanism (Hombach et al. 1995)) are largely, or completely, absent. If NP396 can be processed via a TAP-independent mechanism, then these results may indicate that TAP-independent mechanisms are responsible for the majority of antigen presentation that occurs following vaccination with H_2O_2-LCMV. Future directions for this project would include vaccination of animals that had been lethally irradiated and reconstituted with bone marrow from TAP deficient animals and vaccination of cathepsin deficient animals with H_2O_2-LCMV to determine the effects on vaccination-induced immunodominance. If the NP396-specific $CD8^+$ T cell response to vaccination with H_2O_2-LCMV were abrogated in cathepsin deficient animals, it would be interesting to see if T cell responses to the L156 and L455 peptide epitopes are still present. It would also be interesting to perform LCMV-Arm infections in both of these mouse models to determine the effects that loss of a single pathway might have on immunodominance during live LCMV infection.

Another interesting aspect to the H_2O_2-LCMV vaccine-induced immunodominance hierarchy is the absence of $CD8^+$ T cell responses to the GP33 peptide epitope. This is particularly thought provoking considering that we have some evidence that a weak GP34-specific $CD8^+$ T cell response is primed by H_2O_2-vaccination. One explanation for this may be the affinity of the GP33 peptide for MHC-I. The GP34 (IC50=1.2 nM) peptide has 3 orders of magnitude

higher affinity for its restriction element than the GP33 (IC50=1439 nM) peptide (Table 5.3) (Kotturi et al. 2007). In our experiments we were unable to demonstrate induction of a $CD8^+$ T cell response to any low affinity peptides following vaccination with H_2O_2-LCMV. LCMV peptides that were able to stimulate $CD8^+$ T cells following vaccination in our system had affinities ranging from 0.23-188 nm (IC50 concentration) (Kotturi et al. 2007). In the case of GP34, binding affinity of the peptide for MHC-I may have been too low to be efficiently cross-presented. This may indicate that there is some minimum level of binding affinity that must be achieved between peptide and MHC-I before cross-presentation takes place.

Why are $CD8^+$ T cell responses primed by H_2O_2-LCMV vaccination and subsequently boosted by live viral challenge maintained at such high levels?

One interesting, and quite promising, outcome of our vaccine studies was the discovery that animals that were H_2O_2-LCMV vaccinated and subsequently challenged with either LCMV-Arm or LCMV-Clone 13 achieved very high levels of $CD8^+$ T cells specific for vaccine-induced peptide epitopes. In animals that were H_2O_2-LCMV vaccinated and then challenged with LCMV-Arm, T cell responses to NP396 remained at 20-30% of the $CD8^+$ T cell compartment for at least 42 days after challenge (as shown in Chapter 4). For animals that were H_2O_2-LCMV vaccinated and then challenged with LCMV-Clone 13, $CD8^+$ T cell responses to NP396 constituted 30-50% of the $CD8^+$ T cell compartment for at least 35 days after vaccination. These $CD8^+$ T cell responses were between 2-

and 5-fold greater in magnitude than the responses observed following LCMV reinfection of immune animals.

The magnitude of these $CD8^+$ T cell responses to vaccination with H_2O_2-LCMV followed by either LCMV-Arm or LCMV-Clone 13 challenge are similar to the magnitude of T cell responses induced by a combination of GP33 peptide vaccination and GP33-expressing virus-like particle (VLP) vaccination in a paper by Schwarz et al., 2005 (Schwarz et al. 2005). However, in their system they used a heterologous prime and boost vaccine regimen that required 3 doses of vaccine to achieve the same magnitude of $CD8^+$ T cell responses that we observed in our system after challenge. In addition, the $CD8^+$ T cell immunity elicited by their vaccine was not long lived. After a single dose of their GP33-VLP vaccine, antigen-specific T cell responses returned to baseline levels within 35 days. Animals receiving 5 doses of their GP33-VLP vaccine, at weekly intervals, achieved GP33-specific $CD8^+$ T cell frequencies as high as 25-30%; however, these responses underwent rapid contraction similar to the kinetics of $CD8^+$ T cell responses during acute LCMV infection. These results indicate that our H_2O_2-inactivated vaccine strategy may induce $CD8^+$ T cell immunity that is capable of being boosted at least as efficiently as VLP-based vaccine strategies. Additionally, H_2O_2-inactived vaccines may elicit $CD8^+$ T cell responses that are maintained at higher levels after boosting than VLP-based vaccines.

A mechanism that may account for the massive expansion that the H_2O_2-LCMV vaccine-induced $CD8^+$ T cells undergo following challenge with acute or chronic virus strains is their ability to rapidly produce and respond to cytokine. It

has been documented that immunodominant $CD8^+$ T cell populations have increased ability to both produce and respond to antiviral cytokines (Liu et al. 2004, Whitmire et al. 2005). $CD8^+$ T cells induced by our vaccine strategy are predominantly central memory cells with highly polyfunctional cytokine profiles, and are likely capable of rapid response and proliferation to challenge with their cognate antigen. This may account for the large magnitude of $CD8^+$ T cell responses to vaccine-primed peptide epitopes immediately following challenge. However, it does not explain the large magnitude of the LCMV-specific $CD8^+$ T cell response 35-42 days following challenge. In these animals, which have been vaccinated prior to challenge, LCMV-specific $CD8^+$ T cell responses were 2- to 5-fold larger than in animals that were infected with LCMV-Arm and subsequently challenged.

It is unusual for the frequency of individual antigen-specific $CD8^+$ T cell responses to be as high as 30-50% at time points 35-42 days after challenge; however, this is what we observed in animals vaccinated with H_2O_2-LCMV and then challenged with LCMV-Clone 13 in Chapter 4. One explanation that has been suggested for this finding is the phenomenon previously described by John Harty's group, whereby $CD8^+$ T cells that have undergone expansion in response to secondary *Listeria* infection experience delayed kinetics requiring longer to reach their stable plateau phase than primary T cell responses (Badovinac et al. 2003). However, this phenomenon is not likely to be the explanation for our increased $CD8^+$ T cell memory in our system. In the experiments involving secondary *Listeria* infection and delayed $CD8^+$ T cell contraction, stable memory

levels were achieved by 32 days following infection. Therefore, the increased levels of LCMV-specific $CD8^+$ T cells observed following H_2O_2-LCMV vaccination and subsequent challenge likely represent stable memory levels, and will probably be maintained for the life of the animal. The kinetics of LCMV-specific $CD8^+$ T cell responses in animals that were vaccinated with H_2O_2-LCMV and then challenged most resemble those described in a publication by Masopust et al. 2006 in which animals underwent three rounds heterologous live viral infection (Masopust et al. 2006). In that work, responses to a single VSV peptide epitope were boosted to around 90% of the $CD8^+$ T cell compartment after three heterologous viral infections, and they became a stable memory population that occupied around 50% of the $CD8^+$ T cell compartment. However, in their system $CD8^+$ T cells that were the result of these tertiary infections has less proliferative potential than antigen-specific T cells that had only been through primary or secondary viral infections. A number of experiments could be done to follow up on our results. It would be interesting to determine the level of $CD8^+$ T cell immunity at 6-12 months after LCMV vaccination and challenge. It would also be interesting to determine the proliferative potential of NP396-specific $CD8^+$ T cells following challenge. With this information, we would be able to compare the half-life and proliferative capacity of boosted $CD8^+$ T cell responses to what is typically observed in LCMV-immune animals.

Could peptide cross-reactivity and functional avidity relate to the development of immunodominance?

The degree of cross-reactivity observed among LCMV-specific $CD8^+$ T cell epitopes in Chapter 5 was remarkable. Some $CD8^+$ T cell populations that were generated in response to vaccination with a single peptide were able to cross-react with as many as 4 heterogeneous peptides. Two of these $CD8^+$ T cell populations, cells responsive to GP118 and GP276, are known to respond to peptide epitopes from at least one other virus (VV and influenza A virus respectively) in addition to cross-reacting with multiple LCMV peptide epitopes. One of these $CD8^+$ T cell populations, cells responsive to GP34, is known to be stimulated by LCMV, VV, and influenza A virus infections. In this work, we demonstrated that GP34-specific $CD8^+$ T cells are also capable of being stimulated by the LCMV peptide L156. At this point, it is unknown precisely what the function is of this this level of cross-reactivity among $CD8^+$ T cell responses. In the case of VV infection and PV infection, it has been demonstrated that prior LCMV infection is protective. In the case of PV infection subsequent to LCMV infection, peak viremia is lowered by approximately 10-fold (Selin et al. 1998, Brehm et al. 2002). In the case of VV infection subsequent to LCMV infection, peak viral titers are lowered 1-2 logs (Welsh et al. 2010). Depending on the frequency of this phenomenon, it is possible to imagine a scenario in which many of these broadly cross-reactive $CD8^+$ T cell responses are able to limit replication and pathogenesis of previously unseen virus long enough for a novel adaptive immune response to be mounted against the invading pathogen.

What is more of a mystery is the existence of $CD8^+$ T cell populations that cross-react with multiple peptide epitopes from the same virus. This phenomenon has been observed in VV infection (Welsh et al. 2010), influenza A virus infection (Anderson et al. 1992), and now LCMV infection. In the case of LCMV infection, we observed that peptides that were involved with low functional avidity interactions were capable of cross-reacting with multiple $CD8^+$ T cell populations, while peptides associated with high functional avidity interactions were able to stimulate only one T cell population. Previous studies have shown that low functional avidity $CD8^+$ T cell responses are more commonly generated under high antigenic load, and these responses are not stimulated in situations where antigen stress is low (Alexander-Miller 2005, Kim et al. 2006, Kroger and Alexander-Miller 2007). It has also been shown that antigen density must typically be 1-3 orders of magnitude greater for low functional avidity $CD8^+$ T cells to mediate killing than for high functional avidity $CD8^+$ T cells (Bennett et al. 2007, Zehn et al. 2009). In chapter 5, one potential function we discussed of these multiple low functional avidity $CD8^+$ T cells was to facilitate activation of naïve T cells during the early stages of the adaptive immune response, prior to functional avidity maturation. It is possible that multiple high and low functional avidity cross-reactive peptide-MHC-I interactions are able to more easily overcome the activation threshold of naïve T cells during early infection.

An interesting scenario in which to think about the role of $CD8^+$ T cell cross-reactivity is during chronic viral infection. In the presence of an intact adaptive immune response, naïve animals infected with LCMV-Clone 13 are

ultimately able to clear viremia (Matloubian et al. 1994). This clearance of chronic viral infection has been shown to require the presence of $CD8^+$ T cell immunity (Matloubian et al. 1994). At the same time, the low functional avidity peptide epitope L156 becomes one of the 2 most immunodominant peptides during chronic infection, while the NP396-specific and GP33-specific $CD8^+$ T cell responses (with which the L156 peptide is cross-reactive) are clonally deleted or become functionally anergic. It is possible that these low functional avidity $CD8^+$ T cell responses are protected from clonal deletion and functional anergy in chronic infection. These protected low functional avidity $CD8^+$ T cell responses would then be present under the high antigen loads associated with chronic LCMV infection, and potentially able to mediate clearance of the virus. In the future, it will be important to determine the functional avidity of $CD8^+$ T cells for peptide epitopes other than the one they are vaccinated against (i.e., functional avidity of NP396-specific $CD8^+$ T cells for L156 peptide). It will also be interesting to determine if multiple low functional avidity $CD8^+$ T cell populations are capable of protecting animals from LCMV-Clone 13 serum viremia.

Summary Statement

The work described in this thesis outlines the characteristics, protective ability, and cross-reactivity of $CD8^+$ T cell responses to LCMV infection as well as a novel H_2O_2-inactivated LCMV vaccine. We have demonstrated that it is possible to generate protective and durable $CD8^+$ T cell immunity, using a vaccine that is most likely cross-presented *in vivo* to naïve T cells. This protective $CD8^+$ T cell response has a markedly different immunodominance

hierarchy than what is observed following either acute or chronic infection with live virus. Additionally, we have demonstrated a high degree of cross-reactivity among $CD8^+$ T cells and antigenic determinants from the same virus. These results suggest that inactivated whole virus vaccines may be capable of generating clinically relevant $CD8^+$ T cell immunity, and contribute to our understanding of how future vaccines might be optimized to elicit these responses.

Chapter 7 - Methods

Animals

Female C57BL/6, SCID, and $Rag2^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and used at 6 to 20 weeks of age. All mice were housed at the Vaccine and Gene Therapy Institute according to standards of the Institutional Animal Care and Use Committee (IACUC) and the NIH Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996). The OHSU IACUC approved all animal use protocols in these studies. Results shown from these studies were performed using 1-4 animals per experimental group.

Viruses

LCMV-Arm and LCMV-Clone 13 were grown on BHK-21 cells (ATCC) at an MOI of 0.1. Monolayers were 50-75% confluent at the time of virus infection. Monolayers were overlaid with 1.5 ml of virus stock diluted in completed DMEM and incubated at 37°C, 6% CO_2 for 1 hour. Following incubation, 15 ml of completed DMEM was added to flasks, which were then incubated for an additional 48 hours at 37°C, 6% CO_2. Virus was harvested after 48 hours and purified from tissue culture supernatant via ultracentrifugation at 80,000 X g at 4°C for 3 hours over a 25% glycerol cushion. Pelleted virus was dialyzed, using a 10,000-dalton molecular weight cutoff membrane, against 3 exchanges of PBS (4-12 hours at 4°C per exchange) to remove glycerol.

Virus titer was determined via plaque assay. Plaque assays were performed as described previously (Ahmed et al. 1984). Plaque assay plates were seeded using $3x10^5$ Vero cells per well in 6 well plates. Plates were incubated overnight, media was discarded, and the wells were overlaid with 200 µl of diluted serum and incubated for 1 hour at 37°C, 6% CO_2. Wells were then overlaid with 3 ml 0.5% agarose in EMEM supplemented with 2.5% FBS, antibiotics and 2 mM glutamine and incubated at 37°C, 6% CO_2, for 5 days. Wells were then overlaid with 1 ml 1% agarose containing 0.1-0.2% Neutral Red (Sigma-Aldrich, St. Louis, MO), and plaques were counted 12-18 hours later. Viral titer for LCMV-Clone 13 used in these experiments was 4×10^6 PFU per ml. Viral titers for LCMV-Arm used in these experiments ranged between 8×10^9 and 4×10^{10} PFU per ml. For acute infections, 2×10^5 PFU LCMV-Arm was administered intraperitoneally. In chronic LCMV challenge experiments, animals were injected intravenously with 2×10^6 PFU LCMV-Clone 13.

Vaccines

LCMV-Arm virus to be used for vaccination was inactivated by treatment with 3% H_2O_2 for 4 hours at room temperature. Inactivated virus was then dialyzed, using a 10,000-dalton molecular weight cutoff membrane, against 3 exchanges of PBS (4-12 hours at 4°C per exchange) to remove H_2O_2. Inactivation was confirmed by Vero cell plaque assay, co-culture experiments on BHK cells, and injection (up to 200 µg of material) into highly susceptible SCID or $Rag2^{-/-}$ mice followed by serum plaque assay to test for viremia at 7, 14, and 21 days after injection. Protein concentration of inactivated LCMV was determined

using the modified Lowry protein assay kit from Thermo Scientific (Rockford, IL). Plaque assays were performed as described above. Co-culture experiments were performed in duplicate, using 200 µg of inactivated LCMV. Co-culture flasks were prepared by seeding T150 flasks with 3 X 10^6 BHK cells, and allowing them to incubate over night in completed DMEM. The following day, the flasks were emptied and overlaid with 200 µg of inactivated virus, suspended in 1.5 ml completed DMEM. Treated flasks were incubated at 37°C, 6% CO_2 for 1 hour, at which point 10.5 ml of completed DMEM was added to the flasks. Three days after inoculation of the flasks, supernatants were collected and assayed via plaque assay as described above. Experiments in SCID or $Rag2^{-/-}$ animals were performed by injecting 200 µg of inactivated material into the animals intraperitoneally. Following injection, serum was collected 7, 14, and 21 days later, as described above, and viremia was assessed by plaque assay, as described below. H_2O_2-LCMV vaccine was formulated using 50 µg H2O2 inactivated LCMV-Arm and 5 µg monophosphoryl lipid A (MPL) (InvivoGen, San Diego, CA) in 100 µl RPMI without fetal bovine serum.

LCMV vaccinations were performed by subcutaneously injecting, at the base of the tail, 50 µg H_2O_2-LCMV-Arm formulated with 5 µg MPL. Peptide vaccinations were made using 50 µg of purified peptide (GenScript, Piscataway, NJ) formulated with 10 µg CpG-ODN 1826 (Coley Pharmaceutical Group) and 100 ng recombinant human IL-2 (BioLegend, San Diego, Ca) in incomplete Freund's adjuvant or PBS delivered subcutaneously. Peptide vaccinations were delivered subcutaneously, at the base of the tail. Vaccinations given on days 0

and 3 were formulated in PBS, while vaccinations given on day 6 were formulated in incomplete Freund's adjuvant.

Peptides

The peptides used in these experiments were a subset of the H-2(b)-restricted epitopes to the three major LCMV proteins, nucleoprotein (NP), glycoprotein (GP) and RNA polymerase (L protein), identified previously (Gairin et al. 1995, Oxenius et al. 1995, van der Most et al. 1998, Kotturi et al. 2007, Dow et al. 2008). HPLC-purified (>95% pure) peptides were purchased from Sigma (St. Louis, MO) and GenScript (Piscataway, NJ). These peptides were reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 4×10^{-2} M. For functional avidity experiments, serial dilutions were performed to achieve peptide concentrations between 10^{-4} and 10^{-11} molar for stimulation. Based on the functional avidity curves shown in Figure 4.1 we chose to use peptide concentrations between 10^{-4} and 10^{-6} molar for all further peptide stimulations. The peptides, and optimal concentrations used for T cell stimulation, included: LCMV NP165-175 (SSLLNNQFGTM, 10^{-4} M), NP205-212 (YTVKYPNL, 10^{-6} M), NP238-248 (SGYNFSLGAAV, 10^{-6} M), NP396-404 (FQPQNGQFI, 10^{-6} M), GP33-41 (AVYNFATM, 10^{-6} M), GP61-80 (GLKGPDIYKGVYQFKSVEFD, 10^{-5} M), GP92-101 (CSANNSHHYI, 10^{-6} M), GP118-125 (ISHNFCNL, 10^{-6} M), GP221-228 (SQTSYQYL, 10^{-4} M), GP276-286 (SGVENPGGYCL, 10^{-6} M), GP365-372 (MGVPYCNY, 10^{-5} M), L156-163 (ANFKFRDL, 10^{-4} M), L313-320 (TSTEYERL, 10^{-4} M), L338-346 (RQLLNLDVL, 10^{-4} M), L349-357 (SSLIKQSKF, 10^{-5} M), L455-463 (FMKIGAHPI, 10^{-4} M), L663-671 (VVYKLLRFL, 10^{-6} M), L775-

782 (SSFNNGTL, 10^{-4} M), L1428-1435 (NSIQRRTL, 10^{-4} M), L2062-2069 (RSIDFERV, 10^{-6} M). $HBC_{128-140}$ (TPPAYRPPNAPIL) was obtained from GenScript (Piscataway, NJ).

Peptide Stimulation and Cytokine Staining

In order to measure low frequency antigen-specific responses we utilized peptide stimulation followed by ICCS and a multi-step gating strategy. Intracellular cytokine staining (ICCS) was performed as previously described (Raué and Slifka 2007). Spleens were harvested from experimental animals and stored in 2 ml completed RPMI at room temperature. Splenocytes were harvested by disrupting spleens over 70 µm nylon mesh into 50 ml conical tubes. Cells were washed with 10 ml completed RPMI (centrifugation step was 250 X g for 8 minutes) and red blood cells were lysed by exposure to 0.83% NH_4Cl solution for 2 minutes. Cells were then washed with 10 ml completed RPMI (centrifugation step was 250 X g for 8 minutes) and counted using a hemocytometer. Cells were stimulated for 6 hours at 37°C, 6% CO_2 in completed RPMI with LCMV peptide (concentrations listed above) in the presence of 2 µg/ml brefeldin A. Stimulation volume was 200 µl, and stimulations were performed in 96 well round bottom plates. Stimulation was followed by centrifugation at 250 X g for 3 minutes, and supernatant was discarded. Samples were surface stained with CD8α PerCP-Cy5.5 (clone 53-6.7, eBioscience, San Diego, CA) and Aqua LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen, Carlsbad, CA), overnight at 4°C. The following day, samples were washed with 150 µl wash buffer (PBS containing 1% calf serum) and

centrifuged at 250 X g for 3 minutes. Supernatant was discarded and samples were treated with 100 µl 2% formaldehyde in PBS for 5 minutes. Following fixation samples were washed 3 times with 150 µl wash buffer. Samples were centrifuged at 250 X g and supernatant was discarded between washes. Samples were then washed 3 times with 150 µl perm buffer (0.1% saponin, 0.1% NaN_3, 1% fetal bovine serum, in PBS). Samples were centrifuged at 250 X g and supernatant was discarded between washes. Samples were then stained with IFNγ-FITC (clone XMG1.2, Invitrogen, Carlsbad, CA), TNFα PE (clone MP6-XT22, eBioscience, San Diego, CA), and IL-2 APC (clone JE S6-5H4, BioLegend, San Diego, CA) for 1 hour at 4°C. Samples were then washed 3 times with 150 µl perm buffer, and samples were centrifuged at 250 X g and supernatant was discarded between washes. Samples were then washed 3 times with 150 µl wash buffer, and samples were centrifuged at 250 X g and supernatant was discarded between washes. The samples were then re-suspended in 50 µl wash buffer and analyzed on an LSRII flow cytometer.

Tetramer Staining

Tetramer samples were processed in the same manner as intracellular cytokine stained samples (described above); however, following formaldehyde fixation tetramer samples were re-suspended in 50 µl of wash buffer and analyzed without undergoing the remainder of the staining steps intracellular stained samples were subjected to. Tetramers were obtained from the National Institutes of Health Tetramer Core Facility, Atlanta, GA. Tetramers used in these experiments were D^b-restricted tetramers GP33-41 APC or NP396-404 APC and

K^b-restricted tetramer GP34-41 APC. Tetramer samples were also stained for CD8α PerCP-Cy5.5 (clone 53-6.7, eBioscience, San Diego, CA), Aqua LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen, Carlsbad, CA), and CD11a PE (clone 2D7, BioLegend, San Diego, CA).

Flow Cytometry

ICCS and tetramer samples were acquired using an LSR-2 (BD Biosciences, San Jose, CA) flow cytometer and analyzed using FlowJo software (Treestar, Ashland, OR). Figure 4.2 illustrates the gating strategy used. The FSC-area vs. FSC-height gate was used as a singlet gate to remove clumped cells from analysis. Gating on the Aqua negative population identified the cells that were viable at the conclusion of the assay. Lymphocyte gating was performed based on FSC X SSC characteristics and $CD8^+$ gates were then applied before the percentage of cytokine-positive T cells was determined. The number of NP396 specific $CD8^+$ T cells per spleen shown in Figure 4.3 was determined by performing singlet and live cell gating, described above, on peptide stimulated ICCS samples and then applying a $CD8^+$ T cell gate to this live singlet population. The percentage of $CD8^+$ T cells per spleen was calculated by multiplying the number of cells per spleen (determined by hemocytometer count described above) by the percentage of live singlets that were $CD8^+$. This total number of $CD8^+$ T cells per spleen was then multiplied by the percentage of $CD8^+$ T cells that were NP396-specific, as determined by peptide stimulation and ICCS, to achieve the total number of NP396-specific $CD8^+$ T cells in the spleen of each animal.

Electron Microscopy

High titer LCMV was inactivated using 3% H_2O_2 for 2 hours at room temperature. Control virus was stored at 4° C during the inactivation period. Following inactivation, 90 µl of each sample was treated with 10 µl of 25% gluteraldehyde for 10 minutes. Electron microscopy grids were treated with short-wave UV light for 15 minutes to increase their hydrophobicity. UV treated grids were then placed in 10 µl of sample for 3 minutes. Grids were then wicked dry on filter paper and then placed in 10 µl water for 45 seconds. Samples were then wicked dry on filter paper and then placed in 4% uranyl acetate solution for 45 seconds. Samples were then wicked dry on filter paper and allowed to fully air dry. The OHSU electron microscopy core performed electron microscopy, and images shown in this publication are at 37,000-fold magnification.

Serum Samples

Whole blood was collected from a tail vein and allowed to coagulate at room temperature for 2 hours. Venipuncture was performed using an 18 gauge needle to nick the side veins of the mouse tail. Typically 100-150 µl of whole blood was collected. The blood was centrifuged at 16,000 RCF for 1 min and serum was collected and transferred to a new tube. The serum was again centrifuged at 16,000 RCF for 1 min and serum was collected and stored at -80°C until plaque assays were performed.

$CD8^+$ T cell depletion

$CD8^+$ T cell depletion was performed as previously described (Slifka et al. 1996). Mice were depleted using the anti-CD8α monoclonal antibody 2.43 (BioXCell, West Lebanon, NH) around the time of LCMV-Clone 13 challenge. The anti-CD8α antibody 2.43 is a rat IgG2b monoclonal antibody that was purified to greater than 95% purity using a protein G column. Animals were given 100 µg of antibody, administered intraperitoneally, on days -2, 0, +2, and +4 (where day 0 was when the animals received LCMV-Clone 13 challenge). This regimen has been shown to deplete greater than 95% of circulating $CD8^+$ T cells (Slifka et al. 1996).

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