Analysis of Antigen-Specific Murine CD8 T Cells, Following Vaccination or Murine Cytomegalovirus (MCMV) Infection

by

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ABSTRACT

CD8 T cells are an important arm of the adaptive immune response. They are particularly effective against viruses, and can limit the severity of many viral infections. A primary goal of my thesis work was to better understand CD8 T cell expansion and contraction on a population level. Specifically, I have examined the effects that the co-stimulatory molecule 4-1BB, the MCMV MHC class I immune evasion genes *m04*, *m06* and *m152*, and the NK cell activating receptor Ly49H, have on CD8 T cell activation, CD8 T cell contraction, and secondary CD8 T cell expansion.

The first part of this thesis focuses on improving an anti-viral CD8 T cell vaccine. Stimulation of the tumor necrosis factor receptor (TNFR) superfamily members 4-1BB (CD137) and OX-40 (CD134) has been reported to enhance T cell activation and protect activated T cells from death. To determine whether 4-1BB and OX40 stimulation could improve the CD8 T cell response to a DNA prime, poxvirus boost vaccine, we administered agonist antibodies at the time of the poxvirus boost. 4-1BB stimulation increased effector and memory CD8 T cell numbers by 2-4 fold, and OX-40 stimulation increased the number of antigen-specific CD4 T cells approximately 3-fold. Stimulating both 4-1BB and OX40 enhanced the CD8 T cell response more than 4-1BB alone. Thus stimulating these receptors can improve the response to a powerful virus vector, and may be useful in vaccine development.

Part two of this thesis examines the CD8 T cell response elicited by a natural mouse pathogen, murine cytomegalovirus (MCMV). Infection with MCMV elicits a strong CD8 T cell response in the C57BL/6 strain of mice, and CD8 T cells can effectively control MCMV replication. In order to measure the size and antigen

specificity of the CD8 T cell response, a novel epitope-mapping technique was developed. Using this technique, 16 new CD8 T cell antigens were discovered. This represents the broadest documented CD8 T cell response in any mouse model examined. The epitopes are also useful tools for understanding the biology of MCMV interactions with the mouse immune system. Using these epitopes, I have demonstrated three new findings. First, the immune evasion genes *m04*, *m06*, and *m152*, which strongly inhibit MHC class I antigen presentation in vitro, do not significantly affect CD8 T cell priming in vivo. Second, the CD8 T cell response to some MCMV epitopes, IE3, M38 and m139, expand after a state of viral latency is established, suggesting that these CD8 T cells are in a state of active surveillance, possibly inhibiting MCMV from fully reactivating from latency. Third, I have compared the size and antigen specificity of the CD8 T cell response to MCMV using strains of mice that share *H-2* MHC haplotypes, but have different genetic backgrounds. I found that the B6 background has a much stronger CD8 T cell response than the BALB background. Paradoxically, the strong NK cell response of C57BL/6 mice, which decreases viral replication ~1000-fold, enhances CD8 T cell responses. Additionally, the CD8 T cell immunodominance hierarchy is different between these strains of mice.

Overall, these results demonstrate that the different phases of the CD8 T cell response to a viral vaccine or a viral infection can be altered, but that the effects are often not predictable from experimental results obtained in vitro.

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Abbreviations Used

MCMV, murine cytomegalovirus
HCMV, human cytomegalovirus
LCMV, lymphocytic choriomeningitis virus
MHV-68 or γHV-68, murine herpesvirus-68, a.k.a gamma herpesvirus-68
EBV, Epstein-Barr Virus
HIV, human immunodeficiency virus
SIV, simian immunodeficiency virus
VV, vaccinia virus
MVA, modified vaccinia Ankara
CD, cluster of differentiation
CTL, cytotoxic T lymphocytes
NK, natural killer
NKC, natural killer complex
DCs, dendritic cells
MHC, major histocompatibility complex
IFN-γ, interferon gamma
TNF-α, tumor necrosis factor alpha
TNFR, tumor necrosis factor receptor
mAb, monoclonal antibody
PCR, polymerase chain reaction
ORF, open reading frame
Δ, deleted of
w.t., wild-type
pfu, plaque-forming units
LDA, limiting dilution assay
ICS, intracellular cytokine staining
Tetramers, peptide:MHC class I tetramers
ELISPOT, enzyme-linked immuno spot assay
BMT, bone marrow transplantation
IE or α, immediate-early
E or β, early
L or γ, late
IL, interleukin
ICAM, intercellular adhesion molecule
ERGIC, endoplasmic reticulum - Golgi intermediate compartment

Chapter 1: Introduction

1.1 Overview

The focus of this thesis is the quantification of epitope-specific murine CD8 T cell responses in response to two distinct types of stimulus, recombinant vaccines and viral infection. In the vaccine system, our goal was to improve on a prototypic HIV vaccination regimen, where DNA immunization is used to prime a CD8 T cell response, and a recombinant viral vector is then used to 'boost' the CD8 T cells. We accomplished this by administering agonist antibodies specific for 4-1BB and OX40, tumor necrosis factor receptor (TNFR) family members that contribute to T cell activation, at the time of activation and then monitoring the ensuing T cell response. We found that 4-1BB stimulation and OX40 stimulation each increased CD8 T cell activation and memory, and that there was an additive effect when the two stimuli were combined.

Human cytomegalovirus (HCMV) contributes substantially to morbidity and mortality in humans. Murine cytomegalovirus (MCMV) infection of mice is an excellent animal model for HCMV infection, and CD8 T cells play an important role in protection against viral replication and disease of both HCMV and MCMV. In order to quantify the response to MCMV in C57BL/6 mice more thoroughly, immunodominant CD8 T cell epitopes were mapped, then used to analyze: 1) the role of MHC class I immune evasion genes in vivo; 2) the evolution of the CD8 T cell response over time; and 3) the effect that the genetic background of the host has on the size and antigen specificity of the CD8 T cell response.

1.2 MHC class I antigen processing and presentation pathway

Viruses are obligate intracellular parasites. Because they rely on the host cell machinery for protein synthesis, nascent viral proteins are synthesized in the cytosol. A large proportion of newly-synthesized proteins, termed defective ribosomal products (DRiPs), are rapidly degraded by proteasomes and provide one source of peptides for the MHC class I presentation pathway (Schuber et al. 2000) (Reits et al., 2000). However, it is likely that non-DRiPs also are a source of peptides for the MHC class I pathway, since ubiquitination of CD8 T cell antigens can increase CD8 T cell responses (Rodriguez et al., 1998) (Ye et al., 2002).

Peptides produced by the proteasome are transported into the endoplasmic reticulum (ER) by the transporters associated with antigen processing-1 and -2 (TAP-1 and TAP-2), where they can associate with nascent MHC class I molecules. MHC class I is comprised of two polypeptides, an alpha chain (α) and a beta chain (β). The alpha chain is a transmembrane polypeptide and has three lumenal domains(α1, α2 and α3), a transmembrane domain and a cytosolic tail. The α1 and α2 domains, distal from the transmembrane domain, form the peptide-binding groove consisting of two α helices and a platform of 8 antiparallel β pleated sheets. The MHC class I α chain is partially stabilized by the presence of β–2 microglobulin (β-2m), but a nascent MHC class I molecule is relatively unstable until it associates with a suitable peptide. Complexes of peptide/MHC I are transported through the Golgi apparatus to the cell surface, where they are capable of presenting peptides to CD8 T cells expressing an appropriate T cell receptor (TCR).

CD8 T cells are bone marrow-derived lymphocytes differentiated from lymphoid progenitor cells. As T cells develop, they undergo positive and negative selection in the thymus, based on the ability of the rearranged TCR to interact with MHC class I. Each CD8 T cell has a unique TCR, allowing recognition of its cognate peptide/MHC class I molecule on an antigen presenting cell (APC). In vivo, CD11c+ dendritic cells (DCs) are required for priming naïve CD8 T cells (Jung et al., 2002). A CD8 T cell that recognizes cognate peptide/MHC I on a mature, activated DC will proliferate and differentiate into armed effector and/or memory CD8 T cells.

1.3 CD8 T cell activation, contraction and stable memory

Our fundamental understanding of the size and kinetics of the CD8 T cell response following infections has increased enormously in the last 5 to 10 years, due in large part to the availability of reagents and techniques, peptide/MHC class I tetramers, intracellular cytokine staining (ICS) and ELISPOT, that allow quantification of epitope-specific CD8 T cell responses on a single-cell level (Doherty and Christensen, 2000).

CD8 T cell 'bystander activation'

Before 1998, it was believed that the large majority of CD8 T cells activated by viral or bacterial infection were not pathogen-specific but were instead 'bystander' CD8 T cells. This was believed largely due to the fact that epitope-specific responses could only be quantified by the limiting dilution assay (LDA), which is now known to vastly underestimate the frequency of epitope-specific CD8 T cells. In 1998, this issue was revisited using MHC class I tetramers, ICS and ELISPOT to analyze epitope-specific

CD8 T cell responses after experimental infection of mice with lymphocytic choriomeningitis virus (LCMV) (Murali-Krishna et al., 1998) (Butz and Bevan, 1998).

One study demonstrated by ELISPOT that at least 24% of CD8 T cells were specific for three LCMV epitopes 8 days after infection (Butz and Bevan, 1998). A second study using ICS found that 50-70% of CD8 T cells were specific for defined LCMV epitopes at the height of the CD8 T cell response, in two different strains of mice (Murali-Krishna et al., 1998). These two reports demonstrated that the size of the virus-specific CD8 T cell response was much larger than anticipated.

When OT-I TCR transgenic (Tg) CD8 T cells, specific for an ovalbumin epitope, were adoptively transferred into mice that were subsequently infected with either LCMV, vaccinia virus expressing the influenza nucleoprotein (VVflu-np), or vaccinia virus expressing ovalbumin (VVova), the TCR Tg CD8 T cells only proliferated after infection with the virus expressing ovalbumin (VVova) (Butz and Bevan, 1998). Additionally, LCMV-specific CD8 T cells did not proliferate after heterologous challenge with vaccinia virus (Murali-Krishna et al., 1998). These experiments formally demonstrated that 'bystander activation' is not a prominent feature of the CD8 T cell response to viral infection.

Coordinated regulation of CD8 T cell responses

The kinetics of the CD8 T cell response to all LCMV epitopes consisted of three distinct phases: 1) rapid expansion until day 8; 2) massive loss of ~90% of the activated cells (i.e. contraction); and 3) stable maintenance of memory for over 1 year (Murali-Krishna et al., 1998). This finding, that epitope-specific CD8 T cell responses occur

coordinately, was confirmed very soon afterward with *Listeria monocytogenes* (Busch et al., 1998). Again it was found that CD8 T cells specific for different epitopes underwent expansion, contraction and differentiation into memory cells in unison. Previous work had demonstrated that for different CD8 T cell epitopes, the amount and stability of each epitope presented on the infected cell surface was different (Pamer et al., 1997). This indicated that a property intrinsic to CD8 T cells, not to individual peptide/MHC class I complexes, was most likely responsible for this coordinated CD8 T cell response

A brief exposure to antigen is sufficient for CD8 T cell proliferation and differentiation

A number of reports in the literature strengthened the model that coordinated CD8 T cell responses were due to a property intrinsic to the CD8 T cells. It was discovered that if ampicillin was administered to mice 24 hours after infection with *L. monocytogenes*, viable bacteria were eliminated from the mice within 12 hours, but that ampicillin did not affect the *Listeria*-specific CD8 T cell response (Mercado et al., 2000). The magnitude of the CD8 T cell response, kinetics, effector function and the ability to mount a recall response to *L. monocytogenes* infection were all unaffected. TCR Tg CD8 T cells specific for an *L. monocytogenes* antigen were labeled with CFSE and adoptively transferred into recipient mice. When the recipient mice were infected with *L. monocytogenes*, some TCR Tg CD8 T cells proliferated more than 7 times, while others failed to proliferate at all, indicating that activation was an all-or-nothing event. Shortening the window of bacterial replication to 12 hours reduced the number of naïve TCR Tg CD8 T cells recruited into the response, but did not affect the extent of division

for the recruited CD8 T cells. This further supported the all-or-none model of CD8 T cell activation.

Soon afterward, three related papers were published concurrently. It was confirmed that the antigen dose affected the number of recruited CD8 T cells, but not the extent of proliferation of individual CD8 T cells (Kaech and Ahmed, 2001). Additionally, only 2 or 2.5 hours of exposure to antigen were sufficient to prime naïve CD8 T cells (van Stipdonk et al, 2001) (Wong and Pamer, 2001). This short exposure to antigen in vitro was sufficient to allow differentiation of naïve CD8 T cells into functional effector and memory cells capable of producing IFN-γ (Kaech and Ahmed, 2001). Lytic activity was also noted within 48 to 72 hours (van Stipdonk et al., 2001). Several cytokines were found to affect the number of proliferating cells or the extent of proliferation, including IL-2 (Kaech and Ahmed, 2001) (Wong and Pamer, 2001), IL-7 and IL-15 (Wong and Pamer, 2001). These findings support a model where brief antigen stimulation begins an intrinsic CD8 T cell program of proliferation and differentiation that does not require further antigen stimulation. However, this program can be affected by extrinsic factors as well, such as Il-2, IL-7 and IL-15.

MHC I Antigen presentation in vivo occurs only during a defined window of time

Cumulatively, these studies demonstrated that only a brief period of antigen stimulation is required for priming of naïve CD8 T cells. However, viable *L. monocytogenes* remain in the spleen for up to 6 days (Wong and Pamer, 2003), suggesting that antigen presentation in vivo may persist for much longer. Therefore the duration of antigen presentation in vivo was examined in greater detail, again using the *L.*

monocytogenes system. Naïve TCR transgenic CD8 T cells specific for two different *L. monocytogenes* epitopes were labeled with CFSE. These donor CD8 T cells were then transferred into recipient mice that had been previously infected with *L. monocytogenes* for 0 to 6 days. If the transfer was performed 1 or 2 days after infection of recipient mice, CD8 T cells of both specificities divided rapidly, indicated by dilution of the CFSE label. Transfer of CD8 T cells 3 days after infection resulted in some division, but transfer 4 or more days post-infection did not lead to CD8 T cell division. This indicated that in vivo, priming of naïve CD8 T cells could only occur within a 3-day window after infection, even though viable bacteria were present beyond this 3-day window.

The authors showed that *Listeria*-specific CD8 T cells possessed lytic function as early as 72 hours after infection (Wong and Pamer, 2003). This led them to speculate that primed CD8 T cells lyse antigen-presenting DCs 3 to 4 days after infection, preventing any further CD8 T cells from being primed after this time.

CD8 T cell contraction is due to an intrinsic factor

Other evidence suggests that the contraction phase is also 'programmed' into CD8 T cells at the time of activation. When mice were infected with 100-fold different doses of *L. monocytogenes*, the magnitude of the CD8 T cell response was different, but the kinetics of CD8 T cell contraction were identical (Badovinac et al., 2002). When infection was shortened by antibiotics, or lengthened by further increasing the infection dose, CD8 T cell contraction kinetics were still identical, and contraction even occurred in the continued presence of antigen.

The recall CD8 T cell response to *L. monocytogenes* peaks only five days after a secondary challenge, compared to seven days for the primary response, and also contracts more slowly. The authors transferred naïve TCR transgenic CD8 T cells specific for one antigen, and memory TCR transgenic CD8 T cells specific for a second antigen, into the same mice and then infected the mice. They found that the naïve CD8 T cells peaked at day 7 then rapidly contracted, while the memory CD8 T cells peaked at day 5 and contracted much more slowly. This elegantly demonstrated that the timing of expansion and contraction is hard-wired into the CD8 T cells, and varies between naïve and memory CD8 T cells.

CD8 T cell memory is stable and does not require interaction with antigen or MHC class I

As mentioned previously, LCMV-specific CD8 T cells that have already passed through the expansion and contraction phases after priming are maintained as a stable population of memory CD8 T cells for over 1 year (Murali-Krishna et al., 1998). When LCMV-specific memory CD8 T cells were transferred into naïve $\beta 2m^{-/-}$ recipient mice, or $D^{b-/-}$ X $K^{b-/-}$ X $\beta 2M^{-/-}$ recipient mice, the donor CD8 T cell population was stably maintained for up to 240 days, and these cells had rapid effector function directly ex vivo (Murali-Krishna et al., 1999). This indicates that maintenance of memory CD8 T cells is independent of viral peptide/MHC class I stimulation, or even interactions with MHC class I alone.

It is noteworthy that LCMV and *L. monocytogenes* are cleared infections. However, MCMV is never completely cleared from infected mice. Perhaps because of

this, MCMV-specific CD8 T cells are not stably maintained during the memory phase, but instead accumulate over time, as is discussed more thoroughly below (Chapter 1.10).

1.4 MCMV general virology

Herpesviruses can be divided into three subfamilies: the α-, b- and γ-herpesviruses. Human cytomegalovirus (HCMV), the prototypic member and best-studied β-herpesvirus, is present in 50-90% of the population in North America and up to 100% of the population in developing countries (Mocarski, 1996) (Hanshaw, 1994). HCMV has co-evolved with humans for millions of years and only causes significant morbidity and mortality in immunocompromised individuals, including congenitally-infected fetuses, end-stage AIDS patients and organ transplant recipients.

Because CMVs are species-specific, HCMV does not infect mice. However, murine CMV (MCMV) infection of mice is very similar to HCMV infection of humans, both genetically and biologically. The mouse is the best-developed experimental immunology model, making this an excellent species for studying immune responses to a CMV. MCMV has a 230 kb, linear, double-stranded DNA genome that has been entirely sequenced and was shown to contain 170 ORFs (Rawlinson et al., 1996). The MCMV genome is largely colinear with the HCMV genome (Bankier et al., 1991). Both viruses share a core set of conserved genes, largely devoted to DNA replication and virion assembly. The flanking regions of MCMV and HCMV are not homologous, and many of these genes are important for host immune evasion. CMV virions consist of a DNA-containing core within an icosahedral capsid, surrounded by tegument. The envelope is a lipid bilayer containing viral glycoproteins. The biology of mouse MCMV infection and

human HCMV infection are very similar in terms of cell and tissue tropism, pathogenesis, establishment and reactivation from latency, and immune control. Overall this makes MCMV infection of mice an excellent animal model for understanding the biology, and immune correlates of protection, of HCMV infection of humans.

Like other herpesviruses, MCMV gene expression in tissue culture occurs in three waves. Immediate-early (IE or α) genes are transcribed by cellular RNA polymerase II and do not require de novo viral protein synthesis for expression. The IE protein products transactivate the early (E or β) genes. By definition, E genes require expression of IE proteins. E genes encode proteins necessary for viral DNA replication, as well as proteins that enable the virus to survive in a hostile cell environment. Expression of late (L or γ) genes requires viral DNA replication and typically occurs 12 hours or more after the beginning of E gene expression. L genes mostly encode structural virion components, and proteins necessary for packaging of the viral DNA.

IE gene expression

The major IE region of the MCMV genome is the most actively transcribed region at IE times. Three IE genes are under the control of the major IE enhancer. *ie1* and *ie3* share the $P^{1/3}$ promoter and are differentially spliced from the same transcript. The *ie1* transcript is produced when exons 1, 2, 3 and 4 are spliced together, and IE1/pp89 contributes to but is not sufficient for E gene transcription (Messerle et al., 1992), similarly to HCMV IE1. IE3 is remarkably conserved in terms of amino acid homology, splicing, expression and function compared to its HCMV counterpart, IE2 (Messerle et al., 1992). Exons 1, 2 and 3 are shared between *ie3* and *ie1*, but splicing to exon 5

produces the *ie3* transcript. The IE3 product is sufficient to activate E genes and also auto-represses *ie1/ie3* transcription at E times. IE3 is an essential MCMV gene and IE3 mutant viruses fail to express E or L genes (Angulo et al., 2000). *ie2* is also controlled by the major IE enhancer, but has a separate promoter from *ie1* and *ie3* and is transcribed in the opposite direction (Messerle et al., 1991). IE2 has no direct counterpart in HCMV, and an *ie2* deletion mutant is fully competent to grow, establish latency and reactivate from latency in mice (Cardin et al., 1995). Thus IE3 is the essential E gene transactivator and also represses *ie1/ie3* expression, IE1 is a co-transactivator, and IE2 appears to be completely dispensable.

1.5 Lymphocyte subsets and protection against MCMV

Role of CD8 T cells

In 1974, Brody and Craighead found that cytomegalic inclusion disease (CID), which manifests itself in immunosuppressed humans who are infected with HCMV, could be experimentally recapitulated as overt pulmonary CID in immunosuppressed mice after MCMV infection (Brody and Craighead, 1974). Furthermore, their use of anti-lymphocyte serum to immunosuppress the mice demonstrated that T cells, B cells or NK cells, or a combination, were required for protection against MCMV-induced disease. A few years later it was found that homozygous nu/nu mice died after infection with only 13 pfu of MCMV, while heterozygous nu/wt mice required 1000-fold more virus to result in death, indicating that T cells were indeed required for protection (Starr and Allison, 1977). They also showed that transfer of spleen cells from immune BALB/c mice protected nu/nu mice from death, unless those spleen cells were depleted of T cells before

transfer. Soon after, T cells taken from spleens of BALB/c-infected mice were shown to contain CTL activity that was virus-specific and *H-2* restricted (Quinnan et al., 1978). Taken together, these results suggest that T cells are the most important cell type for controlling MCMV replication, although an important role for antibodies was not ruled out by these experiments.

As is common for viral infections, CD8 T cells play a central role in control of MCMV replication. In the 1980's, the role of CD8 T cells in controlling MCMV infection was further elucidated. Much of this work was performed in a bone marrow transplantation model.

A major complication of HCMV reactivation, especially after bone marrow transplantation (BMT), is interstitial pneumonia. If lethally-irradiated mice reconstituted with syngeneic bone marrow were infected with MCMV, a higher percentage of mice died than if they were not infected with MCMV (Reddehase et al., 1985). In this mouse BMT model, spleen cells from MCMV-immune mice are protective against viral replication in the lungs, liver, spleen, and to a lesser extent salivary gland. By depleting T cell subsets, it was shown that CD8 T cells, but not CD4 T cells, are required for protection against virus-induced pathology in the lungs. In addition, no antibody response was detected in protected mice. Two additional studies (Reddehase et al., 1987) (Reddehase et al., 1988), also performed in the murine BMT model with adoptive transfer of lymphocyte subsets, found that CD8 T cells protect not only the lungs, but also the spleen (Reddehase et al., 1987) and adrenal glands (Reddehase et al., 1988). CD4 T cells alone were not protective in any of these organs, and did not provide 'help' to CD8 T

cells either. Together, these studies clearly demonstrated the ability of CD8 T cells to protect visceral organs against MCMV pathology in immunocompromised mice.

Identification of an IE1/pp89 CD8 T cell epitope in BALB/c mice

Within this same time period, the identification of the first CD8 T cell epitope from MCMV was underway. The ability to restrict MCMV gene expression to IE genes only, IE + E genes only, or IE+E+ L genes, was used to discover that polyclonal CTL from BALB/c mice primarily recognized cells enhanced for L gene expression, but also recognized cells well where IE gene expression was enhanced (Reddehase et al., 1984). Furthermore, the IE antigen and L virion antigen were recognized by distinct CTL clones. In a seminal 1984 report published in *Nature*, limiting dilution analysis was used to estimate that approximately 40% to 50% of CTL precursors that could respond to mitogen could also respond to an antigen expressed at IE times, demonstrating that the IE antigen was immunodominant in the BALB/c strain of mice (Reddehase and Koszinowski, 1984). A clonal CTL line that was specific for the IE antigen was soon isolated (Reddehase et al., 1986) and was used to show that the IE antigen was recognized at IE times and L times but not E times, which correlated with IE gene synthesis, rather than times of protein abundance. That the authors made this observation is noteworthy in light of more recent literature (Schubert et al., 2000) (Reits et al., 2000). A large proportion of newly-synthesized proteins, termed Defective Ribosomal Products (DRiPs), are degraded almost immediately after synthesis (Schubert et al., 2000), and these DRiPs appear to be the predominant source of TAP-transported peptides (Reits et al., 2000). Since the IE antigen was presented best when IE gene synthesis was strongest,

rather than when IE proteins were most abundant, this implies that the IE epitope may be processed primarily from DRiPs rather than degradation of stable IE protein products.

In a quick succession of papers, Koszinowski and colleagues eventually identified the optimal *H-2*^{d}-restricted CD8 T cell epitope. First, they found that transfection of a genomic region encoding IE1, IE2 and IE3 was sufficient to render target cells susceptible to lysis by an IE-specific CTL clone (Koszinowski et al., 1987). Next, an intron-less *ie1* gene was cloned into a recombinant vaccinia virus. Cells infected with this virus were also recognized by the IE clone (Volkmer et al., 1987). Third, a series of recombinant vaccinia viruses expressing *ie1* gene fragments narrowed the epitope to about 100 amino acids, and large synthetic peptides further narrowed the epitope to 20 amino acids (Del Val et al., 1988). Finally, Reddehase et al. (Reddehase et al., 1989) demonstrated that a 5-mer peptide that had the characteristic motif required for L^{d} binding, was the minimal epitope recognized by the IE CTL clone, but that a 9-mer peptide was recognized at 1000-fold lower concentrations. It was then demonstrated, again using recombinant vaccinia viruses, that the 9-mer peptide IE1/pp89 ^{168}YPHFMPTNL^{176} was sufficient to prime an immune response, and that no other CD8 T cell epitope was present within the IE1/pp89 protein (Del Val et al., 1991b).

The protective capacity of IE1-specific CD8 T cells was first clearly demonstrated by Jonjic et al., who showed that immunization with a recombinant vaccinia virus, expressing the MCMV *ie1* gene, was able to protect BALB/c mice against a lethal MCMV challenge (Jonjic et al., 1988). By adoptive transfer into irradiated, MCMV-infected mice, protection was shown to be mediated by CD8 T cells, and could not be transferred by CD4 T cells or serum. The role of CD8 T cells in VV-*ie1*-mediated

protection was further defined when Del Val et al. (Del Val et al., 1991b) constructed a virus expressing only the IE1/pp89 ^{168}YPHFMPTNL^{176} nonamer epitope. When used to immunize mice, this virus elicited protection against lethal MCMV challenge, and also protected mice in the BMT model. Other protocols that could also elicit IE1-specific CD8 T cells, including synthetic peptide plus adjuvant immunization (Scalzo et al., 1995a) and plasmid DNA immunization (Gonzalez Armas et al., 1996), later confirmed the protective role of this antigen.

Role of CD4 T cells in protection against MCMV

Collectively, the above data appear to indicate that CD8 T cells are necessary for protection, while CD4 T cells and antibody are irrelevant, but this is not the case. In the 1990's, the role of CD4 T cells, CD8 T cells, NK cells and B cells under varying experimental conditions was examined in much greater detail. In addition, some initial work was done to elucidate the importance of IFN-γ, TNF-α and perforin as effector cytokines.

In a report by Jonjic et al. (Jonjic et al., 1989) CD4-depleted mice were evaluated for resistance to MCMV. In these mice, CD8 T cell priming was unaffected and CD8 T cells could clear MCMV from the lungs of immunocompromised mice, but clearance of virus from the lungs and spleen was slightly delayed. Therefore, while CD8 T cell priming and effector function is independent of CD4 T cells, CD4 T cells appear to have an additional benefit in viral clearance from spleen and lungs. More importantly, it was discovered that CD4 T cells are essential for viral clearance from the salivary gland, a specialized site of virus production and mucosal shedding. In CD4-depleted mice,

MCMV established a high-titer persistent infection in the salivary gland for more than 5 months, even though virus was cleared from all other organs within 10 weeks.

When MCMV infection was evaluated in CD8-depleted mice, viral clearance in the salivary gland was not delayed, confirming that CD4 T cells are essential in this organ (Jonjic et al., 1989). But surprisingly, the kinetics of viral clearance in the lungs were only slightly delayed compared to immunocompetent mice, and was similar to CD4-depleted mice. This directly contradicted expectations, since CD8 T cells had been repeatedly shown to be important in controlling viral titers in the lungs. Adoptive transfer of lymphocytes that were depleted of either CD4 or CD8 T cells revealed the mechanism for this result. CD4 T cells that were primed against MCMV in the absence of CD8 T cells developed an anti-viral function that was not acquired by CD4 T cells primed in the presence of CD8 T cells. In other words, the full anti-MCMV potential of CD4 T cells did not develop if they were primed in the presence of CD8 T cells.

The anti-viral role of CD4 T cells was corroborated by studies with β2m knockout mice. Although these mice were about 4-fold more susceptible to lethal MCMV challenge, viral clearance from the lungs and spleen, organs known to be strongly affected by anti-viral CD8 T cells, was normal (Jonjic et al., 1989). Viral clearance from the lungs and spleen was partially dependent on CD4 T cells in β2m-/- mice, but not β2m+/- mice, indicating that CD4 T cells developed an anti-viral activity in this model, similarly to the CD8 depletion model system.

Two reports examined the mechanism of viral clearance in the salivary gland by CD4 T cells. In the first report (Lucin et al., 1992), endogenous IFN-γ was neutralized by antibody treatment. In these mice, virus titers in salivary gland were increased compared

to untreated mice, and similar to titers in mice depleted of CD4 T cells. A similar phenomenon was observed in mice treated with neutralizing anti-TNF-α antibodies (Pavic et al., 1993). These reports suggest, but do not prove, that these cytokines are essential effector molecules that CD4 T cells utilize in the salivary gland.

Role of antibodies in MCMV infection

Because CD4 T cells are required for the production of anti-MCMV antibodies, but CD4 T cells are not necessary for control of MCMV in the spleen, lungs or liver, antibodies are not necessary for control of MCMV in these organs (Reddehase et al., 1985) (Reddehase et al., 1987) (Reddehase et al., 1988) (Jonjic et al., 1989). However, antibodies can be protective if given prophylactically. In one of the reports described above (Jonjic et al., 1988), it was noted that a recombinant vaccinia virus expressing *ie1* elicited a protective immune response that was CD8-dependent. Importantly though, sublethal MCMV infection also protected mice against subsequent lethal challenges, but this protection did not require CD8 T cells. Serum from MCMV-immune mice, but not from mice infected with vaccinia virus expressing *ie1*, protected naïve mice from a lethal challenge dose of MCMV. A later report confirmed that MCMV-specific antibodies can be protective if given prophylactically (Farrell, 1991).

Notably, CD4 T cells are absolutely required for salivary gland clearance, which suggested that antibodies may play a role in this organ. Using μMT knockout mice, which lack membrane Ig expression and have defective B cell maturation, the role of CD4 T cells and antibodies could be evaluated independently. These mice cleared MCMV from the salivary gland with normal kinetics, demonstrating that antibodies are

not required for clearance of MCMV from the salivary gland (Jonjic et al., 1994). Antibodies also did not affect the amount of virus that establishes latency in the lungs in this model.

As an interesting aside, a study of natural MCMV infection of Australian wild mice showed that trapped mice often are infected with multiple strains of MCMV. Although antibodies and CD8 T cells are protective against MCMV-induced disease if given prophylactically (described above), this implies that MCMV-specific antibody and T cell immunity are not sufficient to prevent repeated natural super-infection of the same mouse. However, it is possible that mice were co-infected with multiple MCMV strains simultaneously. At the Herpesvirus 2003 meeting, the same group reported on their attempt to induce autoimmunity (and sterility) in wild mice with a recombinant MCMV that expresses ZP-3, a protein found in the ovaries. They made another interesting observation. Not only could mice be super-infected after oral exposure to the recombinant virus, but super-infection occurred more readily than did infection of MCMV-naïve mice. This was due to the presence of antibodies (A Scalzo and G Shellam, personal communication). The evidence is circumstantial, but it appears that in addition to protecting mice against disease, immunity to MCMV morbidity also increases the likelihood of super-infection.

1.6 Identification of additional MCMV CD8 T cell epitopes

The discovery of the IE1/pp89 CD8 T cell epitope is described above (Chapter 1.5). This period of intense searching for the IE1/pp89 epitope was followed by a long lull in identification of further CD8 T cell epitopes. The discovery that MCMV genes

expressed at E times interfere with the MHC class I antigen processing and presentation (described below) was taken as evidence that a CD8 T cell response could only be effectively primed against IE antigens. For a time, this solidified the notion that IE1/pp89 was the most immunodominant antigen. Although both polyclonal and clonal CTL were know to be specific for E antigens (Del Val et al., 1989) (Campbell et al., 1992), no further studies on these antigens appeared in the literature for many years.

Approximately ten years later, the first E-phase CD8 T cell epitope was reported. Holtappels et al. found that pulmonary lymphocytes were lytic directly ex vivo and could be used to evaluate the CD8 T cell response to MCMV directly ex vivo, which would bypass in vitro restimulation and selection (Holtappels et al., 1998). CD3-redirected lysis was used to estimate the total number of activated CD8 T cells. Since it had been recently shown that LCMV infection induced activation of primarily LCMV-specific CD8 T cells (Murali-Krishna et al., 1998), it was implied that effector CD8 T cells elicited by MCMV infection would be primarily MCMV-specific. Only a small proportion of pulmonary CTL lysed cells loaded with the 'immunodominant' IE1/pp89 epitope, suggesting that it was immunodominant relative to other epitopes, but did not elicit most of the MCMV-specific CD8 T cell response. More surprisingly, it was found that pulmonary CTL primarily recognized epitopes expressed at E times, followed by L times and IE times, even though MHC class I immune evasion genes were known to prevent antigen presentation at E times.

After the MCMV genomic sequence was published in 1996 (Rawlinson et al., 1996), the Reddehase lab searched for other CD8 T cell epitopes. By scanning the MCMV genome for peptides with MHC-binding motifs (Falk et al., 1991), 35 candidate

peptides with L^d, K^d or D^d-binding motifs were identified, synthesized and used to generate short-term CTL lines from latently-infected BALB/c mice. In this way the second CD8 T cell epitope, encoded by the immunomodulatory protein m04/gp34, ^{243}YGPSLYRRF^{251} was identified (Holtappels et al., 2000b). Other synthetic peptides that were predicted to bind to *H-2^d* MHC I molecules led to the discovery of epitopes m164 ^{257}AGPPRYSRI^{265} (Holtappels et al., 2002c) m18 ^{346}SGPSRGRII^{354} (Holtappels et al., 2002b) and M45 ^{507}VGPALGRGL^{515} (Reddehase, 2002) of which the epitope from *m164* was found to be codominant with IE1/pp89.

Before the MCMV genomic sequence was published, Debbie Spector's group identified *M83* and *M84* as homologs of the HCMV *UL83* ORF, which encodes the lower matrix phosphoprotein pp65 (Cranmer et al., 1996). pp65 was known to be immunogenic in HCMV, and an *M84* plasmid expression vector used for DNA immunization protected BALB/c mice against sublethal MCMV infection (Morello et al., 2000); the minimal K^d-restricted epitope, M84 ^{297}AYAGLFTPL^{305} was identified soon after (Holtappels et al., 2000c). Although Debbie Spector's group was not able to show protection after DNA immunization of BALB/c mice with a plasmid expressing M83, the second MCMV homolog of UL83, Holtappels et al. (Holtappels et al., 2001) tested the antigenicity of MCMV M83 by synthesizing 12 peptides predicted to bind to either L^d, K^d or D^d and were able to identify M83 ^{761}YPSKEPFNF^{769} as an L^d-restricted CD8 T cell epitope. However, while short-term CTL lines could be generated against each epitope, analysis of CD8 T cells from infected mice indicated that epitopes from m04, M83 and M84 were very subdominant compared to IE1/pp89. Therefore, at present there are seven known CD8 T cell epitopes in BALB/c mice (summarized in Table 1.1). Of these, responses to

IE1/pp89 and m164 are the most dominant. m18 and M45 are subdominant epitopes, but are recognized by CD8 T cells directly ex vivo, while responses to m04, M83 and M84 are primed in vivo but are at or below the threshold of detection in the absence of in vitro restimulation.

Table 1.1 MCMV-Specific CD8 T Cell Responses in BALB/c Mice

A

Time p.i.	Organ	CD3	IE1	m164	m18	m04	M83	M84	Assay
Day 8	PLN	5.0%	0.9%	0.7%	0.4%[a]	0.1%	0.1%	0.1%	ELISPOT
5 Months	Spleen	3.0%	1.0%	1.0%	0.2%[a]	0.1%	0.1%	0.1%	ELISPOT

B

Time p.i.	Organ	CD3	IE1	m164	m18	m04	M83	M84	Assay
Day 7	Spleen		5.5%				0.3%		ICS

C

Time p.i.	Organ	CD3	IE1	m164	m18	m04	M83	M84	Assay
Day 10	Spleen		2.5%	4.0%	0.4%	0.5%	0.2%	0.2%	ICS
Day 300	Spleen		7.5%	7.5%	0.2%	0.1%	0.1%	0.1%	ICS

A = Reddehase Lab, data from Holtappels et al., JV, 2002, Figure 10
B = Spector Lab, data from Ye & Spector, JV,2002, Figure 2
C = Klenerman Lab, data from Karrer et al, 2003, Figure 5
a = from Holtappels et al, J Gen Vir, 2002, Fig 3

Identification of MCMV CD8 T cell epitopes in C57BL/6 mice

In C57BL/6 mice, progress in identifying CD8 T cell epitopes occurred much later. Work performed in our laboratory resulted in the successful isolation of a number of MCMV-specific CD8 T cell clones, but their antigens were not identified after testing a small number of recombinant vaccinia viruses that expressed individual MCMV genes (MC Gold, unpublished observations). To identify CD8 T cell antigens, the MCMV genome was digested into small fragments, the fragments were cloned into a plasmid expression vector, expressed in a fibroblast cell line, and tested for recognition by the

MCMV-specific CD8 T cell clones. In this way, the D^b-restricted epitope recognized by clones 3 and 55 was determined to be M45, ^{985}HGIRNASFI^{993} (Gold et al., 2002). After infection of C57BL/6 mice with MCMV, 6-10% of CD8 T cells were found to be M45-specific (Gold et al., 2002). This was an important finding, but this process failed to identify the antigens recognized by other CD8 T cell clones.

1.7 Immunodominance

Typically after infection with any pathogen, an organism will have strong CD8 T cell responses to a relative handful of epitopes, a phenomenon known as immunodominance (Yewdell and Bennink, 1999). Three primary factors interplay to determine the immunodominance hierarchy to a pathogen. First, an epitope must be presented by MHC I on the surface of the priming APC in sufficient quantity to be recognized by a CD8 T cell. Second, there must be T cells of appropriate specificity and affinity to recognize the peptide:MHC complex and proliferate. Third, potential CD8 T cell responders compete with each other by an unknown mechanism, termed immunodomination, for the 'privilege' to proliferate.

Every step in the antigen processing and presentation pathway represents a barrier to potential peptide epitopes. First, a protein must be translated in relative abundance in infected cells. Second, flanking residues must be present that allow the proteasome to efficiently cleave the C-terminal anchor residue, since no further C-terminal trimming occurs in the ER. Third, the peptide must contain a hydrophobic C-terminal residue in order to be transported by (murine) TAP into the ER. Fourth, the peptide must bind with relatively high affinity to an MHC molecule. This is the most stringent step, and there is a

characteristic motif of peptides that are able to bind to each MHC molecule. For example, 8-mer peptides with an F or Y residue at position five, and L/M/I/V at position eight are most likely to bind well to K^b, and D^b strongly prefers 9-mer peptides with an N at position five and M/I/L at position nine. Currently, it appears that a $K_D > 500$ nM is required for most peptides that are immunogenic. The fifth criteria for presentation of an immunogenic peptide is cell-surface stability.

The combination of all of these factors determines the abundance of peptide:MHC that is present on the cell surface, but abundance alone does not determine immunodominance. Circulating T cells must also be present that have proper specificity and are of sufficiently high avidity to recognize the peptide:MHC. In order for these to exist, the organism must be capable of generating TCR able to recognize the epitope, T cells expressing the TCR must survive both negative and positive selection, and the T cell must encounter one or more APCs presenting a sufficient amount of peptide:MHC to activate the T cell.

All T cells that encounter sufficient amounts of cognate peptide:MHC on a sufficiently mature DC then begin to compete for the privilege of proliferating. This competition occurs not only between CD8 T cells responding to different epitopes, but also between T cells of the same epitope specificity, leading to an immunodominance hierarchy characteristic for that virus or pathogen. It should be noted that immunodominance is always a relative term, made between two or more epitopes. The hierarchy may change under different infection conditions, and an epitope that is immunodominant in the context of one pathogen is often subdominant when expressed heterologously by another pathogen.

Immunodominance hierarchies in viral infections other than MCMV

The shape of the CD8 T cell immunodominance hierarchy can vary substantially between pathogens, and even varies for a single pathogen after infection of different mouse strains. For example, in BALB/c mice infected with LCMV, ~50% of CD8 T cells respond to the immunodominant NP 118 epitope, while fewer than 5% of CD8 T cells respond to the subdominant epitopes NP313 GP 283 and GP 99 (Murali-Krishna et al., 1998) (Rodriguez et al., 2001) (Slifka et al., 2003). But in C57BL/6 mice, over 20% of CD8 T cells respond to NP 396 and GP 33, with substantial additional responses to GP 276 and NP 205 (~7-8%) (Murali-Krishna et al., 1998). The CD8 T cell response to Sendai virus was analyzed before peptide/MHC I tetramers or intracellular cytokine staining were available (Kast et al., 1991), but like LCMV infection of BALB/c mice also appears focused on a single epitope.

HSV-1 is not a natural mouse pathogen but is able to infect mice, and the HSV-1-specific CD8 T cell response is well-characterized in C57BL/6 mice. In the draining lymph node after acute infection, approximately 3% of CD8 T cells produce IFN-γ after stimulation by HSV-1 infected-targets, but only 1% respond to targets infected with a virus missing the gB 498 epitope, suggesting that at least two-thirds of HSV-1-specific CD8 T cells are gB-specific (Wallace et al., 1999). In a separate experiment, 6.3% of CD8 T cells responded to HSV-1-infected targets, 5.8% responded to gB 498, and 0.5% responded to RR 882. This suggests that as many as 90% of HSV-1-specific CD8 T cells recognize a single epitope, however this result should be interpreted with some caution since stimulation with virus-infected targets is not necessarily directly comparable to peptide stimulation.

Epitope-specific CD8 T cell responses have also been evaluated for persistent viral infections in humans. Unfortunately, immunodominance hierarchies are much more difficult to determine in outbred individuals with discordant MHC I molecules and variable immunization and infection histories. For EBV, primary infection often results in clinical disease, acute infection mononucleosis (AIM), making it possible to evaluate EBV-specific CD8 T cell responses during acute and persistent phases of viral infection. EBV infection causes massive monoclonal and oligoclonal expansions of CD8 T cells in AIM patients (Callan et al., 1996). At least 28 CD8 T cell epitopes have been identified for 5 different HLA molecules, and the response to a single EBV epitope can be as large as 44% (Catalina et al., 2001) (Meij et al., 2002) (Callan et al., 1998). CD8 T cells respond predominantly to lytic antigens during the acute phase of infection but these responses are largely culled (Catalina et al., 2001) (Hislop et al., 2002) (Callan et al., 2000). Interestingly, CD8 T cells specific for latent antigens become predominant during the persistent phase of viral infection, most likely due to the unique virus-host interaction (Hislop et al., 2002).

HCMV infection of immunocompetent individuals is usually silent clinically, or remains undiagnosed. Therefore, all CD8 T cell studies published so far have analyzed epitope-specific CD8 T cell responses in chronically-infected subjects. The identification of IE1/pp89 as a dominant CD8 T cell antigen in BALB/c mice infected with MCMV (Reddehase et al., 1989), led to the discovery that CD8 T cells and CD4 T cells in humans respond to IE1/pp72 of HCMV (Alp et al., 1991). pp65, a viral tegument protein, was also found to be immunogenic, as CD8 T cells from 4 of 5 patients evaluated had pp65-specific CD8 T cells (McLaughlin-Taylor et al., 1994). The most comprehensive

analysis of HCMV-specific CD8 T cells was published earlier this year (Elkington et al., 2003). Using predicted HLA-binding motifs for 10 HLA alleles, over 200 peptides were synthesized from fourteen HCMV proteins. The peptides were tested for the ability to bind HLA, induce IFN-γ production from PBMCs, and induce CTL lysis of peptide-coated targets. From these, a total of 63 CD8 T cell epitopes were identified, nearly all of which were previously unknown. Although ten of the 14 proteins were immunogenic, the main message was that pp65 and IE1 induced immunodominant responses across a number of HLA alleles, while CD8 T cell responses to other proteins were significantly weaker. In some individuals, 5-10% of CD8 T cells responded to IE1 epitopes.

1.8 Effects of *m04*, *m06* and *m152* on MHC class I antigen presentation

Shortly after the identification of IE1/pp89 as an antigen in BALB/c mice, Koszinowski and colleagues showed that IE1/pp89 was presented, albeit weakly, 1.5 hours post-infection and 16 hours post-infection, but not from 3-16 hours post-infection (Del Val et al., 1989). This implied that E genes could block IE1/pp89 antigen presentation. Presentation of IE1/pp89 was enhanced if drug blockade was used to allow synthesis of only IE proteins, but a 45-minute window of E gene expression was sufficient to completely inhibit presentation of IE1/pp89. The authors presented evidence that BALB/c CTL clones specific for E antigens, and polyclonal BALB/c and C57BL/6 CTL, were not affected. They thus concluded that the effect was specific for IE1/pp89 antigen, but this proved not to be the case, as presentation of CD8 T cell epitopes derived from SV40 (Campbell et al., 1992) and β-galactosidase (del Val et al., 1992) were later shown to also be affected.

Experimental evidence indicated that equal amounts of IE1/pp89 peptide were associated with L^d under IE or E conditions, but that an E gene retained nascent MHC I molecules in an endoplasmic reticulum - Golgi intermediate compartment (ERGIC), determined by endo-H sensitivity (Del Val et al., 1991a). A gene from the Hind III E region of the MCMV genome appeared to be responsible (Thale et al., 1995), and this was later shown to be *m152* (Ziegler et al., 1997). m152/gp40 expressed by recombinant vaccinia virus was sufficient to retain MHC I molecules in the ERGIC and to prevent CD8 T cell killing of cells expressing IE1/pp89. Given that m152/gp40 and MHC I did not co-immunoprecipitate from infected cells even in weak detergent, and that these two proteins localized to distinct cellular compartments (Ziegler et al., 2000), the mechanism of retention remains a mystery. Kavanaugh et al. (Kavanagh et al., 2001a) confirmed that m152/gp40 retains MHC I molecules in vitro, blocking recognition of D^b-restricted CD8 T cells particularly well.

In vivo, a Δm152 MCMV mutant replicates to ~1 log lower titers in immunocompetent BALB/c mice, compared to wt MCMV or a revertant virus, and this attenuation was abrogated by simultaneous depletion of CD8 and CD4 T cells (Krmpotic et al., 1999). The MCMV mutant displayed no phenotype compared to wild-type MCMV in neonatal CD8-/- and β2m-/- mice, confirming that CD8 T cells were required for the phenotype. The in vivo effect of *m152* on CD8 T cell recognition of MCMV-infected cells was very recently confirmed more elegantly, and in an antigen-specific manner, by Reddehase and colleagues (Holtappels et al., 2004). C57BL/6 mice were irradiated, then infected with w.t. MCMV and Δm152 MCMV on the same day. Using 2-color in situ hybridization, the titer of each virus in the liver could be compared within the same

mouse. It was shown that CD8 T cells specific for the D^b-restricted M45 epitope ^{985}HGIRNASFI^{993} were effective at controlling replication of Δm152 MCMV, but unable to control replication of w.t. MCMV. This demonstrates for the first time that an MHC class I immune evasion gene inhibits CD8 T cell recognition of infected cells in vivo.

On an interesting and related side note, *m152* is also responsible for inhibiting NK cell recognition of MCMV-infected cells (Krmpotic et al., 2002), accomplished by down-regulating cell surface expression of RAE-1α, -β, -δ, -ε and -γ (Lodoen et al., 2003), ligands for the activating NK receptor NKG2D.

In the report (Thale et al., 1995) that defined the genomic location of *m152*, it was also demonstrated that a virus missing the Hind III E region was still able to downregulate surface MHC I molecules at later stages of the early phase. Reusch et al. (Reusch et al., 1999) later demonstrated that on the left side of the genome, *m06* encodes a glycoprotein, m6/gp48, that physically interacts with MHC I. m6/gp48 contains a di-leucine motif in its cytoplasmic tail that targets m6/gp48 and MHC I to the lysosome, where both proteins are degraded. Although no role in CD8 T cell recognition was directly demonstrated at that time, later experiments confirmed that *m06* also prevents antigen presentation to CD8 T cells (LoPiccolo et al., 2003) (Gold, 2002b).

m4/gp34 represents the third MCMV glycoprotein known to affect CD8 T cell recognition of infected cells. It was originally identified as a 34kDa protein that co-immunoprecipitated with K^b and D^b, and was characterized biochemically (Kleijnen et al., 1997). Because m4/gp34 accompanies nascent MHC I molecules from the ER to the cell surface, and expression of m4/gp34 was inversely correlated with ER retention of MHC I molecules, it was first hypothesized that the function of *m04* was to counteract

NK cell recognition of MCMV-infected cells, which had low cell surface expression of MHC I due to the effects of *m06* and *m152*. But at this time, no data exists demonstrating that *m04* affects NK cell recognition of MCMV-infected cells.

However, m4/gp34 does play a role in antigen presentation to CD8 T cells (Kavanagh et al., 2001a). A number of MCMV-specific CD8 T cell clones were isolated. It was found that *m04* was required for evading recognition by CTL restricted by K^b but not D^b. Other experiments demonstrated that m4/gp34 preferentially associates with and accompanies K^b to the cell surface, compared to D^b, thus explaining the differences in CD8 T cell recognition. A separate set of experiments revealed possible mechanisms by which m4/gp34 may affect K^b (Kavanagh et al., 2001b). The majority of K^b that reaches the surface in MCMV-infected cells is associated with m4/gp34, indicating that m4/gp34 may interfere directly with CD8 T cell recognition. However m4/gp34 also forms a low-stability complex with K^b in the ERGIC, detectable only with the weak detergent digitonin, raising the possibility that m4/gp34 interferes with peptide loading or processing.

Function of the immune evasion genes in professional APCs

It has been suggested that the immune evasion genes are unable to function in macrophages (Hengel et al., 2000). However, the functional data provided by Hengel et al. was most dramatic only for transformed macrophages, not primary macrophages; the authors were only able to demonstrate 15-20% CTL lysis using bone marrow (BM) macrophages restricted to IE or E gene expression, while a lack of killing on uninfected targets was not shown. This raises serious questions about the central message of the

paper. The authors stated that macrophages are capable of priming naïve CD8 T cells in vivo, but it has been definitively shown in vivo that CD11c+ dendritic cells are required for priming of CD8 T cells, while macrophages are incapable of priming CD8 T cells (Jung et al., 2002). Hengel et al. concluded that the MCMV MHC class I immune evasion genes would not be expected to affect CD8 T cell priming in vivo. It appears that the data may not be correct, and in any case the rationale for their conclusions is flawed. More recent studies were performed using only primary BM macrophages, derived from C57BL/6 mice, and demonstrated that *m04* and *m152* do affect MHC class I trafficking and antigen presentation, using both biochemical and functional assays (LoPiccolo et al., 2003). There were a number of technical differences between the two systems, but at a minimum it can be concluded that failure of the immune evasion genes to function in primary macrophages, if it occurs at all, is not a general phenomenon.

Possible roles of *m04*, *m06* and *m152* in CD8 T cell priming in vivo

We originally speculated that one function of the MHC class I immune evasion genes in vivo was to alter the spectrum of antigens that prime CD8 T cell responses, or to limit the total size of the CD8 T cell response. This would make some sense teleologically, for example if the virus wants to reduce effective control of replication, limit "bystander" CD8 T cell pathology or 'trick' the immune system into priming CD8 T cell antigen specificities that are not protective. In a review on the mechanisms of immunodominance, Yewdell and Bennink stated that for herpesviruses in general, "a simple prediction is that T_{CD8+} responses will focus on those determinants that for

whatever reason are less affected by the strategy employed by the virus" (Yewdell and Bennink, 1999).

With this in mind, two major categories of antigens would be thought to escape the effects of *m04*, *m06* and *m152*. The first category is antigens expressed before the immune evasion genes, which are all E genes. *m152* transcripts are detectable beginning two hours after infection, increasing for several hours before declining, but are still detectable 24 hours after infection (Holtappels et al., 2002a). Transcripts of *m06* follow similar kinetics. Expression of *m04* transcripts does not occur until 4 hours post-infection, but also continues for 24 hours. Because *m06* and *m152* are among the first E genes expressed, and *m04* is expressed only two hours later, IE genes are the most likely candidate genes to be expressed before *m04*, *m06* and *m152*.

The CD8 T cell immunodominance hierarchy in BALB/c mice would be compatible with this idea. IE1 and m164 are both dominant antigens (Holtappels et al., 2002c). IE1 can partially circumvent the effects of the immune evasion genes by virtue of its timing of expression. In addition , for nearly ten years it was the only known antigen from MCMV, so it was considered 'typical.' On the other hand, m164 is known to be presented despite the effects of the immune evasion genes (Holtappels et al., 2002a), by an unknown mechanism. All other antigens identified thus far are E proteins and very subdominant. Presumably these are subdominant because they are subject to the effects of the immune evasion genes, although technically only m04 epitope presentation has been shown to be blocked in MCMV-infected cells (Holtappels et al., 2000b).

The second category of antigens predicted to escape the effects of *m04*, *m06* and *m152* includes antigens that are restricted by MHC I molecules least affected by the

immune evasion genes. MHC allele-specific effects of all three genes were compared directly (Wagner et al., 2002). *m152* appears to affect all *H-2*^{d} and *H-2*^{b} MHC I alleles, but particularly D^b, a finding first reported by Kavanaugh et al. (Kavanagh et al., 2001a). *m06* also down-regulates all *H-2*^{d} and *H-2*^{b} MHC I alleles, but appears most effective against K^d and D^b, and least effective against D^d. *m04* does not down-regulate surface expression of any MHC I molecules examined, but does preferentially affect antigen presentation by K^b, as discussed above (Kavanagh et al., 2001a) (Kavanagh et al., 2001b). We hypothesized that *m04* would be effective in preventing priming of some K^b-restricted antigens, since *m04* associates with K^b in the ER (Kavanagh et al., 2001b) and may influence the quality of peptides that are loaded onto nascent MHC I molecules.

The overall message is that if *m04*, *m06* and/or *m152* do affect CD8 T cell priming, the results may be complex, but at least partially predictable if the timing of expression and restriction element for each antigen is known. Conflicting with this idea is the observation that *m152* was required to block presentation of M45 in vitro, but w.t. MCMV and Δm152 MCMV were equally effective at priming M45-specific CD8 T cells in vivo. As will be shown by data presented in chapter 4, this finding is not specific for the M45 epitope and the *m152* immune evasion gene.

1.9 Latency and Reactivation

MCMV establishes true molecular latency

Until relatively recently, it was controversial whether MCMV persists at a low level or establishes true latency. Latency is defined as the presence of functional viral genomes in the absence of infectious virus, and three criteria must be met to prove true

molecular latency. First, infectious virus must be undetectable. Second, viral DNA must be present by PCR. And third, viral DNA must be competent for reactivation. Since it is impossible to definitively prove that infectious virus is not simply present below the limit of detection of whichever assay is employed, skeptics argued that MCMV is persistent, not latent.

There is now very strong evidence, though, that MCMV establishes true latency. Kurz et al (Kurz et al., 1997) found that 1 pfu (defined in a standard plaque assay) of sucrose gradient-purified virus was equivalent to ~500 viral genomes, under defined conditions. By infecting cells with centrifugal enhancement, then testing for *ie1* transcripts 72 hours later by RT-PCR, infection with as low as 0.01 pfu, equivalent to ~ 5 viral genomes, was detectable. It was known that MCMV forms both monocapsid and multicapsid virions, and the authors estimated by electron microscopy that the average virion contains ~2.6 genomes. Thus, their infectivity assay could detect infection with approximately 2 virions, which is extremely close to the physical limit of detection, 1 virion. In BALB/c mice infected with MCMV for 12 months, it was demonstrated that no infectious virus was detectable in the lungs using this sensitive infectivity assay, even though approximately 10^6 viral genomes were present, as determined by semi-quantitative PCR. Fourteen days after hemoablative treatment, their infectivity assay could detect infectious virus in all mice, proving that the viral DNA was reactivation-competent. Therefore, the three criteria for latency appear to have been met.

Acute viral titers, latent load and likelihood of reactivation

The mechanisms by which latency is established and maintained, and the process of reactivation from latency are poorly-understood, although some factors that affect these processes are known. Infection of neonatal BALB/c mice, compared to immunocompetent adults, results in higher acute viral titers and delayed viral clearance, particularly in the salivary gland (Reddehase et al., 1994). Importantly, this resulted in establishment of a much higher "latent load" (i.e. the quantity of latent viral genome) in all organs of these mice. The latent load was highest in the lungs and salivary glands, compared to the spleen, which correlated with acute viral titers in these organs. Thus, the latent load was influenced by both the immunocompetence of the mouse and in an organ-specific manner, both correlating with acute viral replication.

It was demonstrated previously that MCMV establishes a 10-fold higher latent load in the lungs than in the spleen, and reactivation is 3 times more likely to occur in the lungs than the spleen (Balthesen et al., 1993). Thus the risk of reactivation in an organ appears to correlate with the latent load in that organ, and this may also explain why interstitial pneumonia is the most frequent manifestation of HCMV reactivation. This finding was extended using the neonatally-infected mice (Reddehase et al., 1994). MCMV reactivation was induced ~10 times more frequently in mice infected as neonates, and reactivation was more frequent in organs with the highest latent load. Notably, reactivation occurred in different organs stochastically (i.e. virus did not appear to reactivate in one organ and then spread to another).

The simple message from this complicated explanation is that acute viral replication is an important determinant of the latent viral load, in each organ and in the

whole mouse. Furthermore, the quantity of latent virus is predictive, in a statistical sense, of the individual mouse and of the organs in which the virus is most likely to reactivate.

Role of lymphocyte subsets in limiting latent load and preventing reactivation

In a mouse model of bone marrow transplantation (BMT), mice that undergo hemoablation by γ-irradiation, and are then infected with MCMV, will survive if transfused with 10^7 bone marrow cells (BMC). Adoptive immunotherapy with MCMV-specific CD8 T cells, while not required for survival, accelerates viral clearance in the adrenal glands, lungs, and to a smaller extent the salivary glands (Steffens et al., 1998). Because virus is cleared more rapidly, the latent viral load is also decreased in these organs by up to 80%. Since it was previously shown that a prolonged productive primary infection increases the latent viral load and the incidence of viral reactivation, it is not surprising that the CD8 immunotherapy also resulted in a greater than 90% decrease in virus reactivation.

As discussed above, in immunocompetent BALB/c mice antibodies do not play any role in resolving primary MCMV infection and do not affect the latent viral load (Jonjic et al., 1994). Furthermore, antibodies may enhance MCMV super-infection in wild mice (A Scalzo and G Shellam, personal communication). But when MCMV was reactivated from latently-infected μMT mice ($Ig^{-/-}$) through irradiation and lymphocyte depletion, it was found that viral titers were 100- to 1000-fold higher in the salivary gland, lungs and spleen than in wild-type controls (Reddehase et al. 1994). This suggests that antibodies play a role either in limiting virus reactivation or limiting viral replication after reactivation. The role of antibodies was definitively proved because the phenotype

was reversed by the transfer of MCMV-specific serum. This is striking when contrasted with the observation that MCMV-specific antibodies are unable to prevent super-infection of a given mouse with multiple strains of MCMV (discussed above in section 1.5).

The finding that antibodies are important in preventing reactivation was used to elucidate the role of NK cells, CD4 T cells and CD8 T cells in immunosurveillance of MCMV. Polic et al. found that in latently-infected μMT mice, after simultaneous depletion of NK cells, CD4 T cells and CD8 T cells, virus was detected in the salivary gland as few as three days after depletion, and was present in lungs and spleen within two weeks of depletion (Polic et al., 1998). By depleting NK cells, CD4 T cells and CD8 T cells in different combinations, it was determined that depletion of any subset alone rarely led to reactivation in any organs, depletion of any two subsets led to frequent reactivation, while depletion of all three subsets allowed reactivation in virtually all mice in all organs examined. Thus the authors concluded that in contrast to α- and γ-herpesviruses, CMV reactivates very frequently and by necessity immunosurveillance is an active process.

Unfortunately this experimental design may be problematic. Antibody depletion of large lymphocyte subsets, rather than constituting a neutral immunological event, might result in the local release of some cytokines. As described below, TNF-α may be a key regulator of reactivation. This potential problem has been discussed previously by Reddehase and colleagues (Kurz et al., 1999), who were unable to detect infectious virus in the lungs of latently-infected BALB/c mice, despite the present of 10^6 viral genomes.

Based on this data, it seems more likely that reactivation is a rare event, and immunosurveillance is not as active as proposed by Polic et al. (1998).

Molecular reactivation from latency

Because MCMV establishes a high latent load in the lungs of BALB/c mice, this is an excellent in vivo system for studying reactivation. Despite the absence of detectable virus, *ie1* and transcripts were found in the lungs (Kurz et al., 1999) or spleen (Henry and Hamilton, 1993) Yuhasv, 1994) of latently-infected mice. Yuhasv et al. (1994) interpreted this as evidence of persistent infection, but Kurz et al. (Kurz et al., 1999) discredited this notion by further demonstrating that although over 12,000 *ie1* transcripts were present in the lungs of some mice, fewer than 10 *ie3* transcripts (recall that IE3 is the essential IE activator of E genes) and fewer than 10 *gB* transcripts (gB is an essential virion protein) were present in the same samples. It appears then that *ie1* transcripts are a very poor indicator of infectious virus. In further work by Kurz et al., after irradiation of latently-infected mice, distinct lung pieces contained either *ie1* transcripts alone, *ie1* and *ie3* transcripts, or *ie1* + *ie3* + *gB* transcripts (Kurz and Reddehase, 1999). Even fewer lung pieces contained infectious virus. This indicates that there are at least four sequential checkpoints that exist between truly latent viral DNA and full reactivation.

HCMV researchers have focused on latency in cells of the bone marrow and blood. For example, long-term culture of allogeneically stimulated monocyte-derived macrophages results in HCMV reactivation from latency (Soderberg-Naucler et al., 1997). With this in mind, MCMV *ie1* transcripts are inducible in latently-infected kidneys after transplantation into allogeneic but not syngeneic mice, although since no

ie3 or *gB* transcripts were detected reactivation was not complete (Hummel et al., 2001). Interestingly, TNF-α was induced by transplantation, and expression of *ie1* transcripts was induced in latently-infected kidneys by treatment with TNF-α alone. This demonstrates that TNF-α may play an important role in initiating *ie1* transcription, but that TNF-α is not sufficient for passing further MCMV reactivation checkpoints such as *ie3* transcript splicing. It is possible that allogeneic transplantation mimics the inflammation that is associated with a natural immune response to infection, and that MCMV has been selected throughout evolution to reactivate at times when the host immune system may be weak or distracted.

1.10 Accumulation of IE1- and m164-specific CD8 T cells during latency

Although MCMV reactivation has been difficult to study at a molecular level, the apparent effects of reactivation are evident in the behavior of MCMV-specific CD8 T cells, as three reports in BALB/c mice have demonstrated.

In the bone marrow transplantation (BMT) model established by Reddehase and colleagues, MCMV titers are very high in the acute phase of infection because the immune system is fighting the virus while in the process of reconstituting itself. Furthermore, replicating virus persists much longer in these mice. In this model system, MCMV-specific CD8 T cells infiltrate the lungs, with peak infiltration occurring four weeks post-infection. CD8 T cells specific for IE1/pp89 and the subdominant epitope M83 were both present in the lungs at this time (Holtappels et al., 2000a). Notably, three months post-infection, when replicating virus is no longer detectable and latency established, the frequency and absolute number of IE1/pp89-specific CD8 T cells had

actually increased, while CD8 T cells specific for M83 had exfiltrated or died. Purification of these IE1/pp89-specific CD8 T cells revealed a predominantly CD62L lo phenotype, indicative of recent activation. The authors proposed a model whereby latent virus infrequently expresses *ie1* transcripts (although pp89 protein could never be detected by immunohistology), that result in proliferation or recruitment of only IE1/pp89 specific CD8 T cells.

It was later found that m164 also encodes a CD8 T cell epitope in BALB/c mice that is co-dominant with IE1/pp89 (Holtappels et al., 2002c). In immunocompetent mice, the response to these epitopes was smaller in the popliteal lymph node during the acute response than in the spleen several months later. This finding was confirmed in the BMT model, where the frequency of m164-specific and IE1/pp89-specific CD8 T cells both increased from the time of peak infiltration to the time when replicating virus was cleared. Thus this phenomenon was not unique but also occurred to m164 (but not to other subdominant epitopes tested).

The authors knew that in CMV seropositive humans, large virus-specific CD8 T cell responses are detected against a comparatively small number of CD8 epitopes. In addition, some human subjects have HCMV-specific CD8 T cell responses that are dominated by oligoclonal or monoclonal CTL, years or decades after infection. In order to determine if a "focusing" of the immune response was occurring, MCMV peptides were acid-extracted from cells infected in vitro, then separated into fractions by HPLC. The fractions were used to stimulate CD8 T cells directly ex vivo from immunocompetent mice infected with MCMV either acutely or latently. Acutely-infected mice responded to the five known epitopes, but also to a number of HPLC fractions not

known to contain any CD8 T cell epitopes (Holtappels et al., 2002c). In contrast, chronically-infected mice responded well only to fractions that contained the dominant IE1/pp89 and m164 epitopes, and subdominant m04 and m18 epitopes. This provided suggestive evidence that the acute CD8 T cell response may be much broader than the memory response, and that this may be analogous to what happens in human HCMV infection.

The phenomenon was revisited in immunocompetent BALB/c mice soon after (Karrer et al., 2003). It was elegantly demonstrated that the IE1/pp89-specific CD8 T cell response occurs in three distinct phases in both the blood and spleen. Rapid expansion was observed from day 0 to day 10, followed by rapid loss of most antigen-specific CD8 T cells by day 15, reminiscent of the kinetics of CD8 T cell responses to cleared infections such as LCMV and *Listeria*. However, this population of cells then underwent a phase of slow, continuous expansion between days 40 and 400, long after viral latency is established, which resulted in an approximate four-fold increase of IE1/pp89 CD8 T cells. The first expansion and contraction phase also occurred in response to infection with vaccinia virus heterologously expressing IE1/pp89, but the second expansion phase did not occur, demonstrating the importance of MCMV's unique biology. This group confirmed that IE1/pp89-specific CD8 T cells persisted in a CD62L lo phenotype even at day 400, and an increased use of TCR Vβ8.1 over time suggested that the expansion may result in increased clonality. Confirming earlier work described just above, the effect was specific for IE1/pp89 and m164 epitopes, but did not extend to m04, M83 or M84 epitopes.

1.11 *Cmv1*^{r}, *Cmv1*^{s}, Ly49H and *m157*

In the early 1980's, beige mice were found to have increased susceptibility to MCMV (Bancroft et al., 1981) (Shellam et al., 1981). The beige mutation was already known to selectively impair NK cell function (Roder and Duwe, 1979), demonstrating that NK cells are important for controlling MCMV. Different mouse strains that showed differing susceptibility to MCMV infection were interbred to produce recombinant inbred mice, and classical Mendelian genetic analysis identified a genetic locus, *cmv1*, that controls MCMV replication in the spleen (Scalzo et al., 1990). In susceptible strains such as BALB/c (*cmv1*^{s}), virus replication in the spleen is from 10^3 to 10^4-fold higher than in resistant strains such as C57BL/6 (*cmv1*^{r}). The *cmv1* locus was linked to the recently-discovered NK complex on mouse chromosome 6, and *cmv1* protection was found to be mediated by NK cells (Scalzo et al., 1992). The NK complex contains a large number of NK-specific proteins, and no in vitro assay existed to determine MCMV susceptibility/resistance, so a positional cloning strategy was undertaken by two separate groups. Low-resolution linkage analysis (Scalzo et al., 1995c), followed by high-resolution linkage analysis (Depatie et al., 1997) (Brown et al., 1997b), closely linked the *cmv1* locus to the *ly49* locus of the NK complex. Finally, overlapping yeast artificial chromosomes (YACs) spanning ~4.7 Mb of DNA were aligned to create a physical map of the NK complex (Brown et al., 1997a) (Brown et al., 1999).

Two years later, three groups simultaneously determined that *cmv1* is equivalent to Ly49H, an activating NK cell receptor (Brown et al., 2001) (Lee et al., 2001) (Daniels et al., 2001). The ligand for Ly49H was unknown, but a previous study showed that antibody-induced cross-linking of Ly49H on NK cells could induce cytotoxicity (Smith

et al., 2000). Notably, Ly49H was absent on CD3+ T cells. Brown et al. (Brown et al., 2001) demonstrated that anti-Ly49H antibody, either by blocking Ly49H function or depleting Ly49H+ NK cells, renders otherwise resistant strains susceptible to MCMV. A year later, Lanier and colleagues found that cells infected with w.t. MCMV expressed the Ly49H ligand, while cells infected with a mutant MCMV missing genes *m150-165* did not (Arase et al., 2002). In vitro, the product of the *m157* gene was determined to bind Ly49H and could confer susceptibility to lysis from NK cells derived from C57BL/6 mice. Another group used a bioinformatics approach to scan the MCMV genome for proteins with MHC-like characteristics, since all known Ly49 ligand are either MHC molecules or MHC-like molecules, and also identified m157 as the ligand for Ly49H (Smith et al., 2002).

It is unknown why MCMV encodes a protein that ultimately limits virus replication. HCMV does not have an *m157* homolog, but rat CMV does. One interesting clue is that 129/J mice express an allele of another Ly49 molecule, the inhibitory Ly49I NK receptor, that also binds to m157. Therefore m157 may have been selected through evolution to bind specifically to Ly49I, while cross-reactivity with the activating Ly49H molecule in some strains of mice may have been a fortuitous but recent evolutionary event. In support of this, nearly all inbred mouse strains except C57BL/6 are phenotypically *cmv1*^{s} (Scalzo et al., 1995c) (Lee et al., 2001) as are most wild mice in Australia (A Scalzo and G Shellam, personal communication).

Chapter 2: Introduction

After activation, CD8 T cells undergo a set program of exponential proliferation, followed by a contraction phase and stable memory (see Chapter 1.3). Following vaccination, we hoped to interfere with the contraction phase in order to improve a CD8 T cell vaccine.

4-1BB and OX40 Stimulation Enhance CD8 and CD4 T Cell Responses to a DNA Prime, Poxvirus Boost Vaccine

CD8 T cells play a major role in containing HIV and SIV infection, and in consequence, the CD8 T cell response is considered an important component of a vaccine for HIV. The task of achieving high numbers of CD8 T cells using heterologous vectors is challenging. A common strategy is to prime a response using plasmid DNA expressing the viral antigen, followed by a boost with a recombinant poxvirus expressing the same antigen (Hanke et al., 2002). Cytokines and co-stimulatory molecules added to either the prime or boost phase have been used to increase the number of responding CD8 T cells with some success, but improvement is needed (Ahlers et al., 2003).

There has been recent interest in two costimulatory molecules of the tumor necrosis factor receptor (TNFR) superfamily expressed on T cells, 4-1BB (CD137) and OX40 (CD134), which are thought to function primarily on CD8 T cells and CD4 T cells, respectively. 4-1BB and OX40 expression is induced on T cells after TCR triggering and these receptors are expressed transiently for 1-5 days (Hurtado et al., 1997) (Cannons et al., 2001) (Gramaglia et al., 1998). Their natural ligands, 4-1BBL and OX40L,

respectively, are transmembrane TNF homologs that are expressed on activated APCs (DeBenedette et al., 1997; Pollok et al., 1994; Stuber et al., 1995) (Weinberg et al., 1999). It is clear that 4-1BB signaling is necessary for optimal CD8 T cell activation in vivo because 4-1BBL-/- mice have reduced CD8 T cell responses after infection with LCMV or influenza virus, and following peptide vaccination (Tan et al., 1999) (DeBenedette et al., 1999) (Bertram et al., 2002) (Tan et al., 2000). 4-1BB stimulation can also be used to enhance T cell proliferation and function (Diehl et al., 2002) (Cannons et al., 2001) (Pollok et al., 1993) (Zhou et al., 1994). OX40 shares sequence homology with 4-1BB and plays an analogous role in CD4 T cell function. In vivo, OX40 blockade ameliorates autoimmune disease and OX40 stimulation increases CD4 T cell proliferation (Weinberg et al., 1999) (Evans et al., 2001). In addition, 4-1BB and OX40 are able to protect activated T cells from death in a number of model systems (Gramaglia et al., 2000; Hurtado et al., 1997; Maxwell et al., 2000; Rogers et al., 2001; Takahashi et al., 1999).

Cumulatively, there is very strong evidence that 4-1BB and OX40 can enhance the development of effective T cell immunity. In addition, they have been used to improve effective immunity in tumor vaccine models (Diehl et al., 2002) (Melero et al., 1997) (Ye et al., 2002b) (Kjaergaard et al., 2001) (Weinberg et al., 2000). However, tumors are inherently weak immunogens, and it was not clear whether these molecules could improve the response to a powerful immunogen such as vaccinia virus. We therefore wanted to determine whether these molecules could be exploited to improve a DNA prime, poxvirus boost vaccine strategy. A practical prophylactic vaccine strategy for stimulating these receptors would probably need to encode their ligands in the plasmid DNA and/or the recombinant poxvirus. However, as a proof-of-principle test, we

used monoclonal antibodies that have previously been shown to enhance T cell responses in vivo, in combination with a standard DNA-prime recombinant poxvirus boost vaccination protocol. Here we report that a 2-4 fold increase in antigen-specific CD8 T cells is achieved by stimulating 4-1BB in vivo. Furthermore, we show that engagement of 4-1BB and OX40 at the same time can have an additive effect on both CD4 and CD8 T cell priming in vivo.

Chapter 2: Results

An anti-4-1BB mAb enhances CD8 T cell activation and memory

Protocols incorporating a DNA-prime followed by a recombinant poxvirus boost are frequently used to generate a CD8 response in experimental vaccine protocols (for a review, see Hanke et al., 2002). Because stimulation through 4-1BB has been reported to enhance CD8 T cell activation, and selectively rescue activated CD8 T cells from death, we wanted to determine if engagement of 4-1BB in vivo during a DNA-prime poxvirus boost strategy would improve the number of activated CD8 T cells and the number that survived to become memory T cells.

BALB/c mice were injected twice, one week apart, with plasmid encoding the H-2D^{d}-restricted HIV-1 envelope glycoprotein peptide (RGPGRAFVTI). Two weeks after the second injection, mice were boosted with MVA expressing the same antigen (MVA.HM), and given a single dose of agonist anti-4-1BB mAb (*see Materials and Methods for rationale*) or control rat IgG. CD8 T cell responses were assessed 6 days and 22 days later by direct ex vivo intracellular cytokine staining (ICS) for IFN-γ. Figure

2.1A shows typical intracellular IFN-γ staining for this experiment; one representative mouse is shown from each group. Figure 2.1B shows the results for the entire experiment, with antigen-specific CD8 T cells expressed as a percentage of total CD8 T cells. Mice that received anti-4-1BB mAb had a small increase in the frequency of antigen-specific CD8 T cells, but it was not statistically significant: 8.2% vs. 5.0% IFN-γ+ CD8 T cells on day 6 ($p=0.09$) and 2.7% vs. 1.7% IFN-γ+ CD8 T cells at 22 days ($p=0.09$) after the MVA.HM boost (Figure 2.1B).

The vaccinated mice developed splenomegaly, and we therefore also determined the total number of antigen-specific T cells per spleen. Six days after the MVA.HM boost, control mice had an average of 2.2×10^5 IFN-γ+ CD8 T cells per spleen, while the mice treated with anti-4-1BB mAb averaged 10.5×10^6 IFN-γ+ CD8 T cells per spleen, a greater than 4-fold increase ($p<0.01$) (Figure 2.1C). Twenty-two days after MVA.HM, control mice had 1.1×10^5 IFN-γ+ CD8 T cells per spleen, while mice treated with anti-4-1BB mAb had 4.0×10^5 IFN-γ+ CD8 T cells per spleen, a difference of nearly 4 fold ($p=0.01$). Stimulating 4-1BB thus had a greater impact on the total number of antigen-specific CD8 T cells than on their frequency. This reflected the fact that mice receiving the 3H3 antibody developed a more profound splenomegaly than mice that received control antibody.

We conclude from these initial experiments that by administering stimulating anti-4-1BB mAb in a vaccine setting, we could significantly increase the total number of antigen-specific CD8 T cells at both the peak of the response, day 6, and after the primary response had resolved, at day 22. Although 4-1BB stimulation has been reported to rescue cells from activation-induced cell death, we did not see a preferential survival of

antigen-specific CD8 T cells after 4-1BB engagement in vivo. However, these experiments were limited by the small numbers of animals per group, which made it difficult to reach statistical significance. In addition, at the 22-day time point used to detect memory cells the mice still had somewhat enlarged spleens, suggesting that a true memory homeostasis had not yet been reached.

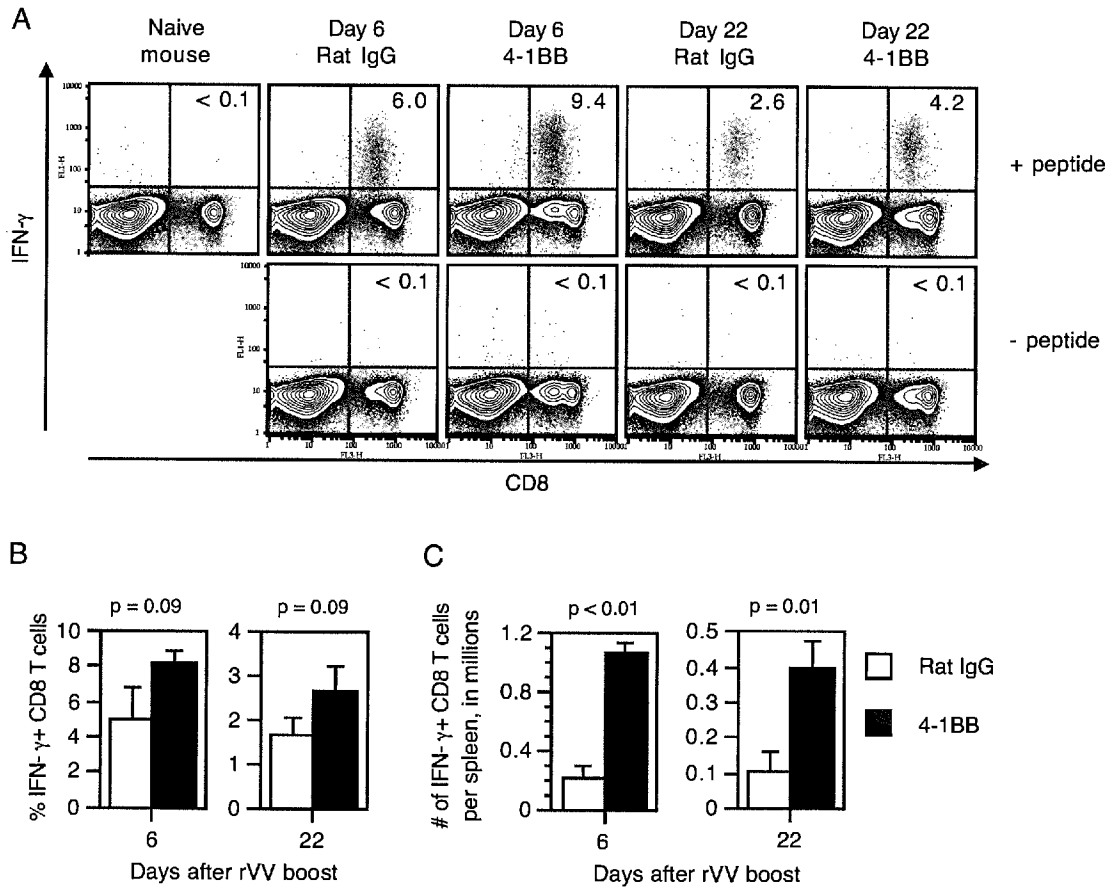

Figure 2.1 4-1BB enhances CD8 T cell activation and memory.

BALB/c mice were primed with 100 μg of plasmid pTH.HM twice, one week apart. Two weeks later, mice were boosted with 1 x 10^6 pfu of MVA.HM and received 200 μg of either purified anti-4-1BB mAb or rat IgG i.p. with the boost. At the indicated number of days later, unfractionated spleen cells were cultured directly ex vivo for 6 hours with brefeldin A, in the presence or absence of 1 μg/ml RGPGRAFVTI peptide, then stained for CD8 and intracellular IFN-γ. One representative is shown from each group (A). Each point represents the average percent (B) and number (C) of antigen-specific CD8 T cells ± SEM (day 6, n=3 mice per group; day 22, n=4 mice per group). A second experiment yielded similar results.

Anti-4-1BB mAb increases long-term CD8 T cell memory

Although CD8 T cell memory is known to be stable after clearance of a viral infection, no data exist as to whether the 4-1BB-enhancement would maintain an increased level of long-lived memory T cells. To assess long term memory, we measured the immune response at 6 and 60 days after the poxvirus boost. For these experiments, we used as our model antigen chicken ovalbumin (OVA), for which the immunodominant K^b-restricted epitope SIINFEKL has been well characterized. Both the DNA prime (pOVA) and the recombinant vaccinia virus boost (rVV-OVA) expressed the whole OVA gene, allowing us to also quantify OVA-specific CD4 T cells by intracellular staining for IFN-γ.

C57BL/6 mice (10 mice per group) were injected twice with the plasmid pOVA, two weeks apart. Four weeks after the second DNA injection, mice were boosted with rVV-OVA together with either anti-4-1BB mAb or control Ab. CD8 and CD4 T cell responses were measured directly ex vivo either 6 days later, at the height of activation, or 60 days later, to evaluate stable long-term memory. The frequency of IFN-γ+ CD8 T cells was twice as high in mice given anti-4-1BB mAb with the rVV-OVA boost compared to control mice at day 6 ($p=0.01$) (Figure 2.2A, upper left) and at day sixty ($p=0.04$) (Figure 2.2A, upper right). SIINFEKL-specific TNF-α production was also assessed and was similarly affected by 4-1BB stimulation (Figure 2.2B, upper panels).

When we compared the numbers rather than the frequency of SIINFEKL-specific CD8 T cells, a 4-fold increase was seen at day 6 ($p<0.01$) (Figure 2.2A, lower left), and an approximately 2-fold difference at day 60 ($p=0.07$) (Figure 2.2A, lower right). This was accounted for by a disproportionate increase in the percentage of CD8+ splenocytes induced by 4-1BB at day 6, compared to control IgG (Figure 2.2C). This is most likely

due to a 4-1BB-stimulated increase in CD8 T cells responding to the vaccinia vector. Again, similar results were obtained regardless of whether SIINFEKL-specific CD8 T cells were quantified using IFN-γ ICS (Figure 2.2A, lower panels) or TNF-α ICS (Figure 2.2B, lower panels).

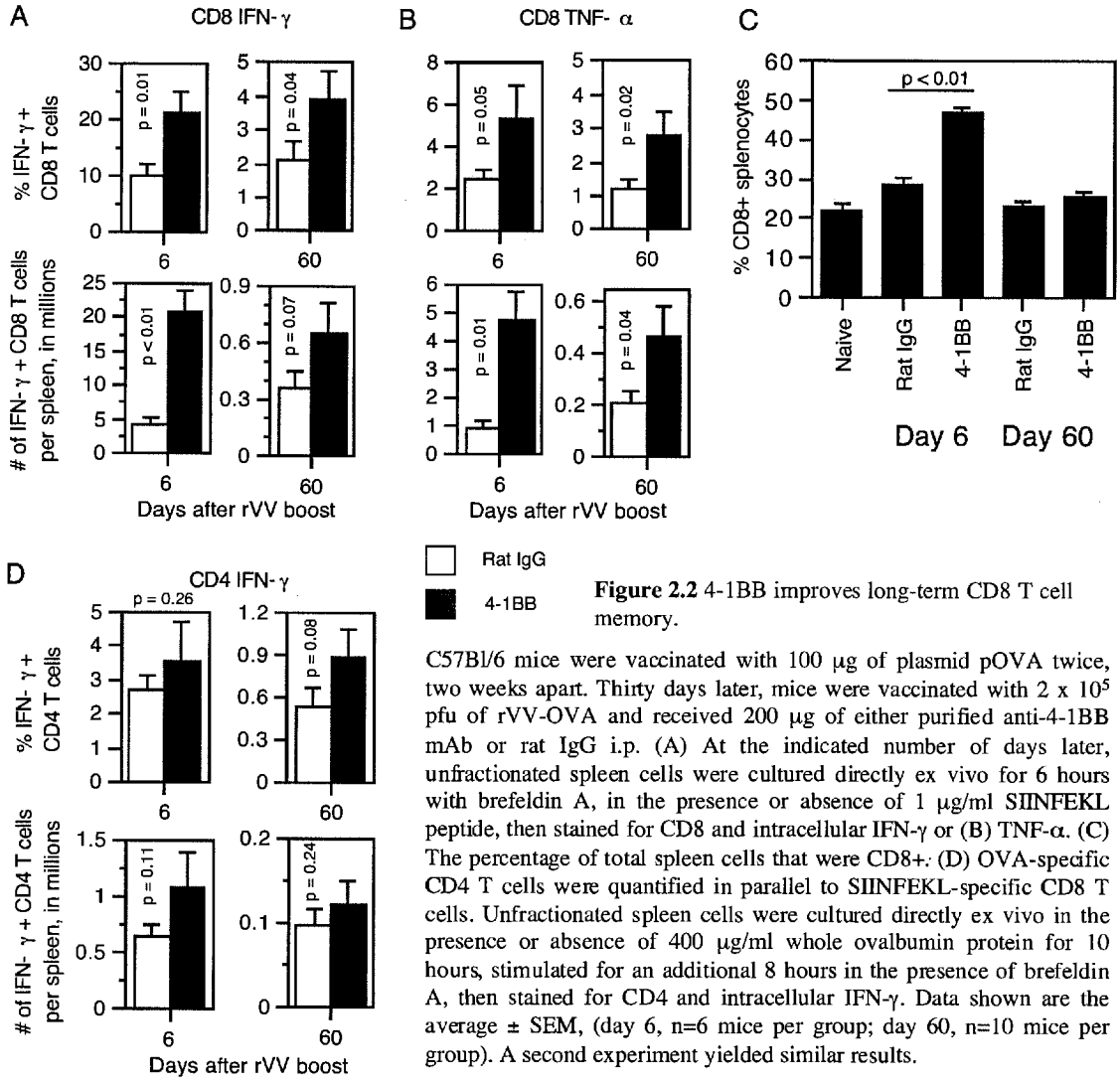

Figure 2.2 4-1BB improves long-term CD8 T cell memory.

C57Bl/6 mice were vaccinated with 100 μg of plasmid pOVA twice, two weeks apart. Thirty days later, mice were vaccinated with 2 x 10^5 pfu of rVV-OVA and received 200 μg of either purified anti-4-1BB mAb or rat IgG i.p. (A) At the indicated number of days later, unfractionated spleen cells were cultured directly ex vivo for 6 hours with brefeldin A, in the presence or absence of 1 μg/ml SIINFEKL peptide, then stained for CD8 and intracellular IFN-γ or (B) TNF-α. (C) The percentage of total spleen cells that were CD8+. (D) OVA-specific CD4 T cells were quantified in parallel to SIINFEKL-specific CD8 T cells. Unfractionated spleen cells were cultured directly ex vivo in the presence or absence of 400 μg/ml whole ovalbumin protein for 10 hours, stimulated for an additional 8 hours in the presence of brefeldin A, then stained for CD4 and intracellular IFN-γ. Data shown are the average ± SEM, (day 6, n=6 mice per group; day 60, n=10 mice per group). A second experiment yielded similar results.

We also analyzed the CD4 T cell response to OVA, using whole ovalbumin protein as antigen, and analyzing IFN-γ+ CD4 T cells by ICS (Fig 2.2D). All vaccinated animals developed a detectable OVA-specific CD4 response, which was strongest at day 6 but still easily detectable at day 60. However, treatment with anti-4-1BB mAb did not

significantly increase the OVA-specific CD4 T cell response, whether assessed as a percentage of total CD4 T cells or as absolute numbers.

We conclude from this data that anti-4-1BB mAb increased the frequency and total number of SIINFEKL-specific CD8 T cells at both day 6 and day 60 after giving the rVV-OVA boost. There was no evidence that activated cells were selectively rescued into the memory compartment. Rather, it appears that 4-1BB increases CD8 T cell expansion during the first 6 days after the rVV-OVA vaccination, and that this leads to a proportional increase in antigen-specific memory CD8 T cells. We further conclude that there was no significant effect of 4-1BB stimulation on CD4 T cell expansion or commitment to memory in this experiment.

Anti-4-1BB mAb and anti-OX-40 mAb increase the number of OVA-specific CD4 T cells, with OX-40 having a more potent effect

The previous experiment indicated that stimulating 4-1BB with anti-4-1BB mAb has a stronger effect on CD8 T cells than CD4 T cells, which was consistent with previous studies performed in vivo (Takahashi et al., 1999) (Tan et al., 1999) (Bertram et al., 2002). Conversely, OX40 is thought to primarily affect CD4 T cells in vivo (Kopf et al., 1999). Based on this we postulated that: 1) stimulating OX40 at the time of vaccination would lead to an enhanced CD4 T cell response; and 2) because CD4 help is often able to enhance CD8 T cell responses, the combination of anti-OX40 and anti-4-1BB mAb would lead to an enhanced CD8 T cell response, above 4-1BB stimulation alone.

In order to test this, we again used the OVA system because we could quantify both CD4 and CD8 T cell responses. C57BL/6 mice were injected twice with plasmid

pOVA, two weeks apart. Thirty days later, mice were vaccinated with rVV-OVA and divided into 4 groups. At the time of the boost, each group was injected with either anti-4-1BB mAb, anti-OX40 mAb, both of these mAb together, or control Ab. Five weeks later, the CD4 T cell response to OVA and the CD8 T cell response to SIINFEKL were quantified directly ex vivo by intracellular IFN-γ staining.

OX40 stimulation caused a 3-fold increase in the frequency of splenic CD4 T cells that were OVA-specific ($p<<0.01$) (Figure 2.3A, top). 4-1BB stimulation caused a small but significant increase in OVA-specific CD4 T cells ($p=0.01$) (Figure 2.3A, top). Mice receiving a combination of both anti-OX40 mAb and anti-4-1BB mAb had the most OVA-specific CD4 T cells, 4-fold more than controls but not significantly more than anti-OX40 mAb alone ($p = 0.11$) (Figure 2.3A, top). In this experiment, there was no significant difference in the average number of spleen cells between any two groups of mice (data not shown). Therefore, changes in the total number of OVA-specific CD4 T cells were similar to the changes in frequency (Figure 2.3A, bottom). Thus in this experiment both OX40 and 4-1BB stimulation increased the OVA-specific CD4 T cell response, but OX40 stimulation was more effective than 4-1BB.

Anti-4-1BB mAb and anti-OX-40 mAb cooperate to enhance CD8 T cells

In the same experiment, the SIINFEKL-specific CD8 T cell response was also quantified. 4-1BB stimulation with the rVV-OVA vaccine significantly increased the frequency of SIINFEKL-specific CD8 T cells ($p=0.03$) (Figure 2.3B, top), as did OX40 stimulation ($p=0.04$) (Figure 2.3B, top). As predicted, mice receiving both anti-OX40 mAb and anti-4-1BB mAb had significantly more SIINFEKL-specific CD8 T cells,

12.7%, than mice receiving either mAb alone (p=0.01 and p=0.03, respectively). As with CD4 T cells, the changes in total numbers of SIINFEKL-specific CD8 T cells were similar to the changes in SIINFEKL-specific CD8 T cell frequency (Figure 2.3B, bottom).

This data further confirms that 4-1BB stimulation is able to improve a vaccine-induced CD8 T cell response, and that this effect is further enhanced by OX40 stimulation.

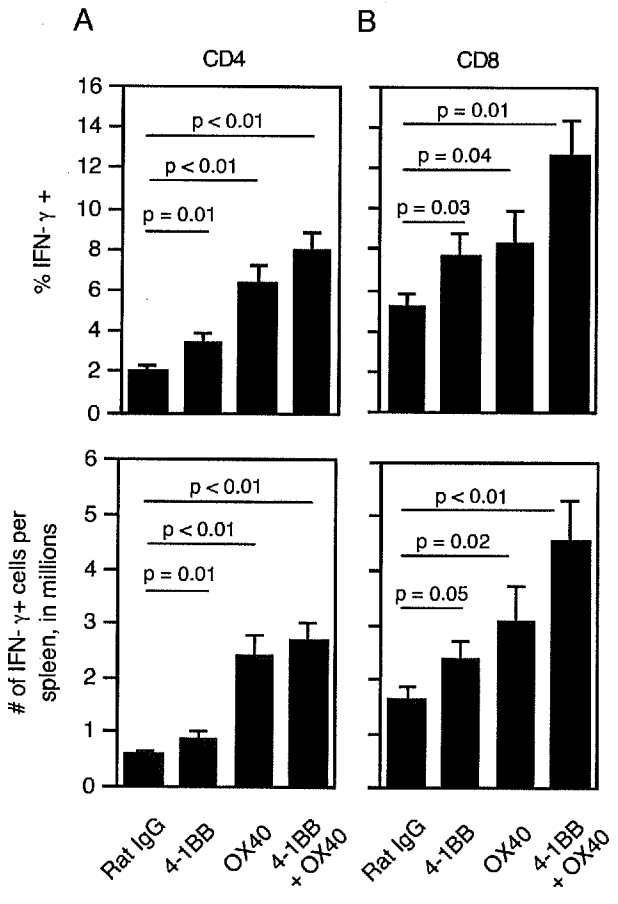

Figure 2.3 4-1BB and OX40 cooperate to enhance CD8 T cell and CD4 T cell responses.
C57Bl/6 mice were vaccinated with 100 μg of plasmid pOVA twice, two weeks apart. Thirty days later, mice were vaccinated with 2 x 10^5 pfu of rVV-OVA i.p. At the time of the rVV boost, mice were divided into four groups and received either 200 μg of anti-4-1BB mAb + 200 μg rat IgG, 200 μg of anti-OX40 mAb+ 200 μg rat IgG, 200 μg anti-4-1BB mAb+ 200 μg anti-OX40 mAb, or 400 μg of rat IgG. Five weeks later, spleen cells were examined for antigen-specific CD4 and CD8 T cell responses. (A) Unfractionated spleen cells were cultured directly ex vivo in the presence or absence of 400 μg /ml whole ovalbumin protein for 10 hours, stimulated for an additional 8 hours in the presence of brefeldin A, then stained for CD4 and intracellular IFN-γ. OVA-specific CD4 T cells are shown as both a percentage of CD4+ T cells and as the number per spleen. (B) Unfractionated spleen cells were cultured directly ex vivo for 6 hours with brefeldin A, in the presence or absence of 1 μg/ml SIINFEKL peptide, then stained for CD8 and intracellular IFN-γ. SIINFEKL-specific CD8 T cells are show as both a percentage of CD8+ T cells and total number per spleen. Data shown are the average ± SEM (n=10 mice per group). A second experiment gave similar results.

Chapter 2: Discussion

We wanted to determine if we could improve a T cell vaccine by administering antibodies that stimulate 4-1BB and OX40 together with a DNA prime, poxvirus boost vaccine. We found that anti-4-1BB mAb was able to increase the number of antigen-specific CD8 T cells induced by vaccination by about 2-4 fold. This was true even though the immunogen, vaccinia virus, is already an extremely potent natural inducer of CD8 T cells. This stimulation was able to increase CD8 T cell activation, but we found no evidence for preferential protection from cell death: the increase in memory CD8+ splenocyte numbers achieved by 4-1BB stimulation was either proportional to or slightly less than the increase seen in the spleen during the acute response. Furthermore, we found that administering anti-OX40 mAb alone enhanced CD4 T cells, and that administering anti-OX40 mAb and anti-4-1BB mAb together increased the numbers of CD8 T cells more than anti-4-1BB mAb alone.

The most significant effect of 4-1BB stimulation was seen at the peak of the response. 4-1BB stimulation increased the total number of CD8+ splenocytes, and the extent of their activation, as well as the number of antigen-specific CD8+ splenocytes. The enhancement of the acute response by 4-1BB was gratifying, given that the stimulus used to activate CD8 T cells, vaccinia virus, is a potent natural immunogen that might have been considered already optimal for CD8 T cell activation. Similar results have been reported very recently in an influenza model; stimulating 4-1BB using a different agonist mAb led to a 2-3 fold increase in the number of influenza-specific CD8 T cells in infected lungs during the acute infection (Halstead et al., 2002). These cells were highly active, and showed enhanced cytotoxicity directly ex vivo. Thus in both that study and

ours, stimulating 4-1BB in the presence of an already strong stimulus, acute virus infection, caused an increase in absolute numbers of activated virus-specific CD8 T cells at the peak of the response.

Because we were primarily interested in prophylactic vaccine development, we were particularly interested in the potential of 4-1BB to enhance the memory response. In studies using a variety of cleared pathogens, the number of CD8 T cells that become memory cells is directly proportional to the number of CD8 T cells seen at the peak of the response, ~5-10% (Doherty and Christensen, 2000). Because of the evidence that 4-1BB engagement can rescue activated cells from death (Hurtado et al., 1997) (Takahashi et al., 1999), we predicted that 4-1BB stimulation would increase the percentage of activated cells that became memory cells. We were therefore surprised when our data failed to support a role for 4-1BB in protection from cell death. However, Hurtado et al. examined T cell death in vitro, which may be controlled by different factors than T cell death in vivo. Furthermore, Takahashi et al. stimulated T cells with superantigen, but T cell activation by superantigen is different from viral antigen stimulation in that the apoptotic signal is dominant over survival (i.e. fewer T cells are present after the response resolves than were initially present), and the kinetics of cell death are also much more rapid. We repeated the experiments performed by Takahashi et al. and also found that 4-1BB stimulation increased the survival of superantigen-activated CD8 T cells (data not shown). We conclude that there is a difference in the nature of T cell activation by superantigen and virus infections, rather than lab-to-lab variation. Our data suggests that 4-1BB stimulation does not increase survival of CD8 T cells activated by vaccinia virus.

A growing number of studies have looked at the role of 4-1BB in T cell activation, particularly CD8 T cells. At the same time, the role of OX40 in CD4 T cell function is also being examined in increasing detail. Although OX40 has been previously shown to enhance CD8 T cell activation, this is the first study we are aware of to examine the ability of 4-1BB and OX-40 to cooperate in both CD8 and CD4 T cell function.

In summary, we found that 4-1BB stimulation increased the size of the CD8 T cell response induced by a vaccine both at the height of the response and in the memory phase. However, there was no preferential survival of activated CD8 T cells into the long-term memory population. We also found that OX40 stimulation was able to increase CD4 T cell memory, and that when OX40 was combined with 4-1BB, the CD8 T cell response was further improved. These experiments were designed as an initial proof-of-principle. We conclude that 4-1BBL and OX40L, encoded within recombinant DNA and viral vectors, may enhance a prophylactic anti-viral vaccine.

Chapter 3: Introduction

CD8 T cell epitope identification in C57BL/6 mice

As discussed in chapter 1.4, BALB/c mice infected acutely with MCMV respond to at least seven CD8 T cell epitopes (Table 1.1). The CD8 T cell responses to IE1/pp89 and m164 are co-dominant, and responses to m04, m18, M45, M83 and M84 are minimal to undetectable. But only one epitope was known in C57BL/6 mice, from M45 (Gold et al., 2002).

In order to identify additional MCMV antigens, we molecularly cloned, expressed and tested ~90% of the known MCMV open reading frames (ORFs) for recognition by our MCMV-specific CD8 T cell clones. Using this method, m141 ^{16}VIDAFSRL^{23} was determined to be the epitope recognized by clone 11, which has been characterized previously (Kavanagh et al., 2001), and represents the second known *H-2^b* CD8 T cell epitope from MCMV. The 'ORF library' was adapted for use with the commonly-employed ICS assay to screen CD8 T cells directly ex vivo from an infected mouse. We were able to identify 16 new CD8 T cell antigens, from which we have defined 13 novel CD8 T cell epitopes. Previous data indicated that at the height of the CD8 T cell response, ~30% of CD8 T cells are MCMV-specific. The known *H-2^b* epitopes account for over 20% of all CD8 T cells in acute infection, or ~65% of the MCMV-specific CD8 T cell response. Compared to other viral infections of inbred mice, this represents the broadest number of known CD8 T cell epitopes for a viral infection.

Chapter 3: Results

Cloning of MCMV ORF library

When we began these studies, the only known CD8 T cell epitope from MCMV was M45 ^{985}HGIRNASFI^{993}, restricted by D^b. This epitope accounted for about 6-8% of total CD8 T cells at the peak of the CD8 T cell response, 7 days post-infection. In the same study, over 10% of CD8 T cells taken from infected mice produced IFN-γ when incubated with MCMV-infected cells. It is likely that some CD8 T cell epitopes were not presented by the MCMV-infected cells, or were not presented well, which suggests that other CD8 T cell epitopes exist. Supporting this, we have recently analyzed CD8 T cells from infected mice to determine the proportion that express activation markers. The data indicate that approximately 30% of CD8 T cells are MCMV-specific in infected mice (MC Gold, manuscript in preparation). Furthermore, we had previously established a panel of MCMV-specific clonal CD8 T cell lines. While clone 3 and clone 5 are D^b-restricted and recognize antigen M45, clones 11, 5 and 96 are K^b-restricted, and recognize infected cells at different time-points (Gold, 2002b) after infection, suggesting that each recognizes a different K^b-restricted epitope. Based on this cumulative evidence, we had good reason to suspect that additional CD8 T cell epitopes remained unknown.

In order to identify the antigens (note that we will use the term 'antigen' to refer to the whole protein, and the term 'epitope' to refer to the peptide that is bound by MHC I and is recognized by the T cell receptor) recognized by other T cell clones, we molecularly cloned and expressed each MCMV gene individually in K41 cells. We then tested each protein product of these genes for its ability to stimulate the T cell clones.

Using the designation by Rawlinson et al., we designed PCR primers that would amplify each MCMV open reading frame (ORF) from the start codon to the last amino acid before the stop codon (see Appendix 1 for primers used). PCR products of the correct size were cloned into plasmid pcDNA3.1, which allows high level expression from the HCMV IE promoter, and screened by restriction analysis for inserts of the correct size. The 5' end of each plasmid was sequenced to verify the identity and orientation of the cloned product. About one-third of the constructs were sequenced only at the 5' end, another one-third were sequenced also at the 3' end, and about one-third were sequenced entirely. Many constructs contained one or more point mutations, but these were not discarded unless they caused a frameshift or stop mutation. On average, less than 1% of amino acids were mutated. Some ORFs were difficult to clone, particularly the large structural glycoproteins, but we have nevertheless managed to clone ~90% of the known ORFs.

Identification of m141 ^{16}VIDASFRL^{23} as the MCMV epitope recognized by CTL clone 11

Plasmids expressing each ORF from MCMV were transfected individually into K41 cells, an *H-2^b* transformed fibroblast cell line. Clone 11, a K^b-restricted MCMV-specific T cell clone, was added to each well for 6 hours, and the production of TNF-α was tested by a sensitive bioassay that has been previously-described (Gold et al., 2002). Clone 11 produced TNF-α when incubated in the presence of K41 cells transfected with *m141*, but not K41 cells that were untransfected, or transfected with any other MCMV

gene (data not shown). m141 encodes a 508 amino acid protein that is necessary for optimal MCMV replication in macrophages (Menard et al., 2003).

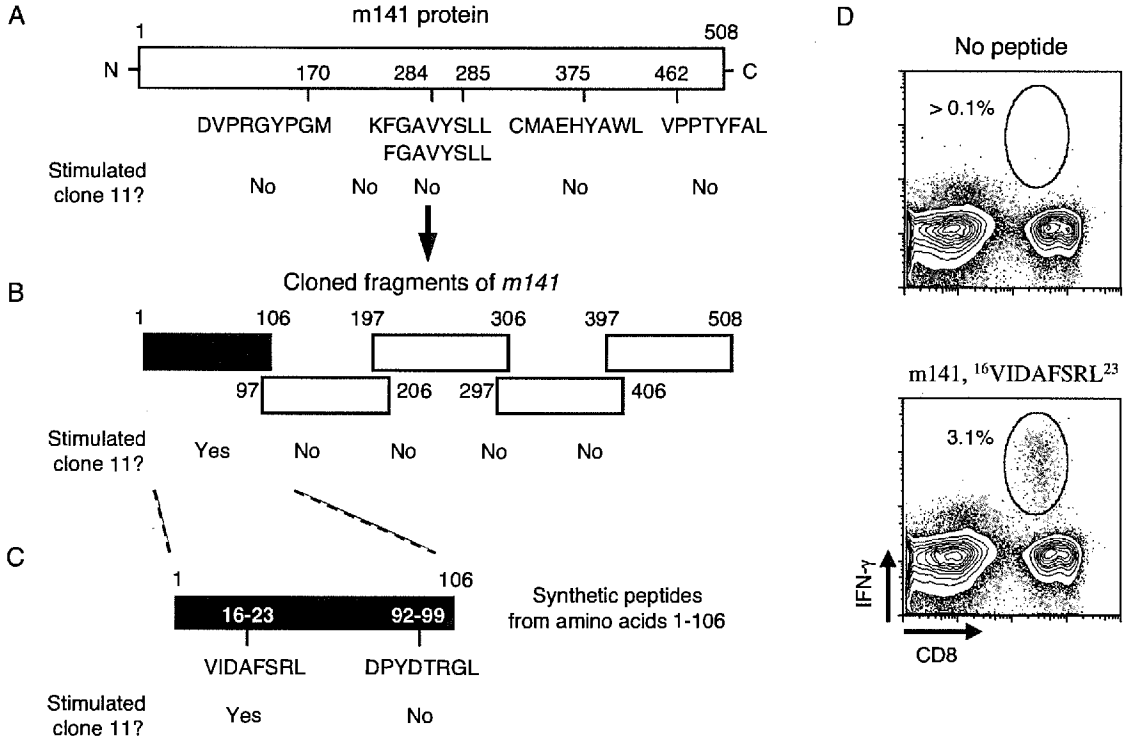

Figure 3.1 Use of the MCMV-specific CD8 T cell clone "clone 11" to identify m141, ^{16}VIDAFSRL^{23} as a CD8 T cell epitope.
Clone 11 produced TNF-α when incubated in the presence of fibroblasts transfected with *m141*. A. The MHC prediction program BIMAS was used to predict K^b-binding peptides from *m141* as candidate epitopes for clone 11. Synthetic peptides were loaded onto K41 cells and tested for the ability to stimulate clone 11 in a TNF-α bioassay. B. Five fragments of *m141* were cloned with an N-terminal Met added to each one. K41 were transfected with each fragment and tested for the ability to stimulate TNF-α production from clone 11. C. Synthetic peptides with intermediate scores, predicted by BIMAS, were loaded onto K41 cells and tested for the ability to stimulate TNF-α production from clone 11. D. C57BL/6 mice were infected intraperitoneally with 1 x 10^6 pfu MCMV. Seven days later, splenic CD8 T cells were evaluated for IFN-γ production by intracellular cytokine staining after stimulation in the presence or absence of synthetic peptide m141, ^{16}VIDAFSRL^{23}.

Because clone 11 is K^b-restricted (Kavanagh et al., 2001), we used the NIH program BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/) to predict peptides from m141 that were likely to bind to K^b, then synthesized the 5 peptides with the highest binding scores. None of these peptides was able to elicit TNF-α secretion from clone 11 (Figure 3.1 A). Five smaller fragments of *m141* were cloned and transfected into K41

cells, each with the necessary start codon, and we found that clone 11 produced TNF-α only in the presence of K41 cells transfected with an *m141* fragment containing amino acids 1-106 (Figure 3.1B). The localization of the epitope within amino acids 1-106 explains why none of the synthetic peptides were able to stimulate clone 11. We synthesized two further peptides from this region, predicted by BIMAS, and found that only one of these, m141 ^{16}VIDAFSRL^{23}, stimulated TNF-α release by clone 11 (Figure 3.1 C). When splenocytes from mice infected with w.t. MCMV for 7 days were stimulated directly ex vivo with this peptide, then analyzed by ICS, 3.1% of CD8 T cells produced IFN-γ, confirming the identity of the epitope (Figure 3.1 D). We concluded that the genomic ORF library could be used in combination with T cell clones to identify CD8 T cell antigens, and had identified the second known *H-2^b* CD8 T cell epitope.

Use of the ORF library to identify antigens directly *ex vivo*

At this stage of the project, we discovered that K41 fibroblasts transfected with *m141* could stimulate IFN-γ production, detectable by the ICS assay, from CD8 T cells taken directly ex vivo from MCMV-infected mice. Therefore, we adapted this assay for use with the ORF library

Each cloned ORF was transfected individually into K41 cells in a 96-well plate. Splenocytes from a mouse infected with w.t. MCMV for 7 days, which is the peak of the CD8 T cell response in the spleen (M Gold and M Munks, unpublished observations), were added to each well of K41 cells and incubated for 6 hours in the presence of brefeldin A. After staining for surface CD8 and intracellular IFN-γ, the cells were analyzed by flow cytometry.

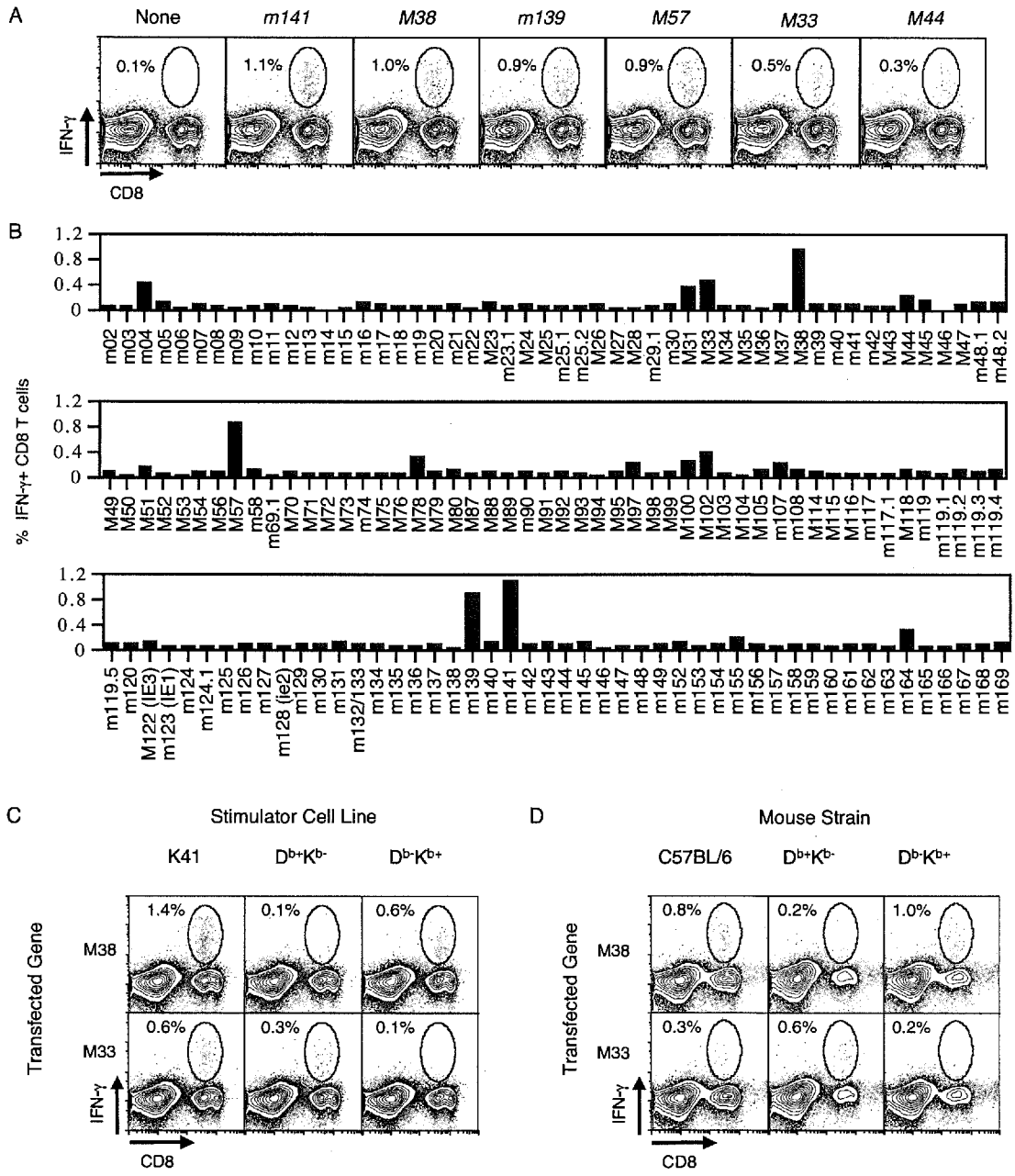

Figure 3.2 Identification and restriction analysis of novel CD8 T cell antigens from MCMV.
A. Plasmid DNA expressing each MCMV ORF was transfected into K41 cells and tested for the ability to stimulate IFN-γ production from splenic CD8 T cells taken directly ex vivo from mice infected with MCMV for 7 days. Displayed are representative intracellular cytokine staining FACS plots showing stimulation using cells that were untransfected, transfected with *m141* as a positive control, or other novel antigens. B. A comparison of all ORFs tested for CD8 T cell stimulation. C. Representative MHC I restriction analysis for each antigen, comparing stimulation with K41 cells to K^b-/- and D^b-/- fibroblast cell lines. D. MHC I restrictions were confirmed by comparing responses in C57BL/6 to K^b and D^b knockout mice, all stimulated with transfected K41 cells.

We identified many other MCMV ORFs likely to encode CD8 T cell antigens, including *M38*, *m139*, *M57*, *M33*, *M44* and others. Figure 3.2A shows some typical

positive FACS plots, and Figure 3.2B shows the results of the entire assay. However, while it was not suspected that MCMV would contain so many CD8 T cell antigens, the fact that the vast majority of genes did not lead to CD8 T cell stimulation (Figure 3.2B) suggested that the effect was specific, rather than artificial.

Proteins with a wide variety of functions are CD8 T cell antigens

A summary of known protein functions for each antigen is listed in Table 3.1. We did not notice any particular trend between protein function and antigenicity. A number of antigens are involved in DNA replication, including M45, a ribonucleotide reductase homolog important for growth in endothelial cells (Brune et al., 2001), M44, the DNA polymerase accessory protein, and M102, a helicase-primase component. Both G-protein coupled receptors, M33 and M78, are antigens, as is the protein kinase M97 and the immune evasion protein m04. MCMV encodes 12 homologs of the HCMV US22 family.

Table 3.1 HCMV homologs, functions and gene families of C57BL/6 antigens.

ORF	HCMV Homolog	Proposed function and/or family
M44	*UL44* (DPAP)	DNA binding protein
M45 [a]	*UL45* (RRL)	Ribonucleotide reductase
M57	*UL57* (MDBP)	Major DNA binding protein
M97	*UL97* (PK)	Phosphotransferase
M102	*UL102* (HP)	Helicase-primase component
M36	*UL36* (GF2)	*US22* family homolog
m139	*US22* (GF2)	*US22* family homolog
m141	*US24* (GF2)	*US22* family homolog
M33	*UL33* (GCR)	Spliced GCR
M78	*UL78*	GCR homolog
m04 [a]		*m02* family, CD8 T cell evasion
m05		*m02* family
M31	*UL31*	?
M38	*UL38*	?
M100	*UL100* (gM)	Glycoprotein M
m155		*m145* family
m164 [a]		TM gp

[a] Genes that are also antigenic in BALB/c mice

While these represent only 7% of all MCMV proteins, they represent 18% of the CD8 T cell antigens. Intriguingly, these three antigens, M36, m139 and m141, are all required for optimal growth in macrophages (Menard et al., 2003), although the significance of this is unknown.

MHC class I restriction analysis of MCMV antigens

The restricting MHC class I molecule for nearly all MCMV antigens was determined by a combination of two techniques. First, we compared CD8 T cell stimulation using transfected K41 cells ($K^{b+}D^{b+}$) to transformed fibroblast cell lines that express only K^b or only D^b (Figure 3.2C). This worked well for strong responses, such as *M38*, but we found that the cell lines expressing only K^b or only D^b were not usually as effective as transfected K41 cells in stimulating CD8 T cells, so for many of the weaker responses, such as *M33*, this made it difficult to be certain of the restricting MHC molecule (Figure 3.2C).

To confirm the restriction element for each antigen, we tested transfected K41 cells for the ability to stimulate CD8 T cells from C57BL/6 mice, $D^{b+/+}K^{b-/-}$ mice and $D^{b-/-}K^{b+/+}$ mice infected for 7 days with MCMV. We observed that while the knockout mice had a higher rate of IFN-γ production in response to untransfected cells, they usually responded more strongly to antigens than did C57BL/6 mice, helping to confirm both the validity of an antigen as well as its restricting MHC molecule (Figure 3.2D). We were able to identify the restriction element for nearly every antigen using these techniques. We also determined that the previously described clone 5 (Kavanagh, 2001) recognizes *M57*, and that clone 96 (Kavanagh, 2001) recognizes *M97*. Since we already knew that

both clones are K^b-restricted, this further confirmed our restriction analysis for these two antigens.

Genetic and bioinformatics mapping of CD8 T cell epitopes

For all ORFs that appeared to contain CD8 T cell antigens, we subcloned and expressed ORF fragments in order to map the epitope to a smaller region of the ORF, as was done with *m141* (Figure 3.1B). For most ORFs, we were able to identify a region of the gene containing the CD8 T cell epitope, for example amino acids 1-246 of M97, which is 643 amino acids in total size, and amino acids 396-606 of M102, which is 812 amino acids in total size (Figure 3.3A). We frequently observed that smaller ORF fragments were at least slightly better, or sometimes significantly better, at stimulating CD8 T cells than was the entire ORF, as was the case for *M97* and *M102*. When *M38* was tested, two non-overlapping fragments elicited a response (Figure 3.3A), indicating the presence of two different CD8 T cell epitopes in the same protein. Both of these were K^b-restricted (data not shown). Similarly, when *m164* was tested, CD8 T cells from both $D^{b+/+}K^{b-/-}$ mice and $D^{b-/-}K^{b+/+}$ mice responded to amino acids 136-286, indicating the presence of both a K^b-restricted and D^b-restricted epitope from this region (data not shown).

We synthesized peptides predicted by the previously-mentioned BIMAS program, and/or the program SYFPEITHI 1.0 (http://syfpeithi.bmi-heidelberg.com/). We typically synthesized three to five peptides from each antigen and tested these for the ability to stimulate IFN-γ production from CD8 T cells taken directly ex vivo from infected mice, using the ICS assay. In this way, we were able to identify the correct peptides for *M97*

and *M38* (Figure 3.3B) as well as *m04*, *M33*, *M36*, *M44*, *m164*, *m139*, *M78* and *M100* (data not shown). However for some antigens, such as *M102*, no peptide epitope was identified, even though the restricting element and the epitope-containing region of the protein was determined (Figure 3.3A). This demonstrated that most but not all CD8 T cell epitopes could be mapped through a combination of genetic and bioinformatic approaches.

Identification of the CD8 T cell epitope from *M57*, which encodes an 1191 amino acid DNA binding protein, was much more laborious, but we were determined to find this epitope because in the initial screen (Figure 3.2A and 3.2B) it appeared to elicit one of the strongest CD8 T cell responses, nearly 1% of CD8 T cells. We first tested 5 synthetic candidate peptides predicted from the entire protein, but these were unable to stimulate CD8 T cells from an infected mouse. Through subcloning, we determined that the epitope was within amino acids 716-966 and tested 9 more candidate peptides from this region, but again none was stimulated CD8 T cells from an infected mouse. A second round of subcloning further narrowed the epitope to within amino acids 781-855, but all predicted epitopes in this small region had already been synthesized and tested for CD8 T cell recognition. Therefore, we synthesized 15-mer peptides, overlapping by 10 amino acids, to cover the entire region. Figure 3.3C shows that two neighboring peptides were both positive, indicating that the correct peptide must be within the 10 amino acid region of overlap.

No peptides within this region were predicted to bind well to K^b, so we synthesized all possible peptide epitopes from the 10 amino acid region of overlap: three 8-mer peptides, two 9-mer peptides and one 10-mer peptide. The 10-mer peptide was

able to stimulate CD8 T cells, but only at a high concentration (Figure 3.3D). One of the 8-mers, CLEFWQRV, also elicited a response, but it was even weaker than the response to the 10-mer. One of the 9-mers, SCLEFWQRV, was able to stimulate an even higher number of CD8 T cells than the 10-mer or 8-mer, and was effective at lower concentrations. Thus we concluded that the optimal peptide was a 9-mer. Notably, the epitope for M38 was identified in a similar fashion and was also found to be a 9-mer.

K^b usually binds to peptides via a Y/F anchor at peptide position 5 and a hydrophobic residue at position 8. A crystal structure of a 9-mer peptide from Sendai virus, SEV-9 FAPGNYPAL, revealed that the extra amino acid was accommodated by a bulge between the peptide's N-terminal anchor residue and the P6 tyrosine anchor (Fremont et al., 1992). M38 SSPPMFRVP may bind to K^b in a similar fashion, with 4 residues between the N-terminal anchor and the P6 phenylalanine, albeit with an unusual C-terminal proline anchor residue. However, M57 SCLEFWQRV may bind to K^b in a distinctly different manner, with a bulge occurring between the P5 phenylalanine and the C-terminal valine anchor.

Table 3.2A and 3.2B, respectively, summarize the D^b and K^b epitopes identified and the predicted binding score of each peptide for K^b or D^b. Of the epitopes that were found, many ranked well for MHC binding by both SYFPEITHI and BIMAS (e.g. *M45* and *m139*); however, some epitopes ranked well only in one program (e.g. *m04* and *m141*), some epitopes were not predicted by either program (e.g. *M38* and *M57*), and some epitopes have not been found yet by using the MHC-binding algorithms (Table 3.2C). Overall, this demonstrates that a genetic approach (subcloning of fragments) and a bioinformatic approach (computer prediction of epitopes) worked well in combination for

identification of most epitopes (Figure 3.3A and 3.3B, Table 3.2A and 3.2B). However, for those peptides that do not fit into any known MHC binding motifs, for example M57 SCLEFWQRV and M38 SSPPMFRVP (Figures 3.3C and 3.3D, and Table 3.2C), it is necessary to use a purely genetic approach to define the minimal CD8 T cell epitope.

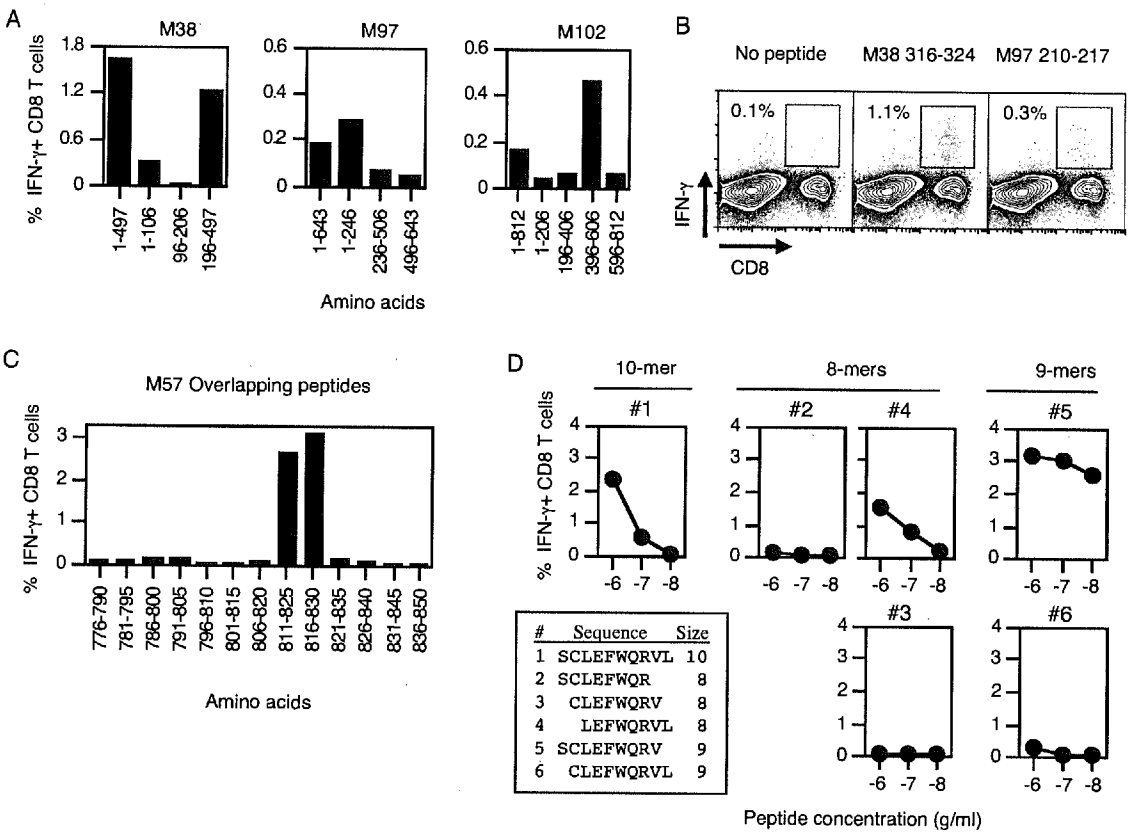

Figure 3.3 Determination of peptide epitopes from antigenic proteins.
A. K41 cells were transfected with each antigen, or fragments of the antigen, then tested by ICS for stimulation of splenic CD8 T cells from mice infected with MCMV for 7 days. *M38*, *M97* and *M102* are shown as representatives. B. Synthetic candidate peptides were tested by ICS. C. For *M57*, 15-mer peptides overlapping by 5 amino acids were used to narrowly define the CD8 T cell epitope. D. All possible peptides from *M57*, 816-825 were tested for their ability to stimulate splenic CD8 T cells from mice infected with MCMV for 7 days.

Table 3.2 Summary of MCMV CD8 T cell epitopes in C57BL/6 mice

A D^b-restricted CD8 T cell antigens

ORF	Residues	Peptide Sequence	%IFN-γ+ CD8 T cells	MHC I	T cell clone	SYFPEITHI Rank	SYFPEITHI Score	BIMAS-9mer Rank	BIMAS-9mer Score	BIMAS-Revised Rank	BIMAS-Revised Score
M45	985-993	HGIRNASFI	4-8%	D^b	3, 55	1	28	1	780	1	762
m04	1-9	MSLVCRLVL	<1%	D^b		13	16	8	9	2	72
M33	47-55	GGPMNFVVL	<1%	D^b		1	27	1	936	3	302
M36	213-221	GTVINLTSV	<1%	D^b	d2A5	2	23	4	36	11	17
M44	130-138	ACVHNQDII	<1%	D^b		T1	25	1	660	1	1003
m164	267-275	WAVNNQAIV	<1%	D^b		5	20	4	39	2	108

B K^b-restricted CD8 T cell antigens

ORF	Residues	Peptide Sequence	%IFN-γ+ CD8 T cells	MHC I	T cell clone	SYFPEITHI Rank	SYFPEITHI Score	BIMAS-8mer Rank	BIMAS-8mer Score	BIMAS 9-mer Rank	BIMAS 9-mer Score
m139	419-426	TVYGFCLL	2-4%	K^b		1	26	1	50		
m141	15-23	VIDAFSRL	2-4%	K^b	11	1	22	20	3		
M78	8-15	VDYSYPEV	1-2%	K^b		2	22	4	10		
M38	316-324	SSPPMFRVP	1-2%	K^b		NP	NP	NP	NP	58	0.3
M57	816-824	SCLEFWQRV	2-4%	K^b	5	NP	NP	NP	NP	T248	0.2
M97	210-217	IISPFPGL	<1%	K^b	96	2	22	4	13		
M100	72-79	RIIDFDNM	<1%	K^b		1	21	1	19		
m164	283-290	GTTDFLWM	<1%	K^b		6	17	3	12		

C Peptide epitopes not identified

ORF	Residues	MHC I
m05	?	D^b
M31	176-366	K^b
M38	31-50	K^b
M102	396-606	K^b
m155	176-377	?

Footnotes:

T indicates a tie

NP = not predicted (SYFPETIHI ranks only 8mer peptides for K^b and 9mer peptides for D^b)

BIMAS has two separate scoring matrices for 9mer peptides binding to D^b, "D^b" and "D^b-Revised"

Bold indicates anchor residues

% of CD8+ T cells that are specific for the peptide

The CD8 T cell immunodominance hierarchy to MCMV is much broader than anticipated

For other mouse models of CD8 T cell responses, typically only one to five immunodominant CD8 T cell epitopes have been described. Even for MCMV infection of BALB/c mice, where 7 CD8 T cell epitopes are known, only 4 of these can be detected reliably directly ex vivo from infected mice (Holtappels et al., 2002c) (M Munks, personal observation). As we performed these studies, we realized that the number of

MCMV CD8 T cell epitopes in *H-2b* mice was much larger. However, in addition to determining the number of CD8 T cell epitopes and their exact identities, we wanted to determine the frequency of CD8 T cells that respond to each epitope, and the shape of the immunodominance hierarchy. Therefore, we performed intracellular cytokine staining on CD8 T cells directly ex vivo mice infected with MCMV for 7 days, the height of the CD8 T cell response (Figure 3.4A). The immunodominant CD8 T cell epitope in all experiments was M45 ^{985}HGIRNASFI^{993}, presented by D^b. CD8 T cells also responded strongly (greater than 1% of CD8 T cells) to four K^b-restricted epitopes, m139 ^{419}TVYGFCLL^{426}, m141 ^{15}VIDAFSRL^{23}, M78 ^{8}VDYSYPEV^{15} and M38 ^{316}SSPPMFRVP^{324} (Figure 3.4A left). CD8 T cells responded relatively weakly (less than 1% of CD8 T cells, but at least double the background response of 0.1%) to a number of K^b- and D^b-restricted epitopes, including M44, M33, M100, M97, m04 and m164, the last of which contains two epitopes, one restricted by K^b and one by D^b. Epitope M57 ^{816}SCLEFWQRV^{824} was only discovered very recently and was only directly compared to the M45 and m139 epitopes, but in a single experiment appears to be second in the immunodominance hierarchy (Figure 3.4A, right side). We conclude from this data that the immunodominant epitope is the D^b-restricted *M45*, followed by a number of less dominant K^b-restricted epitopes from *M57*, *m139*, *m141*, *M78* and *M38*, followed by a larger number of subdominant epitopes restricted by both D^b and K^b.

For almost all antigens, we found that the number of CD8 T cells stimulated by the transfected ORF was proportional to the number of CD8 T cells stimulated by the peptide epitope. Specifically, ~90% of CD8 T cells stimulated by peptide were also stimulated by the transfected ORF, (i.e. stimulation with transfected cells was ~90% as

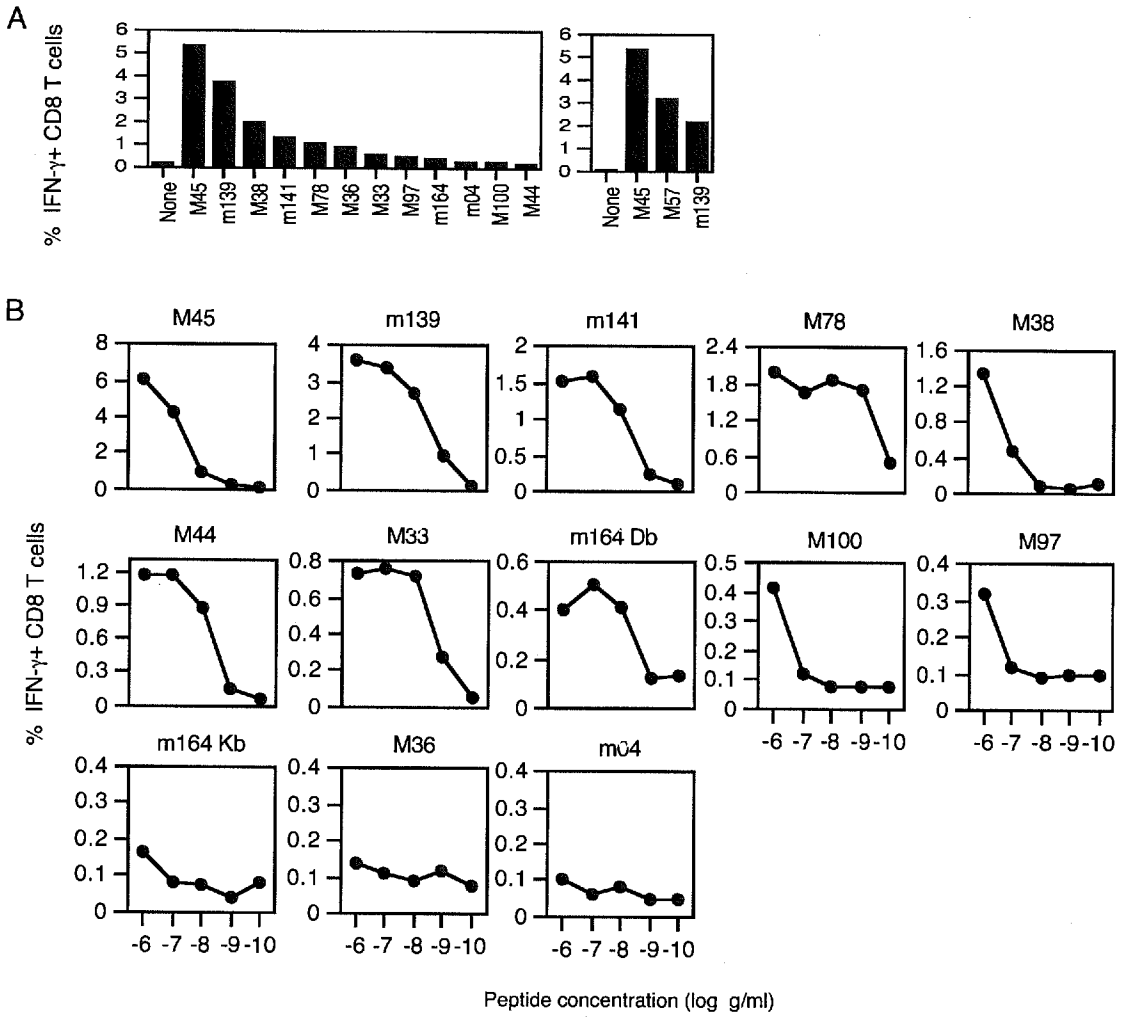

Figure 3.4 Relative immunodominance of C57BL/6 CD8 T cell epitopes.
A. Mice infected with MCMV for 7 days were tested by ICS for CD8 T cell responses to each defined CD8 T cell peptide epitope at 1 μg/ml. B. Splenic CD8 T cells from MCMV-infected mice were tested by ICS for stimulation by each peptide at varying concentrations.

effective as peptide stimulation). However, there were two notable exceptions: *M78* transfection was only about 20% efficient, and we noted throughout several assays that *M45* was never able to stimulate a response (< 1% efficient). Ironically, we initially planned to use *M45* as a positive control for our assays, but encountered difficulties while cloning *M45* and did not determine that it would not stimulate CD8 T cells directly ex vivo until we had already identified a number of other antigens. The reason it is not

presented is unknown, but presumably relates to poor processing in transfected K41 cells since all constructs were expressed by the same promoter.

CD8 T cell functional avidity does not determine immunodominance in MCMV

Many factors contribute to the relative immunodominance of different MHC I epitopes. We wondered if the functional avidity of CD8 T cells for their cognate epitope (i.e. the concentration of peptide required to elicit half-maximal IFN-γ production) would correlate with the relative immunodominance of the epitope. Therefore, we stimulated CD8 T cells from infected mice with varying concentrations of each peptide (Figure 3.4B). While we found that many of the relatively immunodominant epitopes (e.g. *m139*, *m141*, *M78*) were able to elicit IFN-γ production from CD8 T cells at lower concentrations than could less dominant peptides (e.g. *M97*, *M100*, *M36*, *m04*), the most dominant peptide (*M45*) and fourth most dominant peptide (*M38*) were less effective at concentrations below 1 ug/ml. This indicated that CD8 T cell functional avidity may contribute to immunodominance, but the effect is far from absolute. There is a potential for cysteine-containing peptides to form disulfide bonds, potentially interfering with their ability to stimulate CD8 T cells (Chen et al. 1999). Parallel dose-response curves were performed in the presence of a reducing agent (200 μM DTT) for epitopes from m139, M44 and m04, which contain cysteine residues, and indistinguishable results were obtained (data not shown).

Approximately 30% of all CD8 T cells are MCMV-specific, and known peptides account for the majority of these CD8 T cells

CD94/NKG2A is an inhibitory NK cell receptor also expressed on a majority of CD8 T cells after activation in vivo and in vitro (McMahon et al., 2002). Analysis of NKG2A expression on CD8 T cells in uninfected and MCMV-infected mice has demonstrated that 7 days after MCMV infection, 30% of CD8 T cells are specific for MCMV (MC Gold, manuscript in preparation). The sum of the responses to each individual epitope (Fig 3.4A left, excluding the M57 epitope) is ~20%, and when all peptides were used simultaneously (i.e. in a single well), to stimulate IFN-γ production from CD8 T cells, we obtained a similar frequency (data not shown). About 3% of CD8 T cells respond to the M57 epitope (Figure 3.4A right side). Therefore these epitopes cover approximately two-thirds of the MCMV-specific CD8 T cell response, confirming that we are now able to account for the majority of the MCMV-specific CD8 T cell response.

Chapter 3: Discussion

Knowledge of CD8 T cell epitopes from MCMV is useful for studying the anti-viral CD8 T cell response, antigen processing and presentation, and for vaccine development. Unfortunately, identification of these epitopes is severely hampered by the large size of the MCMV genome. We have overcome this difficulty by attacking the problem systematically, by analyzing the antigenicity of each ORF on its own, and comprehensively, by searching ~90% of the genome for antigens. By combining a library of expression plasmids expressing individual MCMV ORFs with a variation of the intracellular cytokine staining assay, we have developed a method that allows rapid

identification of many antigens using CD8 T cells taken directly ex vivo from an infected mouse.

Using this method, we report here the identities of 16 new CD8 T cell antigens from MCMV in C57BL/6 mice. From these, we have identified 13 new CD8 T cell epitopes. The previously-described M45 epitope (Gold et al., 2002) is immunodominant, and the remainder of the epitopes represent a continuous distribution in terms of CD8 T cell recognition. We have estimated that at the peak of the CD8 T cell immune response, these epitopes account for greater than 20% of CD8 T cells, or roughly 65% of MCMV-specific CD8 T cells.

If our defined epitopes account for ~65% of MCMV-specific CD8 T cells, then one unresolved question is "What are the remaining CD8 T cell epitopes?" Many possibilities exist. We have not identified the epitopes from antigens *m05*, *M31*, *M100* and *m155*, but our data suggest that fewer than 1% of CD8 T cells respond to each of these epitopes. It was cost-prohibitive to synthesize overlapping peptides for all antigens, so it is possible that for some antigens identified by our screen, we found only one epitope while other epitopes from the same antigen remain undefined. However, expression of subcloned gene fragments very rarely indicated the presence of more than epitope (with the exception of M38), thus we consider this possibility unlikely. We think it is most likely either that: 1) some of the genes that have not been cloned and tested in our screening assay contain relatively dominant antigens; 2) some ORFs that encode CD8 T cell epitopes were not designated as ORFs by Rawlinson et al.; 3) some epitopes are in alternate reading frames; 4) some dominant antigens will score negative or weakly positive in our screening assay, as is the case for *M45* and *M78*; and/or 5) there are a very

large number of subdominant epitopes that individually are near or below the level of detection in our assay, but cumulatively account for a major proportion of the CD8 T cell response. We consider the last possibility very likely, which leads us to hypothesize that the CD8 T cell response may be much broader even than is documented here. It must also be considered that our estimation of the total MCMV-specific CD8 T cell response may be significantly off. We have used activation markers to estimate the total number of CD8 T cells responding to MCMV antigens, but it is possible for example that we have inadvertently measured activated γδ CD8 T cells and/or NKT cells, which would not respond to any peptide epitopes.

Determinants of Immunodominance

Many factors that affect CD8 T cell immunodominance are known (Yewdell and Bennink, 1999). One might assume *a priori* that in MCMV, the timing of gene expression would affect the CD8 T cell immunodominance repertoire for at least two reasons. First, proteins expressed early in the infection cycle are more likely to be expressed before a cell dies, either from viral infection or from T cell / NK cell cytolysis. Second, the immune evasion genes *m04*, *m06* and *m152*, which severely impede MHC I-restricted antigen presentation, are all E genes. It has been proposed that proteins expressed at IE times will not be subject to the effects of these immune evasion genes, which has long been considered the major reason that IE1 is a dominant CD8 T cell antigen in BALB/c mice (Holtappels et al., 2000b).

However, none of the antigens described here are known to be expressed with IE kinetics. In addition to the reasoning explained above, this was surprising for three

reasons: (1) preliminary experiments were performed in our lab with polyclonal CTL from MCMV-infected mice in order to determine whether IE, E or L genes accounted for the strongest CTL antigens (M.G. unpublished observations). Using fibroblasts infected in vitro, we observed weak but consistent killing of targets restricted to IE gene expression; (2) BALB/c mice recognize IE1 as a dominant CD8 T cell antigen; and (3) IE1 is also strongly immunogenic in HCMV infection. It is possible that either a subdominant antigen expressed with IE kinetics has not been identified, an antigen described here is actually expressed with IE kinetics, or both. We are currently investigating these possibilities.

In the polyclonal CTL assays just described, strong CTL activity was observed in response to fibroblasts restricted by drug blockade to E gene expression, but recognition was not improved under L conditions, indicating that most CD8 T cell antigens are E genes and few are L genes. Previous data by other groups supports this finding (Campbell et al., 1992) (Del Val et al., 1989). Our data here confirms this; the six most immunodominant antigens, M45, M57, m139, m141, M38 and M78 , have all been characterized as E proteins, as have the subdominant antigens M97 and m04. In addition, *M44* and *M102* may be E genes, since their HCMV homologs are transcribed with E kinetics. *UL31* is a L gene in HCMV infection, which suggests that its MCMV homolog *M31* may also be a L gene, but so far only *M100*, which encodes gM, is definitively characterized as a L gene. Therefore, it appears that the overwhelming majority of antigens are E proteins. However, at least one antigen is clearly L, and other unidentified L antigens may exist, since many L genes were unable to be easily cloned due to their size and were therefore not tested in our assay (see Figure 2B for genes tested).

The first step in the antigen processing pathway is proteasomal degradation of proteins. Thus it has been proposed that immunodominant antigens come primarily from cytosolic proteins, which presumably have the most access to proteasomes (Yewdell and Bennink, 1999). We have noted that some antigens identified here are cytosolic proteins, but many are not. For example, M45 is the most immunodominant antigen but is nuclear, as are the subdominant antigens M44 and M102. Notably, influenza nucleoprotein is antigenic across a large number of HLA types in humans, indicating that it is not unusual for nuclear proteins to be antigens. In addition, m04, m05, M33, M78, M100 and m164 are all transmembrane proteins. Together, these data do not support the idea that cytosolic proteins are more immunogenic than other types of proteins.

Three genes that contain CD8 T cell epitopes recognized by C57BL/6 mice also are recognized by BALB/c mice, namely *m04* (Holtappels et al., 2000b), a CD8 T cell immune evasion gene, *m164* (Holtappels et al., 2002c), a gene of unknown function, and *M45* (Reddehase, 2002) a homolog of the cellular ribonucleotide reductase. 10% of the 170 MCMV ORFs are known antigens in C57BL/6 mice. Three of the seven known CD8 T cell antigens in BALB/c mice, 43%, are also antigens in C57BL/6 mice, indicating that some underlying factors probably predispose these genes to be antigenic in more than one mouse strain. For *m04*, antigenicity in both mouse strains may reflect the very high expression level of m04 in infected cells (Kavanagh et al., 2001b), a factor known to affect immunodominance. We also noted that more epitopes are restricted by K^b than by D^b, in particular five of the six most dominant epitopes, however we have no explanation for why there should be a preference for priming of K^b-restricted CD8 T cells.

Patterns of Immunodominance

It has long been postulated that the size of a virus' genome would be a strong determinant of the number of immunodominant epitopes. This hypothesis is intrinsically attractive because there are many hurdles any individual peptide must cross in order to elicit a CD8 T cell response, not the least of which is MHC binding, and a virus with more genes and more potential peptides should end up with more peptides that are able to exceed these hurdles. Our data with C57BL/6 mice, combined with the data from BALB/c mice, seems to support this model, since MCMV is a relatively large virus and also encodes many CD8 T cell epitopes.

On the opposite extreme, LCMV has a small genome and encodes only a single immunodominant CD8 T cell epitopes in BALB/c mice and four epitopes in C57BL/6 mice, even though over 50% of CD8 T cells respond to LCMV at the height of infection. However, the majority of the CD8 T cell response to HSV-1 in C57BL/6 mice is directed against a single gB epitope (Wallace et al., 1999), while there are five known CD8 T cell peptide epitopes from the comparatively small genome of influenza virus. Therefore, any correlation between virus genome size and the number of immunodominant CD8 T cell determinants is still largely speculative and will have to await more detailed characterization of CD8 T cell responses to additional viruses, both large and small.

Epitope-specific CD8 T cell responses have been examined in considerable detail for HCMV. Immunodominance hierarchies within individuals are difficult to establish because most epitopes are defined for a small number of common HLA alleles, but epitopes for other alleles are completely unknown. However, it is clear that CD8 T cells specific for pp65 (McLaughlin-Taylor et al., 1994) and IE1 (Alp et al., 1991) are

dominant in some individuals. The most thorough analysis was published recently (Elkington et al., 2003). Using a bioinformatics approach to search for HLA-binding motifs in14 HCMV proteins, 63 CD8 T cell epitopes were identified, nearly all of which were previously unknown. In 8 healthy HCMV-seropositive subjects, 27 different HLA-A2 epitopes derived from 9 proteins elicited IFN-γ production. This is in accordance with our finding here that at least 17 antigens are restricted by just two mouse MHC class I alleles. Therefore the breadth of the CD8 T cell immune response in humans and mice may be more similar than was previously anticipated.

The CD8 T cell response to MCMV in BALB/c mice has also been characterized in considerable detail. IE1/pp89 and m164 represent co-dominant epitopes, and m18, M45, m04, M83 and M84 contain subdominant epitopes. In addition, published data suggests that there are a number of remaining subdominant CD8 T cell peptide epitopes whose identities are not yet known (Holtappels et al., 2002c). Our data, that C57BL/6 mice respond to a large number of CD8 T cell epitopes, supports the idea that additional subdominant BALB/c CD8 T cell epitopes remain undiscovered.

Size of the CD8 T Cell Response

In addition to defining CD8 T cell epitopes and characterizing the shape of their immunodominance hierarchy, we have also estimated the proportion of the total MCMV-specific CD8 T cell response that is covered by the known epitopes. We have data suggesting that at the peak of the primary CD8 T cell response, ~30% of all CD8 T cells in the spleen are MCMV-specific (MC Gold, manuscript in preparation). This large CD8 T cell response is confirmed here since the known C57BL/6 epitopes account for over

20% of all CD8 T cells after acute infection. In BALB/c mice, where CD3-redirected lysis assays have been used to estimate the total MCMV-specific CD8 T cell response, only 5% of CD8 T cells are MCMV-specific in the acute response and this percentage declines to 2% during memory (Holtappels et al., 2002c). It is possible that some important BALB/c epitopes are yet to be discovered, but we have screened the ORF library with CD8 T cells from BALB/c mice infected for 7 days and failed to find any antigens that elicit strong CD8 T cell responses other than the already-known immunodominant IE1 and m164 antigens. In addition, data presented in Chapter 4 suggests that the MCMV-specific CD8 T cell response is in fact much larger in C57BL/6 mice than BALB/c mice (MM, unpublished observations).

Overview

Success of this CD8 T cell epitope identification method depends primarily on a few significant variables. First, the viral genome must be sequenced and ORFs accurately designated. Second, a transfectable cell line that is able to process and present MHC I antigens must be available. Third, the frequency of virus-specific CD8 T cells must be relatively high compared to the sensitivity of the assay used, although this potential limitation could be overcome by purifying CD8 T cells from an organ with a higher frequency of virus-specific CD8 T cells or else by using clonal T cell lines.

However, this method of CD8 T cell epitope identification has several very important advantages. First, it is not very technically demanding, requiring a solid understanding of molecular biology and cellular immunology techniques, but no other specialized techniques or equipment other than a flow cytometer. Second,

immunodominant antigens are generally identified most clearly in the screening process and can become the focus of epitope identification (for almost all antigens, we found that the number of CD8 T cells stimulated by the whole antigen was proportional to the number of CD8 T cells stimulated by the peptide epitope, with the notable exceptions of *M45* and *M78*). Third, T cell clones are not required. This eliminates both in vitro selection artifacts and the labor- and resource-intensive tasks of isolating, screening, and maintaining T cell clones. Finally, the fact that we can account for most of the MCMV-specific CD8 T cell response implies that this method can be nearly comprehensive.

The epitope-mapping method described here allowed us to systematically and comprehensively screen the MCMV genome for CD8 T cell epitopes. We found that the acute MCMV-specific CD8 T cell response is much broader than anticipated, containing at least 17 antigens. We estimate that these antigens account for at least 65% of the MCMV-specific CD8 T cell response. These antigens will facilitate future studies of anti-viral CD8 T cell responses, antigen processing and presentation, and vaccines. In addition, this method should be generally applicable for other viruses, particularly those with large genomes such as herpesviruses and poxviruses.

Chapter 4: Introduction

Roles of *m04*, *m06* and *m152* in CD8 T cell priming in vivo

It is controversial whether or not the MHC class I immune evasion genes function in professional macrophages. One study suggested that the IE1/pp89 epitope is presented in macrophages in spite of the immune evasion genes (Hengel et al., 2000). However another study done only with MCMV-infected primary bone marrow-derived macrophages found that the immune evasion genes retained MHC I in the ERGIC and prevented antigen presentation to MCMV-specific CTL clones (LoPiccolo et al., 2003). In any case, at minimum it can be concluded that macrophages are not impervious to the effects of *m04*, *m06* and *m152* as a general rule.

We and others presumed that CD8 T cell priming would be affected by *m04*, *m06* and *m152*, based either on the timing of gene expression (e.g. IE expression), or due to MHC I allele-specific effects of the immune evasion genes. In particular, m4/gp34 associates preferentially with K^b in the ER, possibly interfering with peptide loading (Kavanagh et al., 2001b), and also inhibits K^b-restricted CD8 T cell killing (Kavanagh et al., 2001a).

Data obtained in our lab (MC Gold, manuscript in preparation), using cell surface activation markers as surrogate markers of MCMV -specific CD8 T cells, suggested that the size of the CD8 T cell response was similar after infection with w.t. MCMV or various MCMV mutants missing immune evasion genes. But by design this study could not address differences in antigen specificity. In addition, priming of D^b-restricted M45-specific CD8 T cells was unaffected by the immune evasion gene *m152* (Gold et al.,

2002). However, no K^b-restricted antigens were known at this time, making it difficult to test the hypothesis that *m04* would specifically affect K^b-restricted CD8 T cell priming.

The identification of numerous other K^b- and D^b-restricted epitopes (see Chapter 3) allowed us to address this question more rigorously. By comparing antigen-specific CD8 T cell responses from mice infected with w.t. MCMV, or Δm4+m6+m152 MCMV, it is demonstrated here that the immune evasion genes *m04*, *m06* and *m152* do not greatly affect the size or specificity of the MCMV-specific CD8 T cell response in vivo.

Increased frequencies of IE3-, M38- and m139-specific CD8 T cells over time

Following MCMV infection in BALB/c mice, CD8 T cells specific for IE1/pp89 and m164, but not other subdominant epitopes, continue to proliferate long after latency is established (Holtappels et al., 2000a) (Holtappels et al., 2002c) (Karrer et al., 2003). These CD8 T cells have a CD62L lo phenotype, indicative of recent activation (Holtappels et al., 2000a) (Karrer et al., 2003). A very low proportion of these incorporated BrdU label over a 10-day period (Karrer et al., 2003), indicating that viral antigens produced during latency lead to proliferation of MCMV-specific CD8 T cells relatively infrequently.

It was believed that IE1 might partially escape the effects of the immune evasion genes due to its timing of expression, and the E antigen m164 was subsequently shown to be presented on infected cells despite the effects of the immune evasion genes (Holtappels et al., 2002a). These observations led to two hypotheses that are not mutually exclusive. First, that CD8 T cell expansion during the latent phase might occur only for

immunodominant antigens, and/or second, that CD8 T cell expansion might distinguish antigens that wholly or partially escape the effects of the immune evasion genes.

In C57BL/6 mice, 6-8% of CD8 T cells typically respond to M45 in the acute response (Gold et al., 2002), but declines rapidly to below 1% thereafter and we have seen no evidence that it increases again in latently-infected mice. The NK cell response in C57BL/6 mice is much more effective at controlling MCMV replication than NK cells from BALB/c mice (Chapter 1.9), but NK cell depletion in this strain does not result in the accumulation of M45-specific CD8 T cells (S Sierro, personal communication).

Using the newly-identified MCMV epitopes, we have tested three hypotheses: 1) that CD8 T cells, specific for some antigens but not others, would increase during latent infection; 2) that CD8 T cell expansion would occur only in response to immunodominant antigens; and/or 3) that expansion would be strongly influenced by the immune evasion genes *m04*, *m06* and *m152*. Data presented here demonstrate that CD8 T cells specific for some antigens (IE3, M38 and m139) do expand over time, but this appears to be unrelated to immunodominance or the effects of *m04*, *m06* and *m152*.

Effect of host background on the size and specificity of CD8 T cell responses

In BALB/c mice, the frequency of CD8 T cells that respond to the co-dominant IE1/pp89 and m164 epitopes is typically only about 1% (Holtappels et al., 2002c) (see Table 1.1), although other groups have recently reported CD8 T cell responses as large as 5.5% to IE1/pp89 (Ye et al., 2002a) (Karrer et al., 2003). Yet these epitopes are believed to be dominant. We knew that the M45 response is comparatively large, typically 6-8% after acute infection (Gold et al., 2002) (and Chapter 3). In addition, five other C57BL/6

epitopes elicit responses ranging from ~1% to ~4% of CD8 T cells (Chapter 3). We assumed that the size of the total MCMV-specific CD8 T cell response would be similar between the two strains, or possibly larger in BALB/c mice since viral titers reach much higher levels, but this did not seem to fit the data. However, our labs typically use different routes of infection, doses of virus and methods of analysis. In addition, we have found that the response to M45 varies significantly depending on dose of MCMV used (M Munks, unpublished observations). Therefore we decided to compare the response to dominant $H\text{-}2^b$ and $H\text{-}2^d$ epitopes directly in the same mice.

While many of the factors that affect CD8 T cell immunodominance have been discussed throughout this thesis and elsewhere (Yewdell and Bennink, 1999), the genetic background of the host has been largely ignored. We were curious if the genetic background of the mouse, holding the *H-2* haplotype constant, would have a major influence on the size or specificity of the MCMV-specific CD8 T cell response.

Data collected using the dominant $H\text{-}2^d$ epitopes and a limited number of $H\text{-}2^b$ epitopes indicates that the genetic background of the mouse has a significant effect on both the CD8 T cell immunodominance hierarchy and the total size of the CD8 T cell response, with BALB mice having a smaller response than B6 mice. We hypothesized that perhaps an effective NK cell response, although reducing early antigen load, may actually enhance the size of the CD8 T cell response, and a preliminary experiment supports this hypothesis.

Chapter 4: Results

The immune evasion genes *m04*, *m06* and *m152* do not affect CD8 T cell priming in vivo.

At an early point in the antigen identification process, 2 K^b-restricted epitopes from m139 and m141, and 4 D^b-restricted epitopes from M45, M33, M44 and M97, had been mapped precisely. This allowed us to more directly test the hypothesis that one or all of the immune evasion genes affects CD8 T cell priming in vivo. In particular, we suspected that because *m04* preferential affects K^b-restricted CD8 T cells (Kavanagh et al., 2001b), a Δm04 virus would lead to enhanced K^b-restricted CD8 T cell priming.

In order to investigate this, mice were infected with two different w.t. strains of MCMV, BAC-derived w.t. MCMV and the Smith strain of w.t. MCMV, and a BAC-derived MCMV mutant that contains deletions of *m04*, *m06* and *m152*, named Δm04+m06+m152 MCMV. Seven days later, we measured the CD8 T cell response to each antigen directly ex vivo with the ICS assay (Figure 4.1 A). After infection with the BAC-derived w.t. MCMV (left) or the Smith strain of MCMV (center), M45 elicited the strongest response, approximately 6-8% of CD8 T cells. Responses to m139 and m141 were less dominant but were still 2-4% of CD8 T cells, while only ~1% of CD8 T cells responded to M33, M44 and M97. While there were some differences in the magnitude of the response to each epitope, remarkably the hierarchy of CD8 T cell responses was identical in mice infected with Δm04+m06+m152 MCMV. This indicated that *m04* did not affect the CD8 T cell immunodominance hierarchy to these six antigens in vivo, as we had expected, nor did *m06* or *m152*. The experiment was later repeated when a total

of 11 K^b- and D^b-restricted CD8 T cell epitopes were known. Figure 4.1B shows that we again found that the hierarchy of CD8 T cell responses was essentially identical, regardless of whether the mice were infected with w.t. MCMV or Δm04+m06+m152 MCMV. There was a significantly stronger response to M44 in the group infected with Δm04+m06+m152 MCMV, but this was not observed in the experiment shown in Figure 4.1A or a third experiment (data not shown), suggesting that the effect was due to mouse-to-mouse variation.

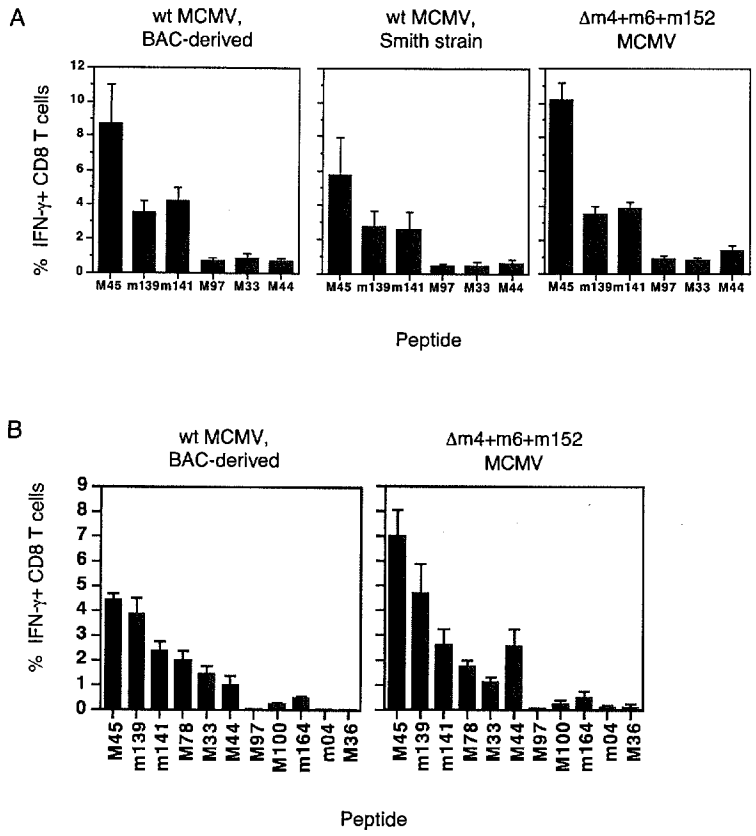

Figure 4.1 The immune evasion genes *m04*, *m06* and *m152* do not affect CD8 T cell priming in vivo. A. C57BL/6 mice were infected with 1 x 10^6 pfu of three different strains of MCMV, BAC-derived w.t. MCMV, Smith-derived w.t. MCMV, or BAC-derived *m04*$^-$*m06*$^-$*m152*$^-$ MCMV. Seven days later, splenocytes were stimulated with peptide and analyzed by ICS. B. A very similar experiment to A, except that more epitopes were analyzed.

We concluded from this set of experiments that while these immune evasion genes have a profound effect on antigen presentation, they do not have a major effect on CD8 T cell priming in vivo. It is unlikely that *m04*, *m06* and *m152* affect MHC class I antigen presentation only in vitro, since Δm152 MCMV replicates to lower titers in vivo

in a CD8-dependent manner (Krmpotic et al., 1999), and Δm152 MCMV, but not w.t. MCMV, is efficiently cleared from infected mice by MCMV-specific CD8 T cells (Holtappels et al., 2004). It was hypothesized previously by Gold et al. (2002) that in vivo, MCMV-infected DCs may play a minor role or no role at all in priming MCMV-specific CD8 T cell responses, suggesting that cross-presentation is the dominant form of CD8 T cell priming in vivo. The data presented here supports this hypothesis, and indicates that the function of these genes in vivo is probably unrelated to CD8 T cell priming. Other possible roles for *m04*, *m06* and *m152* in vivo are presented in the chapter 4 discussion.

The CD8 T cell response to m139 increases over time.

In BALB/c mice, CD8 T cells specific for IE1/pp89 and m164 proliferate at times when MCMV latency is established, while CD8 T cells specific for other epitopes do not (Holtappels et al., 2000a) (Holtappels et al., 2002c) (Karrer et al., 2003). The CD62L lo phenotype of these cells is indicative of recent antigen exposure. We have not observed a similar phenomenon with M45-specific CD8 T cells in C57BL/6 mice, but we wondered if other epitope specificities might display a similar behavior.

At a time when only 6 epitopes were known, we decided to test this hypothesis. Mice were infected with w.t. MCMV for 7 days or 1 year, and the response to each CD8 T cell antigen was determined by ICS (Figure 4.2A). While the CD8 T cell response to M45, m141, M97, M33 and M44 had declined to low or undetectable levels one year post-infection, the frequency of m139-specific CD8 T cells had actually increased slightly. Thus m139 appeared to exhibit a similar phenomenon in C57BL/6 mice as

IE1/pp89 and m164 in BALB/c mice. When more CD8 T cell epitopes were known, the

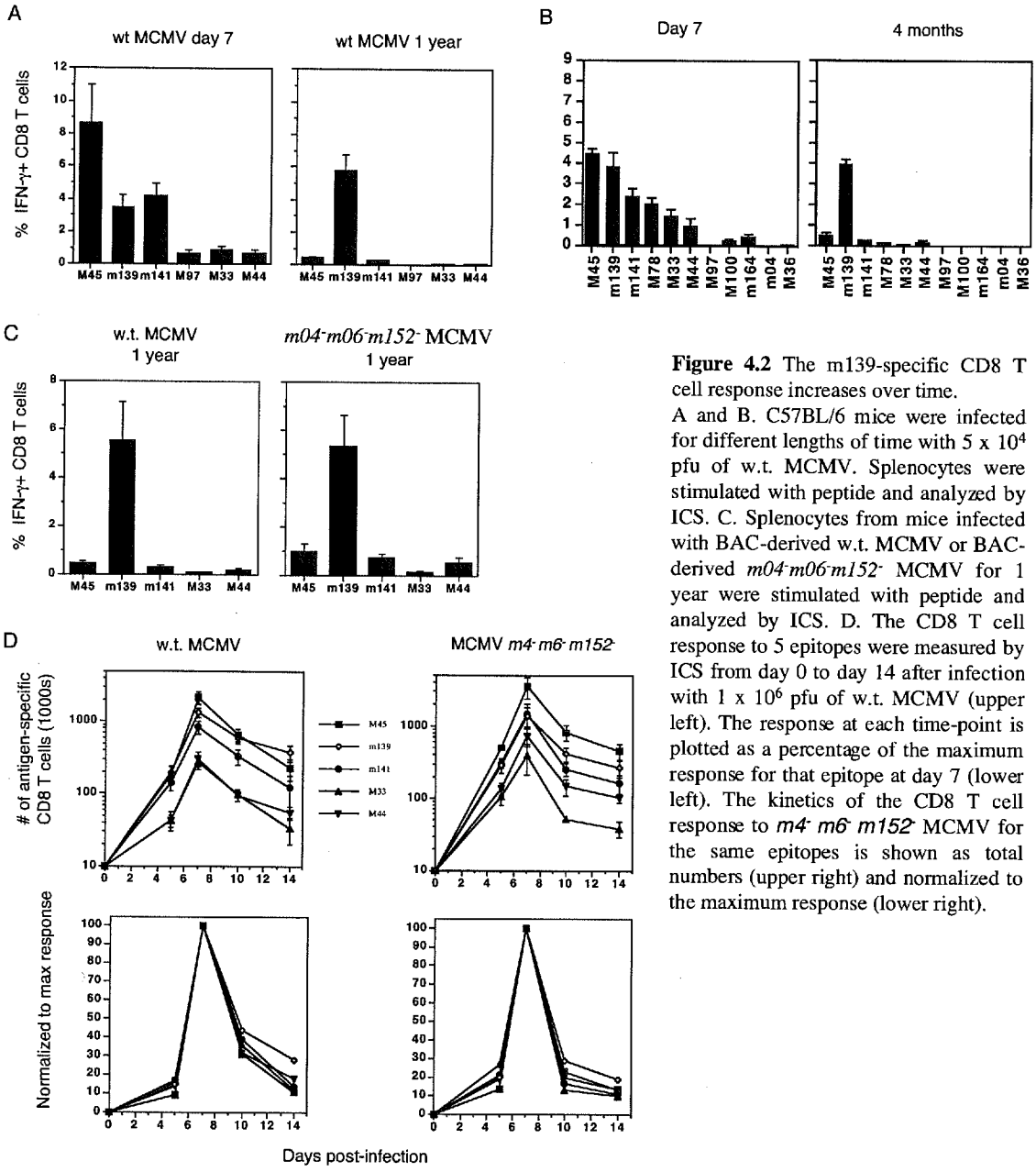

Figure 4.2 The m139-specific CD8 T cell response increases over time.
A and B. C57BL/6 mice were infected for different lengths of time with 5 x 10^4 pfu of w.t. MCMV. Splenocytes were stimulated with peptide and analyzed by ICS. C. Splenocytes from mice infected with BAC-derived w.t. MCMV or BAC-derived *m04-m06-m152-* MCMV for 1 year were stimulated with peptide and analyzed by ICS. D. The CD8 T cell response to 5 epitopes were measured by ICS from day 0 to day 14 after infection with 1 x 10^6 pfu of w.t. MCMV (upper left). The response at each time-point is plotted as a percentage of the maximum response for that epitope at day 7 (lower left). The kinetics of the CD8 T cell response to *m4- m6- m152-* MCMV for the same epitopes is shown as total numbers (upper right) and normalized to the maximum response (lower right).

experiment was repeated. This time though, mice were infected with w.t. MCMV for either 7 days or only 4 months (Figure 4.2B). The frequency of CD8 T cells responding to all epitopes except m139 declined to less than 1%, while m139-specific responses were approximately equal in both groups.

We made three conclusions from these experiments. First, the expansion of m139-specific CD8 T cells does not also occur in response to all epitopes, since 10 other epitopes displayed a different pattern. Second, although expansion during the chronic phase of infection in BALB/c mice occurs only for the co-dominant IE1/pp89 and m164 epitopes, in C57BL/6 mice CD8 T cells specific for the non-immunodominant m139 epitope also expand over time. Third, this experiment demonstrated that this phenomenon begins to occur less than 4 months after infection in C57BL/6 mice.

m04, *m06* and *m152* do not affect the chronic CD8 T cell response to MCMV

We wondered if the m139 epitope, similar to the m164 epitope in BALB/c mice, might be presented on the surface of infected cells despite the effects of *m04*, *m06* and *m152* (Holtappels et al., 2002a), while presentation of all other antigens was blocked by these same genes. In other words, could the m139 epitope escape the immune evasion genes that affected most other epitopes?

No CD8 T cell lines specific for m139 existed at this time, so we could not test this hypothesis directly, in vitro. However if this model was correct, infection of mice with Δm04+m06+m152 MCMV should allow CD8 T cells of other specificities to also increase in frequency over time. Figure 4.2 C shows that infection of mice with Δm04+m06+m152 MCMV for 1 year did not cause CD8 T cells specific for M45, m141, M33, M44 or M97 to increase in frequency over time.

This indicates that in addition to having little or no effect on CD8 T cell priming (Figure 4.1A), these immune evasion genes do not greatly affect the CD8 T cell response to m139 or other antigens during MCMV latency either. Therefore, expansion of m139-

specific CD8 T cells during chronic infection is not due to presentation in the presence of *m04*, *m06* and *m152*.

CD8 T cells specific for m139 do not fail to undergo apoptosis

These data strongly suggest, but do not prove, that m139-specific CD8 T cells expand throughout chronic infection. While unprecedented, it was possible that after expanding until day 7 post-infection, m139-specific CD8 T cells simply failed to undergo apoptosis. To test this possibility, we compared the early kinetics of the m139-specific CD8 T cell response to 4 other antigens. Mice were infected for five to 14 days with w.t. MCMV, and the response to 5 epitopes was measured by ICS. For all epitopes, the CD8 T cell response peaked at day 7 and declined substantially by day 14 (Figure 4.2D, upper left). When the response to each epitope was normalized to the peak response to that epitope at day 7 (Figure 4.2D, lower left), it is clear that all epitope-specific responses followed nearly identical kinetics for the first 14 days post-infection. For each epitope, 70% to 80% of the CD8 T cells had died by day 14. Notably, Figure 4.2D (lower left) shows that m139-specific CD8 T cells contracted slightly less than CD8 T cells of other specificities, but we do not yet know if this is reproducible or biologically significant. We concluded that the m139-specific CD8 T cells undergo similar expansion and death within the first 14 days following infection, but that thereafter m139-specific CD8 T cells expand again while CD8 T cells of most other specificities do not.

To further examine any potential role of *m04*, *m06* and *m152* in CD8 T cell activation or apoptosis, the kinetics of 5 antigen-specific CD8 T cell populations were also determined after infection of mice with Δm04+m06+m152 MCMV (Figure 4.2D,

right side). The response to each antigen peaked at day 7, and the majority of each CD8 T cell population underwent apoptosis by day 14. This further confirmed that *m04*, *m06* and *m152* have little or no effect on CD8 T cell activation or apoptosis in vivo.

CD8 T cells specific for M38 ^{316}SSPPMFRVP^{324} behave similarly to CD8 T cells specific for m139 ^{419}TVYGFCLL ^{426}

While searching for an epitope within M38, we noticed two interesting things: 1) this antigen encodes two separate K^b-restricted epitopes; and 2) the CD8 T cell response to one epitope increases over time, but the response to another does not. K41 cells were transfected either with the entire *M38* ORF, or with fragments of *M38*, then used to stimulate CD8 T cells from mice infected for 7 days with w.t. MCMV. Figure 4.3B (left side) shows that CD8 T cells from acutely-infected mice produced IFN-γ when stimulated with the entire *M38* ORF, and that two non-overlapping fragments of *M38* were also recognized, with a larger response to amino acids 196-497 than to 1-106. When the same analysis was performed with splenocytes from mice chronically infected with w.t. MCMV, the response to fragment 1-106 had declined dramatically while the response to 196-497 had increased in size (Figure 4.3B, right side). This suggests that CD8 T cells responding to one M38 epitope may behave similarly to m139-specific CD8 T cells. The fact that a second epitope within the same ORF follows a different pattern has important implications for the possible mechanism leading to this phenomenon.

We synthesized synthetic peptides from M38 based on the epitope prediction programs BIMAS and SYFPEITHI, but none stimulated IFN-γ production by CD8 T cells from MCMV-infected mice. Subcloning of *M38* 196-497 into smaller fragments led

to the discovery of M38 ^{316}SSPPMFRVP^{324} as a CD8 T cell epitope (see Chapter 3, Table 1B). Overlapping peptides of amino acids 1-100 allowed us to narrow down the weaker M38 epitope to within amino acids 31-50 (data not shown), but we have so far been unsuccessful in identifying the optimal peptide (see Chapter 3, Table 3.1C).

When M38 ^{316}SSPPMFRVP^{324} was used to stimulate CD8 T cells from mice that were infected either acutely or chronically with w.t. MCMV, we found that these CD8 T cells displayed a similar pattern as m139-specific CD8 T cells (Figure 4.3B). The response to M38 ^{316}SSPPMFRVP^{324} increased from approximately 2% of CD8 T cells in acute infection, to 9% of CD8 T cells during chronic infection, strongly suggesting that these CD8 T cells follow a similar pattern of expansion, contraction and re-expansion as do m139-specific CD8 T cells.

At this point in time, the peptide epitopes from antigens m05, M31, M57, M102 and m155 had not been determined. We wondered if the responses to any of these antigens also increased over time. To test this, K41 cells were transfected with these antigens and used to stimulate CD8 T cells from mice chronically infected with MCMV, or CD8 T cells from acutely-infected mice as a control. Only weak responses were elicited from each antigen (data not shown), suggesting that the CD8 T cell response against these antigens does not increase over time. We concluded from this that CD8 T cells specific for M38 ^{316}SSPPMFRVP^{324} or m139 ^{419}TVYGFCLL ^{426} are able to proliferate after the initial expansion and contraction phases have occurred, while CD8 T cells specific for all other known antigens do not.

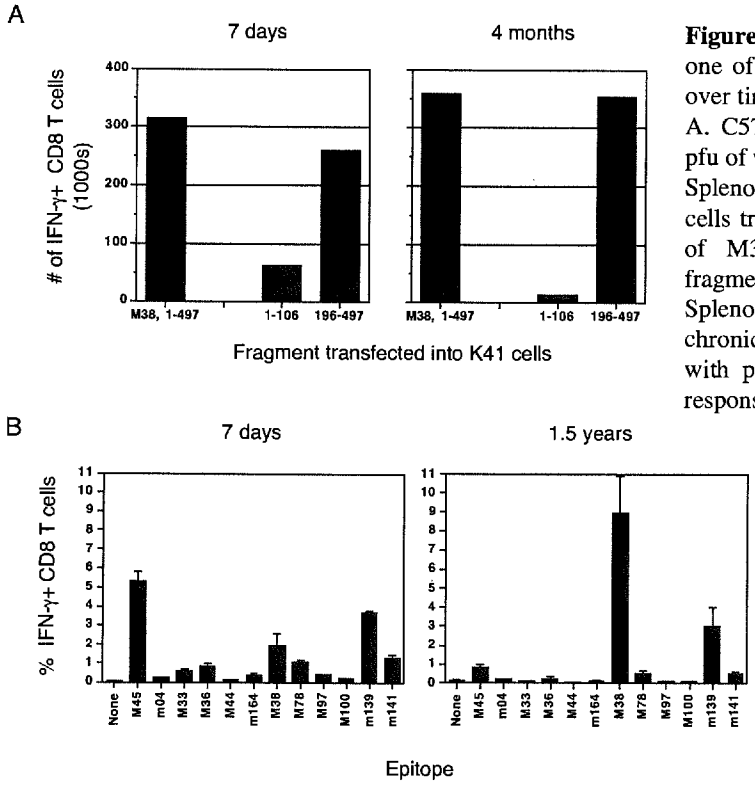

Figure 4.3 The CD8 T cell response to one of two epitopes from M38 increases over time.
A. C57BL/6 mice infected with 1 x 10^6 pfu of w.t. MCMV for 7 days or 4 months. Splenocytes were stimulated with K41 cells transfected with all 497 amino acids of M38, or else the indicated M38 fragments and analyzed by ICS. B. Splenocytes from acutely-infected or chronically-infected mice were stimulated with peptide, then analyzed by ICS for responses to 12 CD8 T cell epitopes.

CD8 T cells from mice chronically infected with MCMV recognize IE3 as an antigen, but CD8 T cells from acutely-infected mice do not

Data collected previously in our lab indicates that the size of the MCMV-specific CD8 T cell response is similar in acute and chronic infection (MC Gold, manuscript in preparation). However, the data presented in Figure 4.2 and 4.3 demonstrate that CD8 T cells specific for most epitopes, except epitopes M38 $_{316\text{-}324}$ and m139 $_{419\text{-}426}$, decline dramatically between acute and chronic infection. There appears to be a discrepancy between these findings, since it does not appear that the expansion of CD8 T cells specific for these two epitopes is equal to the contraction that occurs for CD8 T cells of other specificities. Therefore it seemed likely that there may be antigen-specific CD8 T

cell responses that are weak or undetectable in acute infection that become dominant during chronic infection.

To test this hypothesis directly, we screened the ORF library, essentially as described in Chapter 3, with splenocytes from mice chronically-infected with w.t. MCMV. K41 cells were transfected with plasmid DNA expressing each cloned ORF from MCMV (~90% of ORFs have been cloned). These transfected K41 cells were then used to stimulate splenocytes from mice infected with MCMV for 1.5 years, and CD8 T cell responses were analyzed by ICS. The results are shown in Figure 4.4A. *M38* elicited the strongest IFN-γ production from CD8 T cells, consistent with our previous results, and *m139* also elicited a strong response (both indicated by a single *). Minor responses were elicited by genes known to encode antigens, including *M31*, *M57*, *m141* and *m164*, although responses to many genes encoding other antigens, such as *M36*, *M44*, *M97* and *m04*, were similar to background levels (all previously-known antigens except *M38* and *m139* are indicated by a +). However, significant responses were also detected in response to *ie3* and *M35* (indicated by a double **). The response to *ie3* was confirmed in an identical screen, indicating that *ie3* is likely to encode a CD8 T cell epitope recognized well in chronic infection, but not acute infection. The response to *M35* was not confirmed when the experiment was repeated, although minor responses to a number of other ORFs were observed (not shown). This may indicate that some CD8 T cells in an individual chronically-infected mouse respond to an epitope that is not recognized by a significant number of CD8 T cells in other chronically-infected mice.

We wondered if *ie3* was somehow missed when we first screened the ORF library with splenocytes from mice acutely infected with MCMV (Chapter 2), but soon found

that this was not the case. Figure 4.4B shows that *ie3* stimulates CD8 T cell IFN-γ production from chronically-infected mice but not acutely-infected mice. Importantly, the acutely-infected mice did respond to K41 cells transfected with M38, indicating a robust CD8 T cell response.

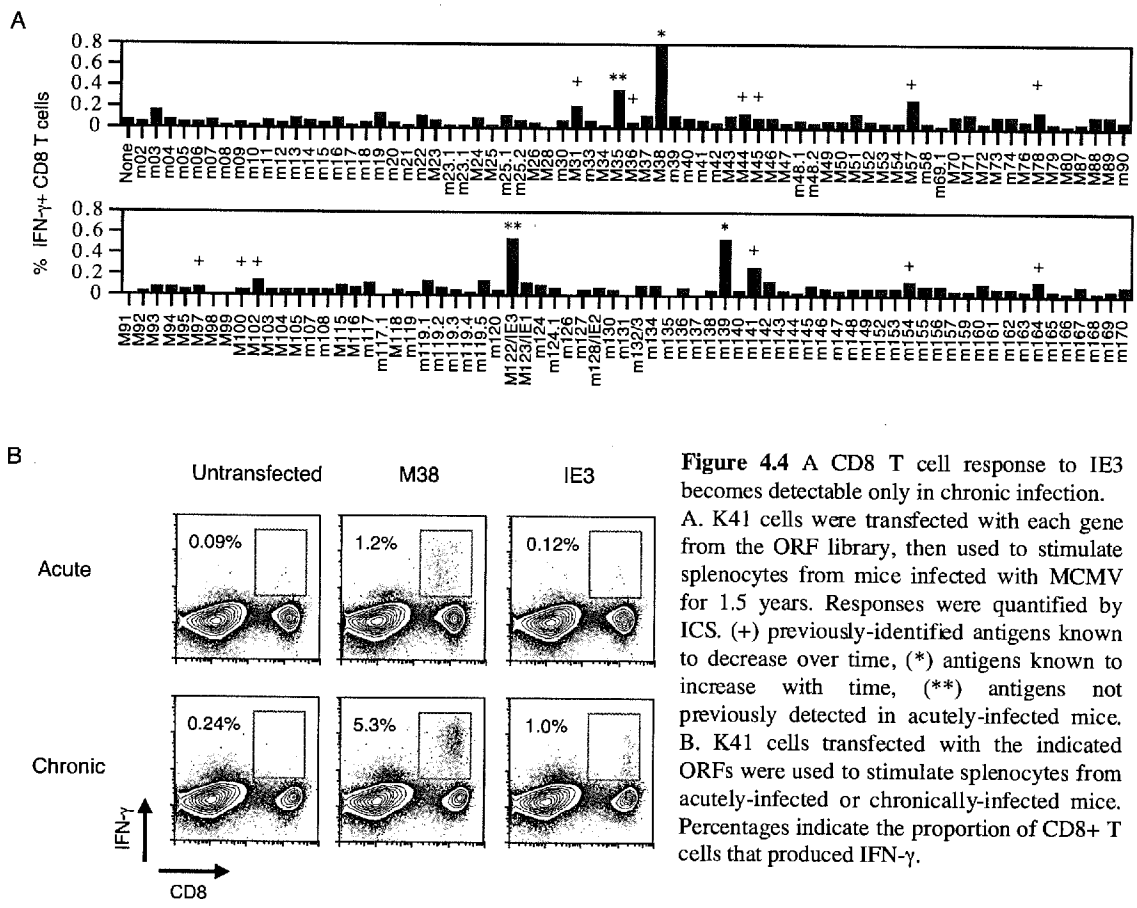

Figure 4.4 A CD8 T cell response to IE3 becomes detectable only in chronic infection. A. K41 cells were transfected with each gene from the ORF library, then used to stimulate splenocytes from mice infected with MCMV for 1.5 years. Responses were quantified by ICS. (+) previously-identified antigens known to decrease over time, (*) antigens known to increase with time, (**) antigens not previously detected in acutely-infected mice. B. K41 cells transfected with the indicated ORFs were used to stimulate splenocytes from acutely-infected or chronically-infected mice. Percentages indicate the proportion of CD8+ T cells that produced IFN-γ.

The overall conclusion from this set of experiments is that IE3 represents a third CD8 T cell antigen, along with M38 and m139, that elicits a response of equal or higher magnitude in chronic infection than in acute infection. Additionally, since the response to IE3 is not detectable early after infection, this adds considerable weight to the hypothesis that CD8 T cells responding to these antigens are proliferating over time. Future experiments will identify the IE3 epitope, and formally test if IE3-, M38- and m139-

specific CD8 T cells proliferate over time while CD8 T cells responding to other antigens do not.

Mice with identical MHC haplotypes, but different genetic backgrounds, have MCMV-specific CD8 T cell responses of differing sizes and antigen specificities

We decided to test the influence that the genetic background of the mouse has on immunodominance, if the *H-2* haplotype is held constant. C57BL/6 mice and BALB.B mice, both of the $H\text{-}2^b$ haplotype, were infected with w.t. MCMV. Seven days after infection, BALB.B mice responded most strongly to the m139 epitope (Figure 4.5A). Responses to M45 and m141 were less dominant, while responses to M33, M44 and M97 were very subdominant. This is in contrast to C57BL/6 mice, in which M45-specific CD8 T cells were most dominant (Figure 4.5A), a finding we have consistently observed in more than 10 separate experiments. In addition, the frequency of CD8 T cells responding to M45, m139 and m141 is much lower in BALB.B mice (note that the scales on each graph are different), even though MCMV replicates to 1000-fold higher titers in mice with a BALB background (Scalzo et al., 1995) (see Chapter 1.9). While this experiment directly comparing the immunodominance hierarchy in these two strains of mice has not been repeated exactly as described, similar experiments are consistent with these results and suggest that the immunodominance hierarchy is altered in BALB.B mice, and that the size of the response is also smaller, compared to C57BL/6 mice.

Importantly, we wanted to directly compare the dominant antigens from BALB/c mice, IE1/pp89 and m164, to the dominant antigen in C57BL/6 mice, M45. To accomplish this goal, BALB/c mice were bred to BALB.B mice, resulting in F1 mice

with a pure BALB background, but heterozygous at the *H-2* locus. These will be referred to as BALB $H\text{-}2^{dxb}$ mice. Conversely, C57BL/6 mice were bred to B6.BALB-H2^{d} mice, resulting in F1 mice with a pure B6 background, but heterozygous at the *H-2* locus. These will be referred to as B6 $H\text{-}2^{dxb}$ mice. The resulting BALB $H\text{-}2^{dxb}$ mice and B6 $H\text{-}2^{dxb}$ mice have identical MHC I molecules, and should both be capable of responding to CD8 T cell epitopes restricted by K^{d}, K^{b}, D^{d}, D^{b} or L^{d} (the L locus is absent in the B6 background).

BALB $H\text{-}2^{dxb}$ mice and B6 $H\text{-}2^{dxb}$ mice were infected with w.t. MCMV, and splenic CD8 T cells were analyzed by ICS after stimulation with peptides restricted by both $H\text{-}2^{d}$ and $H\text{-}2^{b}$ (note that M45 encodes a D^{b}-restricted epitope that is dominant in C57BL/6 mice, but also a D^{d}-restricted epitope that is very subdominant in BALB/c mice). BALB $H\text{-}2^{dxb}$ mice responded most strongly to the dominant $H\text{-}2^{d}$ epitopes pp89 and m164 (Figure 4.5B). Their response to the $H\text{-}2^{b}$ epitopes M45 and m139 was on par with the response to m18, a subdominant $H\text{-}2^{d}$ epitope. Responses to the D^{d}-restricted M45 epitope, and to m141 and M97, were minimal. In contrast, B6 $H\text{-}2^{dxb}$ mice had a very different immunodominance hierarchy (Figure 4.5B, right side), responding strongly to the $H\text{-}2^{b}$-restricted M45 epitope and the $H\text{-}2^{d}$ restricted m164 epitope. IE1/pp89 and m139 also elicited strong responses, and smaller but significant frequencies of CD8 T cells responded to all other epitopes examined.

These two preliminary experiments suggest that the CD8 T cell immunodominance hierarchy is significantly affected by the genetic background of the host. Specifically, it appears that in a relative sense, the B6 background favors D^{b}- and D^{d}-restricted responses to M45, and the K^{b}-restricted response to m139, while the BALB

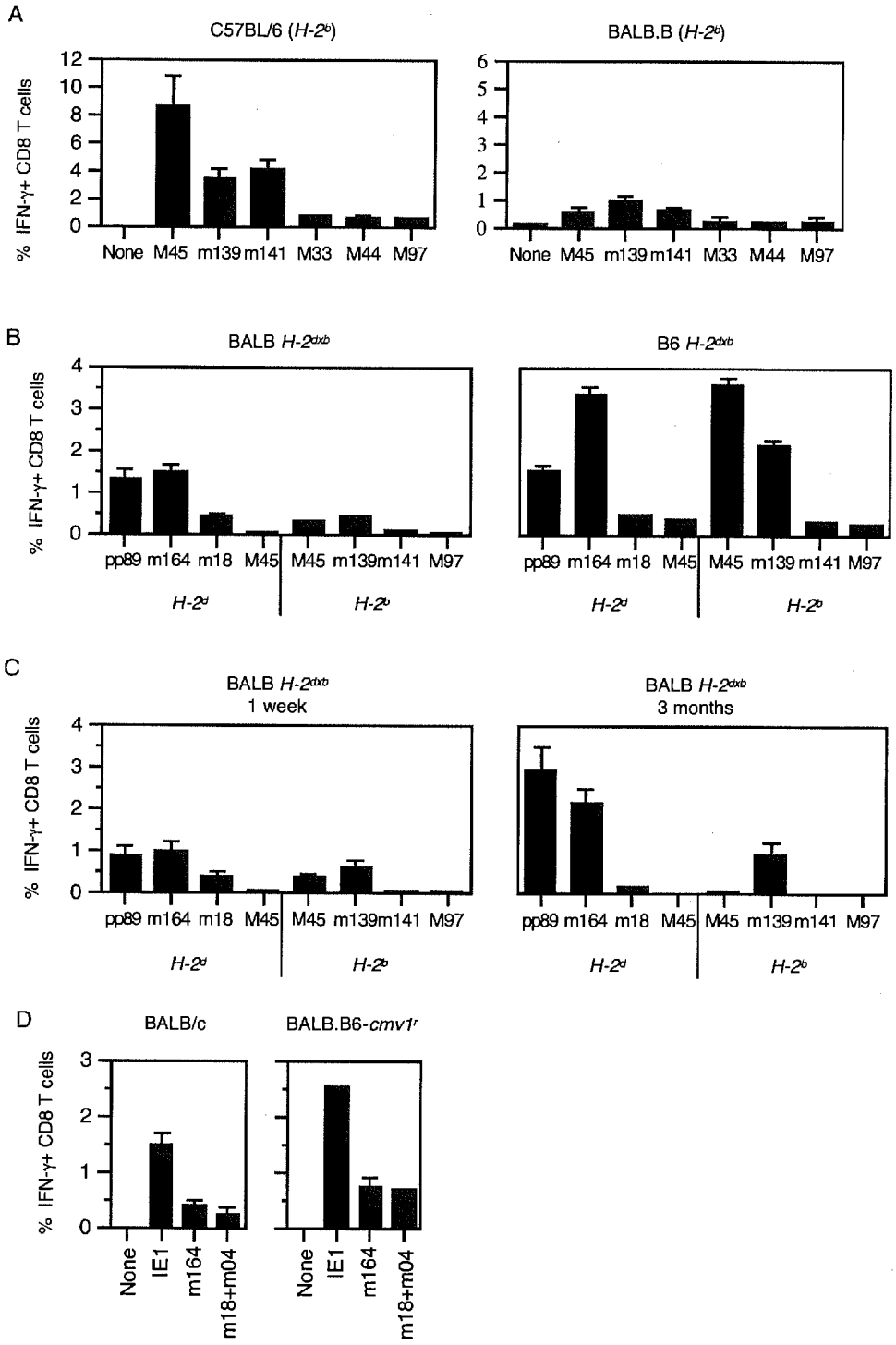

Figure 4.5 The size and antigen specificity of the CD8 T cell response to MCMV is affected by the genetic background of the host.

A. C57BL/6 or BALB.B mice were infected with 1 x 10^6 pfu of w.t. MCMV for 7 days, and splenocytes were then analyzed by ICS for antigen-specific responses. B. BALB *H-2*^{dxb} or B6 *H-2*^{dxb} mice were analyzed for antigen-specific responses 7 days after infection with 1 x 10^6 pfu of w.t. MCMV infection. C. BALB *H-2*^{dxb} mice infected for 1 week or 3 months with 5 x 10^4 pfu of w.t. and then tested for antigen-specific responses by ICS. D. BALB/c mice or BALB/c *cmvR* mice were infected with 1 x 10^6 pfu of w.t. MCMV, then tested 7 days later for antigen-specific responses by ICS.

background disfavors them. Conversely, the B6 background may disfavor IE1/pp89 compared to the BALB background. Somewhat surprisingly, neither mouse strain responded well to m141, even though it is normally one of the most dominant *H-2*^{b} epitopes.

As is strikingly seen in Figure 4.5B, the sum of all epitope-specific responses was much greater in B6 *H-2*^{dxb} mice (~13%) than in BALB *H-2*^{dxb} mice (~5%) further suggesting that the B6 background is more conducive to strong MCMV-specific CD8 T cell responses than the BALB background.

On a BALB background, increasing CD8 T cell responses to IE1 (pp89), m164 and m139 may be preferential for *H-2*^{d} epitopes

Two groups have shown that the CD8 T cell responses to both IE1/pp89 and m164 increase with time (Holtappels et al., 2000a) (Holtappels et al., 2002c; Karrer et al., 2003), and we have demonstrated a similar phenomenon with m139 in C57BL/6 mice (Figure 4.2). Figure 4.5B demonstrated that BALB *H-2*^{dxb} mice respond relatively poorly to m139, which is normally the second or third most dominant CD8 T cell antigen in C57BL/6 mice, but respond well to IE1/pp89 and m164, which are normally co-dominant after infection of BALB/c mice. In light of these observations, we wondered if the CD8 T cell response to these three antigens would increase in parallel after infection of BALB *H-2*^{dxb} mice, or if IE1/pp89-specific and m164-specific CD8 T cells would proliferate more significantly than m139-specific CD8 T cells.

The result was somewhat ambiguous. Within 3 months of infection, the CD8 T cell responses to all three antigens increased, but IE1/pp89-specific and m164-specific

CD8 T cell responses were the largest (Figure 4.5C). However, this finding is not totally conclusive, given that the m139-specific response was also smaller one week after infection (Figure 4.5, left). The IE1/pp89-specific population of CD8 T cells expanded 3.2-fold over this period, and m164-specific CD8 T cells expanded 2.2-fold, while CD8 T cells specific for m139 expanded only 1.5-fold. Although preliminary, this data suggests that CD8 T cells of some antigen specificities may expand more rapidly than others.

The size of the CD8 T cell response to MCMV is affected by the presence of Ly49H on NK cells

For some time, we had assumed that mouse strains where MCMV replicated to the highest titers would result in a larger total amount of viral antigen, which would ultimately result in large CD8 T cell responses. To us, this implied that an efficient NK cell response could indirectly 'compete' with CD8 T cells by reducing the viral load. It is well-documented that NK cells in C57BL/6 mice efficiently control MCMV infection, due to an interaction of Ly49H on NK cells with its ligand m157 on virus-infected cells (see Chapter 1.7). Our model predicted that if there were any differences in the size of the CD8 T cell response between BALB/c and C57BL/6 mice, the higher MCMV titers in BALB/c mice would equate with a stronger CD8 T cell response. However, the cumulative evidence, in the literature and presented above, is fairly convincing that MCMV elicits a larger CD8 T cell response in C57BL/6 mice than in BALB/c mice.

This presented a paradox, and we therefore formulated a newer model where NK cells have a dual role, to limit virus replication early in infection and to simultaneously promote a Th1 inflammatory environment, resulting in a stronger anti-viral CD8 T cell

response. BALB.B6-*cmv1*^{r} mice are identical to BALB/c mice except that they are congenic for the C57BL/6 NK complex, including *ly49h* (Scalzo et al., 1995b). MCMV titers in the spleens of BALB.B6-*cmv1*^{r} mice are decreased 100-10,000 fold between days 2 and 6 compared to MCMV titers in BALB/c mice (Scalzo et al., 1995b). One prediction of the hypothesis that NK cells both limit viral replication and promote a Th1 response is that BALB.B6-*cmv1*^{r} mice will have a larger CD8 T cell response to MCMV than do BALB/c mice. We infected BALB/c mice and BALB.B6-*cmv1*^{r} mice with w.t. MCMV, then quantified antigen-specific CD8 T cell responses by ICS. In a single experiment, the response to IE1 and m164 was increased about 1.5-fold in the BALB.B6-*cmv1*^{r} mice compared to BALB/c mice (Figure 4.5D). Responses to m18 and m04, minor *H-2*^{d} antigens, were measured together in a single sample and were similarly affected.

Conclusive delineation of the role of Ly49H awaits complementing experiments, such comparing the CD8 T cell response after infection of C57BL/6 mice with w.t. MCMV or Δm157 MCMV, but this result suggests that NK cells promoting a stronger anti-viral CD8 T cell response, despite decreasing the antigen load.

Chapter 4: Discussion

Overview

Identification of CD8 T cell antigens from MCMV, described in chapter 3, was an important goal. It allowed us to more definitively measure the size and specificity of the acute CD8 T cell response to MCMV. More importantly, however, CD8 T cell epitopes are useful tools for further characterization of CD8 T cell responses after MCMV

infection under a variety of experimental conditions. The data described here were obtained relatively rapidly once these tools were available.

Five significant observations were made: 1) The immune evasion genes *m04*, *m06* and *m152* do not greatly affect the size or specificity of the MCMV-specific CD8 T cell response in vivo; 2) CD8 T cells specific for IE3, M38 and m139 appear to accumulate over time, while CD8 T cells of other specificities do not, but this is not affected by the presence or absence of *m04*, *m06* and *m152*; 3) The CD8 T cell immunodominance hierarchy in MCMV-infected mice differs between C57BL/6 mice and BALB.B mice, which have identical MHC haplotypes, and between BALB $H\text{-}2^{dxb}$ and B6 $H\text{-}2^{dxb}$ mice, indicating that the genetic background of the host affects immunodominance; 4) The size of the CD8 T cell response to MCMV is also strongly affected by the strain of the mouse; 5) A strong NK cell response may increase the size of the CD8 T cell response.

m04, *m06* and *m152* do not affect CD8 T cell immunodominance

We were very surprised that the immune evasion genes do not have a noticeable effect either on the size or antigen specificity of the MCMV-specific CD8 T cell response, as seen in Figure 4.1. The experiments shown here will be repeated to include the recently-identified M57 epitope, and there may be some minor differences that are reproducible, but our overall impression at this time is that these genes do not affect CD8 T cell priming in a substantial way.

One possible explanation is that in MCMV-infected DCs, these genes are either not expressed, are non-functional, or their effects are overcome by the high level of MHC class I processing and presentation. While the data are conflicting, it appears more likely

that *m04*, *m06* and *m152* are indeed expressed in professional APCs (Hengel et al., 2000) (LoPiccolo et al., 2003), and are able to prevent MHC class I antigen presentation at least in primary bone marrow-derived macrophages (LoPiccolo et al., 2003). Since these genes do not appear to affect CD8 T cell priming in vivo, the underlying implication is that cross-presentation of viral antigens from dead and dying MCMV-infected cells is the primary mechanism of CD8 T cell priming in vivo. Apparently, direct CD8 T cell priming from MCMV-infected DCs has little or no role in MCMV biology.

Then what is the role of these immune evasion genes in vivo? One possibility is that the immune evasion genes may delay viral clearance during the window when effective CD8 T cells have been primed but infectious virus persists in many organs, including the lungs and salivary glands. A Δm152 virus replicated to lower titers after acute infection of neonatal BALB/c mice, due to enhanced CD8 T cell control (Krmpotic et al., 1999), supporting this model. Additionally, Reddehase and colleagues have now shown very clearly and elegantly that in vivo, *m152* is required to prevent clearance of MCMV by CD8 T cells specific for the D^b-restricted M45 epitope ^{985}HGIRNASFI^{993} (Holtappels et al., 2004). These CD8 T cells were effective at controlling replication of a Δm152 MCMV mutant, but unable to control replication of w.t. MCMV, from infected liver tissue within the same mouse. This demonstrates for the first time that an MHC class I immune evasion gene inhibits CD8 T cell recognition of infected cells in vivo, during the period when virus is actively replicating.

Many authors have speculated that the immune evasion genes also contribute to MCMV's ability to 'fly below the radar' during latency, avoiding CD8 T cell immune surveillance and persisting within the host for life. This model is appealing because the

function of the immune evasion genes may be impaired in the inflammatory environment of acute infection, but more effective during the latent phase of infection when inflammation is reduced. At the extreme, it has been proposed that elimination of immune evasion genes from HCMV or MCMV would create a virus incapable of establishing latency. But work in our lab suggests that Δm4+m6+m152 MCMV does establish latency and is capable of reactivation. CD8 T cells specific for m139 continue to expand regardless of whether w.t. MCMV or Δm4+m6+m152 MCMV is used for infection, further supporting the model that these genes are not necessary for the establishment of latency, although they may affect the amount of latent virus.

Another possible function is to delay CD8 T cell recognition of latently-infected cells when virus has begun to reactivate but no infectious virions have yet been produced. The immune evasion genes might give MCMV a 'running-start' at reactivation, increasing the likelihood of virion production and spread to a new host. This would be particularly useful for MCMV if it occurred in the salivary gland, the site thought to be most important for virus transmission. A reactivation assay is still under development in our lab, but the results should provide very important information, and a collaborative study is planned to compare transmission of w.t. MCMV to Δm4+m6+m152 MCMV.

The CD8 T cell response to IE3, M38 and m139 increases from acute infection to latency in C57BL/6 mice

CD8 T cells responding to a subset of C57BL/6 epitopes appear to expand over time, similar to what is observed in latently-infected BALB/c mice (Holtappels et al., 2000a) (Holtappels et al., 2002c) (Karrer et al., 2003). During acute infection, responses

to M38 and m139 are easily detectable, while a response to IE3 is not. The evidence so far shows that CD8 T cells specific for m139 rapidly expand and contract within the first 14 days of infection, and suggest that expansion occurs thereafter. We will formally demonstrate the kinetics of the response to IE3, M38 and m139 after the IE3 epitope is identified. Data collected in our lab indicates that the size of the CD8 T cell response is similar during acute and chronic infection (MC Gold, manuscript in preparation) This indicates that there may be additional 'IE3-like' epitopes that remain undiscovered, since the currently known 'acute' epitopes are capable of stimulating more CD8 T cells than the currently known 'chronic' epitopes.

So what is the potential mechanism for this phenomenon? BALB/c IE1/pp89 and m164 epitopes share two notable properties. First, CD8 T cell responses to both epitopes are dominant after acute infection. Second, both epitopes are able to partially escape the effects of the immune evasion genes. The IE1/pp89 epitope is presented well under IE conditions, but not under conditions that allow E gene expression (Del Val et al. 1989), and the m164 epitope escapes the effects of the MHC class I immune evasion genes by an unknown mechanism (Holtappels et al., 2002a). We hypothesized that either or both of these characteristics might be important for CD8 T cell expansion during latency. The finding that M38 and IE3 epitopes, which are moderately and extremely subdominant respectively, elicit a similar behavior discounts the importance immunodominance in acute infection. And since expansion of m139-specific CD8 T cells was similar in mice infected with w.t. MCMV or Δm4+m6+m152 MCMV, a role for the immune evasion genes is also excluded.

A well-done study with influenza virus raises another possible model for MCMV. During the primary response to influenza virus infection of C57BL/6 mice, CD8 T cell epitopes NP366-374 and PA224-233 are equally dominant. However, CD8 T cells specific for NP366-374 preferentially undergo a large recall expansion, while PA224-233-specific CD8 T cells do not (Belz et al., 2000). Primary bone marrow DCs, infected in vitro or in vivo, present both epitopes, leading to equal priming of naïve CD8 T cells of both specificities (Crowe et al., 2003). But primary macrophages, B cells and epithelial cells present only NP366-374 and not PA224-233, leading to expansion of memory CD8 T cells specific for only the NP epitope. An elegant adoptive transfer experiment revealed that within the same mouse, naïve CD8 T cells of both specificities proliferate while memory CD8 T cells only proliferate extensively if they are specific for NP366-374 but not PA224-233 (Crowe et al., 2003). This confirms that the APCs that are responsible for priming of naïve CD8 T cells present a different set of peptides than the APCs that are responsible for expansion of memory CD8 T cells. As an interesting side-note, vaccinating mice with PAA224-233 leads to higher viral titers after infection than occurs in unvaccinated mice.

Could an analogous situation be occurring after MCMV infection? It is tempting to speculate that a similar phenomenon may be occurring after MCMV infection, but at present no data exists to substantiate or contradict this model. It is still controversial which cell types are infected during MCMV latency, but macrophages and endothelial cells are prime candidates. One group used PCR in-situ hybridization (PISH) to sensitively and specifically demonstrate that in latently-infected mice, the liver, spleen, heart and kidneys contain MCMV DNA within capillary endothelial cells (Koffron et al.,

1998). In the lungs, a major site of MCMV latency, DNA was not detected in endothelial cells but was found in alveolar macrophages. We should be able to address the role of macrophages and endothelial cells in expansion of IE3-, M38- and m139-specific CD8 T cells by determining if these epitopes are presented by infected macrophages and endothelial cells but not by other cell types.

A second model for the observed CD8 T cell phenomenon is based on timing of gene expression. If CD8 T cell immune surveillance of latently-infected cells is an active process, capable of detecting antigen and interrupting the reactivation process, then those CD8 T cells specific for antigens expressed earliest during reactivation will be more likely to receive antigen "hits" and proliferate. There is strong evidence that gene expression during MCMV reactivation is a sequential process with at least four checkpoints (Kurz and Reddehase, 1999). (discussed in chapter 1.7). In light of this, there are five antigens that result in CD8 T cell expansion in either C57BL/6 or BALB/c mice, and two of these are IE genes (IE1 & IE3).

While reviewing the literature, it has become clear that while IE1, IE2 and IE3 represent a genomic region that is very active at IE times, these are not the only genes transcribed under IE conditions. This is best-characterized in HCMV infection, where no less than four regions of the genome appear to be expressed at IE time. Notably, a number of IE transcripts originate from the *UL36-38* region of HCMV. This corresponds to M36-M38 of MCMV, (M38 represents a third mouse antigen whose CD8 T cells expand over time). In MCMV, the Campbell lab has examined transcription of genes *m139-m145* (Hanson et al., 1999). While *m139* transcripts were not detected until E times in infected cells in vitro, it is nonetheless very intriguing that expression of a number of

other transcripts in this region are present at IE times (m139 represents the fourth mouse antigen that expands over time). Finally, *m164* transcripts are detectable under IE conditions during productive lytic infection in vitro, although protein products were not detected until E times (Holtappels et al., 2002a) (m164 represents the fifth mouse antigen whose CD8 T cells expand over time). The overall point of this discussion is that of the five antigens known to cause CD8 T cell expansion over time in BALB/c or C57BL/6 mice, transcripts for at least three of these are known to be expressed at IE times (*ie1*, *ie3* and *m164*), and the other two genes are from genomic regions associated with IE transcription (*UL36-38* and *m139-m145*). While certainly not conclusive, this is an interesting coincidence that merits investigation.

There is one confounding factor for this model. The CD8 T cell epitope from M38 that expands over time, M38 ^{316}SSPPMFRVP^{324}, is not the only epitope from this protein that is recognized by CD8 T cells; an M38 epitope between amino acids 31-50 also exists. As seen in Figure 4.3A, the CD8 T cell response to one of these increases over time, while the response to the other epitope (so far unidentified), apparently does not. Thus any model that attempts to explain presentation of only specific antigens must also be able to explain how different epitopes within the same protein elicit CD8 T cell populations with different behaviors. It is possible that splicing of mRNAs occurs differentially during productive infection and latency.

The situation with M38 may not be unique, either. The m164 $_{257\text{-}265}$ epitope in BALB/c mice elicits a population of CD8 T cells that expands over time, but in C57BL/6 mice there appear to be two weak m164 epitopes, m164 $_{267\text{-}275}$ and m164 $_{283\text{-}290}$, that elicit CD8 T cell responses that do not increase over time. I will need to determine if CD8 T

cells specific for each m164 epitope have similar or different behavior in latently-infected B6 *H-2*^{dxb} mice BALB *H-2*^{dxb} mice.

Thus the primary conclusion at this time is that the underlying mechanism governing preferential accumulation of CD8 T cells specific for a subset of epitopes might be quite complex.

Chapter 5: Discussion and Future Directions

Patterns of CD8 T cell responses differ, depending on virus lifestyles

CD8 T cell responses to pathogens with different lifestyles appear to fall into roughly four distinct categories. The immune response to cleared pathogens in mice have been characterized by far in the most detail. After infection of either C57BL/6 or BALB/c mice with LCMV, CD8 T cell populations of different specificities expand, contract, and then stably persist over time (Murali-Krishna et al., 1998). A similar pattern occurs after infection with Listeria monocytogenes (Busch et al., 1998). CD8 T cell responses to HIV or SIV follow a different pattern, because these viruses encode error-prone polymerases (Goulder and Walker, 1999). Strong CD8 T cell responses occur after infection, and effectively limit viral replication, but "escape variants" randomly occur and are strongly selected because they contain mutated CD8 T cell epitopes that are no longer recognized by virus-specific CD8 T cells. A third distinct CD8 T cell response occurs after infection with Epstein-Barr Virus (EBV). γ-herpesviruses have two very different phases of gene expression, lytic and latent. During acute infection, dominant CD8 T cell responses are primed primarily against lytic antigens. However, these responses are culled (Callan et al., 2000), similarly to cleared viral infections such as LCMV, and responses against antigens expressed in the latent phase increase over time and become dominant (Hislop et al., 2002).

CD8 T cell responses to MCMV do not follow any of these patterns. The data presented here, combined with studies in the literature (Holtappels et al., 2002c), suggest that CD8 T cell priming occurs to a large number of antigens during acute infection. But

CD8 T cells responding to all antigens are rapidly culled by day 14 (Chapter 4) (Karrer et al., 2003), similarly to what happens after infection with a cleared pathogen. Following this loss, CD8 T cells specific for a select few antigens proliferate again, continuously expanding over time (Chapter 4) (Holtappels et al., 2000a) (Holtappels et al., 2002c) (Karrer et al., 2003). The acute CD8 T cell response to HCMV has not been documented, but CD8 T cell responses in some chronically-infected people have remarkably shown that often only a few clonotypes represent a very large proportion of detectable HCMV-specific CD8 T cell responses (Khan et al., 2002). Furthermore, CMV seropositive status is associated with an increased number of oligoclonal expansions in the elderly, and CMV-specific oligoclonal CD8 T cells can account for up to one-fourth of CD8 T cells in some elderly individuals (Khan et al., 2002). Thus it is tempting to speculate that what occurs within the first few months of MCMV infection in mice, extrapolated over years and decades of HCMV infection of humans, are related observations. It seems possible or even likely that some HCMV-specific CD8 T cells primed during acute infection are restimulated by antigen each time the virus abortively or productively reactivates, and proliferate for the person's lifetime.

Direct antigen presentation by infected DCs, and cross-presentation

The presence or absence of *m152* does not affect M45-specific CD8 T cell priming in vivo, even though *m152* is required to prevent M45 presentation in vitro (Gold et al., 2002). Based on this finding, it was proposed by Gold et al. that cross-priming of CD8 T cells, rather than direct priming from infected DCs, was not only possible but may be the dominant priming mechanism in MCMV infection (Gold et al., 2002). Data

presented here, testing more than ten CD8 T cell epitopes against a virus missing all three known MHC class I immune evasion genes, strongly support this initial conclusion.

The most important question in this respect might shift from "Does cross-priming ever occur in natural infection?" to "In MCMV infection, does direct priming ever occur?" JAWS II, an immature DC cell line, is infectable by MCMV and capable of presenting antigens (Gold et al., 2002). And primary bone marrow macrophages are capable of supporting productive infection, presenting antigen, and are subject to the effects of the immune evasion genes (LoPiccolo et al., 2003). Thus while productive infection of primary DCs and the effectiveness of the immune evasion genes in DCS has not been formally proven, all evidence suggests that this will be the case. Therefore, if direct infection of DCs does occur in vivo, they should be subject to the effects of the immune evasion genes.

As discussed above, removal of the immune evasion genes *m04*, *m06* and *m152* does not appear to restore direct priming by infected DCs. What additional factor(s) could be responsible for preventing MCMV-infected cells from priming CD8 T cells in vivo? One potential clue is that HCMV encodes an IL-10 homolog. At the CMV 2003 meeting, one presenter demonstrated that infected human DCs express viral IL-10, which induces host IL-10 in a positive feedback loop (Chang, 2003, HV abstract 9.33). The HCMV-infected DCs down-regulated MHC class II expression, did not mature after CD40L stimulation, failed to secrete pro-inflammatory cytokines, and failed to express costimulatory molecules, due to the feedback between viral and cellular IL-10. Although MCMV does not encode an IL-10 homolog, MCMV infection of macrophages in vitro does induce IL-10 expression, which results in decreased MHC class II surface

expression (Redpath et al., 1999). Furthermore, MCMV infection of IL-10$^{-/-}$ mice in vivo induces MHC class II up-regulation on both F4/80+ macrophages and CD11c+ DCs, but in w.t. mice leads to MHC class II down-regulation on macrophages and DCs (Redpath et al., 1999). This indicates that MCMV may be preventing activation or differentiation of DCs (and macrophages) in vivo, via IL-10 induction. Thus MCMV infection may block DC maturation and prevent direct CD8 T cell priming through IL-10 induction. If this hypothesis is true, then DCs in IL-10$^{-/-}$ mice may be capable of directly priming CD8 T cell responses, and we would therefore observe an effect of *m04*, *m06* and *m152* on CD8 T cell immunodominance. We have already infected IL-10$^{-/-}$ mice with w.t. MCMV and Δm4+m6+m152 MCMV, and we should have the answer to this question in a few weeks.

At the Herpesvirus 2003 meeting, data were also presented that MCMV encodes a previously-unrecognized protein capable of down-regulating B7-2 from the surface of infected cells (Loewendorf, HV 2003 Abstract 11.06). Other MCMV proteins have also been identified that are capable of internalizing and destroying other costimulatory and adhesion molecules (M Messerle, personal communication). Additional work has recently demonstrated that MCMV infection of bone marrow-derived macrophages prevents the increases in ICAM-1 surface expression that is normally induced by TNF-α (Popkin and Virgin, 2003).

Together, these findings imply that MCMV may strike at multiple layers, from antigen presentation to costimulatory and adhesion molecules to DC maturation, in order to prevent infected DCs from priming CD8 T cells. Clearly, a lot of work remains to fully understand how MCMV prevents infected DCs from effectively priming CD8 T cells in

vivo. One of our goals will be to see if enough of these layers can be peeled off to restore direct DC priming in vivo.

What is the mechanism that causes expansion of IE3-, M38- and m139-specific CD8 T cells over time in C57BL/6 mice?

First and foremost, we must identify the optimal IE3 peptide and formally demonstrate that CD8 T cell proliferation for all three of these populations is indeed occurring. Careful analysis of the data indicates that some expansion may be occurring in the persistent phase of infection. Therefore we will also need to determine the relative proportion of proliferation during the persistent vs. latent phases of infection.

But more importantly, what is the mechanism that causes expansion of IE3-, M38- and m139-specific CD8 T cells over time in C57BL/6 mice? Our first impression was that the MHC class I immune evasion genes were involved, but similar expansion occurs after infection with w.t. MCMV or Δm4+m6+m152 MCMV, making this very unlikely. It is interesting that all three of these antigens are K^b-restricted. But a similar phenomenon occurs in BALB/c mice with IE1/pp89 and m164, which are restricted by separate MHC I molecules, L^d and K^d respectively, so this is probably merely a coincidence in C57BL/6 mice. Furthermore, the fact that IE3 becomes a substantial target of the memory response, although undetectable in acutely-infected mice, implies that the phenomenon is unrelated to immunodominance in acute infection.

In the chapter 4 discussion, I explained an analogous finding that occurs after secondary infection of mice with influenza virus. In that system, DCs prime equal numbers of CD8 T cells specific for NP and PA epitopes, but memory CD8 T cells

primarily interact with non-DCs in the recall response, which present only the NP epitope. This leads to expansion of only NP-specific CD8 T cells. It is possible that the a similar phenomenon is occurring in MCMV infection, but the very different biology of the two viruses should be kept in mind. Thus it is possible that macrophages and endothelial cells, two cell types implicated in harboring latent virus, are incapable of presenting most epitopes that are dominant in acute infection. Equally likely, latently-infected cells may express only a cell-specific subset of genes. This would be an interesting finding, given that m139 plays an important role in macrophage-specific growth, and m139-specific CD8 T cells expand during latent infection (Koszinowski, 2003).

Another alternative, our currently-favored hypothesis, is that latently-infected cells undergo relatively frequent periods of abortive reactivation, resulting in expression of some genes relatively frequently compared to other genes. This is based on the model of Kurz et al. whose data implies the existence of numerous check-points in the process of reactivation (Kurz et al., 1999b). We propose that competition is occurring between CD8 T cells of different antigen specificities. Those CD8 T cells that recognize antigens expressed earliest (and most often) during reactivation are stimulated to either lyse the infected cell or release cytokines, and some of these CD8 T cells go on to proliferate. CD8 T cells that recognize antigens expressed later in the process of reactivation are unlikely to be stimulated if the reactivation process has been interrupted. In support of this model, all antigens that elicit this CD8 T cell phenomenon are encoded by genes expressed under IE conditions or else genomic regions implicated in IE gene transcription (see Chapter 4 Discussion).

We plan to test this model in vitro by examining the order of expression for each CD8 T cell antigen. Ideally we would examine the order of gene expression in latently-infected mouse tissues, but this is a very technically-demanding procedure so we will use cells infected in vitro as an approximation. Fortunately, there is no evidence for HCMV or MCMV that the order of gene expression differs between productive lytic infection and reactivation from latency, as is the case for γ-herpesviruses. In fact, for the transcripts that have been examined during reactivation from latency in vivo, the expression order is similar to that observed in vitro (Kurz et al., 1999b) (Grzimek et al., 2001). Reddehase and colleagues have offered to produce mutant viruses that contain point mutations within defined CD8 T cell epitopes. If competition is occurring during latent infection between CD8 T cells of different specificities, we would expect that CD8 T cells of other specificities will expand instead after infection with a virus containing mutations in the IE3, M38 and m139 epitopes.

Reddehase and colleagues have already produced a mutant MCMV with a point mutation in the IE1/pp89 epitope, and found that CD8 T cells of a previously-unknown specificity infiltrate the lungs in their BMT model after infection with this virus. This implies that competition between CD8 T cell specificities does in fact occur, at least in acute infection. In order to investigate the specificity of this population, they will use the ORF library described in Chapter 3 in an attempt to determine the antigen(s) recognized by this new CD8 T cell population.

On a related note, it is conceivable that CD8 T cells will represent an extremely sensitive tool for evaluating the molecular process of reactivation. If competition between CD8 T cells is proven to occur based on the order of gene expression during reactivation,

it would be theoretically possible to determine the order of gene transcription during reactivation by evaluating differential proliferation rates of CD8 T cells specific for defined antigen (transferred after CFSE labeling), analogous to how metabolic pathways in bacteria are determined by comparing strains with different genetic mutations.

There is one serious complication for any model that attempts to explain why CD8 T cells specific only for selected epitopes accumulate during latency. CD8 T cell expansion during latency must be restricted to particular CD8 T cell epitopes, not whole proteins, since M38 and m164 both appear to contain multiple epitopes, but only one epitope that induces CD8 T cells to continue to expand over time. Formal proof of this divergence of behavior will be possible once final mapping of the second M38 epitope is complete. And it will be enlightening to determine if CD8 T cells specific for each m164 epitope behave similarly or differently in latently-infected B6 $H\text{-}2^{dxb}$ mice and BALB $H\text{-}2^{dxb}$ mice.

I have considered that differential splicing may result in expression of one epitope without the other. This is possible for M38, but less likely in the case of m164, where all peptides are very near each other within the predicted m164 protein. I would speculate that early expression of antigens during reactivation might be required, but not sufficient, for inducing CD8 T cell expansion. Perhaps efficient antigen processing, or the presence of CD8 T cells of sufficiently high functional avidity for a particular epitope, will also be required. In other words, the mechanism may involve multiple degrees of complexity.

NK cells and the size of CD8 T cell response

The data strongly suggest that MCMV infection results in a larger CD8 T cell response in C57BL/6 mice than in BALB/c, even thought virus replicates to 1000-fold higher titers in BALB/c mice. Although other differences undoubtedly contribute, one factor that appears to significantly affect the strength of the CD8 T cell response is efficient NK cell activation. This indicates a fundamental link between the innate and adaptive immune response that to my knowledge has not been previously appreciated.

A very recent paper demonstrated that when the poorly-immunogenic A20 lymphoma was transfected with the MCMV genes *m06* and *m152*, which decreases MHC class I surface expression, NK cell activation was enhanced, most likely due to "missing self" on the tumor cells. This increased tumor-specific CD8 T cell activation in an NK & IFN-γ dependent manner and resulted in rejection of an otherwise lethal tumor.

These observations raise questions that are experimentally testable. To what degree are NK cells required for generating effective MCMV-specific CD8 T cell responses? Are there other MHC-like MCMV genes, in addition to *m144* (Rawlinson, 1996, Farrell, 1997), that are potential ligands for inhibitory NK cell receptors? Does deletion of these genes result in enhanced CD8 T cell responses? What about adding recombinant genes to MCMV that activate NK cells, for example NKG2D ligands?

These questions are not only interesting from an academic standpoint, but have very practical implications as well since a live attenuated strain of HCMV is being strongly considered as a prophylactic vaccine strategy. I predict that deleting genes whose protein products bind directly to inhibitory NK cell receptors will have two distinct beneficial effects. First, NK cells will more effectively control viral titers in the

first few days following infection, leading to reduced viral pathology in the short-term. Second, NK cells will promote a stronger CD8 T cell response, which will also reduce viral titers and pathology in the long-term. Two candidates for this approach are *m144*, which inhibits NK cells in both BALB/c and BALB.B6-*cmv1*^{r} mice (Farrell, 1997), and *m157*, which binds to the inhibitory Ly49I receptor in 129/J mice in addition to the activating Ly49H receptor in C57BL/6 mice (Arase, 2002). Adding recombinant genes to MCMV (*H-60*, *RAE-1*) whose protein products bind to activating NK cell receptors (NKG2D) would be expected to have a similar effect. Of course in this instance *m152* would have to be deleted, as gp40/m152, down-regulates cell surface expression of RAE-1 proteins (Lanier, 2003).

A primary goal of rational vaccine design is to decrease virulence and also enhance protective immunity. Unfortunately, for most pathogens attenuation of virulence also limits induction of protective T cell memory. The strategy proposed above might accomplish both goals at the same time. In addition, this approach might be particularly effective in specific situations where CD8 T cells are much more protective than antibodies, since typically live viral vectors elicit the strongest CD8 T cell responses.

Final comments

Many of the immunological principles that are widely believed to be true were defined largely with data derived in vitro. However, experimental results reported in this dissertation show that the function of costimulatory molecules and MHC class I immune evasion genes in vivo is not always easily determined from these in vitro studies. In addition, complex phenomena such as CD8 T cell immunodominance and viral latency

do not occur in vitro. Therefore, we need to deliberately move towards in vivo and directly ex vivo studies. Our current challenge is to integrate in vitro and in vivo data into a coherent model that can be further tested and refined.

We are just beginning to unravel many secrets of the mammalian immune system, but MCMV has been quietly and slowly accomplishing the same goal for millions of years. Importantly, MCMV has been perfecting its understanding of the host in the ideal system, in vivo. It is time for us to take a cue from MCMV and study the virus-host interaction as much as possible in its natural environment, the living mouse.

Chapter 6: Materials and Methods

Mice

Six- to 12-week-old female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Simonsen Laboratories (Gilroy, CA). $K^{b-/-}$ and $D^{b-/-}$ mice were a gift from F. Lemmonier. BALB.B10-H2b, B6.BALB-H-2d, and BALB.B6-cmvr1 were purchased from Jackson Laboratories. All mice were housed in the OHSU animal care facility.

Cell lines and culture medium

K41 cells, an SV-40 transformed *H-2^b* fibroblast cell line (gift of Marek Michalek), were cultured in DMEM complete (Dulbecco's minimal eagle medium supplemented with 10% fetal bovine serum and penicillin/streptomycin). $K^{b-/-}D^{b+/+}$ and $K^{b+/+}D^{b-/-}$ cells were derived from skin fibroblasts of adult C57BL/6 mice deficient for either K^b or D^b. They were transformed by transfection with a plasmid expressing the SV40 T antigen (gift of Marek Michalek) and were also grown in DMEM complete. The previously-described TNF-α-sensitive WEHI 164/clone 20 cell line (Gold et al., 2002) was cultured in RPMI complete (RPMI 1640 supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol and penicillin/streptomycin). All cells were grown at 37°C in the presence of 5% CO_2.

Antibodies

Rat IgG was obtained from Sigma (St. Louis, MO). Anti-4-1BB stimulating mAb from the 3H3 hybridoma, and anti-OX40 stimulating mAb from the OX86 hybridoma

(European Cell Culture Collection, Porton Down, U.K.) were purified on a Protein-G sepharose column (Pharmacia, Piscataway, NJ).

Plasmid DNA vaccine constructs

Plasmid pTH.HM (24) was a gift from Tomas Hanke, Oxford, U.K. pTH.HM encodes a string of CD8 T cell epitopes behind the HCMV IE promoter, including the H-2D^{d}-restricted murine epitope RGPGRAFVTI from HIV-1 envelope glycoprotein. Plasmid pOVA was constructed from the pIRES plasmid (Invitrogen, Carlsbad, CA), which contains two multiple-cloning sites (MCS) separated by an internal ribosomal entry site (IRES). Gene expression is controlled by the HCMV IE promoter. The full-length ovalbumin reading frame, which encodes the H-2K^{b}-restricted CD8 T cell antigen SIINFEKL, was amplified by PCR and cloned into the 'A' site. The B site contains the Ad5E4 ORF4 gene in reverse orientation, resulting in a non-functional ORF (this plasmid is used as a control plasmid for other studies).

Milligram quantities of plasmid DNA were obtained using Qiagen Megaprep columns (Qiagen, Valencia, CA) according to the manufacturer's protocols, quantified by UV absorbance, and the purity verified by $A_{260/280}$ and agarose gel electrophoresis.

MCMV ORF Cloning

The sequence of the MCMV genome has been published (Rawlinson et al., 1996). Primers were designed for each ORF to be 18 to 24 bp in length, with 50-75% GC content. All 5' primers included the sequence CACC ATG at the 5' end in order to allow unidirectional cloning into the plasmid DNA vector pcDNA3.1 (Invitrogen). 3' primers

included the final amino acid of the protein but not the stop codon, leaving the gene in-frame with the 3' tags from the vector.

For about 20 genes, DNA purified from the Hind III fragments of MCMV was used as the PCR template. However, sequencing revealed that a large number of point mutations were present compared to the published sequence. Therefore for the vast majority of genes, BAC-derived w.t. MCMV DNA was used as the template and proved to be very similar to the published sequence.

High-fidelity PCR was performed with *pfu* polymerase (Fermentas), using 1 round of denaturation at 95° C for 2 minutes, followed by 35 rounds of amplification at 95° C for 1 minute, 62° C for 1 minute and 72° C for 3 minutes, then 1 round of 72°C for 10 minutes. All reactions were performed in the presence of 8% DMSO, which greatly improved the yields. PCR products were analyzed by 1.5% agarose gel electrophoresis. Products of the correct size were gel-purified using QIAquick columns according to the manufacturer's protocol (Qiagen). Purified PCR products were ligated into pcDNA3.1 using the TOPO cloning kit (Invitrogen).

DNA was prepared from individual ampicillin-resistant colonies using QIAprep 8 strips with a QIAvac 6S manifold (Qiagen), and screened by restriction digestion for correct size. All ORFs were sequenced at the 5' end to confirm that the gene was inserted in the correct orientation and reading frame. About one-third of the genes were sequenced from the 5' end only, another one-third were sequence from both the 5' and the 3' end, and the last one-third were sequenced completely. ORFs that contained mutations resulting in frame-shifts or stop codons were discarded. Many ORFs contained point mutations, but

the mutations constituted less than 1% of amino acids and so these ORFs were not discarded.

Antigen fragment subcloning

Sixteen genes considered likely to contain CD8 T cell epitopes (Figure 3.2A & 3.2B) were subcloned into three to five smaller fragments, essentially as described above for each ORF. 5' and 3' primer pairs were designed so that each fragment would have an N-terminal methionine start codon, and would overlap neighboring fragments by 11 amino acids. Plasmid DNA encoding the ORF was used as template DNA, and all constructs were sequenced in entirety.

Recombinant vaccinia viruses

Recombinant modified vaccinia virus Ankara (MVA) containing the same murine CD8 T cell antigens as the plasmid pTH.HM, named MVA.HM, was a gift from Tomas Hanke, and has been previously described. Recombinant vaccinia virus (VV) containing the full-length ovalbumin ORF, rVV-OVA, was a gift from J. Yewdell, NIH, Bethesda, MA, and has been previously described.

Vaccination protocols

DNA was injected intramuscularly (i.m.). Mice were briefly anesthetized with isoflurane. The lower hind legs were shaved and 50 μg of plasmid DNA was injected into the calf muscle of each leg (100 μg total DNA per mouse each time vaccinated). Recombinant viruses were injected intraperitoneally (i.p.) using either 1 x 10^6 pfu. of MVA.HM or 2 x

10^5 pfu. of rVV-OVA. All antibodies were injected as a single dose i.p. on the same day as mice were injected with virus. For the experiments shown in Figures 2.1 and 2.2, 200 μg of mAb were injected per mouse. In the experiment shown in Figure 2.3, each animal received a total of 400 μg of Ab: 1) 400 μg Rat IgG; 2) 200 μg 3H3 and 200 μg Rat IgG; 3) 200 μg OX86 and 200 μg Rat IgG; or 4) 200 μg 3H3 and 200 μg OX86.

The rationale for this protocol is as follows. The in vivo half-life of another mAb, 1D8, which is also specific for 4-1BB and is the same isotype (rat IgG2a) as the 3H3 mAb, is approximately 7.5 days. Because 4-1BB is only expressed for a few days after T cell activation and then returns to a state of low or no expression, we administered a single dose of anti-4-1BB mAb at the same time as antigen, and assumed that maximal 4-1BB stimulation would be achieved at the time of 4-1BB expression on CD8 T cells over the following three to four days.

Because rat IgG induces an anti-rat IgG antibody response in mice, we were limited to administering one dose of antibody. We assumed that stimulating 4-1BB would rescue activated CD8 T cells from death, so we chose to administer the antibody at the time of the poxvirus boost, when the maximum number of activated antigen-specific CD8 T cells were obtained. We performed one pilot experiment administering anti-4-1BB mAb at the time of the DNA prime and found that the final result was similar to that observed when administered at the time of the poxvirus boost (data not shown).

Peptide predictions and synthesis

ORFs containing likely CD8 T cell epitopes were translated into amino acid sequences using MacVector (Accelrys, Inc), and candidate MHC-binding peptides were predicted

using SYFPEITHI version 1.0 (http://syfpeithi.bmi-heidelberg.com/) and/or the NIH program BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/). All 8-mer, 9-mer and 10-mer peptides were synthesized by GeneMed (South San Francisco, CA). HPLC analysis showed that all crude peptides were 65-95% pure. The mass of each peptide was confirmed by mass spectrometry.

Peptides were dissolved at 2 mg/ml in DMSO, stored at –20° C and used at 1 ug/ml in all experiments except where otherwise indicated. Overlapping 15-mer peptides were synthesized by Jerini (Berlin, Germany), at 50 nmol scale. These were dissolved in 50 µl of DMSO to yield an approximately 1 mM solution, and used at a final concentration of 10 µM for CD8 T cell stimulation.

Spleen cell preparations

Spleen cells were mechanically dissociated into single-cell suspensions through a nylon mesh, washed twice in Hank's balanced salt solution (HBSS) supplemented with 1% fetal bovine serum and penicillin/streptomycin, counted, then resuspended in complete T cell medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 200 mM L-glutamine, 50 µM 2-mercaptoethanol and penicillin/streptomycin).

Stimulation with peptides

Spleen cells were cultured in 200 ul of complete T cell medium in 96-well round-bottom plates at a density of 2-4 x 10^6 cells/well. For analysis of peptide-specific CD8 T cells, spleen cells were cultured for 5-6 hours in the presence of brefeldin A (GolgiPlug, BD Pharmingen, San Diego, CA) with or without peptide. Except where noted otherwise, 1

ug/ml synthetic peptide was used. For analysis of OVA-specific CD4 T cells, spleen cells were cultured with or without 400 ug/ml ovalbumin protein (Sigma) for 18 hours total, the last 8 hours in the presence of brefeldin A.

Intracellular cytokine staining

Cells were washed in FACS buffer (PBS, pH 7.4, supplemented with 1% fetal bovine serum and 0.1% sodium azide) and surface stained with PE-Cy5-conjugated anti-CD8α (clone CT-CD8α, CalTag Labs, Burlingame, CA), CyC-conjugated anti-CD8α (clone 53-6.7, eBioscience) or CyC-conjugated anti-CD4 (clone H129.19, BD Pharmingen). Cells were then washed twice with FACS buffer, fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's protocol, and stained intracellularly with FITC-conjugated anti-mouse IFN-γ (clone XMG1.2, eBioscience) or anti-mouse TNF-α (clone MP6-XT22, BD Pharmingen). All FACS samples were acquired on a FACSCalibur flow cytometer (BD Pharmingen) using CellQuest software. Data were analyzed using FloJo software (TreeStar, Inc., San Carlos, CA). To calculate the total number of antigen-specific CD8 T cells in a mouse spleen, the total number of spleen cells was multiplied by the percentage of spleen cells that were CD8+, which was multiplied by the percentage of CD8 T cells that were specific for antigen.

Direct ex vivo screening of ORF-specific CD8 T cells

K41 cells were plated at 4,000 cells per well in 96 well flat bottom plates. One day later, each well was transfected with approximately 500 ng of plasmid DNA and 0.5-1.0 μl of

FuGene 6 (Roche) according to the manufacturer's protocol. Two days later, the day of the T cell stimulation, the culture medium was replaced with RPMI complete. 0.8 x 10^6 splenocytes, prepared as described above, were added per well onto the K41 cells. Immediately afterwards, 1X brefeldin A (GolgiPlug, BD Pharmingen) was added to the cells to prevent cytokine secretion. After a 6 hour incubation at 37° C, 5% CO_2, duplicate wells were combined into a single well in 96 well round bottom plates. Cells were analyzed by intracellular cytokine staining as described above.

TNF-α Bioassay

The TNF-α bioassay has been previously described (Gold et al., 2002). K41 cells were plated at 4,000 cells per well in 96-well flat bottom plates, then transfected the following day, as described above, with each MCMV gene individually. On the day of the assay, 10^4 T cell clones were added to each well and incubated for a total of 6 hours. In a separate 96-well flat bottom plate, 4 x 10^4 WEHI 164 cells were plated in each well for use as the indicator cell line. Just prior to transfer of supernatent from the T cell clones to the WEHI 164 cells, medium containing the transcription inhibitors $LiCl_2$ (25 mM) and actinomycin D (2 μg/ml) were added to the WEHI 164 cells, which renders them susceptible to death in the presence of TNF-α. After an overnight incubation, Alamar Blue was added to the WEHI 164 cells at a ratio of 1:10 and the cells were incubated for 24 hours. Live WEHI 164 cells convert the dye from blue to pink, while dead cells are unable to metabolize the dye. The presence of TNF-α was determined 24 hours later by visual examination.

Statistical tests

p values were determined using a one-tailed Student's t-test.

References Cited

Ahlers JD, Belyakov IM, Berzofsky JA. (2003). Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines. Curr Mol Med *3*, 285-301.

Alp, N. J., Allport, T. D., Van Zanten, J., Rodgers, B., Sissons, J. G., and Borysiewicz, L. K. (1991). Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. J Virol *65*, 4812-4820.

Angulo, A., Ghazal, P., and Messerle, M. (2000). The major immediate-early gene ie3 of mouse cytomegalovirus is essential for viral growth. J Virol *74*, 11129-11136.

Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., and Lanier, L. L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science *296*, 1323-1326.

Badovinac, V. P., Porter, B. B., and Harty, J. T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat Immunol *3*, 619-626.

Balthesen, M., Messerle, M., and Reddehase, M. J. (1993). Lungs are a major organ site of cytomegalovirus latency and recurrence. J Virol *67*, 5360-5366.

Bancroft, G. J., Shellam, G. R., and Chalmer, J. E. (1981). Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. J Immunol *126*, 988-994.

Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Chee, M. S., Hutchison, C. A., 3rd, Kouzarides, T., Martignetti, J. A., Preddie, E., and et al. (1991). The DNA sequence of the human cytomegalovirus genome. DNA Seq *2*, 1-12.

Belz, G. T., Xie, W., Altman, J. D., and Doherty, P. C. (2000). A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-

specific CD8(+) T-cell response is much less apparent following secondary challenge. J Virol *74*, 3486-3493.

Bertram, E. M., Lau, P., and Watts, T. H. (2002). Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. J Immunol *168*, 3777-3785.

Brody, A. R., and Craighead, J. E. (1974). Pathogenesis of pulmonary cytomegalovirus infection in immunosuppressed mice. J Infect Dis *129*, 677-689.

Brown, M. G., Dokun, A. O., Heusel, J. W., Smith, H. R., Beckman, D. L., Blattenberger, E. A., Dubbelde, C. E., Stone, L. R., Scalzo, A. A., and Yokoyama, W. M. (2001). Vital involvement of a natural killer cell activation receptor in resistance to viral infection. Science *292*, 934-937.

Brown, M. G., Fulmek, S., Matsumoto, K., Cho, R., Lyons, P. A., Levy, E. R., Scalzo, A. A., and Yokoyama, W. M. (1997a). A 2-Mb YAC contig and physical map of the natural killer gene complex on mouse chromosome 6. Genomics *42*, 16-25.

Brown, M. G., Scalzo, A. A., Matsumoto, K., and Yokoyama, W. M. (1997b). The natural killer gene complex: a genetic basis for understanding natural killer cell function and innate immunity. Immunol Rev *155*, 53-65.

Brown, M. G., Zhang, J., Du, Y., Stoll, J., Yokoyama, W. M., and Scalzo, A. A. (1999). Localization on a physical map of the NKC-linked Cmv1 locus between Ly49b and the Prp gene cluster on mouse chromosome 6. J Immunol *163*, 1991-1999.

Brune, W., Menard, C., Heesemann, J., and Koszinowski, U. H. (2001). A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science *291*, 303-305.

Busch, D. H., Pilip, I. M., Vijh, S., and Pamer, E. G. (1998). Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity *8*, 353-362.

Butz, E., and Bevan, M. (1998). Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity *8*, 167-175.

Callan, M. F., Fazou, C., Yang, H., Rostron, T., Poon, K., Hatton, C., and McMichael, A. J. (2000). CD8(+) T-cell selection, function, and death in the primary immune response in vivo. J Clin Invest *106*, 1251-1261.

Callan, M. F., Steven, N., Krausa, P., Wilson, J. D., Moss, P. A., Gillespie, G. M., Bell, J. I., Rickinson, A. B., and McMichael, A. J. (1996). Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. Nat Med *2*, 906-911.

Callan, M. F., Tan, L., Annels, N., Ogg, G. S., Wilson, J. D., O'Callaghan, C. A., Steven, N., McMichael, A. J., and Rickinson, A. B. (1998). Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. J Exp Med *187*, 1395-1402.

Campbell, A. E., Slater, J. S., Cavanaugh, V. J., and Stenberg, R. M. (1992). An early event in murine cytomegalovirus replication inhibits presentation of cellular antigens to cytotoxic T lymphocytes. J Virol *66*, 3011-3017.

Cannons, J. L., Lau, P., Ghumman, B., DeBenedette, M. A., Yagita, H., Okumura, K., and Watts, T. H. (2001). 4-1BB ligand induces cell division, sustains survival, and

enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol *167*, 1313-1324.

Cardin, R. D., Abenes, G. B., Stoddart, C. A., and Mocarski, E. S. (1995). Murine cytomegalovirus IE2, an activator of gene expression, is dispensable for growth and latency in mice. Virology *209*, 236-241.

Catalina, M. D., Sullivan, J. L., Bak, K. R., and Luzuriaga, K. (2001). Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. J Immunol *167*, 4450-4457.

Chen W., Yewdell J.W., Levine R.L., and Bennink J.R. (1999). Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants. J Exp Med *189*, 1757-1764.

Cranmer, L. D., Clark, C. L., Morello, C. S., Farrell, H. E., Rawlinson, W. D., and Spector, D. H. (1996). Identification, analysis, and evolutionary relationships of the putative murine cytomegalovirus homologs of the human cytomegalovirus UL82 (pp71) and UL83 (pp65) matrix phosphoproteins. J Virol *70*, 7929-7939.

Crowe, S. R., Turner, S. J., Miller, S. C., Roberts, A. D., Rappolo, R. A., Doherty, P. C., Ely, K. H., and Woodland, D. L. (2003). Differential antigen presentation regulates the changing patterns of CD8+ T cell immunodominance in primary and secondary influenza virus infections. J Exp Med *198*, 399-410.

Daniels, K. A., Devora, G., Lai, W. C., O'Donnell, C. L., Bennett, M., and Welsh, R. M. (2001). Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. J Exp Med *194*, 29-44.

DeBenedette, M. A., Shahinian, A., Mak, T. W., and Watts, T. H. (1997). Costimulation of CD28- T lymphocytes by 4-1BB ligand. J Immunol *158*, 551-559.

DeBenedette, M. A., Wen, T., Bachmann, M. F., Ohashi, P. S., Barber, B. H., Stocking, K. L., Peschon, J. J., and Watts, T. H. (1999). Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. J Immunol *163*, 4833-4841.

del Val, M., Hengel, H., Hacker, H., Hartlaub, U., Ruppert, T., Lucin, P., and Koszinowski, U. H. (1992). Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J Exp Med *176*, 729-738.

Del Val, M., Munch, K., Reddehase, M. J., and Koszinowski, U. H. (1989). Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. Cell *58*, 305-315.

Del Val, M., Schlicht, H. J., Ruppert, T., Reddehase, M. J., and Koszinowski, U. H. (1991a). Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. Cell *66*, 1145-1153.

Del Val, M., Schlicht, H. J., Volkmer, H., Messerle, M., Reddehase, M. J., and Koszinowski, U. H. (1991b). Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. J Virol *65*, 3641-3646.

Del Val, M., Volkmer, H., Rothbard, J. B., Jonjic, S., Messerle, M., Schickedanz, J., Reddehase, M. J., and Koszinowski, U. H. (1988). Molecular basis for cytolytic T-

lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp89. J Virol *62*, 3965-3972.

Depatie, C., Muise, E., Lepage, P., Gros, P., and Vidal, S. M. (1997). High-resolution linkage map in the proximity of the host resistance locus Cmv1. Genomics *39*, 154-163.

Diehl, L., van Mierlo, G. J., den Boer, A. T., van der Voort, E., Fransen, M., van Bostelen, L., Krimpenfort, P., Melief, C. J., Mittler, R., Toes, R. E., and Offringa, R. (2002). In vivo triggering through 4-1BB enables Th-independent priming of CTL in the presence of an intact CD28 costimulatory pathway. J Immunol *168*, 3755-3762.

Doherty, P. C., and Christensen, J. P. (2000). Accessing complexity: the dynamics of virus-specific T cell responses. Annu Rev Immunol *18*, 561-592.

Elkington, R., Walker, S., Crough, T., Menzies, M., Tellam, J., Bharadwaj, M., and Khanna, R. (2003). Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. J Virol *77*, 5226-5240.

Evans, D. E., Prell, R. A., Thalhofer, C. J., Hurwitz, A. A., and Weinberg, A. D. (2001). Engagement of OX40 enhances antigen-specific CD4(+) T cell mobilization/memory development and humoral immunity: comparison of alphaOX-40 with alphaCTLA-4. J Immunol *167*, 6804-6811.

Falk, K., Rotzschke, O., Deres, K., Metzger, J., Jung, G., and Rammensee, H. G. (1991). Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. J Exp Med *174*, 425-434.

Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A., and Wilson, I. A. (1992). Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. Science *257*, 919-927.

Gold, M. C., Munks, M. W., Wagner, M., Koszinowski, U. H., Hill, A. B., and Fling, S. P. (2002). The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by M45-specific CTL but does not alter the immunodominance of the M45-specific CD8 T cell response in vivo. J Immunol *169*, 359-365.

Gold, M. C. (2002b). The effects of murine cytomegalovirus immunomodulatory genes on the CD8 T cell response to murine cytomegalovirus in H-2(b) mice. Dissertation.

Gonzalez Armas, J. C., Morello, C. S., Cranmer, L. D., and Spector, D. H. (1996). DNA immunization confers protection against murine cytomegalovirus infection. J Virol *70*, 7921-7928.

Goulder, P. J., and Walker, B. D. (1999). The great escape - AIDS viruses and immune control. Nat Med *5*, 1233-1235.

Gramaglia, I., Jember, A., Pippig, S. D., Weinberg, A. D., Killeen, N., and Croft, M. (2000). The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J Immunol *165*, 3043-3050.

Gramaglia, I., Weinberg, A. D., Lemon, M., and Croft, M. (1998). Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J Immunol *161*, 6510-6517.

Halstead, E. S., Mueller, Y. M., Altman, J. D., and Katsikis, P. D. (2002). In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. Nat Immunol *3*, 536-541.

Hanke, T, McMichael, AJ, Mwau, M, Wee, EG, Ceberej, I, Patel, S, Sutton, J, Tomlinson, M, Samuel, RV. (2002). Development of a DNA-MVA/HIVA vaccine for Kenya. Vaccine *20*, 1995-8.

Hanshaw, J. B. (1994). Congenital cytomegalovirus infection. Pediatr Ann *23*, 124-128.

Hanson, L. K., Dalton, B. L., Karabekian, Z., Farrell, H. E., Rawlinson, W. D., Stenberg, R. M., and Campbell, A. E. (1999). Transcriptional analysis of the murine cytomegalovirus HindIII-I region: identification of a novel immediate-early gene region. Virology *260*, 156-164.

Hengel, H., Reusch, U., Geginat, G., Holtappels, R., Ruppert, T., Hellebrand, E., and Koszinowski, U. H. (2000). Macrophages escape inhibition of major histocompatibility complex class I-dependent antigen presentation by cytomegalovirus. J Virol *74*, 7861-7868.

Henry, S. C., and Hamilton, J. D. (1993). Detection of murine cytomegalovirus immediate early 1 transcripts in the spleens of latently infected mice. J Infect Dis *167*, 950-954.

Hislop, A. D., Annels, N. E., Gudgeon, N. H., Leese, A. M., and Rickinson, A. B. (2002). Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. J Exp Med *195*, 893-905.

Holtappels, R., Podlech, J., Pahl-Siebert, M., Julch, M., Thomas, D., Simon, C.O., Wagner, M., Reddehase, M.J. (2004). Cytomegalovirus misleads its host by priming of CD8 T cells specific for an epitope not presented in infected tissues. J Exp Med *199*, 1-7.

Holtappels, R., Grzimek, N. K., Simon, C. O., Thomas, D., Dreis, D., and Reddehase, M. J. (2002a). Processing and presentation of murine cytomegalovirus pORFm164-derived peptide in fibroblasts in the face of all viral immunosubversive early gene functions. J Virol *76*, 6044-6053.

Holtappels, R., Grzimek, N. K., Thomas, D., and Reddehase, M. J. (2002b). Early gene m18, a novel player in the immune response to murine cytomegalovirus. J Gen Virol *83*, 311-316.

Holtappels, R., Pahl-Seibert, M. F., Thomas, D., and Reddehase, M. J. (2000a). Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62L(lo) memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. J Virol *74*, 11495-11503.

Holtappels, R., Podlech, J., Geginat, G., Steffens, H. P., Thomas, D., and Reddehase, M. J. (1998). Control of murine cytomegalovirus in the lungs: relative but not absolute immunodominance of the immediate-early 1 nonapeptide during the antiviral cytolytic T-lymphocyte response in pulmonary infiltrates. J Virol *72*, 7201-7212.

Holtappels, R., Podlech, J., Grzimek, N. K., Thomas, D., Pahl-Seibert, M. F., and Reddehase, M. J. (2001). Experimental preemptive immunotherapy of murine cytomegalovirus disease with CD8 T-cell lines specific for ppM83 and pM84, the two homologs of human cytomegalovirus tegument protein ppUL83 (pp65). J Virol *75*, 6584-6600.

Holtappels, R., Thomas, D., Podlech, J., Geginat, G., Steffens, H. P., and Reddehase, M. J. (2000b). The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells. J Virol *74*, 1871-1884.

Holtappels, R., Thomas, D., Podlech, J., and Reddehase, M. J. (2002c). Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2d haplotype. J Virol *76*, 151-164.

Holtappels, R., Thomas, D., and Reddehase, M. J. (2000c). Identification of a K(d)-restricted antigenic peptide encoded by murine cytomegalovirus early gene M84. J Gen Virol *81*, 3037-3042.

Hummel, M., Zhang, Z., Yan, S., DePlaen, I., Golia, P., Varghese, T., Thomas, G., and Abecassis, M. I. (2001). Allogeneic transplantation induces expression of cytomegalovirus immediate-early genes in vivo: a model for reactivation from latency. J Virol *75*, 4814-4822.

Hurtado, J. C., Kim, Y. J., and Kwon, B. S. (1997). Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. J Immunol *158*, 2600-2609.

Jonjic, S., del Val, M., Keil, G. M., Reddehase, M. J., and Koszinowski, U. H. (1988). A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. J Virol *62*, 1653-1658.

Jonjic, S., Mutter, W., Weiland, F., Reddehase, M. J., and Koszinowski, U. H. (1989). Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J Exp Med *169*, 1199-1212.

Jonjic, S., Pavic, I., Polic, B., Crnkovic, I., Lucin, P., and Koszinowski, U. H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J Exp Med *179*, 1713-1717.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K.,Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E., Littman, D., Lang, R. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Karrer, U., Sierro, S., Wagner, M., Oxenius, A., Hengel, H., Koszinowski, U. H., Phillips, R. E., and Klenerman, P. (2003). Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J Immunol *170*, 2022-2029.

Kast, W. M., Roux, L., Curren, J., Blom, H. J., Voordouw, A. C., Meloen, R. H., Kolakofsky, D., and Melief, C. J. (1991). Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. Proc Natl Acad Sci U S A *88*, 2283-2287.

Kavanagh, D. G., Gold, M. C., Wagner, M., Koszinowski, U. H., and Hill, A. B. (2001a). The multiple immune-evasion genes of murine cytomegalovirus are not redundant: m4 and m152 inhibit antigen presentation in a complementary and cooperative fashion. J Exp Med *194*, 967-978.

Kavanagh, D. G., Koszinowski, U. H., and Hill, A. B. (2001b). The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment. J Immunol *167*, 3894-3902.

Khan, N., Shariff, N., Cobbold, M., Bruton, R., Ainsworth, J. A., Sinclair, A. J., Nayak, L., and Moss, P. A. (2002). Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol *169*, 1984-1992.

Kjaergaard, J., Peng, L., Cohen, P. A., Drazba, J. A., Weinberg, A. D., and Shu, S. (2001). Augmentation versus inhibition: effects of conjunctional OX-40 receptor monoclonal antibody and IL-2 treatment on adoptive immunotherapy of advanced tumor. J Immunol *167*, 6669-6677.

Kleijnen, M. F., Huppa, J. B., Lucin, P., Mukherjee, S., Farrell, H., Campbell, A. E., Koszinowski, U. H., Hill, A. B., and Ploegh, H. L. (1997). A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. Embo J *16*, 685-694.

Koffron, A. J., Hummel, M., Patterson, B. K., Yan, S., Kaufman, D. B., Fryer, J. P., Stuart, F. P., and Abecassis, M. I. (1998). Cellular localization of latent murine cytomegalovirus. J Virol *72*, 95-103.

Kopf, M., Ruedl C., Schmitz N., Gallimore A., Lefrang K., Ecabert B., Odermatt B., and Bachmann M.F. (1999). OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. Immunity *11*, 699-708.

Koszinowski, U. H., Reddehase, M. J., Keil, G. M., and Schickedanz, J. (1987). Host immune response to cytomegalovirus: products of transfected viral immediate-early genes are recognized by cloned cytolytic T lymphocytes. J Virol *61*, 2054-2058.

Krmpotic, A., Busch, D. H., Bubic, I., Gebhardt, F., Hengel, H., Hasan, M., Scalzo, A. A., Koszinowski, U. H., and Jonjic, S. (2002). MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. Nat Immunol *3*, 529-535.

Krmpotic, A., Messerle, M., Crnkovic-Mertens, I., Polic, B., Jonjic, S., and Koszinowski, U. H. (1999). The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. J Exp Med *190*, 1285-1296.

Kurz, S., Steffens, H. P., Mayer, A., Harris, J. R., and Reddehase, M. J. (1997). Latency versus persistence or intermittent recurrences: evidence for a latent state of murine cytomegalovirus in the lungs. J Virol *71*, 2980-2987.

Kurz, S. K., Rapp, M., Steffens, H. P., Grzimek, N. K., Schmalz, S., and Reddehase, M. J. (1999). Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. J Virol *73*, 482-494.

Kurz, S. K., and Reddehase, M. J. (1999). Patchwork pattern of transcriptional reactivation in the lungs indicates sequential checkpoints in the transition from murine cytomegalovirus latency to recurrence. J Virol *73*, 8612-8622.

Lee, S. H., Girard, S., Macina, D., Busa, M., Zafer, A., Belouchi, A., Gros, P., and Vidal, S. M. (2001). Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. Nat Genet *28*, 42-45.

Lodoen, M., Ogasawara, K., Hamerman, J. A., Arase, H., Houchins, J. P., Mocarski, E. S., and Lanier, L. L. (2003). NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J Exp Med *197*, 1245-1253.

LoPiccolo, D. M., Gold, M. C., Kavanagh, D. G., Wagner, M., Koszinowski, U. H., and Hill, A. B. (2003). Effective inhibition of K(b)- and D(b)-restricted antigen presentation in primary macrophages by murine cytomegalovirus. J Virol *77*, 301-308.

Lucin, P., Pavic, I., Polic, B., Jonjic, S., and Koszinowski, U. H. (1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J Virol *66*, 1977-1984.

Maxwell, J. R., Weinberg, A., Prell, R. A., and Vella, A. T. (2000). Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. J Immunol *164*, 107-112.

McLaughlin-Taylor, E., Pande, H., Forman, S. J., Tanamachi, B., Li, C. R., Zaia, J. A., Greenberg, P. D., and Riddell, S. R. (1994). Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol *43*, 103-110.

McMahon, C.W., Zajac A.J., Jamieson A.M., Corral L., Hammer G.E., Ahmed R., and Raulet D.H. (2002). Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells. J Immunol *169,* 1444-1452.

Meij, P, Leen, A, Rickinson, AB, Verkoeijen, S, Vervoort, MB, Bloemena, E, Middeldorp, JM (2002). Identification and prevalence of CD8(+) T-cell responses directed against Epstein-Barr virus-encoded latent membrane protein 1 and latent membrane protein 2. Int J Cancer *99*, 93-9.

Melero, I., Shuford, W. W., Newby, S. A., Aruffo, A., Ledbetter, J. A., Hellstrom, K. E., Mittler, R. S., and Chen, L. (1997). Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med *3*, 682-685.

Menard, C., Wagner, M., Ruzsics, Z., Holak, K., Brune, W., Campbell, A. E., and Koszinowski, U. H. (2003). Role of murine cytomegalovirus US22 gene family members in replication in macrophages. J Virol *77*, 5557-5570.

Messerle, M., Buhler, B., Keil, G. M., and Koszinowski, U. H. (1992). Structural organization, expression, and functional characterization of the murine cytomegalovirus immediate-early gene 3. J Virol *66*, 27-36.

Messerle, M., Keil, G. M., and Koszinowski, U. H. (1991). Structure and expression of murine cytomegalovirus immediate-early gene 2. J Virol *65*, 1638-1643.

Morello, C. S., Cranmer, L. D., and Spector, D. H. (2000). Suppression of murine cytomegalovirus (MCMV) replication with a DNA vaccine encoding MCMV M84 (a homolog of human cytomegalovirus pp65). J Virol *74*, 3696-3708.

Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J., Zajac, A. J., Miller, J. D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177-187.

Pamer, E, Sijts, A., Villanueva, M., Busch, D., and Vijh, S. (1997). MHC class I antigen processing of Listeria monocytogenes proteins: implications for dominant and subdominant CTL responses. Immunol Rev *158,* 129.

Pavic, I., Polic, B., Crnkovic, I., Lucin, P., Jonjic, S., and Koszinowski, U. H. (1993). Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection. J Gen Virol *74 (Pt 10)*, 2215-2223.

Polic, B., Hengel, H., Krmpotic, A., Trgovcich, J., Pavic, I., Luccaronin, P., Jonjic, S., and Koszinowski, U. H. (1998). Hierarchical and redundant lymphocyte subset control

precludes cytomegalovirus replication during latent infection. J Exp Med *188*, 1047-1054.

Pollok, K. E., Kim, Y. J., Hurtado, J., Zhou, Z., Kim, K. K., and Kwon, B. S. (1994). 4-1BB T-cell antigen binds to mature B cells and macrophages, and costimulates anti-mu-primed splenic B cells. Eur J Immunol *24*, 367-374.

Pollok, K. E., Kim, Y. J., Zhou, Z., Hurtado, J., Kim, K. K., Pickard, R. T., and Kwon, B. S. (1993). Inducible T cell antigen 4-1BB. Analysis of expression and function. J Immunol *150*, 771-781.

Popkin, D. L., and Virgin, H. W. t. (2003). Murine cytomegalovirus infection inhibits tumor necrosis factor alpha responses in primary macrophages. J Virol *77*, 10125-10130.

Quinnan, G. V., Manischewitz, J. E., and Ennis, F. A. (1978). Cytotoxic T lymphocyte response to murine cytomegalovirus infection. Nature *273*, 541-543.

Rawlinson, W. D., Farrell, H. E., and Barrell, B. G. (1996). Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol *70*, 8833-8849.

Reddehase, M. J. (2002). Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat Rev Immunol *2*, 831-844.

Reddehase, M. J., Balthesen, M., Rapp, M., Jonjic, S., Pavic, I., and Koszinowski, U. H. (1994). The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. J Exp Med *179*, 185-193.

Reddehase, M. J., Buhring, H. J., and Koszinowski, U. H. (1986). Cloned long-term cytolytic T-lymphocyte line with specificity for an immediate-early membrane antigen of murine cytomegalovirus. J Virol *57*, 408-412.

Reddehase, M. J., Jonjic, S., Weiland, F., Mutter, W., and Koszinowski, U. H. (1988). Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors. J Virol *62*, 1061-1065.

Reddehase, M. J., Keil, G. M., and Koszinowski, U. H. (1984). The cytolytic T lymphocyte response to the murine cytomegalovirus. II. Detection of virus replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur J Immunol *14*, 56-61.

Reddehase, M. J., and Koszinowski, U. H. (1984). Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature *312*, 369-371.

Reddehase, M. J., Mutter, W., and Koszinowski, U. H. (1987). In vivo application of recombinant interleukin 2 in the immunotherapy of established cytomegalovirus infection. J Exp Med *165*, 650-656.

Reddehase, M. J., Rothbard, J. B., and Koszinowski, U. H. (1989). A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. Nature *337*, 651-653.

Reddehase, M. J., Weiland, F., Munch, K., Jonjic, S., Luske, A., and Koszinowski, U. H. (1985). Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J Virol *55*, 264-273.

Redpath, S., Angulo, A., Gascoigne, N. R., and Ghazal, P. (1999). Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. J Immunol *162*, 6701-6707.

Reits, E. A., Vos, J. C., Gromme, M., and Neefjes, J. (2000). The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature *404*, 774-778.

Reusch, U., Muranyi, W., Lucin, P., Burgert, H. G., Hengel, H., and Koszinowski, U. H. (1999). A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. Embo J *18*, 1081-1091.

Roder, J., and Duwe, A. (1979). The beige mutation in the mouse selectively impairs natural killer cell function. Nature *278*, 451-453.

Rodriguez, F., Slifka, M. K., Harkins, S., and Whitton, J. L. (2001). Two overlapping subdominant epitopes identified by DNA immunization induce protective CD8(+) T-cell populations with differing cytolytic activities. J Virol *75*, 7399-7409.

Rogers, P. R., Song, J., Gramaglia, I., Killeen, N., and Croft, M. (2001). OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity *15*, 445-455.

Scalzo, A. A., Elliott, S. L., Cox, J., Gardner, J., Moss, D. J., and Suhrbier, A. (1995a). Induction of protective cytotoxic T cells to murine cytomegalovirus by using a nonapeptide and a human-compatible adjuvant (Montanide ISA 720). J Virol *69*, 1306-1309.

Scalzo, A. A., Fitzgerald, N. A., Simmons, A., La Vista, A. B., and Shellam, G. R. (1990). Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. J Exp Med *171*, 1469-1483.

Scalzo, A. A., Fitzgerald, N. A., Wallace, C. R., Gibbons, A. E., Smart, Y. C., Burton, R. C., and Shellam, G. R. (1992). The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. J Immunol *149*, 581-589.

Scalzo, A. A., Lyons, P. A., Fitzgerald, N. A., Forbes, C. A., and Shellam, G. R. (1995b). The BALB.B6-Cmv1r mouse: a strain congenic for Cmv1 and the NK gene complex. Immunogenetics *41*, 148-151.

Scalzo, A. A., Lyons, P. A., Fitzgerald, N. A., Forbes, C. A., Yokoyama, W. M., and Shellam, G. R. (1995c). Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene complex-associated loci Ly49 and musNKR-P1. Genomics *27*, 435-441.

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature *404*, 770-774.

Shellam, G. R., Allan, J. E., Papadimitriou, J. M., and Bancroft, G. J. (1981). Increased susceptibility to cytomegalovirus infection in beige mutant mice. Proc Natl Acad Sci U S A *78*, 5104-5108.

Slifka, M. K., Blattman, J. N., Sourdive, D. J., Liu, F., Huffman, D. L., Wolfe, T., Hughes, A., Oldstone, M. B., Ahmed, R., and Von Herrath, M. G. (2003). Preferential escape of subdominant CD8+ T cells during negative selection results in an altered antiviral T cell hierarchy. J Immunol *170*, 1231-1239.

Smith, H. R., Chuang, H. H., Wang, L. L., Salcedo, M., Heusel, J. W., and Yokoyama, W. M. (2000). Nonstochastic coexpression of activation receptors on murine natural killer cells. J Exp Med *191*, 1341-1354.

Smith, H. R., Heusel, J. W., Mehta, I. K., Kim, S., Dorner, B. G., Naidenko, O. V., Iizuka, K., Furukawa, H., Beckman, D. L., Pingel, J. T., *et al.* (2002). Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc Natl Acad Sci U S A *99*, 8826-8831.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell *91*, 119-126.

Starr, S. E., and Allison, A. C. (1977). Role of T lymphocytes in recovery from murine cytomegalovirus infection. Infect Immun *17*, 458-462.

Steffens, H. P., Kurz, S., Holtappels, R., and Reddehase, M. J. (1998). Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence. J Virol *72*, 1797-1804.

Stuber, E., Neurath, M., Calderhead, D., Fell, H. P., and Strober, W. (1995). Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity *2*, 507-521.

Takahashi, C., Mittler, R. S., and Vella, A. T. (1999). Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. J Immunol *162*, 5037-5040.

Tan, J. T., Whitmire, J. K., Ahmed, R., Pearson, T. C., and Larsen, C. P. (1999). 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. J Immunol *163*, 4859-4868.

Tan, J. T., Whitmire, J. K., Murali-Krishna, K., Ahmed, R., Altman, J. D., Mittler, R. S., Sette, A., Pearson, T. C., and Larsen, C. P. (2000). 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination. J Immunol *164*, 2320-2325.

Thale, R., Szepan, U., Hengel, H., Geginat, G., Lucin, P., and Koszinowski, U. H. (1995). Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility complex class I molecule transport. J Virol *69*, 6098-6105.

Volkmer, H., Bertholet, C., Jonjic, S., Wittek, R., and Koszinowski, U. H. (1987). Cytolytic T lymphocyte recognition of the murine cytomegalovirus nonstructural immediate-early protein pp89 expressed by recombinant vaccinia virus. J Exp Med *166*, 668-677.

Wagner, M., Gutermann, A., Podlech, J., Reddehase, M. J., and Koszinowski, U. H. (2002). Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J Exp Med *196*, 805-816.

Wallace, M. E., Keating, R., Heath, W. R., and Carbone, F. R. (1999). The cytotoxic T-cell response to herpes simplex virus type 1 infection of C57BL/6 mice is almost entirely directed against a single immunodominant determinant. J Virol *73*, 7619-7626.

Weinberg, A. D., Rivera, M. M., Prell, R., Morris, A., Ramstad, T., Vetto, J. T., Urba, W. J., Alvord, G., Bunce, C., and Shields, J. (2000). Engagement of the OX-40 receptor in vivo enhances antitumor immunity. J Immunol *164*, 2160-2169.

Weinberg, A. D., Wegmann, K. W., Funatake, C., and Whitham, R. H. (1999). Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. J Immunol *162*, 1818-1826.

Wong, P., and Pamer, E. G. (2003). Feedback regulation of pathogen-specific T cell priming. Immunity *18*, 499-511.

Ye, M., Morello, C. S., and Spector, D. H. (2002a). Strong CD8 T-cell responses following coimmunization with plasmids expressing the dominant pp89 and subdominant M84 antigens of murine cytomegalovirus correlate with long-term protection against subsequent viral challenge. J Virol *76*, 2100-2112.

Ye, Z., Hellstrom, I., Hayden-Ledbetter, M., Dahlin, A., Ledbetter, J. A., and Hellstrom, K. E. (2002b). Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB. Nat Med *8*, 343-348.

Yewdell, J. W., and Bennink, J. R. (1999). Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol *17*, 51-88.

Zhou, Z., Pollok, K. E., Kim, K. K., Kim, Y. J., and Kwon, B. S. (1994). Functional analysis of T-cell antigen 4-1BB in activated intestinal intra-epithelial T lymphocytes. Immunol Lett *41*, 177-184.

Ziegler, H., Muranyi, W., Burgert, H. G., Kremmer, E., and Koszinowski, U. H. (2000). The luminal part of the murine cytomegalovirus glycoprotein gp40 catalyzes the retention of MHC class I molecules. Embo J *19*, 870-881.

Ziegler, H., Thale, R., Lucin, P., Muranyi, W., Flohr, T., Hengel, H., Farrell, H., Rawlinson, W., and Koszinowski, U. H. (1997). A mouse cytomegalovirus glycoprotein

retains MHC class I complexes in the ERGIC/cis-Golgi compartments. Immunity *6*, 57-66.