# The effects of murine cytomegalovirus immunomodulatory genes on the CD8 T cell response to murine cytomegalovirus in H-2$^b$ mice

by

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## Abstract

Murine cytomegalovirus (MCMV) is a herpesvirus family member and characteristically remains with its host for life. Herpesviruses have evolved myriad mechanisms to evade the immune system, and specifically, some of these genes interfere with the MHC class I pathway. MCMV encodes three genes that are known to interfere the class I pathway as assessed by $^{51}Cr$ release assays and biochemical methods. m6/gp48 redirects class I to the lysosome for degradation and functionally interferes with the ability of CTL clones to kill virus-infected fibroblasts. m4/gp34 binds class I molecules in the ER and at the cell surface, and blocks the ability of $K^b$ but not $D^b$-restricted CTL to kill virus-infected fibroblasts. This *m4* effect is dependent on a third known immunomodulatory gene *m152*. m152/gp40 retains class I molecules in the ER cis-Golgi intermediate compartment. *m152*'s effects are profound. None of the MCMV-specific CTL clones we have isolated are able to kill wildtype MCMV-infected fibroblasts while removal of *m152* alone is sufficient to restore good killing of those target cells. Because *m152* is so effective at inhibiting recognition by CTL clones *in vitro*, we hypothesized that the CTL response to MCMV would be affected by *m152*. Furthermore, we hypothesized that the MCMV-specific CTL response would be constrained to respond to antigens least affected by *m152* resulting in an alteration of the CTL repertoire. We addressed these hypotheses by examining the CTL response to wildtype MCMVs or mutant MCMVs lacking *m152*. Although *m152* completely blocks antigen presentation of MCMV antigens in infected fibroblasts, the size and the kinetics of the MCMV-specific CTL response *in vivo* was not altered by *m152*. Furthermore, *m152* did not prevent the establishment of latency. Finally, the CD8 response to the M45 antigen was not altered by *m152* indicating that although the MCMV immunomodulatory gene *m152* severely inhibits antigen presentation to CTL *in vitro*, *m152* does not alter the immunodominance of the M45 antigen. This work requires us to reconsider the assumption that class I immune evasion genes modulate the

CD8 T cell response *in vivo* and furthermore, leaves open the question of the purpose of class I immune evasion genes in the virus-host relationship.

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## List of Abbreviations

AICD-activation-induced cell death

β2m -β2microglobulin

B6 –C57Bl/6

CDR-complementarity determining region

CTL- Cytotoxic T lymphocyte

DCs-dendritic cells

EBV-Epstein-Barr virus

HC-class I heavy chain

HSV-herpes simplex virus

ICS-intracellular cytokine staining

IM-infectious mononucleosis

ITAM-immunoreceptor tyrosine-based activation motif

LDA-limiting dilution analysis

LM-Listeria monocytogenes

LPS-lipoploysaccharide

MEF-mouse embryo fibroblast

m.o.i.- multiplicity of infection

MHV-68-murine γ-herpesvirus 68

NKs-Natural killer cells

ORFs-open reading frames

pfu-plaque forming unit

TCR-T cell receptor

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**Title: The effects of MCMV immunomodulatory genes on the CD8 T cell response to MCMV in H-2$^b$ mice.**

## Introduction.

The cytomegaloviruses are ubiquitous viruses that cause little to no pathology in their natural hosts unless they are immunocompromised. As a result, the importance of human cytomegalovirus (HCMV) as a pathogen has dramatically grown due to the AIDS epidemic and the increasing numbers of people who receive immunosuppressive treatments following transplantation (Britt and Alford, 1996). Both HCMV and murine cytomegalovirus (MCMV) [a] are betaherpesvirus subfamily members. As such, they share many characteristics, and MCMV has been used as a model for HCMV infection. MCMV, like other herpesviruses, establishes latency and must reactivate in the face of a primed immune response to spread to a new host.

CD8 T cells are an important aspect of host immune control to both HCMV and MCMV infections. Not so coincidentally perhaps, the betaherpesviruses, as well as all studied herpesviruses, encode genes that interfere with the major histocompatibility complex (MHC) [a] class I pathway, the pathway used by CD8 T cells to recognize virus-infected cells. Although it is clear that the mechanisms used by different herpesviruses differ in their molecular intricacies, the outcomes are similar with the resulting effect inducing a disruption of the MHC class I pathway as assessed by *in vitro* assays. However, studies are limited on how these immunomodulatory genes affect the host immune response *in vivo*. Moreover, no studies have specifically focused on the role of such immunomodulatory genes on the size and specificity of the CD8 T cell response *in vivo*; this topic has been the focus of my thesis work.

Limitations inherent in studying pathogenesis in humans lead us to determine the role of immunomodulatory genes on the CD8 T cell response to MCMV in mice. Because studies on the CTL response to MCMV in C57Bl/6 mice were limited, I first

began characterizing the CD8 T cell response to MCMV in this mouse strain. Furthermore, I placed a strong emphasis on studying the role of class I immunomodulatory genes encoded by MCMV, and their effects on H-$2^b$-restricted CD8 T cells *in vivo* and *in vitro*. This introduction comprises an overview on MCMV, the CTL response to MCMV and other viruses, and a description of immune evasion strategies with an emphasis on those that interfere with the MHC class I antigen processing and presentation pathway.

## CMV

### Classification.

The *Herpesviridae* family is divided into three subfamilies: the *Alphaherpesvirinae* (the herpes simplex-like viruses), the *Betaherpesvirinae* (the cytomegaloviruses), and the *Gammaherpesvirinae* (the lymphocyte-associated herpesviruses). The cytomegaloviruses are the principal members of the betaherpesvirus subfamily. They share some characteristics with other herpesviruses such as virion structure, a large double-stranded linear DNA genome, and importantly, the ability to establish persistent and latent infections. However, the qualities that originally established the cytomegaloviruses as a separate subfamily were: a tropism for the salivary glands, their slow growth in cultured cells, and their species specificity. There are two noteworthy aspects of this species specificity. First, most mammalian species are infected by a CMV, a demonstration of their ubiquity and their highly successful evolutionary strategy. Second, a CMV specific for one species cannot cross the species barrier and infect other species (Mocarski, 1996). Phylogenetic analyses of the *Herpesviridae* family have confirmed the three subfamily classification based on molecular studies (McGeoch et al., 1995). Evolutionary studies concluded that the three subfamilies of herpesviruses arose about 200 million years ago, and that co-speciation with host lineages took place alongside the mammalian radiation

over the last 80 million years (McGeoch et al., 2000). Thus, each CMV has closely co-evolved with its host species.

**Genome.**

Both HCMV and MCMV genomes have been sequenced, are of 230 and 235 kbp, and have 208 and 170 predicted ORFs respectively (Chee et al., 1990; Rawlinson et al., 1996). The HCMV genome is the only betaherpesvirus with an "E" genome structure: an arrangement of unique and inverted repeats (Mocarski, 1996). Other betaherpesviruses, including MCMV, have less complex genome arrangements, which include a single unique sequence with short direct repeats at either end (Rawlinson et al., 1996). Both genomes have a high G+ C content of approximately 59%. HCMV and MCMV genomes are collinear. MCMV genes are named based on predicted open reading frames (ORFs) starting from the left end of the genome with "m1" and ending to the right with "m170". HCMV and MCMV share homology in 4 of 6 gene families (78% nucleotide homology) that are located in the center of the MCMV genome. However, two sections at either the left and the right ends of the genome are only found in MCMV (Rawlinson et al., 1996). These sections encode genes that may be the result of pressures from the host immune response; these include the viral immune evasion genes *m4, m6*, and *m152*.

Prior to the availability of the MCMV sequence, the MCMV genome was mapped using the HindIII restriction enzyme (Ebeling et al., 1983). Sixteen fragments were generated and named A through P according to size with A being the largest fragment. Fifteen of these fragments were cloned into pUC-based plasmids and were used as source material for the MCMV genomic DNA library described in chapter 2.

**Virus growth cycle.**

Attachment and penetration of the virions is poorly defined. Interestingly, the restricted host range of the CMVs is not due to these preliminary steps but rather a block in viral gene expression that occurs post penetration. DNA replication occurs after circularization of the viral DNA. Genes are transcribed and translated in a coordinately regulated fashion

with the sequential expression of three different classes of genes: immediate early (IE), early (E), and late (L). These designations are dependent on both the timing of gene expression as well as the sensitivity of genes to specific inhibitors of transcription, translation, and DNA replication. A host RNA polymerase initially transcribes IE genes from the major immediate early enhancer-promoter. This results in IE gene products that act as trans-activators for E viral promoters as well as host cellular promoters. While some E gene products trans-activate the expression of other E genes, some result in products directly responsible for DNA synthesis. L gene products are primarily structural, and by definition, require that DNA synthesis occur before their expression (Mocarski, 1996). Transcription and translation of genes from IE,E, and L stages can be manipulated *in vitro* with the use of drugs, to allow the dissection of stage-specific events. Infection of cells with MCMV, in the presence of the protein synthesis inhibitor cycloheximide, allows the enhanced transcription of IE genes only. To then allow the selective expression of those IE proteins, but prevent new transcription of E and L genes, the drug is switched to the transcription inhibitor actinomycin D. Because E genes are dependent on IE ones, it is impossible to selectively express E genes without IE genes. However, L gene expression can be blocked with the use of phosphonoacetic acid, a DNA synthesis inhibitor, resulting in the selective expression of the combination of IE and E genes. As will be apparent later, we and others have used these drug blockades to identify the stage of gene expression responsible for specific MCMV antigens (Del Val et al., 1989).

CMVs encode functions to achieve viral DNA replication and packaging which is thought to occur after capsids are formed. Virion egress is poorly understood but the resulting product is a mature nucleocapsid surrounded by a tegument, enveloped by a lipid bilayer that contains modified viral glycoproteins (Mocarski, 1996). The complex and coordinated gene expression strategy used by CMVs is an important aspect of the virus life cycle. Whether viral genes eventually result in the expression of antigens or immune evasion genes, they are subject to this complex genetic control. Therefore, it is

likely that subsequent immunological responses are intimately linked to the timing of specific gene expression; potential consequences of the timing of gene expression will be discussed later in this dissertation.

**Infection and pathogenesis.**

HCMV infects 50 to 90% of the human population and is associated with lower socioeconomic status (Stagno et al., 1986). Infection usually occurs within the first few years of life. CMVs are spread by direct contact with infectious bodily fluids such as blood, urine, and saliva as well as CMV-infected transplanted organs. Although CMVs cause no overt pathology in the immunocompetent host, severe morbidity and mortality can occur in immunocompromised individuals (Britt and Alford, 1996). Congenital infection of HCMV can cause severe birth defects; HCMV is the most common congenital virus infection occurring in 0.2 to 2.2. % of live births, (Yow and Demmler, 1992). This is in contrast to MCMV, where no evidence exists for cross-placental transmission resulting in congenital infection (Mocarski, 1996).

Once the acute phase of disease is resolved, HCMV and MCMV, like most herpesviruses, establish latency in their hosts. MCMV DNA is detectable in diverse organs including the heart, kidney, liver, lung, spleen, brain, and salivary glands (Collins et al., 1993). Latency is defined as the presence of viral DNA without the production of infectious virions, while in persistence, infectious virus remains present. The apparent lack of productive virions, as assessed by the most sensitive methods available, argues for MCMV establishing a latent rather than a persistent infection in the immunocompetent host (Kurz et al., 1997; Pollock and Virgin, 1995). As will be discussed later, the pattern of resolution of MCMV in different organs varies with the specific mouse strain due to the presence of a polymorphic NK receptor. Differences exists in the kinetics of resolution of acute MCMV infection in different mouse strains although in most organs, replicating MCMV can be detected no later than three weeks post-infection. However, MCMV persists in the salivary glands up to 8 weeks post infection before resulting in the

establishment of latency (Pollock and Virgin, 1995). Both macrophages and endothelial cells harbor latent virus (Koffron et al., 1998; Pollock et al., 1997; Soderberg-Naucler et al., 1997). In both humans and mice, macrophages are thought to act as a reservoir and disseminate virus (Fish et al., 1995; Koffron et al., 1998; Soderberg-Naucler et al., 1997). Localization of CMV in the vascular endothelium may be related to the increased association of transplant vascular sclerosis in rats (De La Melena et al., 2001) and humans (Dummer et al., 1994; Grattan et al., 1989). CMV is a potential risk factor for atherosclerosis in humans and mice where CMV DNA in the vascular endothelium co-localizes with inflammatory cells (Berencsi et al., 1998).

Both HCMV and MCMV have been described as immunosuppressive (Koszinowski et al., 1990; Rasmussen, 1990). This phenomenon does not refer to the ability of CMV to elicit an adequate immune response, which is in fact quite effective, but refers to the ability of CMV to prevent T cell responses to other viruses or impair allogeneic T cell responses (Andrews et al., 2001; Slater et al., 1991 ). Although no specific mechanism has been identified as the cause for the generalized immune-suppression, some studies in MCMV implicate a defect in hematopoiesis (Mutter et al., 1988). Moreover, the defect in priming of both CD4s and CD8s in MCMV infection described by Cambell's group (Slater et al., 1991), could potentially be explained by results from a recent study. Andrews et al. showed that MCMV could infect dendritic cells (DCs) both *in vivo* and *in vitro*; *In vitro*, the infected DCs were impaired in their ability to mature, upregulate the surface expression of costimulatory molecules, produce cytokines, and stimulate an allogeneic T cell response, all in response to the activating lippopolysaccharide (LPS) treatment (Andrews et al., 2001).

Although how cytomegaloviruses establish and maintain latency is poorly understood, viral factors, as well as pressures from the host immune response, are both involved. Reactivation from latency may be controlled by the virus by a series of checkpoints, the first being the requirement for transcription from the IE1 promoter

enhancer (Kurz and Reddehase, 1999). Moreover, the ability of MCMV to establish latency in endothelial cells may be dependent on the anti-apoptotic function conferred by the MCMV gene *M45*, a gene not essential for productive infection in fibroblasts (Brune et al., 2001). Control of virus reactivated from latency is clearly immune-associated; in CMV-infected individuals, immunosuppression often leads to virus dissemination, resulting in a chronic disease state and even death (Riddell, 1995; Shanley et al., 1979). Treatment of CMV disease under those circumstances can be accomplished by the infusion of therapeutic lymphocytes, the primary efficacious subset being CD8 T cells (Lucas et al., 2000; Riddell, 1995; Steffens et al., 1998). Although CD8 T cells are crucial in the maintenance of latency, Polic et al. showed that maintenance of latency in MCMV-infected mice, was due to cooperative control from different lymphocyte subsets (Polic et al., 1998). These subsets fit into a hierarchy of effectiveness with CD8s at the top, followed by CD4s, and then natural killer (NKs) cells; however, any two of these three subsets are sufficient to maintain latency. These findings underscore the complexity of latency and the need for different immune functions in the control of reactivated virus.

Although the immune system clearly plays a role in the maintenance of latency, what is not clear is how the immune response exerts its control. Is reactivation from latency a stochastic event that results in low virus production that is immediately controlled by the immune response, or does the immune response prevent reactivation from occurring at all? Technical limitations in the ability to detect small amounts of infectious virus, and the need to ablate the immune response to detect disseminating virus, make this question difficult to resolve. Nevertheless, data from this thesis suggests that CD8 T cells, in mice infected with MCMV, express some activation markers that bear a phenotype similar to CD8 T cells that were recently stimulated by antigen. If this proves correct, the immune response would not be responsible for preventing reactivation per se, but rather for the control of disseminating virus.

## The immune response to CMV.

### Natural killer cells.

Natural killer cells are an important component of the innate immune response. They were originally found to offer protection against virus-infected cells and tumor cells. The reduction of class I molecules on the surface of such target cells was associated with increased killing by NKs; this idea became known as the missing-self hypothesis (Ljunggren and Karre, 1990). NKs are non-B, non-T lymphocytes that do not express a rearranged antigen receptor nor the CD3 complex. NK activity is regulated by positive or negative signals that either activate or inhibit effector functions (Lanier, 1998), including proliferation, cytokine production and killing of target cells (Biron et al., 1999). Many of these negative signals are received from polymorphic class I molecules which provide an inhibitory signal to NK cells (Lanier, 1998). Although the role of NK cells in HCMV is unknown, individuals with NK deficiencies have difficulties controlling herpesvirus infections (Biron et al., 1989). Nonetheless, studies show that NKs are clearly important in the innate immune response to MCMV. *In vitro* expanded NK cells, adoptively transferred into MCMV-infected NK-deficient mice, provided resistance to MCMV (Bukowski et al., 1985). In the early 1980's, the resistance to MCMV was associated with a genetic susceptibility (Shellam et al., 1981). This resistance was later correlated with the *Cmv-1* locus, a non-MHC encoded locus, present in the NK gene complex on the mouse chromosome 6 (Scalzo et al., 1992). Relevant to this thesis work is that C57Bl/6 mice, the mouse strain we study, encodes the *Cmv-1*$^r$ gene, which is associated with increased resistance to MCMV, while the susceptible BALB/c strain of mice are known as *Cmv-1*$^s$ (Scalzo et al., 1992). The resistance gene has now been conclusively associated with the activating receptor Ly49H, whose ligand has not yet been identified (Dokun et al., 2001). MCMV infection induced the selective expansion of a Ly49H$^+$ NK

subset (Dokun et al., 2001) that controlled MCMV in the spleen via a perforin-dependent mechanism (Daniels et al., 2001). Moreover, MCMV-infected mice, depleted of Ly49H$^+$ NK cells, had increased MCMV titers demonstrating that this specific subset of NK cells can control MCMV infection (Daniels et al., 2001). These papers demonstrate novel findings about NK cells: 1) that a specific NK cell subset can expand through a receptor-specific stimulation and 2) that a specific NK subset can control a pathogen. However, Dokun et al. also describe the expansion of IFN-γ producing NKs which are independent of Ly49H expression and which appear earlier after MCMV infection than the Ly49H$^+$ subset.

These recent data can be reconciled with work from Christine Biron's group who also studies MCMV in C57Bl/6 mice. She showed that NKs control MCMV in different organs by means of different effector mechanisms that are ultimately triggered by different upstream events. In the spleen, MCMV control by NKs occurs via a perforin-dependent mechanism, which is dependent on NK cytotoxicity induced by IFNα/β (Biron et al., 1999). This NK response is consistent with the response from the Ly49H$^+$ subset described above, which expands in response to MCMV infection. In the liver however, IFN-γ produced by NKs is dependent on IL-12, probably delivered by macrophages (Orange and Biron, 1996). These IFN-γ producing NKs appear to be the same Ly49H-independent cells Dokun et al. described as the nonspecifically stimulated NKs. The generation of these two different NK subsets is intriguing. Furthermore, why is the Ly49H$^+$ subset not expanding in the liver? Studies on CD8 T cells, which like NKs have both cytolytic and cytokine-secreting capabilities, demonstrate that cytolysis is not a commonly used effector mechanism to combat viral infections in the liver; this trend suggests that mechanisms are in place to minimize immune-associated liver damage (Harty et al., 2000). Nevertheless, the expansion of the Ly49H$^+$ NK subset in MCMV infection may potentially be the result of a host evolutionary strategy to specifically combat MCMV with whom it has long been co-evolving.

It has been suggested that NK-produced cytokines play an indirect role in amplifying and modulating the subsequent acquired immune response (Biron, 1999; Guidotti and Chisari, 2001). For example, IFN-γ, which is known to increase class I surface expression, is likely involved in augmenting class I-restricted responses by CD8 T cells. Although NKs may play a role in linking the innate and acquired immune responses, no data exist to prove such a hypothesis in MCMV infection. Finally, MCMV encodes *m144,* a class I homolog. Mice infected with a mutant MCMV lacking *m144* demonstrate reduced viral titers which could be attributed to better control by NKs (Farrell et al., 1997). Whether *m144* in fact directly inhibits NK cells *in vivo* remains to be demonstrated. Nevertheless, these data suggest that MCMV has evolved mechanisms to specifically interfere with NKs, a mechanism that appears to be commonly used among viruses that interfere with the MHC class I pathway (Tortorella et al., 2000).

**Acquired immunity.**

**Antibodies.**

Although antibodies to MCMV are generated with the help of CD4 T cells (Jonjic et al., 1989), they do not affect the establishment of latency, the clearance kinetics, or the DNA burden after primary infection (Jonjic et al., 1994). Nevertheless, antibodies can limit the dissemination of reactivating virus *in vivo* (Jonjic et al., 1994). This is supported by the finding that B cell-deficient mice have 2-3 logs higher viral titers after reactivation compared to control mice (Polic et al., 1998). Treatment of immunocompromised CMV-infected individuals with anti-CMV serum immunoglobulins has limited and unpredictable therapeutic outcome and is therefore not routinely used (Farrell and Shellam, 1991; Sullivan, 1989).

**CD4 T lymphocytes.**

CD4 s play a limited but important role in the control of MCMV infection. Although CD8s are primarily responsible for controlling virus, only IFNγ-producing Th1 CD4s appear to be able to control viral replication in the salivary glands (Lucin et al., 1992). In CD4-deficient mice, MCMV persists in the salivary glands resulting in a chronic infection (Jonjic et al., 1989). However, more than IFN-γ from the CD4s is responsible for this effect; IFN-γ administered systemically was not sufficient to induce a latent rather than a persistent state in the salivary glands. Therefore, some other unidentified CD4-associated component may be required to prevent persistence in the salivary glands. Mice deficient in perforin or granzymes A and B, molecules involved in cytolysis, showed higher virus titers in the salivary gland than the wildtype mice 15 days after MCMV infection (Riera et al., 2000). Thus, cytolytic CD4s might contribute to clearance of virus in the salivary gland, however, not enough information exists to exclude NKs and CD8s in this effect since both also have cytolytic effector functions. Because MCMV is disseminated through saliva, it is likely that it has secured its survival through the use of immune evasion strategies that target the salivary glands. CD8 T cells control MCMV in all organs except in the salivary glands during acute infection (Jonjic et al., 1989; Lucin et al., 1992). This may be due to class I immune evasion strategies encoded by MCMV, a topic I will discuss later in this introduction. Finally, CD4 T cells can also act in a compensatory fashion for CD8s in an experimental model where CD8 T cells are depleted prior to infection with MCMV (Jonjic et al., 1990). Although this result is intriguing, it is difficult to assess the importance of such findings.

Because the topic of this thesis is the CD8 T cell response to MCMV, I will first review some background on CD8 T cells and the MHC class I pathway before returning to the literature on CD8 T cells in MCMV infection.

**Overview of MHC class I-restricted CD8 T cell responses.**

CD8 T cells continually survey the internal contents of cells through their interactions with MHC class I molecules that are present on the surface of cells. The MHC class I pathway is the constitutive process by which host or pathogen-derived proteins are degraded into peptides, loaded onto class I molecules in the ER, and then presented as a unit on the cell surface. Because viruses are obligate intracellular parasites, which depend on the host machinery for the generation of their proteins, CD8 T cells contribute greatly to resistance against viruses. CD8 T cells are activated when they receive an optimal signal through their T cell receptors (TCR) by engaging their respective peptide/ MHC complex. This CD8 T cell activation results in the clonal expansion of naïve T cells to form a primary CD8 effector population that helps resolve acute infections; this effector population makes up a large proportion of all of the CD8 T cells, the extent to which has only recently been appreciated. As antigen is cleared, the majority of the antigen-specific CD8 T cells die by apoptosis. However, some of the effector CD8 T cells survive to form a long-lived CD8 T cell memory population that provides a more vigorous and effective response than the primary one upon re-exposure to the same pathogen (Sprent and Tough, 2001).

**Class I molecules.**

MHC class I molecules (or class I molecules) are expressed on almost all nucleated cells in mammals (Pamer and Cresswell, 1998). They are encoded in the MHC complex on chromosome 6 in humans and chromosome 17 in the mouse where MHC is called H-2. The MHC genes are polygenic, a term which refers to the fact that 3 different MHC loci are present in both humans and mice. This feature, which is the result of gene duplication, allows multiple peptide-binding capabilities. Moreover, the MHC genes are the most polymorphic genes known, such that multiple alleles of each MHC gene exist (Janeway and Travers, 1994). Both attributes are responsible for the ability of the immune system

within a species to cope with a multitude of pathogens. Furthermore, as will be discussed later, these attributes are presumably the selective pressures responsible for the evolution of redundant viral immune evasion strategies. The cell surface class I molecule is a trimeric complex made up of two polypeptide chains, the class I heavy chain, the non-MHC encoded light chain known as $\beta_2$microglobulin ($\beta_2$m), and a peptide of 8-10 amino acids. The heavy chain is made up of three domains: $\alpha_1$, $\alpha_2$, and $\alpha_3$ from which the $\alpha_1$ and $\alpha_2$ domains combine to form the peptide-binding cleft. Each MHC allele binds a peptide that encodes specific anchor residues, a consequence of MHC polymorphism. The resulting product is a ligand for the MHC class I specific TCR, which recognizes the MHC/peptide molecules in an allele-specific fashion, and is the basis for the concept of MHC restriction.

**The MHC class I pathway.**

In the classical class I pathway, peptides are derived from proteins that are synthesized in the cytosol and degraded by the proteasome, a large multicatalytic protease. However, the source of proteins and the signals that direct them to the proteasome for degradation are unclear. For instance, it has been shown that peptides commonly originate from defective translation products (Schubert et al., 2000). Furthermore, although the requirement for peptides to be derived from proteins that are targeted for degradation through ubiquitination is still controversial, the addition of a ubiquitinated targeting sequence to various proteins has increased the efficiency of antigen processing and presentation from those proteins (Rodriguez et al., 1997). Ultimately though, the proteasome is essential in the generation of peptides that bind class I molecules although this does not exclude the fact that other proteases are also likely to be involved. Peptides resulting from proteasomal degradation are then translocated into the ER by the transporter associated with antigen processing and presentation (TAP), a heterodimeric ATP-dependent protein that is encoded in the MHC complex (Pamer and Cresswell, 1998). Although chaperones subsequently assist in the proper folding of class I molecules, and in providing peptide

quality control, their roles are incompletely resolved and discrepancies exist between humans and mice. In the mouse ER, newly synthesized class I heavy chain (HC) binds the ER-retained transmembrane chaperone calnexin, which in humans appears inessential (Zhang et al., 1995). The class I HC then associates non-covalently with $\beta_2$m, at which point, calnexin is exchanged for the chaperone calreticulin, a soluble homolog of calnexin, which appears important in both humans and mice (Zhang and Salter, 1998). In addition to its role as a chaperone, calreticulin may also provide peptide quality control. We found that in MCMV-infected target cells, the lack of calreticulin affected two $K^b$-restricted MCMV-specific CD8 T cell clones differently; one was unaffected by the absence of calreticulin while the other was no longer able to kill the target cell (manuscript in press and chapter 2.1). Eliott and colleagues suggest that calreticulin could provide peptide quality control by augmenting a tapasin-dependent peptide optimization function (manuscript in press). Tapasin is another chaperone, essential to the class I assembly complex, that acts as a bridge for the calreticulin-associated class I HC/$\beta_2$m dimers and TAP. However, tapasin's function appears to be more than simply facilitating peptide loading by enhancing the proximity of class I to TAP. Cells lacking tapasin have an increased rate of peptide-receptive class I on their cell surface (Lehner et al., 1998), and in insect cells, tapasin retained peptide-empty class I in the ER (Schoenhals et al., 1999). Moreover, tapasin increases the rate of TAP-dependent peptide translocation, suggesting that tapasin stabilizes TAP and thus coordinates peptide translocation and HC/$\beta_2$m-TAP association (Pamer and Cresswell, 1998). Once HC/$\beta_2$m associates with peptide, and is released from the assembly complex, this trimolecular complex matures through the ER and Golgi and travels to the cell surface to be detected by CD8 T cells.

**The T cell receptor and the CD3 complex.**

CD8 T cells express a clonotypic T cell receptor that is made up of two transmembrane glycoprotein chains, the $\alpha$ and $\beta$ chains, which are linked by a disulfide-bond. The TCR is responsible for recognizing peptide/MHC molecules, but associates with the CD3

complex in order to signal the cell it has bound antigen. Each TCR chain is made up of a constant region and a variable region. Only 1 and 2 genes encode the constant regions for the α and the β chain constant regions respectively. Diversity in the TCR comes from the variable region. The variable region is the result of rearranged V, D and J genes that are joined by somatic recombination during T cell development. Specifically, the antigen-binding site, the site that interacts with peptide and MHC, is encoded largely by the D and J gene segments; this is homologous to the CDR3 loops that makes up the antigen binding site of immunoglobulins. The multitude of J segments, of which there are at least 61 genes, and N-region diversity, contribute to the diversity of TCRs that are generated and required to cope with many pathogens displayed in the form of peptide/MHC(Janeway and Travers, 1994).

The CD3 complex is made up of 4 different CD3 chains, 1δ, 1γ, 2ε, and the ζ homodimer that combined, are responsible for transmitting the signal that antigen binding has occurred. Each CD3 chain and the ζ homodimer contain sequences called immunoreceptor tyrosine-based activation motifs (ITAMs). These ITAMs associate with protein tyrosine kinases that become phosphorylated upon antigen binding. A series of events such as the recruitment of other kinases, the activation of molecules such as phospholipase C-γ, and the translocation of transcription factors to the nucleus, allow new gene transcription that can result in the differentiation, proliferation, and effector functions of T cells (Janeway and Travers, 1994).

**Direct priming and cross-priming.**

Naïve CD8 T cells that circulate in the periphery are the result of positive and negative selection in the thymus where T cell development occurs. The result of thymic selection is a repertoire of millions of CD8 T cell clones each with a singular TCR (Janeway and Travers, 1994). These naïve T cells circulate continuously from the blood to the lymph through the lymphoid organs where they first encounter their antigen and become activated or primed. Naïve CD8 T cells, unlike antigen-experienced CD8s, require

stimulation by professional APCs, such as the dendritic cell, in order to become activated. CD8 T cell responses to viruses can be directly primed by virus-infected professional APCs through the class I pathway described above. However, some viruses do not infect professional APCs and a mechanism called cross-priming appears important in the activation of naïve CD8 T cells in vivo (Sigal et al., 1999). Cross-priming is the ability of a professional APC to present antigens acquired exogenously on its own newly synthesized MHC class I molecules (Heath and Carbone, 2001).

Although the phenomenon of cross-priming was discovered over 20 years ago (Bevan, 1976), the mechanism is still poorly understood. A recent review by Heath and Carbone concludes that little is known about the source and quantity of antigen required for cross-presentation, the identity of the APC, and the cellular and molecular signals involved (Heath and Carbone, 2001). Nevertheless, the antigen dose is crucial and a minimum threshold level is apparently required for cross-priming (Kurts et al., 1998). Antigens from apoptotic cells or associated with heat shock proteins have been able to prime CTL responses (Albert et al., 1998; Janetzki et al., 1998). Dendritic cells have been shown, in *in vitro* studies, to be capable of cross-priming (Albert et al., 1998; den Haan et al., 2000). Furthermore, they are the most likely APCs responsible for cross-presentation because of their co-stimulatory capabilities. However, macrophages have also been implicated (Bellone et al., 1997), and ultimately, the APC responsible for cross-presentation *in vivo* is unknown (Heath and Carbone, 2001).

There may be advantages and disadvantages to eliciting CD8 T cells by the cross-priming mechanism as opposed to direct priming. For example, cross-priming might allow presentation of antigen "X" that would normally be prevented from being presented in some virus-infected cells by viral immune evasion mechanisms that interfere with the MHC class I pathway. The generation of these CTL specific for antigen "X" would be useful in clearing virus-infected cells in which evasion genes do not prevent the presentation of antigen "X". If however, none of the infected cells ever present antigen

"X", then the immune system has spent resources on generating a useless CTL population. Ultimately, as Jonathan Yewdell describes, it is important that the priming by professional APCs generate CTL of specificities that are capable of acting as effector CTL on the virus-infected cells whether they be professional APCs or non hematopoietic cells (Yewdell and Bennink, 1999). The CMVs encode immune evasion genes that interfere with the class I antigen processing and presentation pathway and the importance of cross-priming in these infections remains to be determined.

**Immunodominance.**

Immunodominance is a phenomenon that is commonly seen in CD8 T cell responses to pathogens. Of the many peptides from any pathogen that could be presented to CD8 T cells via the MHC class I pathway, only a few of these actually elicit measurable CD8 T cell responses (Yewdell and Bennink, 1999). Many factors that range from the generation of peptide, to the availability of CTL with the appropriate TCR, can contribute to this process. However, one important factor is the affinity of peptide for its MHC molecule; peptides must bind MHC at a minimum threshold level of $K_d$> 500nM (Yewdell and Bennink, 1999). Although more difficult to quantify, the efficiency of antigen processing and presentation is likely to play a role. For example, the residues that flank an epitope affect the liberation of antigenic peptides (Del Val et al., 1991). Also, targeting of an antigen to the ER increases presentation of that peptide; the addition of an ER-targeting sequence to a recombinant vaccinia virus expressing OVA could increase the number of OVA-specific peptide/ MHC complexes on the cell surface 10 fold (Porgador et al., 1997). Furthermore, CD8s specific for a peptide/MHC antigenic complex must be available in order to generate a response. However, whether immunodominant CD8 responses are the result of CTL precursors that are present at higher numbers is not known due to our inability to quantify such small numbers of T cells. Finally, the ability of CD8 T cells to suppress CTL of other specificities, has been described as immunodomination. One possible explanation for such suppression could be that CD8s

that respond more rapidly are able to outcompete CTL of other specificities by decreasing the antigen load. Immunodomination may also be mediated by IFN-γ. Mice deficient for IFN-γ and infected with Listeria showed a shift in the natural immunodominance hierarchy (Badovinac et al., 2000). Specifically relevant to this thesis work is how immunodominance might be affected by viruses such as the CMVs which interfere with antigen processing and presentation. Jonathan Yewdell in his review hypothesizes that such immune evasion genes would affect immunodominance: "The effects on immunodominance of such global interference with antigen processing has yet to be investigated in detail, but a simple prediction is that CD8 T cell responses will focus on those determinants that for whatever reason are less affected by the strategy employed by the virus (Yewdell and Bennink, 1999)." The question of whether class I immune evasion genes affect immunodominance is addressed experimentally in chapter 2.

**CD8 T lymphocytes in MCMV infection.**

The requirement for lymphocytes to control MCMV infection was determined in the 1970's. Brody and Craighead reported that mice died with doses as low as 100 pfu of MCMV if they were depleted of lymphocytes (Brody, 1974). Furthermore, nude mice infected with 10 pfu of MCMV could be rescued from death by the administration of $10^7$ splenocytes from a previously-infected BALB/c mouse (Starr and Allison, 1977). Quinnan et al. then showed that CD8 T cells were the protective subset; cytotoxic lymphocytes, isolated from the spleen of mice infected with MCMV for 5 to 10 days, could specifically kill MCMV-infected target cells, in a class I-restricted fashion (Quinnan et al., 1978). Subsequent to these initial studies, Ulrich Koszinowki and colleagues began characterizing the CTL response to MCMV in BALB/c (H-$2^d$) mice, and over the years, have provided a large majority of what is known about this topic.

Early studies to characterize the CTL response to acute MCMV infection (day 8 post infection) showed that two populations of CTL could be distinguished based on their direct *ex vivo* cytolytic capacity. Although both CTL populations required IL-2 as a

growth factor, only one of the populations was directly cytolytic while the other required restimulation with MCMV antigen to perform cytolytic functions (Reddehase et al., 1984). Based on updated information about CD8 T cells, it is clear that these populations represent what we now think of as effector CTL and memory CTL respectively.

In order to determine from which stage the CTL antigens were derived, i.e.: immediate early (IE), early (E), or late (L), day 8 effector CTL, treated with IL-2, were tested for their ability to kill fibroblasts treated to only express IE genes, or genes from all 3 stages (Reddehase et al., 1984). They found that when target cells were specifically enhanced for IE proteins, they were killed most effectively. About 50% of the polyclonal CTL population was specific for antigens expressed at the IE phase. However, cells expressing all 3 gene families were well recognized indicating that E or L genes were also contributing to the response since without selective enhancement for IE antigens, IE expression levels would be low. To then differentiate between E or L-specific responses, UV-inactivated virus, which would predominantly represent L structural proteins, was tested as a source of antigen. They found that input structural virions contributed little to the CTL response compared to the E antigen-specific response (Reddehase et al., 1984). This is in contrast to the HCMV-specific CTL response in which the response to the tegument protein pp65 (UL84), a structural protein which does not need new viral gene expression to be presented, has been thought to be immunodominant (McLaughlin-Taylor et al., 1994). However, if HCMV is like MCMV, immunodominant antigens may not yet have been identified. Further experiments done in the presence of PAA definitively ruled out newly synthesized L genes as a significant source of antigen. PAA is a drug that prevents viral DNA synthesis and therefore blocks L gene expression. These experiments showed that CD8 T cells from MCMV-infected mice kill target cells to the same extent whether they are treated with PAA or not ((Del Val et al., 1989) and MC Gold unpublished data). Hence, L genes make up a small to undetectable portion of the CD8 T

cell epitopes in MCMV while both IE and E antigens predominate as targets for the CTL response in both H-$2^d$ and H-$2^b$-restricted mice.

An interesting observation was made in the attempt to identify the antigenic stages responsible for eliciting MCMV-specific CTL. Although cells selectively enhanced for IE antigens were killed efficiently by IE-specific clones, the IE-specific killing was abrogated once E genes were expressed (Reddehase et al., 1986). Furthermore, IE-specific recognition was restored once L gene expression commenced (Del Val et al., 1989). This phenomenon was the first suggestion that MCMV early gene(s) interfered with the class I antigen processing and presentation pathway. So far, three MCMV genes, *m4*,*m6*, and *m152*, have been shown to prevent lysis of infected cells by CD8 T cells *in vitro*. (Kavanagh et al., 2001; Reusch et al., 1999; Ziegler et al., 1997). I will elaborate on those mechanisms in the viral immune evasion section.

Although CTL clones specific for antigens from all 3 stages (IE, E, and L) could be isolated (Del Val et al., 1989; Reddehase et al., 1986; Reddehase et al., 1987), and therefore be used to identify the antigen they recognized, an attempt to identify the IE antigenic peptide was made due to the fact that only 3 genes (IE1, IE2, and IE3) are predominantly expressed at IE times. The IE1 gene, *m123*, encodes a 89kD nonstructural protein that stimulated the IE-specific CTL clones only if the class I molecule $L^d$ was also expressed (Volkmer et al., 1987). The specific 9 amino acid antigenic sequence (YPHFMPTNL) was identified using vaccinia viruses expressing portions of the IE1 gene (Del Val et al., 1991; Del Val et al., 1988 ). Adoptive transfer studies demonstrated that the IE-specific CTL were protective and confirmed the *in vivo* efficacy of pp89-specific CTL (Reddehase et al., 1987). Furthermore, a vaccinia virus expressing the antigenic peptide from pp89 could also be used to immunize mice against a subsequent lethal dose of MCMV (Del Val et al., 1991; Jonjic et al., 1988 ).

No antigens from MCMV were identified for years following the identification of the antigenic peptide from MCMV pp89. This, along with the difficulty in identifying

MCMV-specific antigens, may have both contributed to the idea that pp89 is immunodominant. However, the frequency of pp89-specific CD8s in acutely (day 8) infected BALB/c mice is only 1-2% as measured using direct *ex vivo* splenocytes in the ELISPOT assay (Holtappels et al., 2000; Holtappels et al., 2002). While this number may represent a significant proportion of the CD8 T cell response in other virus infections, this does not appear to be the case in MCMV infections where at least 10% of the CD8s are thought to be specific for the virus. Using virus-infected APCs as stimulators, I have shown that at least 10% of CD8s from day 7 infected mice are MCMV-specific (MC Gold chapter 2.2). Holtappels et al. however, use αCD3 stimulation to conclude the same findings, a point I will return to later (Holtappels et al., 2002). Recently, Holtappels et al. described three other MCMV antigens. These are: the $D^d$-restricted epitope from m4, the $L^d$-restricted epitope from M83, and the $K^d$-restricted epitope from M84 (Holtappels et al., 2001; Holtappels et al., 2000; Holtappels et al., 2000). However, the importance of these epitopes in the MCMV-specific CTL response is unclear for two reasons. The first is that the frequency of CD8 T cells specific for peptides from m4, M83, and M84 combined, makes up less than 1% of the H-$2^d$-restricted CD8 T cell response in MCMV-infected BALB/c mice; these combined frequencies make up an even smaller contribution to the MCMV-specific CD8 T cell response than the IE antigen does (Holtappels et al., 2002). The second reason these epitopes are of questionable biological relevance is likely to be due to the manner in which they were selected. The peptides from the m4, M83, and M84 proteins were identified using 1) the predicted amino acid sequence of all known MCMV ORFs based on the MCMV DNA sequence (Rawlinson et al., 1996) and 2) allele-specific peptide binding motifs for each H-$2^d$ class I allele based on the work by Rammensee and colleagues (Rammensee et al., 1993). In this manner, all the peptides in the genome that fit the predicted peptide binding motifs were synthesized and tested as potential epitopes. It is worth pointing out that specificities could be missed using this methodology as even well-characterized epitopes do not always adhere to the

predicted binding motif. Nevertheless, the synthesized peptides were used to restimulate splenocytes from MCMV-infected mice to generate peptide-specific CTL lines. CTL specific for m4, M83, and M84 were expanded indicating that CTL of all three specificities had been primed *in vivo*. However, when the frequencies of the m4, M83 and the M84-specific responses were determined using the ELISPOT using *ex vivo* splenocytes from MCMV acutely infected mice, they were either very low, 0.1% of CD8s for m4 and M83, or undetectable in the case of M84 (Holtappels et al., 2001; Holtappels et al., 2000). Interestingly, a study to determine if M83 and M84-specific CTL had antiviral properties showed that when adoptively transferred, the CTL were able to protect mice infected with MCMV in a bone marrow transplantation model (Holtappels et al., 2001). The fact that M83 and M84-specific CTL are primed *in vivo* and have protective qualities but do not make up a substantial, or even detectable proportion of the CTL response is an interesting illustration of the enigma of immunodominance.

Even more recently, a $D^d$-restricted epitope from the m164 protein was identified, which is responsible for eliciting close to 1-2% of the CD8s in acute MCMV infection, a number that rivals the anti-pp89 CD8 frequency (Holtappels et al., 2002). Although this proportion is noteworthy, the claim made by Reddehasse and colleagues that both peptides from pp89 and the m164 are immunodominant, and these quantitatively dominate the CD8 T cell memory response in chronic MCMV infection is questionable. Their claim is based on a method called the CD3-redirected ELISPOT assay which they use to measure the total MCMV-specific CD8 response. They make two assumptions. The first is that all CD8 cells that can be triggered through CD3 to produce IFN-γ represent MCMV-specific CD8 T cells. The second is that all MCMV-specific CD8s will produce IFN-γ in response to αCD3 stimulation. While close to 10% of CD8s in acute infection could be stimulated through αCD3, and were therefore considered to be MCMV-specific, about 2% of CD8s were stimulated by this method in chronic infection (Holtappels et al., 2002). However, based on results presented in chapter 2.2 of this

thesis, stimulation through αCD3 appears to underestimate the numbers of MCMV-specific CD8s, especially in chronic infection; about 10% of the CD8s from C57Bl/6 mice were found to be MCMV-specific in both the acute and memory responses using virus-infected APCs as stimulators in the intracellular cytokine staining assay. Stimulation through αCD3 is not conventionally used to enumerate antigen-specific responses. Furthermore, the ability of effector versus memory CD8s to respond to stimulation via αCD3 stimulation may be different. Moreover, as will be discussed in the next section, a trend is emerging showing that not uncommonly, close to 10% of the CD8s are specific for viruses that persist once the acute phase of the infection is resolved (Doherty and Christensen, 2000).

Conversely, the antigen-specific responses elicited with either the IE1 or the m164 peptides showed similar and modest results. Both the IE1 and m164-specific responses in acute infection made up 1% of the CD8s and about 0.8% in memory response using peptide stimulation in the ELISPOT assay. These data, obtained using peptide restimulation, support the idea that the bulk of the CD8 T cell response in BALB/c mice is dominated by an antigen or antigens that remain to be identified, if in fact 10% of the CD8s are MCMV-specific. Finally, the claim that the CD8 T cell response to MCMV focuses over time on a few select antigens is also unanswered by the data described above. Nevertheless, this raises the question of the role of antigen in the shaping of the CTL repertoire in MCMV infection and is an important question that remains to be answered.

In summary, the CD8 T cell response to MCMV infection offers protection in clearing acute virus, in limiting the viral burden, and in preventing reactivation from latency. Although IFN-γ (Presti et al., 1998) and mediators of the perforin pathway (Riera et al., 2000) control MCMV infection, it is not clear if these are due to the action of CD8 or NKs which also have similar effector mechanisms. Ultimately, the effector mechanisms by which the T cells mediate their protection from MCMV infection *in vivo*

are unknown, and studies to examine these functions would enhance our understanding of MCMV-specific CD8 T cell responses. The CTL response to MCMV in BALB/c mice is focused on antigens from both the IE and E stages while the response in C57Bl/6 mice is predominantly due to E antigens (MC Gold chapter 2). In humans (Riddell et al., 1993),as well as in mice (Podlech et al., 1998; Steffens et al., 1998), CD8 T cells are therapeutic and can control infection in CMV-infected individuals that are immunocompromised, underscoring once again the importance of CD8 T cells in immunity to CMV infections. Although both MCMV and HCMV encode mechanisms to interfere with the MHC class I antigen processing and presentation pathway, a good CTL response to CMV provides a balanced control of the virus while still allowing it to spread to a new host.

**CD8 T cell responses to intracellular pathogens.**

As described earlier, the CD8 T cell response to a virus goes through several stages. Naïve CD8 T cells are first activated by antigen. However, the number of naïve CD8 T cells that are recruited into any one antigen-specific response cannot be quantified as they are below the limit of detection of any of the currently available assays unless TCR transgenics are used. Nevertheless, these activated cells multiply and differentiate into a massive effector CD8 T population that can kill virus-infected target cells or secrete cytokines; the massive expansion of the CD8 T cell response was under-appreciated until recently as will be described below. Ensuing viral clearance causes the majority of the effector cells to become unnecessary and undergo apoptosis. This death phase of the CD8 T cell response, although quantifiable as a whole, is difficult to follow on a per cell basis as apoptotic cells are very labile. Ultimately though, a population of CD8 T cells, larger than the original number of naïve CD8s that were originally recruited, survive to become memory cells that can respond more quickly upon secondary exposure to the same pathogen (Ahmed and Gray, 1996; Sprent and Tough, 2001).

**Quantification of CD8 T cell responses.**

As outlined above, CD8 T cell responses include 3 phases: activation and expansion, death, and stability or memory (Ahmed and Gray, 1996). This generalized view appears applicable to most viral infections and intracellular pathogens, and has been confirmed by the quantification of CD8 T cell numbers throughout the course of an infection. In fact, CD8 T cell biology has recently received a great boon from the emergence of new techniques and tools to quantify antigen-specific CD8 T cells. In the following section, I will first describe the methods used to enumerate T cells in order to subsequently compare and contrast T cell responses to various intracellular pathogens.

**The limiting dilution analysis (LDA).**

Previously, CD8 T cell precursor frequencies were estimated by the LDA in conjunction with the chromium release assay. In the LDA, CD8 T cells from an infected host are plated under limiting dilution conditions, and are required to not only survive but also proliferate in response to antigen *in vitro*. The CD8s are then tested for their ability to kill target cells in an antigen-specific fashion using the chromium release assay. Based on data from the new techniques described above, it is now clear that the LDA grossly underestimated, up to 10 fold in some cases, the number of CD8 T cells responding to any single virus during the acute phase of an infection (Doherty and Christensen, 2000). That the LDA underrepresented CD8 T cell responses can be explained by the fact that CTL in the acute phase of infection are primarily composed of effector cells; these are easily triggered to undergo apoptosis, or activation-induced cell death (AICD) in response to repeated antigen stimulation (Ahmed and Gray, 1996; Russell, 1995). In contrast, memory CD8 T cells require restimulation with antigen to proliferate, and unlike effector CD8s, are more refractory to AICD, most likely due to increased levels of anti-apoptotic genes such as Bcl-2 (Grayson et al., 2001; Grayson et al., 2000). With underestimations around 2-3 fold, the LDA did provide reasonably accurate numbers of memory CTL (Doherty and Christensen, 2000). Moreover, the LDA also provided

valuable information on the kinetics of CTL responses although some re-evaluation will likely be required once again due to underestimates (Doherty and Christensen, 2000; Murali-Krishna et al., 1998).

**Tetramers.**

In 1996, the development of tetrameric complexes of MHC class I molecules+peptide, known as tetramers, conjugated to a fluorochrome, allowed the direct quantification of antigen-specific CD8 T cells using flow cytometric analysis (Altman et al., 1996). This technique, along with the intracellular cytokine staining (ICS) assay, (Butz and Bevan, 1998; Murali-Krishna et al., 1998), another FACS-based technique which allows multicolor-analysis, and the ELISPOT (Murali-Krishna et al., 1998), have all contributed to the realization that the numbers of CD8 T cells responding to any virus studied are significantly greater than was ever thought based on the limiting dilution analysis (LDA) (Doherty and Christensen, 2000).

Tetramers are a very powerful tool that allow the direct staining of CD8 T cells isolated from an animal or whole blood from humans. This last fact alone has great implications for monitoring patients in a clinical setting. Although CD8 T cell frequencies obtained using tetramers most often correlate with frequencies obtained using assays that assess CD8 T cell function, the $CD8^+$ $tetramer^+$ phenotype cannot be used to conclude that the T cell is functional; other assays must accompany tetramer staining to specifically test the cytolytic or cytokine-secreting capacities of those CD8 T cells. Cases exist that demonstrate that tetramer positivity does not always correlate with functionality (Zajac et al., 1998). Furthermore, a recent report shows that not all functional CD8 T cells bind to tetramers; CD8 T cells that produced IFN-$\gamma$ and killed target cells in response to a polyomavirus peptide, did not react with tetramers of the same specificity (Moser et al., 2001). It remains to be seen if this isolated example will prove to be more common as tetramer usage becomes more widespread. Therefore, although tetramers

require prior antigen identification, they provide a quantitative assessment of CD8 T cells, but cannot be used *a priori* to comment on their functional attributes.

**The intracellular cytokine staining assay.**

The intracellular cytokine staining (ICS) assay, although first described to enumerate CD4 T cells (Waldrop et al., 1997), is a FACS-based method that measures CD8 T cells that produce a cytokine in response to a short (5-6 hrs) restimulation with antigen (Butz and Bevan, 1998; Murali-Krishna et al., 1998). In this assay, cells isolated from mice or a patient are incubated with antigen, either in the form of peptide (Butz and Bevan, 1998; Murali-Krishna et al., 1998), or as in this thesis work, with virus-infected APCs. The addition of brefeldin A during the *in vitro* restimulation prevents export of the cytokine which can then be labeled after the cells have been permeabilized. Most commonly, the cytokines IFN-$\gamma$ and TNF-$\alpha$ are detected, but variations on this assay can be used to detect other intracellular molecules such as perforin or CTLA-4 (Slifka et al., 1999; Slifka and Whitton, 2000). Although both the ICS assay and the tetramer technology can be used with other antibodies coupled to other fluorochromes to allow further phenotypic characterization of cells, the ICS assay is powerful tool to assess the numbers of functional cytokine-producing cells. Limitations in this assay result from the fact that CD8 T cell numbers may be underrepresented due to the fact that not all antigen specific CD8 T cells produce cytokines (Greten et al., 1998). Furthermore, as with all FACS-based assays, the ICS assay is subject to a fairly high limit of detection (must be greater than 0.5% of CD8s) (Doherty and Christensen, 2000). Finally, although peptide-stimulated cultures probably elicit the large majority of CD8s specific for that peptide, stimulation with virus-infected APCs may underestimate the total number of CD8 T cells, an argument I will return to in the discussion.

**The ELISPOT.**

The ELISPOT is a another assay used to enumerate antigen-specific CD8 cells by their ability to secrete IFN-$\gamma$ in an antigen-specific manner and was also described in the late

1990's (Murali-Krishna et al., 1998). This assay is similar to the ICS assay except the cells are not prevented from secreting cytokines. Instead, the ELISPOT is much like an ELISA where a membrane is coated with a capture antibody for the cytokine to be detected, most commonly IFN-γ. Cells are restimulated with either peptide or APCs and the antigen-specific cells, which produce IFN-γ, are detected as spots on the membrane through a colorometric detection assay and counted with a microscope. As in the ICS assay, cells can be used directly *ex vivo*, however, the primary advantage to the ELISPOT is that a smaller number of cells can be analyzed.

**Kinetics of CD8 responses to intracellular pathogens.**

In the following section, I would like to highlight some recent findings about CD8 T cell responses to intracellular pathogens emphasizing results obtained using the techniques I described above. In some cases, the results have allowed confirmation of old paradigms while others have led to their reevaluation. Even more exciting are the new paradigms that have emerged from these studies. I will focus on CD8 T cell responses to intracellular pathogens emphasizing studies on influenza, LCMV, MHV-68, and a few other viruses, in addition to the well-studied intracellular bacterium *Listeria monocytogenes* (LM).

**The acute response.**

The initial priming of individual CD8 clones in the secondary lymphoid organs results in the expansion of antigen-specific CD8 T cells; in the spleen, antigen-specific CD8s for LM, LCMV, Flu, MHV-68 and MCMV become detectable around 5 days after those infections (Busch et al., 1998; Flynn et al., 1998; Murali-Krishna et al., 1998; Stevenson and Doherty, 1998)(MC Gold unpublished data). Although the magnitude of different antigen-specific CD8s may vary, the kinetics of these antigen-specific populations within a single infection appear similar and independent of dose (Belz et al., 2000). The synchrony of CD8 T cell responses was nicely illustrated using the Listeria model although this has also been shown with LCMV (Murali-Krishna et al., 1998) and

influenza (Belz et al., 2000). Busch et al. demonstrate that the CD8 response is coordinately controlled; four different tetramer positive populations followed the same population dynamics as seen by a coordinated expansion and contraction of the different antigen-specific subsets (Busch et al., 1998). Because the kinetics of the CD8 responses to the immunodominant and subdominant epitopes were similar, these authors favor the hypothesis that precursor frequency is the major determinant in immunodominance. The acute CD8 T cell response to different intracellular pathogens continues to expand and often peaks around 7 to 9 days post infection. Although the lack of available antigen precedes the massive CD8 die-off, and is thought to be responsible for the end of the acute response (Sprent and Tough, 2001), CD8s continue to cycle 2-3 days beyond the point of detectable antigen; this leaves open the question of what regulates and sustains this continued concerted CD8 response.

Due to the new technologies described above, the numbers of antigen-specific CD8s can now be quantified with more accuracy and more specificity. New data generated over the last several years show that CD8s can massively expand in response to viral infections. Furthermore, most of the expanding CD8s are antigen-specific and not bystanders as had been previously thought. The CD8 T cell response to LCMV best illustrates this striking expansion: up to 70% of all CD8s cells ($2X10^7$ $CD8^+$ splenocytes) are specific for LCMV at the peak of infection (Murali-Krishna et al., 1998). Another example is seen in (EBV) infections resulting in the syndrome of infectious mononucleosis. In the peripheral blood of patients with EBV-induced infectious mononucleosis (IM), up to 44% of CD8s were specific for a single epitope (Callan et al., 1998). It is worth noting that IM is seen in a minority of EBV infections and that the acute CD8 T cell response to the more usual subclinical EBV infection has not been described. However, other viruses elicit more modest responses. For example, the acute responses to MHV-68 (Doherty et al., 2001), or MCMV(MC Gold chapter 2) range closer to 10 or 20% of the CD8s. Even more modest response are elicited by LM and influenza

where only 3% of CD8s are specific for those pathogens (Busch et al., 1998; Doherty and Christensen, 2000). Whether these lower CD8 frequencies are due to technical limitations in identifying all antigen-specific cells or biological differences due to antigen load, the types of cells that are infected, and whether the virus is lytic or not remains to be established.

The quantification of antigen-specific CD8s from mice has routinely been done using the spleen as the source of CD8s while blood is used in human studies. Above, I highlighted CD8 T cell numbers obtained from the spleen. However, more careful studies are now being performed to enumerate CD8 T cells present at peripheral sites. From these studies, it is becoming clear that the frequencies of antigen-specific CD8s at the site of infection can vary greatly from the frequencies in the secondary lymphoid organs. For example, in an acute respiratory infection with Sendai virus, the frequencies of antigen-specific CD8s in the spleen were 5-6% while in the lungs they made up 60% of CD8s, a site where normally, few CD8s reside (Woodland et al., 2001). Nevertheless, while the frequencies may vary greatly at these different sites, the numbers of antigen-specific cells may be equivalent. In a day 8 PR8 influenza infection, the frequency of $NP_{366}$-specific CD8s was 10% in the spleen and 20% in the lungs, however this translated to roughly equal numbers $NP_{366}$-specific CD8s around $5X10^5$ cells (Haanen et al., 1999). Therefore, although the frequency of antigen-specific CD8s may be much higher at sites of localized infections, the numbers of antigen-specific CD8s appear to be evenly distributed between the secondary lymphoid organs and in the periphery (Doherty and Christensen, 2000; Haanen et al., 1999). Furthermore, the CD8s in the periphery appear to primarily result from the mobilization of CD8s from the lymphoid tissue to sites of antigen rather than result from *in situ* proliferation in the periphery (Doherty and Christensen, 2000). Do CD8s from both locations have an equal chance of entering the memory population? Peter Doherty hypothesizes that it's the CD8s from the secondary lymphoid organs, not the ones in the lungs after influenza infection, that will go on to form the memory CD8

population. This hypothesis is based on the idea that repeated antigenic stimulation of CD8s, which with influenza would occur in the lung, but not in the spleen, would cause CD8 in the lung to die through AICD while those in the spleen would survive and could become memory cells. How this might differ in infections such as LCMV and MCMV, which cause systemic infections and replicate in the secondary lymphoid organs, is unknown.

**The memory response.**

Once the acute phase of the CD8 T cell response resolves a fraction of the effector population survives to become long-lived memory CD8s. The controversy over whether memory cells are derived from effector CD8s or are derived from a separate lineage has been resolved recently. Several studies show that memory CD8s are the result of effector CD8s which must undergo a minimum number of divisions before they can become memory CD8s (Jacob and Baltimore, 1999; Kaech and Ahmed, 2001; van Stipdonk et al., 2001). However, what instructs effector cells to die or live and become memory cells is not known. Jonathan Sprent hypothesizes that the CD8s that are generated late in the acute CD8 T cell response survive to become memory cells because they are spared repeated stimulation which causes most cells to die, an argument similar to the one described earlier by Peter Doherty. Evidence for this was presented by David Woodland (VGTI, 12/01) who showed using BrdU-incorporation, that the surviving CD8s, which will presumably make up the CD8 memory pool specific for Sendai, were generated late, rather than early in the acute phase of infection.

What determines the size of the memory pool? As mentioned above, once the acute CD8 response terminates, over 90% of the antigen-specific effector CD8s die by apoptosis. In LCMV infection 5-10% of CD8s at the peak of the response survive to make up the memory CD8 T cell response (Sourdive et al., 1998). It appears however, that the memory CD8 response to LCMV does not reflect the norm for cleared viral infections. In influenza for example, the lack of a massive expansion as that seen with

LCMV prevents quantification of the numbers of resulting memory CD8s (Doherty and Christensen, 2000). Nevertheless, it is clear from LDAs, CTL assays and recall responses, that an influenza specific long-lived memory CD8 population persists (Belz et al., 2000; Belz et al., 2000). Although the decline of CD8s after antigen is cleared is common to all viral infections, the number of CD8s that are maintained, or the "set point" appears higher in viral infections that persist. In HIV (McMichael and Rowland-Jones, 2001), HTLV-1(Greten et al., 1998), MHV-68 (Stevenson and Doherty, 1998) , SIV (Kuroda et al., 1998), and EBV (Tan et al., 1999), close to 10% of all CD8s are antigen-specific. This suggests that chronic antigenic stimulation provides the maintenance of a memory population at higher set-point than those seen in most cleared viral infections such influenza (Doherty et al., 2000). Whether this higher "set point" is the result of a smaller number of cells dying after viral clearance, or the result of continued proliferation as a result of chronic viral stimulation remains to be sorted out.

Where do all the memory CD8 T cells reside once infection has been cleared and do they perform the same functions? As mentioned above, recent studies show that as in acute infection, memory CD8s are distributed between the spleen and the other tissues. Using a recombinant LM expressing OVA or vesicular stomatitis virus, Masopust et al. showed that substantial frequencies of CD8s were found in the fat pad, kidney, and peritoneal cavity. Furthermore, while close to half of all antigen-specific CD8s were found in the spleen and showed no direct cytolytic activity, an equal numbers of CD8s were found in a combination from the lung, liver, and lamina propia and interestingly these exhibited direct *ex vivo* killing (Masopust et al., 2001). Other studies with Sendai virus also show that a substantial number of CD8s remain in the lung and these also demonstrate an effector phenotype based on phenotypic markers (Woodland et al., 2001). So far we know that MHC class I is not required for the maintenance of memory CD8s in vivo (Murali-Krishna et al., 1999) while the cytokine IL-15 appears crucial for their survival (Sprent and Tough, 2001). Nevertheless, many studies now need to be done to

address the role of these different CD8 subsets *in vivo*, and what maintains these cells if in fact antigen is completely cleared.

As mentioned above a significant advantage of the two FACS-based techniques is the ability to further characterize the tetramer$^+$ or cytokine-producing populations by co-staining the cells with antibodies to cell surface molecules. Although no single cell surface marker can be used to conclusively define a CD8 T cell as naïve, effector, or memory, the combination of several cell surface markers can be used to define subsets within those categories. Table 1 shows a list of the markers that have been used to phenotypically characterize CD8s. For example, recent activation of stimulated naïve and memory cells can been detected by the early and brief expression of CD69 and CD25 (Pihlgren et al., 1996). Naïve CD8s can be differentiated from memory cells by CD11a, Ly6C and CD44 which are upregulated on CD8 memory cells (Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000; Pihlgren et al., 1996; Slifka and Whitton, 2000). However, only recently have markers been identified which can differentiate effector CD8s from memory CD8s. For example, effector CD8s upregulate a cell specific O-glycan epitope, which can be detected with an antibody to CD43, that becomes downregulated on memory CD8s (Harrington et al., 2000). Moreover, the level of expression of some constitutively expressed markers can also be used as markers of recent activation. For instance, low CD8 expression levels on CD8s suggest they have been recently activated (Busch et al., 1998; Flynn et al., 1998; Galvan et al., 1998; Murali-Krishna et al., 1998; Pihlgren et al., 1996; Slifka and Whitton, 2000). However, the use of phenotypic markers to analyze stage-specific CD8s has mostly been done in infections that are fully cleared and do not persist although some controversy still exists as to whether antigen is ever fully cleared (Klenerman et al., 1997). Nevertheless, in infections where virus persists, and CD8s are continually restimulated by antigen, it is not clear what these phenotypic markers can allow us to conclude about the CD8s. Are these CD8s effectors, memory, or something in between? Clearly, further characterization of

CD8s in persist infections is required in order to allow the use of phenotypic markers to assess antigen-specific CD8s in the memory phase of persistent infections.

In conclusion, the new techniques described above have allowed more accurate quantitation of effector and memory virus-specific CD8 T cells. Most relevant to this work is the consensus that high numbers of CD8 T cells, about 10% of CD8s, persist in response to a single chronic infection. However, the role of antigen in shaping the memory CD8 T cell response is unclear; given that mice and humans are infected by several ubiquitous herpesviruses, it is unclear how the T cell repertoire is affected by the these infections. Furthermore, can markers which have been used to define effector CD8s be used to conclude that long-lived CD8s with those same markers are in fact continually stimulated by antigen? Finally, although a combination of techniques allows us to conclude different CD8 T cell subsets exist based on their functional and phenotypic attributes, much remains to be discovered about their *in vivo* function.

**Table 1.** Expression of markers on murine CD8 T cells at different stages in the T cell response.

| | naïve | effector | | | memory | Ref. |
|---|---|---|---|---|---|---|
| | 0 | 3-5 | 6-7 | 8-14 | | |
| Size by FSH | small | | large | large | small | |
| CD3/TCR | hi | lo | lo | hi | hi | (Pihlgren et al., 1996) |
| CD11a(LFA-1) | lo | hi | hi | hi | hi | (Slifka and Whitton, 2000) |
| CD11b(Mac-1) | lo | | hi | hi | lo | (Christensen et al., 2001) |
| CD11c | lo | hi | hi | hi | ? | (Huleatt and Lefrancois, 1995; Kim et al., 1999) |
| CD8 | hi | hi | hi | lo | hi | (Slifka and Whitton, 2000) |
| CD25 (IL-2Rα) | lo | hi | lo | lo | lo | (Harrington et al., 2000) |
| CD43 | lo | hi | hi | int | lo | (Harrington et al., 2000) |
| CD44 | lo | hi | hi | hi | hi | (Cerwenka et al., 1998) |
| CD62L(L-selectin) | hi | | lo | lo | hi/lo-tissue dependent | (Hogan et al., 2001) (Busch et al., 1998) |
| CD69 | lo | hi | lo | lo | lo | (Harrington et al., 2000) |
| CD122(IL-2Rβ) | lo | hi | ? | ? | lo/int | (Murali-Krishna and Ahmed, 2000); (Pihlgren et al., 1996) |
| CD152(CTLA-4) | lo | hi | hi | int | lo | (Slifka and Whitton, 2000) |
| PNA | lo | hi | hi | hi | hi-int | (Galvan et al., 1998) |
| Ly6C | lo | lo | lo | int | hi | (Goldrath et al., 2000; Pihlgren et al., 1996) |
| Fas | lo | hi | ? | ? | lo | (Pihlgren et al., 1996) |
| NKG2A | lo | | hi | hi | hi | MC Gold/C McMahon |
| perforin | lo | | hi | hi | int | (Slifka et al., 1999) |
| IFN-γ (+ag) | neg | | + | + | + | (Slifka and Whitton, 2000) |
| TNF-α(+ag) | neg | | +/- | +/- | + | (Slifka and Whitton, 2000) |

**Viral interference with the MHC class I pathway.**

The immune system has evolved to deal with dangerous pathogens such as viruses. The MHC class I pathway allows the continual surveillance of the internal contents of cells by CD8 T cells through the external expression of antigens displayed by MHC class I molecules. It is not surprising therefore that viruses have also evolved strategies to counteract the host MHC I tactic used for defense. Although viruses exhibit a multitude of evasion strategies such as the prevention of complement-mediated lysis, the inhibition of infected cells by anti-apoptotic mechanisms, the incorporation of cytokine inhibitors, and the blocking of antigen-processing genes, I will focus here exclusively on strategies that interfere with the MHC class I pathway. Almost each step in the MHC class I pathway, from the processing of peptides to recognition by CD8 T cells, has been targeted by viruses (Alcami and Koszinowski, 2000). DNA viruses with large coding capacities can block a range of polymorphic class I molecules by interfering with specific steps in the pathway with blockades that prevent the generation of functional antigenic complexes. This blockade strategy is not usually used by smaller RNA viruses which generally benefit from their rapid replication rate, in combination with the high error-rate intrinsic to RNA, that results in antigenic variation ultimately preventing CD8 T cell recognition. HIV is an exception to this rule and uses both strategies described above. While both strategies appear designed to disarm the host of the antiviral CD8 T cell response, and proof exists that these interfering functions provide a benefit to the virus *in vivo*, certain aspects of this field have made difficult the discovery of the *in vivo* relevance of some of the viral immune evasion strategies. In large part this is due to the fact that many of the viruses that are studied infect humans making *in vivo* experimentation impossible. However, this constraint is removed in the study of MCMV in mice where animal experimentation allows the testing of assumptions that are thought to be a given in the study of immune evasion. An example of such a hypothesis is that

viruses encode class I evasion genes to interfere or alter the CD8 T cell response to the virus. However, this teleological argument remains to be tested. In chapter 2, I attempt to address how the *in vivo* MCMV-specific CTL response might be affected by an MCMV gene which has been shown *in vitro* to have potent inhibitory effects on class I antigen presentation. Furthermore, complex interactions between sometimes multiple viral genes and the host response result in a web that is difficult to untangle. Nevertheless, the study of immune evasion mechanisms is invaluable for several reasons. For example the large DNA viruses in the herpesvirus family and poxviruses are thought to have about 50% of their genome dedicated to host control (Alcami and Koszinowski, 2000). Many immune evasion genes are homologs of host genes and some viral immune evasion strategies, such as the mechanism by which HIV causes MHC I internalization, have taught us about less well-known cell biological pathways (Piguet et al., 1999). Finally, the knowledge of immune evasion mechanisms may result in the development of more effective vaccines or immune therapies to combat viral infections. In this introduction, I will present some of the class I immune evasion genes and strategies used by several viruses that interfere with steps throughout the antigen processing and presentation pathway, and then review what is known about MCMV.

**Preventing peptide generation.**

Epstein Barr virus (EBV), a large DNA virus, is a member of the γ-herpesvirus subfamily. Although all latently infected B cells express the EBNA-1 protein, EBNA-1-specific CD8 T cells were initially undetectable. This defect was found to be due to a 238 amino acid sequence of repeated Gly and Ala residues that prevents the generation of peptides from EBNA-1. The transfer of the Gly-Ala sequence into other proteins resulted in blocking peptide generation from those proteins and removal of the Gly-Ala sequence was sufficient to restore peptide generation and presentation to EBNA-1-specific CD8 T cells (Blake et al., 1997; Levitskaya et al., 1995). Although EBNA-1-specific CD8 T cells are elicited in humans, probably through cross-priming (Blake et al., 1997; Tellam et al.,

2001), the presence of the Gly-Ala sequence appears to attenuate the EBNA-1-specific CD8 T cell response to latently-infected cells. As mentioned earlier, cross-priming CTL to antigens that are never presented on infected cells might be the down-side of cross-priming. Whether the CTL response to the EBNA-1 protein is a demonstration of this quandary remains to be demonstrated.

The HCMV gene product pp65 blocks presentation of peptides from the HCMV IE protein in association with many MHC alleles (Gilbert et al., 1993). pp65 is a Ser/Thr kinase that is required to phosphorylate IE, as well as block generation of peptides from IE. The mechanism by which pp65 inhibits IE peptide generation is unknown, however, the inhibition of IE proteolysis is likely to be the cause (Yewdell and Bennink, 1999). Interestingly, pp65 itself elicits a substantial number of CD8 T cells (McLaughlin-Taylor et al., 1994). However, pp65 is a structural protein, which does not require new gene transcription for the generation of antigenic pp65 peptides; this is likely the reason why pp65-specific CTL were discovered and why pp65 is thought to be immunodominant. However the interference of peptide generation by pp65 or other gene products is certainly involved in the lack of identification of other HCMV CTL epitopes.

**Blocking TAP.**

Because TAP is dedicated to transporting peptides derived from the proteasome to class I molecules in the ER, blocking TAP allows viruses to selectively interfere with the class I pathway while minimizing effects on other cellular processes. Furthermore, over 90% of all peptides presented by class I molecules are TAP-dependent (Yewdell and Bennink, 1999).

Both herpes simplex virus (HSV), a member of the α-herpesvirus subfamily, and HCMV interfere with peptide transport through TAP. The HSV gene ICP47 blocks peptide transport by binding to the TAP1/TAP2 complex in the cytosol (Hill et al., 1995; York et al., 1994) while the HCMV US6 protein interferes with TAP transport in the ER lumen.(Ahn et al., 1997; Hengel et al., 1997). So far, products from four HCMV genes,

US2, US3, US6, and US11 have been shown in *in vitro* assays to prevent the generation of class I antigenic complexes (Tortorella et al., 2000). Interestingly, HCMV US6 is an E gene that is highly expressed at L times and later into the virus growth cycle than the other 3 class I immunomodulatory genes (Hengel et al., 1998).One hypothesis that is commonly put forth is that HCMV uses the staggered expression of these four genes to interfere with the class I pathway throughout the virus growth cycle; however, this hypothesis remains to be proved.

**Retention of MHC class I molecules.**

The retention of MHC class I molecules in the secretory pathway is used by several viruses .The HCMV IE gene US3 product associates with peptide-loaded classical class I molecules and prevents their egress to the cell surface (Ahn et al., 1996; Jones et al., 1996). The adenovirus E3/19K protein, the first identified immune evasion gene, contains an ER-retention motif in its cytoplasmic tail that selectively retains some newly synthesized class I molecules. (Tortorella et al., 2000; Yewdell and Bennink, 1999). Finally, the MCMV encoded *m152* gene product also retains class I in the ER cis-Golgi and will be described below in the section on MCMV.

**Proteins that direct class I molecules to the cytoplasm for proteasomal degredation.**

HCMV US2 and US11 are both E proteins that induce the retrograde transport of newly synthesized class I molecules back to the cytosol to be degraded by the proteasome. The mechanisms used by US2 and US11 are different although the outcomes are similar in that both target HLA-A and B but leave HLA-C, E and G unaffected (Loenen et al., 2001). Furthermore, US2 and US11 bind different mouse class I molecules (Machold et al., 1997) suggesting that the functional redundancy afforded by these two proteins is required to cover the range of polymorphic class I molecules. Deciphering the mechanism used by US2 and US11 lead to the discovery of a constitutive process by which proteins that are misfolded in the ER get retrograde translocated through the Sec61 pore, the channel which originally delivered them to the ER (Wiertz et al., 1996).

The most recent additions to the long list of class I immune evasion genes are K3 and K5, genes encoded by both human and mouse γ-2herpesviruses, specifically the Kaposis's sarcoma-associated herpesvirus and the murine γ-herpesvirus 68 (MHV-68) respectively. Recently, Boname et al. determined that the murine K3 downregulates MHC class I by a ubiquitination mechanism, ultimately resulting in class I degradation (Boname and Stevenson, 2001). Allele-specific binding was associated with the C-terminal of the K3 molecule while ubiquitination and degradation was associated with a conserved N-terminal sequence that could be substituted by the homologous sequence from the human K3. These results suggest that both the human and the mouse K3 genes, and possibly the human K5 gene, all operate by a similar ubiquitination mechanism, a hypothesis that was also suggested based on previous reports (Lorenzo et al., 2001).

**Internalization of cell surface class I molecules.**

The HIV Nef protein downregulates the cell surface expression of MHC class I molecules as well as the CD4 molecule, which is used for HIV entry. As with other viral proteins described above, Nef selectively downregulates HLA-A and B, but not HLA-C which can act as an inhibitory ligand for NK cells (Cohen et al., 1999).

**MCMV immune evasion genes.**

MCMV encodes three genes that are known to interfere with interfere with antigen presentation to CD8 T cells: *m4*,*m6*, and, *m152* . All three genes, as with the HCMV immune evasion genes, are encoded within two gene families that primarily encode glycoproteins and that flank the viral genome. Interestingly, these are the two regions that are not homologous in MCMV and HCMV. The effects of the *m152* gene led to the discovery of a protein that interfered with recognition by CD8 T cells (Del Val et al., 1989). *m152* encodes a 40 kDa glycoprotein, and is the class I evasion gene that is expressed earliest, at 2 hours post infection (Holtappels et al., 2000). m152/gp40 decreases class I surface expression and retains class I in the ER-cis Golgi, however, the

mechanism by which this occurs is still undefined (Ziegler et al., 2000). Furthermore, m152/gp40 preferentially retains $D^b$ over $K^b$ class I molecules in H-2b mice (Kavanagh et al., 2001). This result lead to the hypothesis that *m4* might make up for the incompletely retained $K^b$ molecules, a topic I will revisit in the discussion.

Although three MCMV genes are known to interfere with the class I pathway, *m152* appears to be dominant and severely inhibits antigen presentation *in vitro*. Mutant MCMVs lacking *m152* are highly visible to CTL in $^{51}Cr$ release assays under conditions in which the wildtype virus is not detected (Kavanagh et al., 2001; Krmpotic et al., 1999; Ziegler et al., 1997). The only previously published *in vivo* study on the effects of *m152* showed that a virus deleted for the *m152* gene demonstrated reduced virulence as demonstrated by a two-fold increase in the number of surviving neonatal BALB/c mice after a Δm152 infection (Krmpotic et al., 1999). Also, the Δm152 virus showed an attenuated replication capacity; mice infected with the Δm152 virus had 1 log less virus titers compared to wildtype-infected mice. Furthermore, this effect could be attributed to T cells. Finally, adoptive transfer of CD8 T cells from mice infected with wildtype MCMV into irradiated mice infected either with the Δm152 MCMV or the wildtype MCMV showed that the Δm152 infection was slightly better controlled. In summary, deleting *m152* interfered with optimal viral growth through a modest increase in CD8 T cell control. In this study, viral titers were determined at an early time point in infection suggesting that *m152* plays a modest role in interfering with the efficacy of the acute CD8 T cell response. Whether *m152* functions later in infection and exert its effects primarily during reactivation of latency remains to be established. This highlights another general hypothesis in the field of immune evasion: that immune evasion genes primarily interfere with the chronic stage of viral infections. This argument, although unproven, is based on the observation that evasion genes that directly interfere with class I pathway have so far only been associated with viruses that cause chronic infections (Yewdell and Bennink, 1999).

The m4/gp34 protein was originally shown to bind class I molecules in the ER, and travel with them to the cell surface in a confirmation that is stable in the moderate detergent NP-40 (Kleijnen et al., 1997). Recent work by Daniel Kavanagh showed that in addition its tight association with class I, m4/gp34 loosely associates with class I in the ER, in an NP40-unstable, but digitonin-stable form (Kavanagh et al., 2001). Before this thesis work, no function was associated with *m4*. Chapter 2 will describe the studies that established *m4* as a CTL evasion gene.

The *m6* gene encodes a 48 kDa glycoprotein that, like *m4* , is expressed later than *m152* in a second set of early genes (Hengel et al., 1998). m6/gp48, through its lumenal/transmembrane portion, associates with class I molecules in the ER to form a complex. The *m6* cytoplasmic tail contains a functional dileucine motif that directs the m6/class I complex to exit the ER, pass through the Golgi, and get redirected to the lysosome for degradation (Reusch et al., 1999). Whether the class I molecules actually reach the cell surface and then get redirected is still unresolved. Nevertheless, cells transfected with *m6* show decreased class I surface expression. Furthermore, cells infected with a mutant MCMV lacking *m6* are better killed in $^{51}$Cr release assays (MC Gold unpublished data) than are wildtype-infected targets demonstrating that all 3 known MCMV immune evasion genes functionally interfere with antigen presentation to CD8 T cells.

This now takes me to the questions I addressed in my thesis. My thesis project can be divided broadly into two parts: 1) the effects of MCMV immunomodulatory genes *m4* and *m152* on antigen processing and presentation as assessed *in vitro* and 2) the effects of immunomodulatory gene *m152* on the *in vivo* CTL response to MCMV. In the data presented in chapter 2.1, I will first describe the isolation of MCMV-specific H-2$^b$-restrictedCD8 T cell clones which allowed me to ask whether *m4* functions as an immune evasion gene *in vitro*. Furthermore, the CTL clones were instrumental in the identification of the first H-2$^b$ epitope I describe in chapter 2.3. However the focus of this

thesis is on studies that address the relevance of *m152 in vivo*. As I mentioned earlier, many assumptions are made about the *in vivo* functions of immune evasion genes. Furthermore, it is assumed that there are clear advantages to the virus to encode such genes. Since we work with mice, we have the opportunity to address questions about the role of immune evasion genes in MCMV infection, a biologically relevant animal model. Because *m152* severely inhibits antigen presentation *in vitro* as shown by others and in chapter 2.2, we hypothesized that *m152* might affect both the quantity of CTL and the quality of CTL in response to MCMV infection. These questions were tested in C57Bl/6 mice using MCMV mutant viruses lacking *m152*. However, the CTL response to MCMV was previously poorly characterized. Therefore to ask whether *m152* affected the size and the kinetics of the CTL response to MCMV, we compared the CTL responses in mice infected with wildtype MCMV to those from mice infected with Δm152 viruses. Furthermore, we questioned whether *m152* would affect the establishment of latency in chapter 2.2.; was the CTL response to MCMV Δm152 infection qualitatively different and if so, could it be so effective as to prevent the establishment of latency? Finally, in chapter 2.3, with the identification of the first H-2b-restricted MCMV CTL epitope, we addressed the assumption that antigens affected by immune evasion genes would be altered in the immunodominance hierarchy.

# Chapter 2

### *Introduction to the data chapters.*

Experiments were performed to address the role of the MCMV immunomodulatory genes *m4* and *m152* on antigen presentation *in vitro*, and the role of *m152* on the CD8 T cell response *in vivo*. These studies are contained in the three sections that follow. Section 2.1 describes the isolation and characterization of MCMV-specific CD8 T cell clones. Using these clones we discovered that *m4* is a CTL evasion gene. Furthermore, the clones displayed phenotypes that may allow further elucidation on the role of calreticulin in the class I assembly complex. Section 2.2 contains the bulk of the *in vivo* studies on the CD8 T cell response to MCMV infections with and without *m152*. In this section, the size and kinetics of the CTL response to MCMV is described. Moreover, we demonstrate that *m152* is not necessary for the establishment of latency. Furthermore, characterization of CD8s from chronically MCMV-infected mice suggests that these CD8s appear to have characteristics of both effector and memory CD8s. This raises the question: are these cells continually seeing antigen and if so, how often? Finally, section 2.3 describes the identification of the first H-$2^b$ epitope from MCMV by screening an MCMV genomic DNA library with the MCMV-specific CTL clones described in section 2.1. The identification of this epitope allowed us to question whether *m152* alters the immunodominance of an antigen whose presentation is severely impaired by the presence of *m152* in *in vitro* assays. The surprising answer is that it did not.

## Chapter 2.1- *m4* and *m152* interfere with antigen presentation to CTL *in vitro*

### *Polyclonal CTL from MCMV-infected mice demonstrate variation in their ability to kill wildtype-MCMV-infected targets*

In order to study the CD8 T cell response to MCMV we chose to work on C57Bl/6 mice, which have the H-$2^b$ haplotype, for the primary reason that knockout mice have the same H-2 haplotype. In the future, we plan to perform studies that address the role of host genes in MCMV infection using mice lacking specific genes. However, most previous work on CD8 immunity to MCMV, and the identification of *m152* as an inhibitor of antigen presentation was performed using BALB/c mice, which have the H-$2^d$ haplotype. To study MCMV in B6 mice, it was necessary to perform a series of experiments that paralleled the previous studies in BALB/c mice. To this end, I isolated MEFs from embryos of C57Bl/6 mice. Therefore, to first test whether MCMV-specific CD8 T cells were functionally inhibited by different immunomodulatory genes we used the $^{51}Cr$ release assay and restimulated polyclonal effectors. To prepare these effectors, mice were infected with MCMV for at least 12 weeks, sacrificed and splenocytes were restimulated with MCMV for 5 days *in vitro*, as described in the materials and methods. We first assessed the ability of polyclonal CTL to lyse MEF targets infected with wildtype virus. Figure 1A shows three separate assays that illustrate the range of results that we obtained. Although occasional assays detected MCMV-specific CTL, many simply showed non-specific lysis of uninfected cells which frequently exceeded the lysis of infected cells. Wildtype virus was occasionally well detected, more commonly it was seen no better than uninfected cells; frequently, wildtype virus infected cells were lysed less well than uninfected cells. These polyclonal cultures can comprise a number of specificities including both classic virus-specific CD8s and a population of NK-T cells that lyse uninfected cells in preference to infected cells as will be discussed later (data not shown).

Polyclonal cultures from MCMV-infected mice are thus highly variable and very difficult to interpret.

### *MCMV and mutant viruses.*

In order to address the role of immunomodulatory genes on antigen presentation *in vitro* and on the CD8 response *in vivo*, we used mutant viruses which were deleted for the immunomodulatory gene(s) of interest. All mutant viruses were constructed in Ulrich Kozsinowski's laboratory as described in the materials and methods. Table 2 lists the viruses used in this thesis. Two methods were used to generate the viruses. The first approach made use of a plasmid encoding Lac Z as a marker, flanked by sequences homologous with the flanking sequences of the gene of interest. Successful homologous recombination of the plasmid with wildtype MCMV DNA in transfected cells resulted in an MCMV mutant lacking the gene which was now replaced by Lac Z. The more recent bacterial artificial chromosome (BAC) technology was used to construct the other MCMV mutants. In the BAC system, the bacterial "F" plasmid encodes the entire MCMV genome and maintains the capability of replicating in bacteria containing the necessary recombinases. Homologous recombination between the gene of interest encoded by the BAC and a DNA insert encoding a selection marker results in the deletion of the gene of interest with the substitution of the selection marker.

### *m152 inhibits antigen presentation to restimulated polyclonal effectors from mice infected with wildtype MCMV or a virus lacking m152*

Although it was clear from the literature that MCMV infection elicits virus-specific protective CD8 responses, we reasoned that it might be easier to detect such responses using target cells infected with a virus that lacked immune evasion gene(s). The mutant ΔMS94.5 was initially used to map the principle immune evasion gene *m152* by Thäle et al.(Thale et al., 1995); it lacks 15.8kb of the Hind III region of the (230kb) MCMV

genome (Table 2). The deleted region encodes the predicted ORFs m150 to m165. As this was the first MCMV mutant available to us at the time we began this work, we compared the ability of polyclonal CTL to lyse targets infected with ΔMS94.5 or wildtype virus. Figure 1 shows that ΔMS94.5-infected cells were recognized better than wildtype virus-infected cells. We also infected mice with ΔMS94.5 and found that polyclonal CTL cultures generally showed better virus-specific lysis (figures 1, 2, and 3) Furthermore, the preference for ΔMS94.5 over wildtype was usually even more pronounced in these cultures.

### *Polyclonal CTL kill Δm4-infected targets at an intermediate level*

Also intriguing was the intermediate level of killing seen on targets infected with a virus lacking the *m4* gene using the same polyclonal effectors (figure 2). *m4* had previously been shown to bind class I in the ER and remain tightly associated with it on the cell surface (Kleijnen et al., 1997). However, no function had been ascribed to *m4* before this work. Viruses lacking *m4* were clearly recognized better than wildtype virus, implying that *m4* interferes with antigen presentation. Because responses from polyclonal effectors yielded variable results, we could not determine whether *m4* was moderating the level of killing of all MCMV-specific CTL, or if only a portion of the CTL were affected by *m4*. Nevertheless, this data was the first functional evidence of *m4* interference with antigen presentation.

### *The CTL response to MCMV appears to mature over time.*

As a first step towards understanding the equilibrium between MCMV infection and the CD8 response in chronic/latent infection, we wanted to describe the development of the CTL response over time. Furthermore, I noted over the course of several experiments, that CTL isolated from mice infected for longer periods of time appeared more potent in $^{51}Cr$ release assays. Figure 3 shows two independent CTL assays performed identically to

those from figure 2, using mice that had been infected for various intervals (between one and ninety weeks) prior to the assay. The results from these assays highlight the experiment to experiment variation that we find with polyclonal CTL assays. For instance, the lack of any lytic activity from the wildtype infected mice at weeks 1 and 4 in the second experiment is atypical; more commonly we saw non-specific lysis. Nevertheless, some consistent trends emerged. First, we note again that ΔMS94.5-infected targets were always better detected than wildtype-infected targets. Second, the response from wildtype virus-infected mice appeared to steadily improve over time. In the first assay, lysis of wildtype infected cells was below that of uninfected cells until 32 weeks post-infection. The second assay was more dramatic, showing virus-specific lysis first detectable at 40 weeks post infection, and good recognition of both wildtype and ΔMS94.5-infected cells at 90 weeks post infection. Because MCMV establishes a persistent/latent infection, these results are suggestive of a continued maturation of the CD8 response as a consequence of recurrent antigen exposure. However, given the uncertain composition of these polyclonal cultures, the difficulty separating virus-specific from non-specific lysis, and potential additional variation imposed by the 5 day in vitro culture, it was not possible to make a strong conclusion about the size or specificity of the CD8 population from these assays. For that reason we turned to intracellular cytokine staining assays for further experiments as will be described in data chapter 2.2 below.

### *m152 inhibits antigen presentation to CTL clones isolated from mice infected with wildtype MCMV or a virus lacking m152.*

To compare the recognition of ΔMS94.5 and wildtype more accurately without the confounding influence of non-specific lysis, we isolated CTL clones from mice infected with MCMV. As described in the materials and methods, splenocytes from C57Bl/6 mice, infected for 12 weeks or more with wildtype MCMV (Smith) or MCMV mutants lacking *m152*, were plated under limiting dilution conditions, assessed for viability, and

screened for their ability to kill target cells infected with MCMV lacking *m152*. Table 3 shows the panel of clones that were generated. Figure 4 shows that none of CTL clones we isolated killed wildtype-infected fibroblasts while all of the CTL clones killed Δm152-infected targets. The inability of wildtype-infected targets to be killed was seen even with clone 96, which was derived from a mouse infected with wildtype MCMV. These results demonstrated that *m152* interfered with antigen presentation in H-$2^b$ MEFs and confirmed the role of *m152* as a CTL evasion gene. Nevertheless, if the clones are representative of the larger polyclonal population, we might expect to find some with the ability to kill wildtype-infected fibroblasts since a low level of killing was occasionally seen on wildtype-infected targets using restimulated polyclonal effectors; however, no clones of this phenotype have yet been isolated as will be discussed later.

### *Characterization of MCMV-specific CTL clones.*

The clones were characterized using different methods. In the first set of experiments, we wanted to determine from which stage of the virus life cycle the antigens they recognized were derived. In the second set of experiments, we determined the restricting class I molecule for several clones. And in the third set of experiments, HPLC-purified peptide fractions from MCMV-infected cells were used to sensitize targets to determine if clones of different specificities had been isolated. Together, this information could be useful in epitope identification.

### *MCMV specific CTL clones recognize E antigens.*

To determine from which stage of the virus life cycle the antigens the clones recognized were derived, we performed a CTL assay using targets infected with ΔMS94.5 under conditions where either only IE genes were expressed or both IE and E genes were expressed. These conditions can be achieved with the use of metabolic inhibitors as described in the materials and methods. Furthermore, the addition of PAA prevented L

gene expression. Figure 5a shows that clones 3, 5, 11, and 96 are all able to recognize PAA-treated targets but do not recognize cells expressing IE genes only. We therefore concluded that clones recognized E antigens. Because E genes are further temporally regulated and differentially expressed over time, we infected cells over a timecourse and used these as APCs in the ICS assay to examine the percent of clone cells that could respond to the APCs by secreting IFN-γ. In this assay, brefeldin A is added to not only inhibit secretion of the cytokine IFN-γ, but also prevent further antigen presentation. Figure 5b confirms that all clones recognized antigens at times consistent with E gene expression. None of the clones secreted IFN-γ at 2 hours post infection which is when we would predict IE antigens would be recognized. Clone 11 detected an antigen expressed early in the E phase starting at 4 hours post infection. Clone 3 cells responded after 6 hours of infection while clones 55 and 96 were only stimulated after 8 hours of infection. These results supported the conclusion that the MCMV-specific CTL clones were specific for E antigens.

### *Some MCMV-specific CTL clones are $K^b$-restricted, others are $D^b$-restricted CTL.*

A different way to examine whether we had isolated clones of different specificities was to determine their MHC restriction element. To define the restriction element used by each clone, I isolated MEFs from congenic mice that either express the $K^b$ locus (B10.A5R) but not $D^b$, or the $D^b$ locus (B10.A2R) without $K^b$, and used these as targets in a $^{51}Cr$ release assay (no L locus exists in these mice). Figure 6 shows that clones 5, 11, and 96 were $K^b$-restricted while clone 3 and 55 were $D^b$-restricted.

### *Clones 11 and 96 recognize different HPLC peptide fractions.*

We then wanted to determine if some of the different $K^b$ or $D^b$-restricted clones we had isolated were of different specificities. We used HPLC purified peptide fractions isolated

from MEFs infected with ΔMS94.5, to sensitize RMA-S targets in a $^{51}Cr$ release assay as described in materials and methods. Figure 7 shows that clone 11 recognized targets sensitized with peptide fraction 31 while clone 96 recognized targets sensitized with fractions 35 and 36. Clone 3 was not stimulated by any of the peptide fractions isolated in this preparation. This therefore demonstrated that clones of at least three different specificities had been isolated; at least one $D^b$-restricted antigen (clones 3 and 55), and two $K^b$-restricted antigens (clones 11 and 96) could now be used to assess CD8 responses to three different MCMV antigens.

A note here: as we continued to propagate the clones, they became infected with mycoplasma. We have continued to propagate these cells in mycoplasma removal agent and these cells maintain their specificity. On the occasions when the clones kill poorly or non-specifically, they are discarded and newly thawed cells are used.

### *m4 is a CTL evasion gene which acts through $K^b$-restricted CTL clones but not $D^b$-restricted CTL.*

Figure 2 showed that Δm4-infected targets were killed at an intermediate level by polyclonal effectors. To address whether *m4* differentially affected some of the CTL in the polyclonal population or if *m4* was moderating the level of killing of all MCMV-specific CD8s we tested the ability of MCMV-specific CTL clones to kill Δm4-infected targets. As we began assessing the ability of different MCMV-specific CTL clones to kill Δm4-infected targets, we discovered that some clones but not others could kill the Δm4-infected targets. Because we did not have a revertant virus to confirm the *m4* phenotype, several different Δm4 mutants were used to infect target cells. Figure 8A, B, and C shows that clones 11 and 96 killed Δm4-infected targets while clone 3 did not. The observation that only some clones were affected by *m4* coincided with two other results. One was the characterization of the restricting class I allele for each of the clones tested. Figure 8D shows that the clones that were affected by *m4* were $K^b$-restricted while the unaffected

clones were $D^b$-restricted. The other was an observation made by Daniel Kavanagh, who showed through biochemical analyses that *m152* was able to completely retain $D^b$ but not $K^b$ class I molecules. The CTL assay results allowed us therefore to conclude that *m4* functions as a CTL evasion gene. Furthermore, the combination of the functional data and Daniel Kavanagh's biochemical analyses allowed us to conclude that *m4* and *m152* inhibit antigen presentation in a complementary and cooperative manner.

### *Calreticulin plays a selective role in antigen processing and presentation.*

As described in the introduction, the role of calreticulin in the loading complex is unclear. To ask whether calreticulin was necessary for presentation to MCMV-specific CTL clones, calreticulin$^{-/-}$ fibroblast cells were infected with ΔMS94.5 and used as targets in a $^{51}Cr$ release assay. Figure 9 shows that of 5 MCMV-specific CTL clones tested, only clone 11 was able to kill infected calreticulin$^{-/-}$ targets. We could not attribute the calreticulin independent killing to a class I allele-dependent phenomenon as both clones 11 and 96 are $K^b$-restricted (figure 6). Furthermore, it did not appear that the need for calreticulin was based on a quantitative effect. Clones 96 and 5 both showed better killing on infected targets than clone 11, however, they were not able to overcome their need for calreticulin. These data furthermore show that in the same targets cells, in the same assay, clones differ in their dependence on calreticulin. These data therefore suggest that calreticulin selectively affects some peptides but not others. Further studies are required to determine the role of calreticulin in the loading complex.

### *Tapasin is required for antigen presentation to all clones tested*

We have also begun to study the role of tapasin in the loading complex. As described in the introduction, tapasin acts as a bridge between TAP and complexed class I/$\beta_2$m/calreticulin, however, the role of tapasin in peptide quality control is unclear. Figure 10 shows that clones 3 and 94 were unable to kill ΔMS94.5-infected tapasin$^{-/-}$

targets, furthermore, none of the several MCMV-specific CTL clones tested have ever been able to kill cells lacking tapasin (data not shown). Although these data demonstrate that tapasin is required for presentation to clones 3 and 94, they do not address the separate roles tapasin may have. This question will need to be addressed using different tapasin mutants as will be discussed later.

**Figure 1. Polyclonal CTL from MCMV-infected mice kill Δm152-infected targets better than wildtype-infected targets .** Restimulated polyclonal effectors from mice infected with wildtype MCMV or ΔMS94.5 were tested for their ability to kill wildtype or ΔMS94.5- infected B6 targets. Three assays are shown to demonstrate the assay to assay variation.

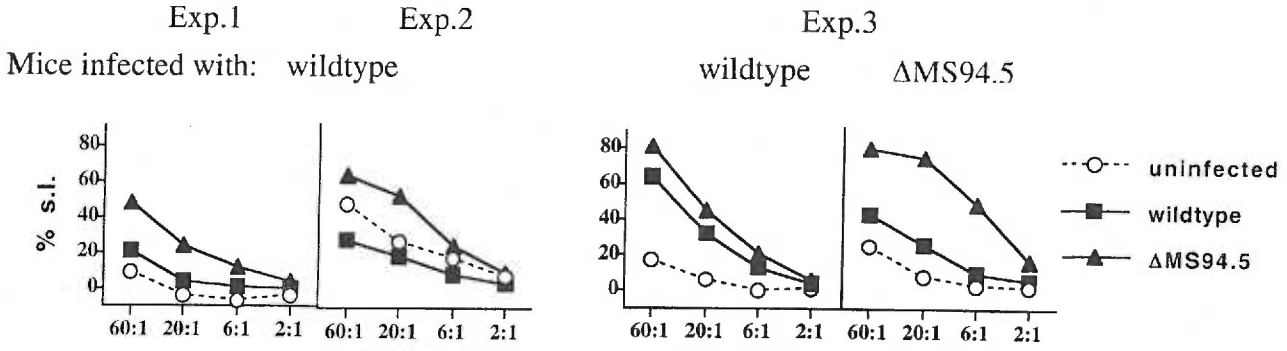



**Figure 2. Polyclonal CTL from ΔMS94.5-infected mice kill Δm4-infected targets at an intermediate level .** Restimulated polyclonal effectors from mice infected with ΔMS94.5 were tested for their ability to kill wildtype, ΔMS94.5 or Δm4-infected B6 targets.

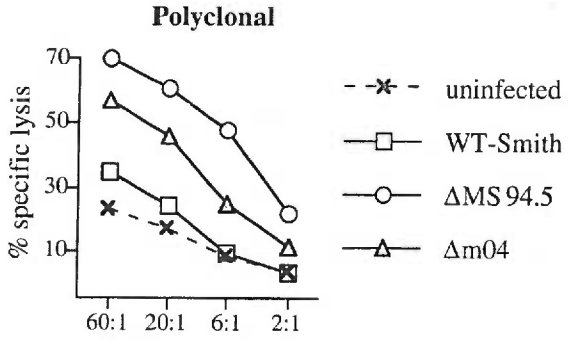


**Figure 3. The CTL response to MCMV appears to mature over time.** Restimulated polyclonal effectors from mice infected with wildtype MCMV or ΔMS94.5 over a period of 32 weeks in A) or up to 90 weeks in B) were tested for their ability to kill wildtype, or ΔMS94.5- infected targets.

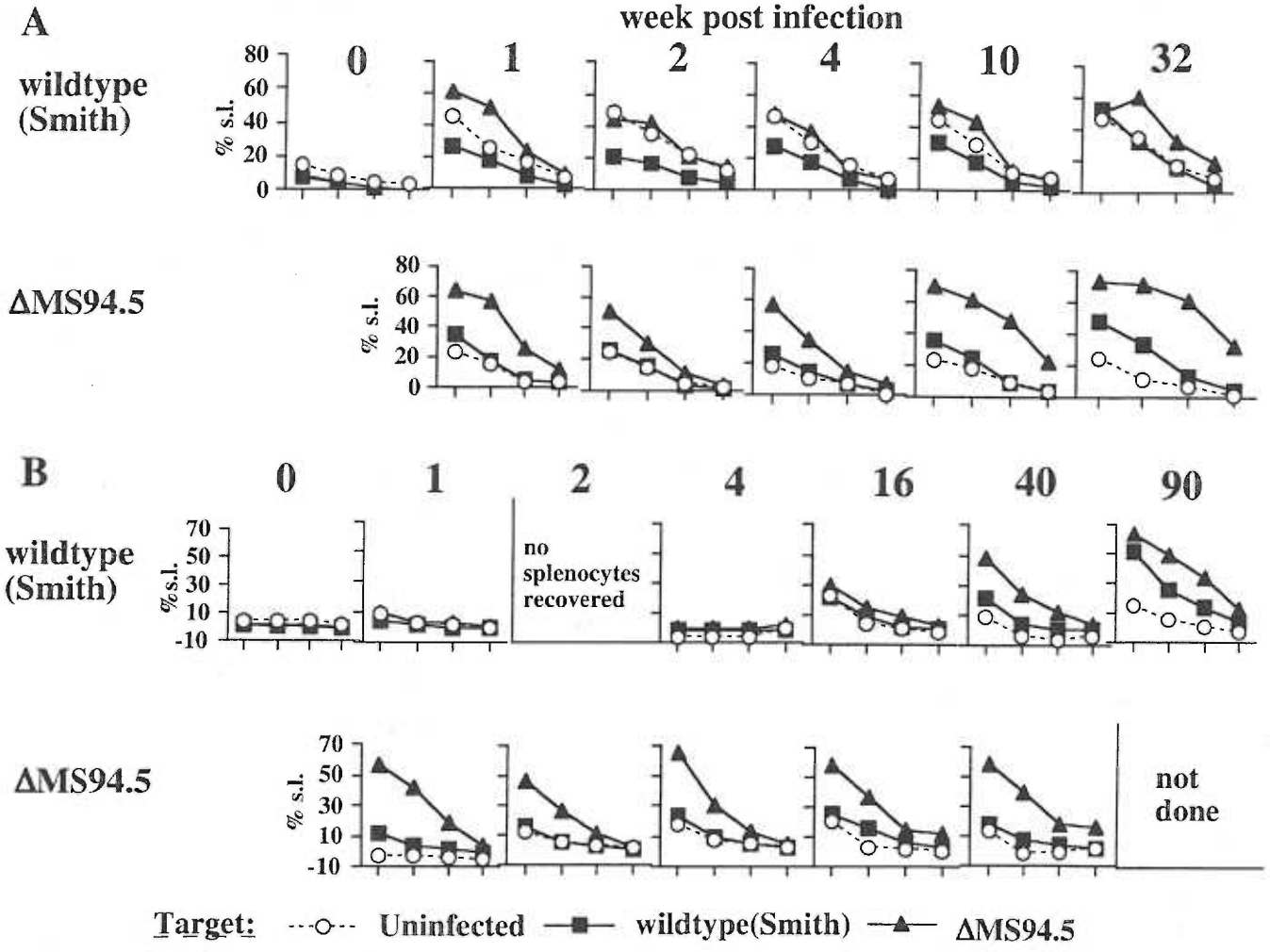


**Figure 4. *m152* prevents antigen presentation to MCMV-specific CTL clones.** MCMV-specific CTL clones were tested for their ability to kill B6 MEFs infected with wildtype MCMV(Smith) or ΔMS94.5, a virus lacking *m152*. Six clones are shown and none are capable of killing targets infected with wildtype MCMV.

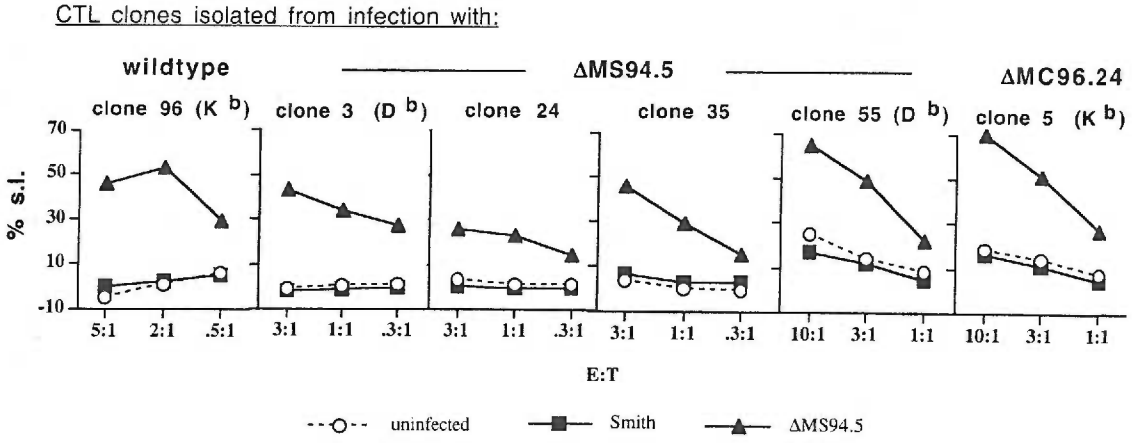
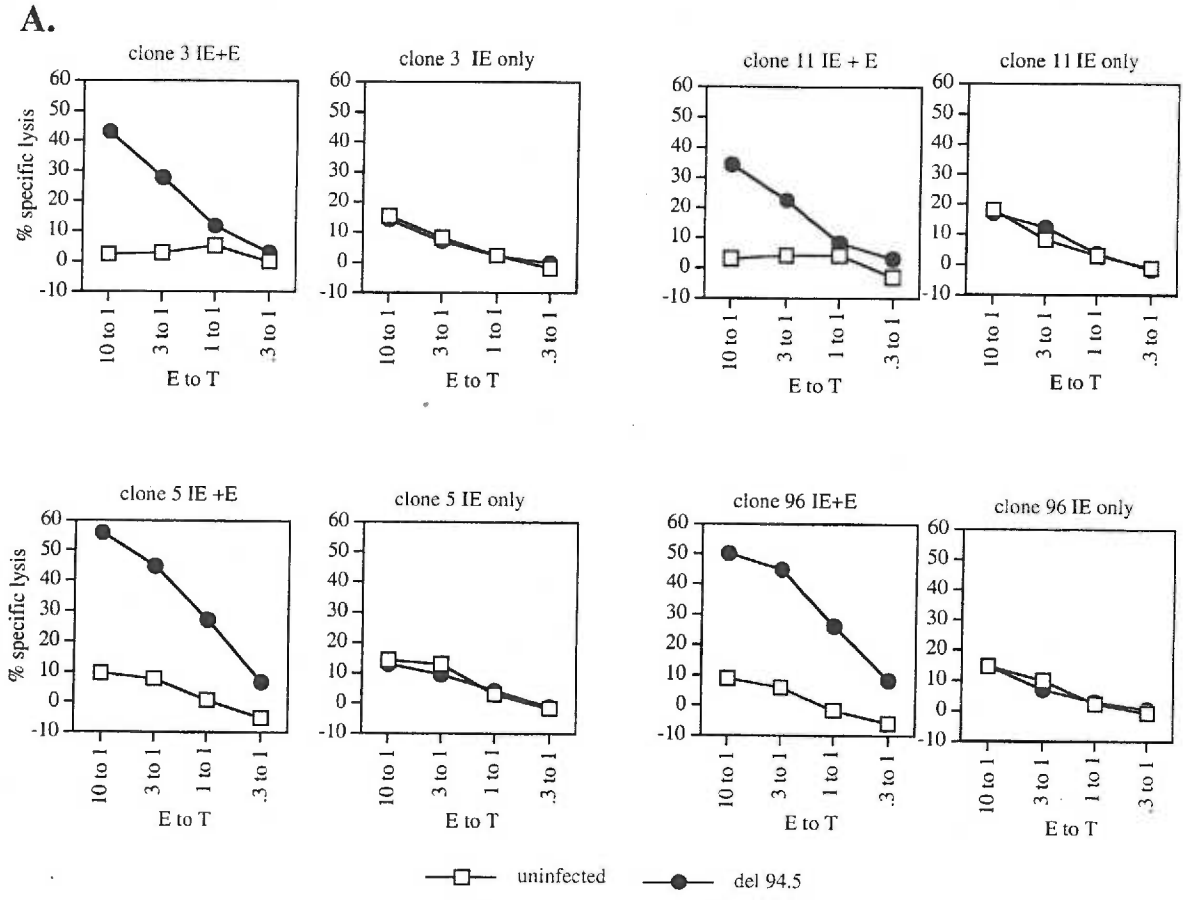


**Figure 5. MCMV-specific CTL clones recognize E antigens expressed at different times in the E phase of infection. A.** MCMV-specific CTL clones were tested for their ability to recognize IE or E antigens. B6 MEFs were infected with ΔMS94.5 in the presence of metabolic inhibitors to enhance IE and prevent E gene expression or recognize targets expressing both IE and E genes. **B.** CTL clones were then tested for the ability to secrete IFN-γ in the ICS assay in response to APCs infected with ΔMS94.5 for 0,2,4,6,8, or 16 hours+PAA.



**B.**

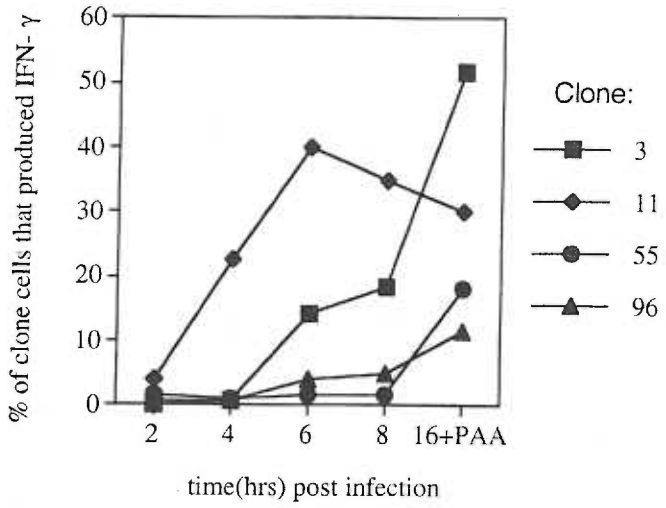

% of clone cells that produced IFN- γ
60
50
40
30
20
10
0
2
4
6
8
16+PAA
time(hrs) post infection
Clone:
3
11
55
96

**Figure 6. Some MCMV-specific CTL clones are $K^b$-restricted and others $D^b$-restricted.** The class I restriction element used by MCMV-specific CTL clones 3,5,11,55,and 96, derived from MCMV-infected B6 mice, were determined by testing the ability of each clone to lyse fibroblasts from B10A.2R ($K^k$ $D^b$) or B10A.5R ($K^b$ $L^dD^d$) mice. Fibroblast targets were either uninfected or infected with MCMV ΔMS94.5.

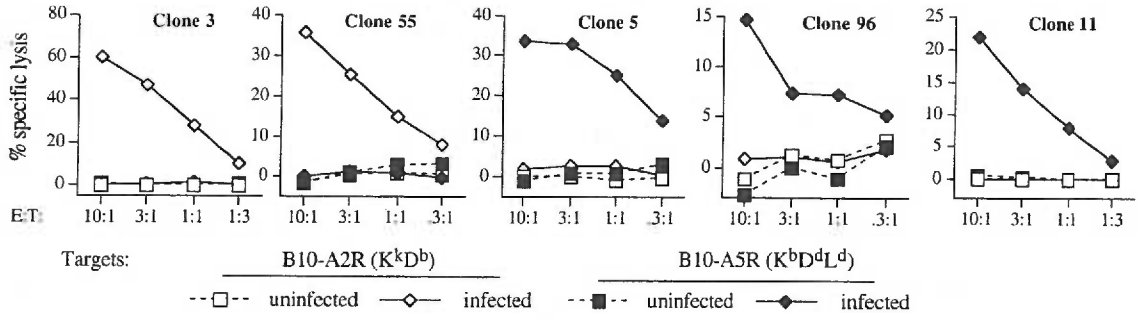


**Figure 7. MCMV-specific CTL clones 11 and 96 have different specificities.** B6 MEFs were infected with MCMV ΔMS94.5 in the presence of PAA to prevent L gene expression. Cells were harvested, and peptides were extracted and purified using HPLC as described in materials and methods. Peptides were used to sensitize RMA-S targets in a $^{51}Cr$ release assay. Clones 11 and 96, which are both Kb-restricted, recognize different peptide fractions. Clone 3 was not stimulated by any of the peptide fractions.

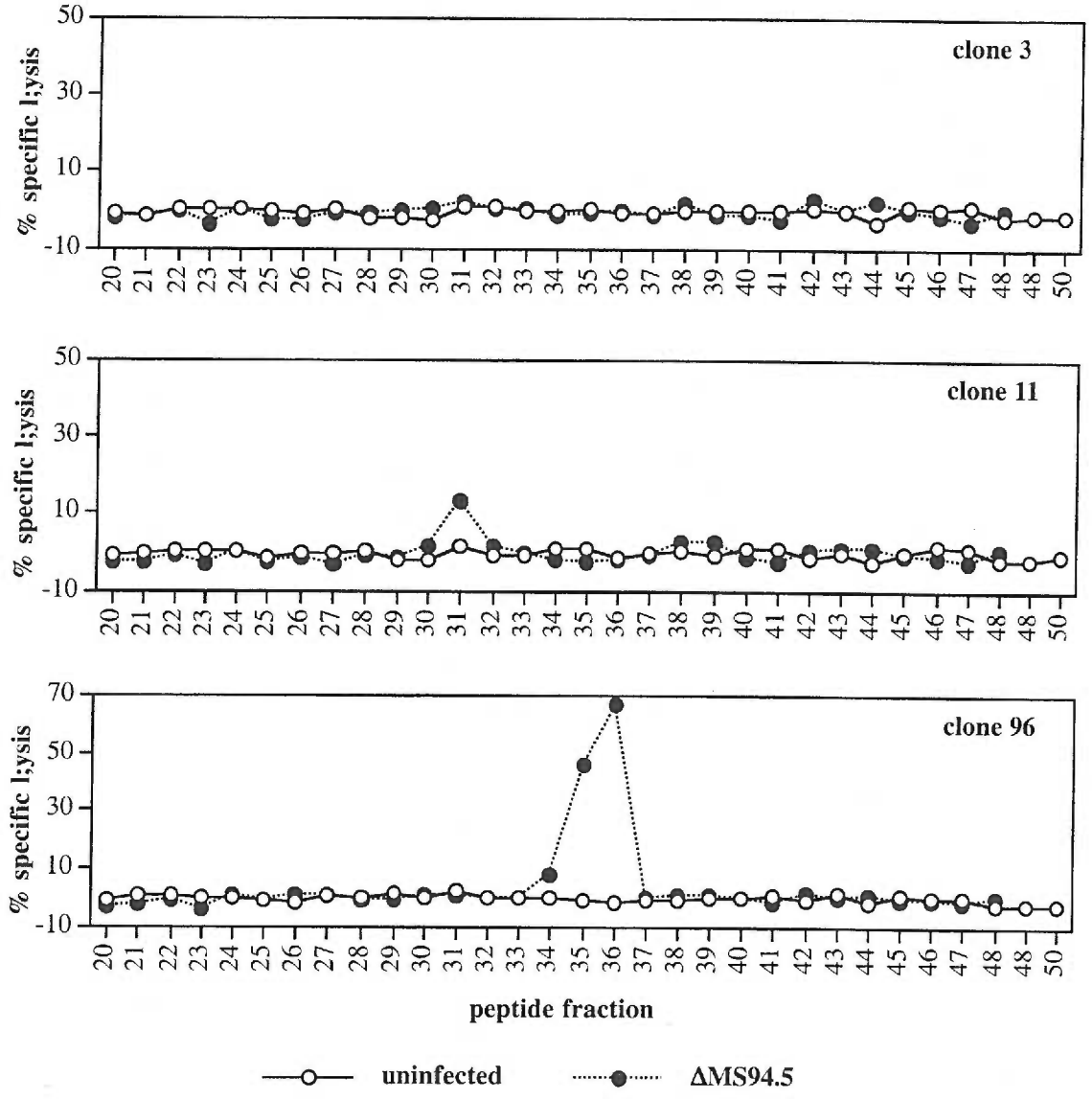


**Figure 8. *m4* is an immune-evasion gene necessary for evasion from $K^b$- but not $D^b$-restricted CTL clones.** CTL clones were tested for their ability to lyse B6 MEFs infected with the viruses shown. **A-C:** MCMV-specific CTL clones were tested against Smith or the wildtype BAC virus MW97.01 and various *m4* deletion mutants, as follows. **A**: Δm4-MC95.33, ΔMS94.7 (lacking ORFs 1-17), ΔMS94.5 and Smith. **B**: Δm4-MW99.03, ΔMS94.5, and Smith. **C**: Δm152-99.05, MW97.01 (wt) and three *m4* deletion viruses: m4Tn3514, m4Tn3516 and m4TnP. **D.** Only $K^b$-restricted clones but not $D^b$-restricted clones were able to kill targets lacking *m4*.

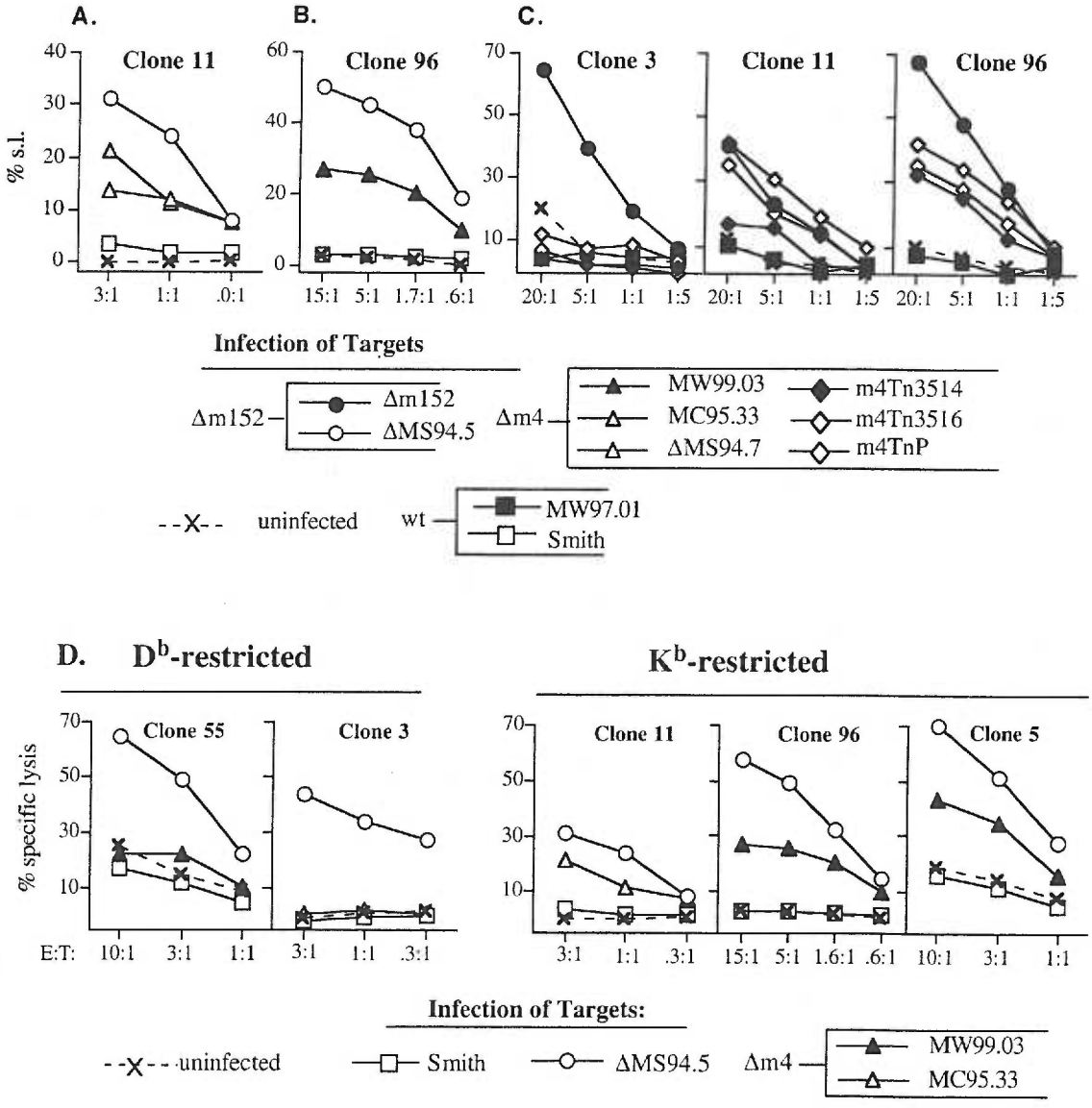


**Figure 9. Calreticulin is not necessary for antigen presentation to MCMV-specific CTL clone 11 but is required for clones 3, 5, 55, and 96.** MCMV-specific CTL clones were tested for their ability to kill B6 calreticulin$^{-/-}$ fibroblasts infected with ΔMS94.5. Only clone 11 does not require calreticulin.

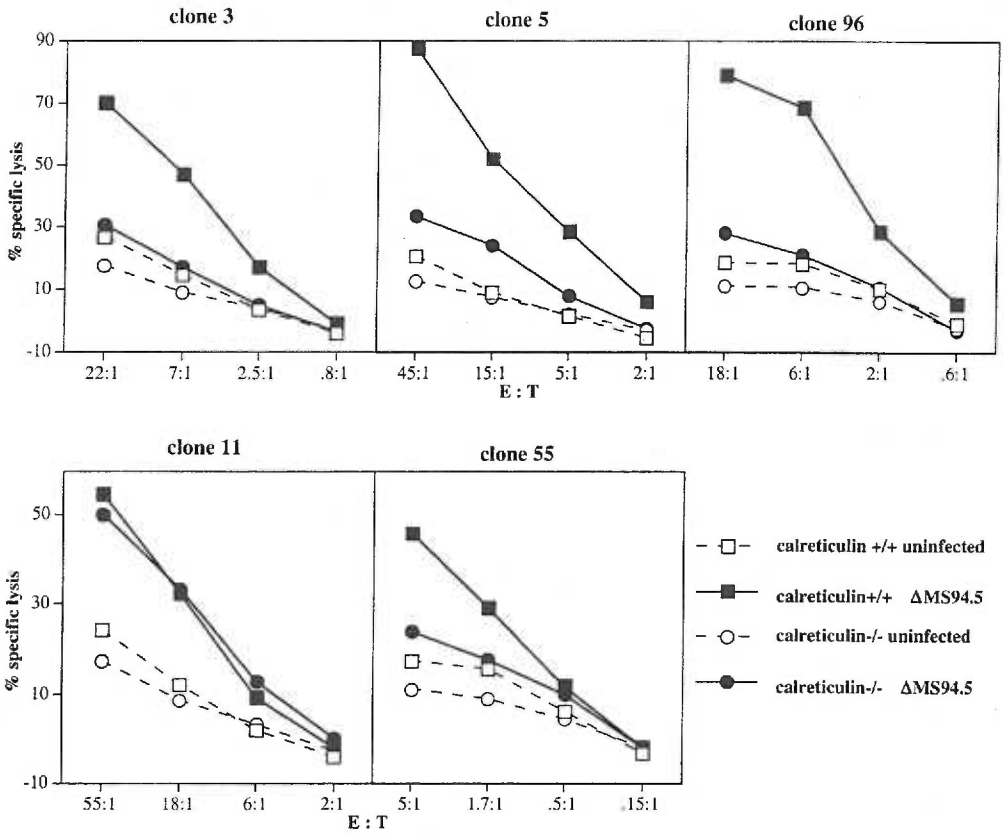


**Figure 10. Tapasin is required for antigen presentation to MCMV-specific CTL clones 3 and 94.** CTL clones 3 and 94 were tested for their ability to kill B6 tapasin$^{-/-}$ fibroblasts infected with ΔMS94.5. A positive control is included to show that clones were capable of killing wildtype B6 targets infected with ΔMS94.5 virus.

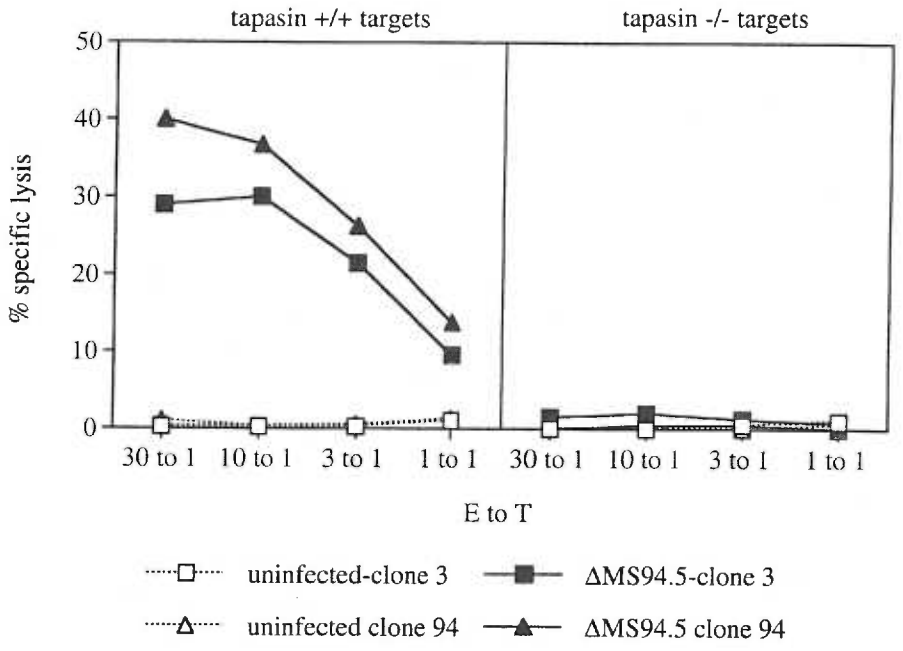


**Table 2.** Viruses used in this thesis.

| VIRUS: | METHOD | GENOTYPE | INSERTION |
|---|---|---|---|
| Smith strain | natural isolate | wildtype | none |
| MW97.01 | BAC derived | wildtype | none |
| ΔMS94.5 | mutagenesis in cells[1] | ΔORFs m150-m165 | lacZ |
| ΔMC96.24 | mutagenesis in cells | Δm152 | none |
| rMC96.27 | mutagenesis in cells | wildtype, revertant of ΔMC96.24 | none |
| ΔMS94.7 | spontaneous mutant | ΔORFs 1-17 | none |
| Δm4-MC95.33 | mutagenesis in cells | Δm4 (nt 3250-4041) | lacZ |
| Δm4-MW99.03 | BAC/recombination[2] | Δm4 (nt 3270-4067) | $kan^r$ |
| m4Tn3514 | BAC/transposon[3] | *m4* disrupted | Tn1721 into *m4* at nt 3514 |
| m4Tn3516 | BAC/transposon | *m4* disrupted | Tn1721 into *m4* at nt 3516 |
| m4TnP | BAC/transposon | putative *m4* promoter disrupted | Tn1721 into putative *m4* promoter at nt 3099 |
| Δm4+m152-MW99.04 | BAC/recombination | Δm4+m152 (nt 3270-4067 and 210244-211377) | $kan^r$/$zeocin^r$ |
| Δm152-MW99.05 | BAC/recombination | Δm152 (nt 210244-211377) | $zeocin^r$ |

The methods used to create mutant MCMVs are described in Materials and Methods.

[1] recombination between plasmid and wildtype MCMV in transfected cells.
[2] homologous recombination in *E. coli* between MCMV bacterial artificial chromosome (BAC) and insert.
[3] transposon-mediated mutagenesis of MCMV BAC in *E. coli* .

**Table 3**. MCMV-specific CTL clones isolated throughout thesis work.

| Clone | From mice infected with: [a] | Cloning # [b] | Restricted by: [c] | m4 phenotype [d] | Comments [e] |
|---|---|---|---|---|---|
| 3 | ΔMS94.5 | 1 | $D^b$ | No | Recognizes HGIRNASFI/$D^b$<br>Requires calreticulin and tapasin<br>TCR-Vβ7 |
| 11 | ΔMS94.5 | 1 | $K^b$ | Yes | Recognizes an early E antigen.<br>Does not require calreticulin<br>Requires tapasin<br>Recognizes peptide fraction 31<br>TCR-Vβ5.1/5.2 |
| 24 | ΔMS94.5 | 1 | | No | |
| 35 | ΔMS94.5 | 1 | | Yes | |
| 40 | ΔMS94.5 | 3 | | No | Did not survive repeated restimulation |
| 55 | ΔMS94.5 | 2 | $D^b$ | No | Recognizes HGIRNASFI/$D^b$<br>Requires calreticulin<br>TCR-Vβ7 |
| 5 | Smith | 1 | | | NK1.1 positive; did not survive repeated restimulation |
| 14 | Smith | 1 | | | Low level killing on Smith-infected targets; did not survive repeated restimulation |
| 29 | Smith | 1 | | | Low level killing on Smith-infected targets; did not survive repeated restimulation |
| 94 | Smith | 4 | | | Requires tapasin |
| 96 | Smith | 4 | $K^b$ | Yes | Only surviving Smith clone<br>Requires calreticulin<br>Recognizes peptide fraction 35/36<br>TCR-Vβ8 |
| 102 | Smith | 4 | | | |
| 204 | Smith | 5 | | | Did not survive repeated restimulation<br>TCR-Vβ8 |
| 5 | ΔMC96.24 | 1 | $K^b$ | Yes | TCR-Vβ9<br>Requires calreticulin and tapasin |
| d2A5 | Δm4-MW99.03 | ? | $D^b$ | No | Requires tapasin |

[a] CTL clones were isolated from mice infected with ΔMS94.5, Smith, ΔMC96.24, or Δm4

[b] This indicates the cloning attempts from which the clones were derived.

[c] Indicates by which class I allele the clone is restricted (when known)

[d] Indicates whether the clone recognized Δm4-infected MEFs.

[e] Comments on the requirement of the clone for tapasin and calreticulin are included. Other information on vβs, and antigen specificity are included when known.

## Data chapter 2.2-The CD8 T cell response to MCMV and MCMVΔm152 mutants *in vivo*

### *Quantitative assessment of MCMV-specific CD8s by intracellular cytokine staining.*

To further characterize the CD8 T cell response to MCMV and mutants lacking *m152*, we wanted to quantify the number of MCMV-specific CD8s directly ex vivo. In data chapter 2.1, I showed that qualitative CTL assays required a 5 day in vitro restimulation, and we believe that this is likely responsible for much of the variability we see within and between polyclonal CTL experiments. We therefore adapted the intracellular cytokine staining assay (ICS) for use with virus-infected APCs. As APCs we initially used MEFs that had been infected in the same way as those used for targets in $^{51}Cr$ release assays, and incubated them with splenocytes from infected mice in the presence of brefeldin A. After 6 hours, the cells were stained for surface expression of CD8 and then fixed and permeabilized before being stained with an anti-IFN-γ antibody and analyzed by flow cytometry. Figure 11A demonstrates the gating sequence for a typical positive sample. CD8+ live lymphocytes were gated as shown, and the percentage of cells expressing IFN-γ was measured as shown in the histogram. We wanted to determine the best APC conditions to allow us to detect highest numbers of MCMV-specific CTL. Figure 11B shows an analysis using ICS of the ability of CD8s from wildtype or ΔMS94.5-infected mice to respond to wildtype or ΔMS94.5-infected MEFs. The percentage of $CD8^+$ splenocytes that expressed IFN-γ is indicated in the upper right quadrant. 2% of $CD8^+$ splenocytes from wildtype-infected mice made IFN-γ in response to wildtype-infected targets, and 4.8% responded to ΔMS94.5-infected targets. Interestingly, the stronger response of mice infected with ΔMS94.5 that we had noted in the polyclonal CTL assays was also seen in this assay: 3.8% of CD8s from those mice responded to wildtype virus,

and 10% to ΔMS94.5. We concluded that ICS could be used to quantify the CD8 response to MCMV, and that using ΔMS94.5 in APCs allowed the detection of a higher percentage of MCMV-specific CD8s. Although ΔMS94.5 lacks 16 ORFs and could in theory lack antigens recognized by CD8s from wildtype-infected mice, ΔMS94.5 stimulators always allowed us to detect more virus-specific CD8s than wildtype-infected APCs, even in wildtype-infected mice.

Although the relative percentages of CD8 T cells responding to wildtype and ΔMS94.5 were always seen, the assay shown in Figure 11B gave the highest numbers of responders that we saw in a series of assays using infected MEFS as APCs. We therefore sought to improve the efficiency of restimulation by optimizing our APCs. For this purpose we tested JAWSII cells, an H-$2^b$ dendritic cell line that expresses $K^b$ and $D^b$ but does not express any mouse class II (U.S. Pat. 5,648,219 and U.S. Pat. 5,830,682). Dendritic cells are infectable by MCMV. In Figure 11C, we compared the ability of JAWSII and MEFs to act as stimulators for MCMV-specific CTL. More than twice as many $CD8^+$ T cells responded to ΔMS94.5-infected JAWSII as responded to MEFs. We therefore used ΔMS94.5-infected JAWSII as APCs in ICS assays for the rest of the experiments that follow.

### *Quantitation of the CD8 response to MCMV over time by ICS.*

Results from figure 3 in chapter 2.1 suggested that the CTL responses to both wildtype MCMV and ΔMS94.5 matured over time. We questioned whether this was due to a quantitative increase in MCMV-specific CD8s and therefore used ICS to quantify the CD8 response to MCMV over a protracted time course. Two such experiments are shown in Figure 12. For each experiment, mice were infected with either wildtype (Smith) or ΔMS94.5 MCMV for the periods shown. Mice were purchased from the same vendor and infected with the same stock of virus for each experiment. MCMV-specific CD8s were quantified by ICS; results are shown for individual mice both as a percentage of total

CD8+ splenocytes (upper panel) or total numbers of MCMV-specific CD8+ splenocytes (lower panel). MCMV causes a pronounced splenomegaly which is most marked at week 1 post infection; hence the decline in total virus-specific splenocytes between acute and chronic infection exceeds the decline in the percentage of CD8s which are virus-specific.

At day 7 post infection we detected about 15% of CD8s (average 5 million cells) that made IFN-γ in response to MCMV-infected APCs. At longer time periods (out to 44 weeks) about 1 million CD8s were MCMV specific. There are several points to notice from these experiments. As discussed in chapter 1, it appears the numbers of memory CD8s that result in response to a virus that is never eradicated is different from the numbers of memory CD8s that result from a virus that is cleared. For example, in several models of chronic infection such as HTLV-1(Greten et al., 1998), SIV (Kuroda et al., 1998), and EBV (Tan et al., 1999), the number of memory CD8s is 10 to 20% of the number seen at the peak of the response. In contrast, in several models of resolved infections such as LCMV, Listeria, and influenza, the numbers of memory CD8s are in general around 5% of the peak numbers. We interpret the higher numbers seen in chronic MCMV infection to indicate ongoing antigenic stimulation due to viral replication; this is pursued further below. Second, after the first month, the numbers of CD8s remained fairly constant. This suggests that a stable equilibrium is reached. We did not see a gradual decline in numbers that might indicate increasing immune control. Neither did we see an increase that could account for the apparent improvement in the CTL response seen in our earlier CTL assays shown in figure 3 of data chapter 2.1. However, as shown in figure 12B, CTL assays parallel to those performed in figure 3 of data chapter 2.1 were carried out using splenocytes from the same mice used in the ICS assay , and these also did not indicate the apparent maturation of the response seen in the original experiments. Thus the suggestion of maturation of the CD8 response over time is at best an inconsistent feature of these assays; and more sensitive assessments of CD8 avidity will be needed to determine whether maturation can occur with time in this infection.

However, the most striking feature of these assays is the CD8 response to ΔMS94.5. This virus continued to provoke high numbers of CD8s- over 20% of the total CD8s, seen in animals 44 weeks after infection. Again, this suggested that despite being readily detected by CD8s, ΔMS94.5 was not eradicated by this response but established a vigorous chronic infection.

### *Is the higher frequency of virus-specific CD8s seen in ΔMS94.5 compared to Smith strain a consistent difference between wildtype virus and viruses lacking* m152*?*

The experiments described above demonstrate clearly that ΔMS94.5 continues to evoke a population of activated T cells, suggesting that *m152* is not necessary for virus persistence. A consistent feature of these experiments was that the CD8 response in chronic infection with ΔMS94.5 was always higher than the response to wildtype (Smith) virus. While we could make various interesting speculations as to the reason for this, we could not be at all confident that this would be a consistent difference between wildtype viruses and viruses lacking *m152*. ΔMS94.5 lacks 16 ORFs; it was derived from the original Smith strain (ATCC VR-194/1981) that had been serially passaged in Ulrich Koszinowski's laboratory for some years. During the course of these studies we obtained access to two matched pairs of wildtype viruses and single *m152* deletants. The virus Δm152-MC96.24 was derived by insertional mutagenesis in cells from Smith (ATCC-VR-194); rMC96.27 is the revertant for this virus. Δm152-MW99.05 was derived by shuttle mutagenesis in the BAC plasmid. Wildtype MW97.01 is the virus reconstituted from the wildtype BAC.

In order to determine whether a deletion of *m152* alone would result in similar differences in the CTL population, mice were infected with each of these viruses, and the number of virus specific CD8s was assessed by ICS. Figure 13A shows two different experiments directly comparing two different Δm152 viruses and their parental wildtype.

In the first experiment, the Δm152 virus elicited CD8 numbers comparable to those seen in ΔMS94.5 infection. Surprisingly, however, the matched revertant to the Δm152 virus (rMC96.27) also elicited numbers of CD8s equivalent to those seen in the Δm152 strain(ΔMC96.24). Wildtype Smith however, elicited the lowest CD8response of the 4 strains. In experiment 2 in figure 13A, although the response the wildtype BAC (MW97.01) was lower than the CD8 response from the Δm152 virus (MW99.05) both viruses elicited a good CTL response that was higher than the one to wildtype Smith strain; of these six strains, the one that elicited the lowest number of CD8s in chronic infection was our original wildtype virus. The wildtype virus used in our studies was Smith strain (ATCC-VR-1399); theoretically identical to the parental strain for ΔMS94.5 but potentially diverging during an unknown number of serial passages in Germany, and also at ATCC when the stock was cleaned of contaminating mycoplasma. There is thus some variation in the behavior of multiply laboratory passaged strains of Smith. The most valid comparisons are those between mutants and their direct parental and revertant strains. However, the significant result from all of these studies was that viruses lacking *m152*, that are highly visible to CTL *in vitro*, continue to elicit CD8s *in vivo* that are as high as those seen in wildtype virus infection.

### *MCMV-specific CD8s express a memory-effector phenotype suggestive of recurrent activation.*

The high numbers of virus-specific CD8s detected in chronic infection suggested that the population was experiencing some level of repeated antigenic stimulation with consequent activation. To analyze this further we looked at some activation makers on CD8s in chronic infection. Upon TCR engagement, T cells express various "markers" of activation with different kinetics. For instance, CD69 and CD25 are upregulated immediately and downregulated within hours to days. CD62L is downregulated upon activation of naive cells, remains low during the effector stage and is again upregulated

on a subpopulation of CD8 memory cells. Ly6C is thought to be a good marker for memory CD8s and has also been used to differentiate effectors from memory CD8s (Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). The mean fluorescence intensity of CD8 is decreased upon activation, and this CD8 low phenotype persists for some time. Thus, a CD8 low phenotype is indicative of antigen exposure within the past 60 days (Slifka and Whitton, 2000). We therefore looked at Ly6C expression and CD8 downregulation to give an indication of the activation status of the MCMV-specific CD8s in chronic infection. Figure 14B displays Ly6C and IFN-γ staining on CD8 cells after exposure to virus infected APCs. Consistent with previous reports, there is an increase in the percentage of antigen-specific cells that express high levels of Ly6C, a memory phenotype, versus the intermediate or low levels of Ly6C expressed by effector CD8s. The percentage of antigen-specific cells with an Ly6C low or intermediate phenotype at week 22 post infection was less than 1% for Smith and 1% for ΔMS94.5. However, even at week 1 post infection, the majority (88% and 86%) of antigen-specific CD8s had an Ly6C high memory phenotype. We conclude that this marker is very rapidly upregulated to a memory phenotype after antigen exposure an is not a useful marker for effector CD8s.

In order to distinguish effector from memory CD8s, we have looked at CD8 downregulation on CD8s after MCMV infections in several experiments. In experiment 1 of figure 13B, we compared CD8 expression levels on individual animals infected for 30 weeks with wildtype (Smith), ΔMS94.5,ΔMC96.24, rMC96.27. A similar degree of CD8 downregulation was observed in the matched Δm152 and parental pair. Once again, the odd one was Smith which showed restored CD8 levels in contrast to CD8s from ΔMS94.5-infected mice which reliably express a CD8 $^{lo}$ phenotype. In experiment 2, CD8 levels on CD8s from wildtype MW97.01 and Δm152-MW99.05-infected mice were compared. Although CD8 downmodulation was not as pronounced in this experiment, no differences were seen among the Δm152 virus and its matched parental. Figure 14B

shows CD8 expression levels after 1 and 22 weeks from individual mice infected either with wildtype (Smith) or ΔMS94.5. CD8 is downregulated on the CD8s from wildtype-infected mice at week 1 but CD8 levels are restored at week 22 post infection. In contrast, CD8 T cells from mice infected with ΔMS94.5 have lower levels of CD8 both at weeks 1 and 22 post infection. As with the enumeration of antigen-specific CD8s in MCMV-infected mice, only matched Δm152 and revertant pairs allow us to draw some conclusions since CD8 responses from Smith do not match those of other wildtype infections. CD8 expression levels from mice infected with either Δm152 viruses and their matched wildtype showed the same level of downregulation indicating that *m152* is not responsible for this phenotype. Furthermore, the increased CD8 downregulation seen on MCMV-specific CD8s leave open the question as to whether these are continually being stimulated by antigen.

### *Mice infected with MCMV have high numbers of CD8s that are NKG2A+*

While we were quantifying MCMV-specific CD8s we began a collaboration with David Raulet. He provided us with an antibody to the NK receptor NKG2A which he had recently found was also expressed on CD8 T cells. On NK cells, NKG2A combines with CD94 to form a dimer that acts as an inhibitory receptor when it binds its non-classical class I molecule Qa-1 ligand. However, the role of NKG2A on CD8 T cells is still undefined. Figures 13D and 14 C show the frequency of CD8s that express NKG2A. About 6% of CD8s from naïve mice are NKG2A+. Upon infection with MCMV, the number of CD8s expressing NKG2A increases to between 15 and 20% as shown in 14C. Although the frequency of NKG2A+ CD8s drops to 7% in a chronic infection with Smith, the numbers increase to 24% in the ΔMS94.5-infected mice. High numbers of NKG2A+ CD8s are also maintained in mice infected with single Δm152 deleted viruses and their revertants where close to 30% of the CD8s are NKG2A+ at 30 weeks post infection. When analyzing the antigen-specific CD8s, close to 70% expressed NKG2A

(figures 13D and 14D) in all infections except Smith where closer to 50% were NKG2A+. The number of CD8s expressing NKG2A also increases after other viral infections such as with vaccinia (MC Gold unpublished observation) and with LCMV (Chris McMahon personal communication). Furthermore, although the total number of NKG2A+ CD8s decreases after LCMV clearance, 70% of LCMV-specific CD8s remain NKG2A+ (Chris McMahon personal communication). This suggests that NKG2A is upregulated on effector CD8s following viral infection. Furthermore, like Ly6C, NKG2A remains present on most memory cells and is likely to be a marker for antigen-experienced CD8s.

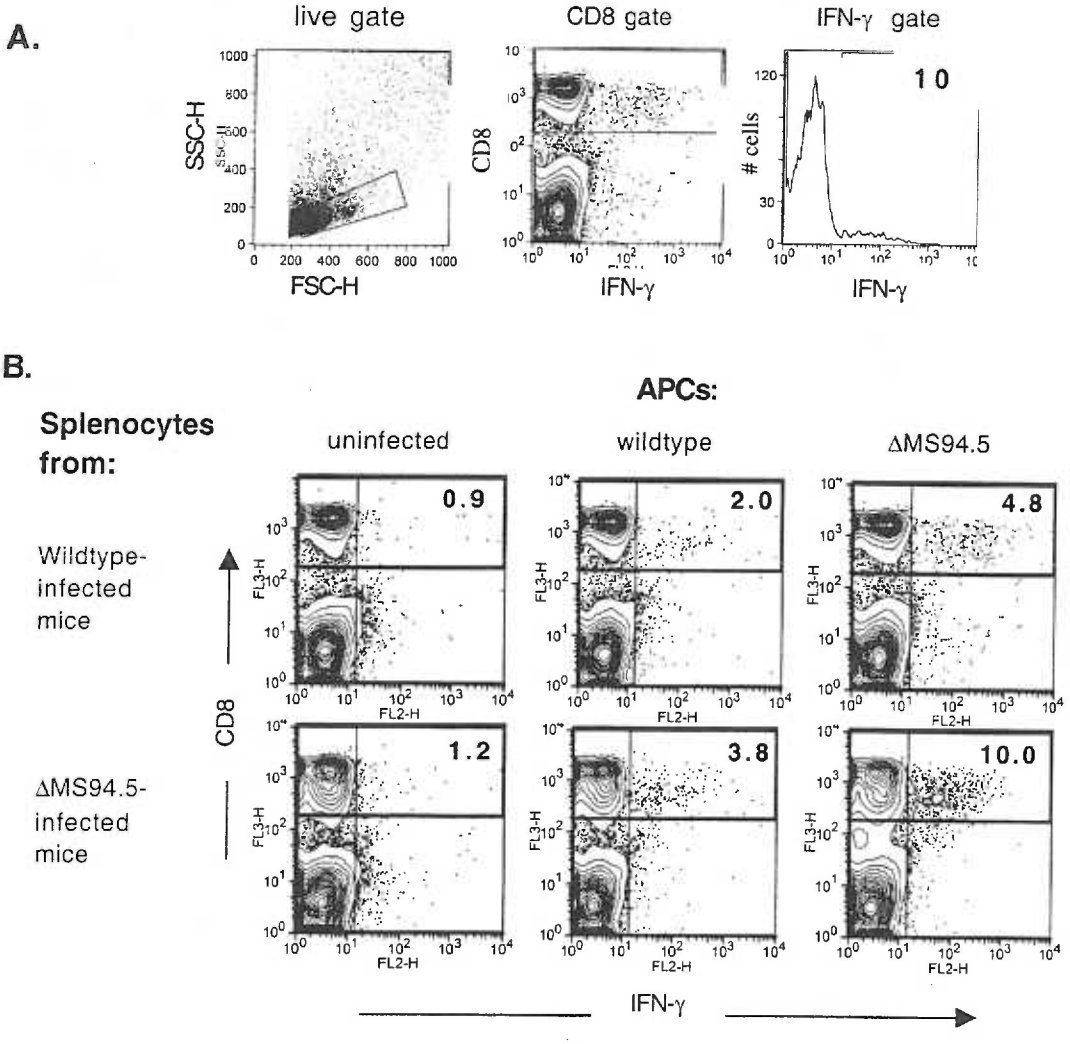
### *CD8s in chronic MCMV infection are not rapidly dividing.*

In order to address whether the activated phenotype of CD8s from ΔMS94.5-infected mice correlated with an increase in proliferation by those cells, we infected mice with either wildtype MCMV or ΔMS94.5 for 1 or 30 weeks and fed BrdU to mice 1 week prior to sacrificing the mice. Splenocytes from these mice were restimulated as for the ICS assay and the cells were stained for CD8 expression, IFN-γ, and after nuclear wall permeabilization, the DNA was stained with an antibody to BrdU. In this fashion we were able to look at the level of cycling, as detected by BrdU incorporation, in MCMV-specific CD8 T cells. Figure 15 shows the expression of IFN-γ in conjunction with BrdU on CD8 T cells. One week after infection with either wildtype or ΔMS94.5, over 90% of antigen-specific cells have incorporated BrdU indicating they had recently replicated their DNA. However, CD8 T cells from mice chronically infected with MCMV showed little BrdU incorporation. This suggests that CD8s in mice chronically infected with MCMV do not undergo massive cycling. Furthermore, mice infected with either Smith or ΔMS94.5 had equally low levels of BrdU staining suggesting that there is no discernable difference in the cycling of antigen-specific CD8s regardless of the infecting virus.

### *m152 is not necessary for the establishment of latency.*

Viral activity during chronic MCMV infection is relatively difficult to detect even using sensitive molecular techniques. We hypothesize that the numbers and activation state of CD8 T cells described above represent the most sensitive indicator of virus activity; and these parameters indicated that viruses lacking *m152*, that are readily visible to effector CTL, show no impairment of the establishment or maintenance of latent infection. To confirm this impression, we performed a realtime quantitative PCR (Taqman) analysis of genome copies of viral DNA present in the lungs and livers of animals chronically infected with various virus strains. Table 4 shows the results of three separate experiments. The level of detection was around the level of sensitivity of this assay; so multiple individual animals are included. The maximum number of genome copies seen from naïve mice was never more than 10. We therefore assumed that a number of 10 or higher indicated the presence of virus. Virus was detected in mice infected for 1 week after either infection with Smith or ΔMS94.5 although higher levels of DNA were detected in Smith-infected infected mice in two out of three experiments. In chronically infected mice, equally low levels of virus were detected after both infections. However, because the level of DNA in chronic infection with viruses lacking *m152* was at least as high as that seen in wildtype virus infection we concluded that *m152* is not necessary for the establishment of latency.

**Figure 11. The ICS assay allows the quantification of MCMV-specific CD8s A.** Gating strategy used to define the live lymphocyte gate, the CD8 gate and the IFN-γ positive CD8s which we consider to be antingen-specific. **B.** Uninfected MEFs or MEFs infected with Smith or ΔMS94.5 were used as stimulators in the ICS assay. Effectors were splenocytes from mice infected for more than 12 weeks with either wildtype MCMV or ΔMS94.5. The number in the upper right quadrant indicates the frequency of CD8s that produced IFN-γ. **C.** JAWS stimulate MCMV-specific CD8s better than MEFs. MEFs and JAWS II cells were infected with ΔMS94.5 are compared in their ability to stimulate MCMV-specific CD8s.



C.

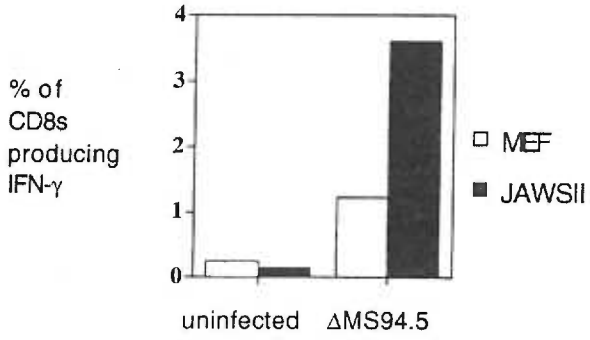


**Figure 12. Kinetic analysis of the MCMV-specific CD8 response in mice infected with wildtype MCMV or ΔMS94.5 using the ICS assay. A.** Splenocytes from mice infected over a period of 64 weeks were tested for their ability to secrete IFN-γ in response to ΔMS95.5-infected JAWS II stimulators. **B.** Splenocytes from mice infected over a period of 44 weeks were tested for their ability to secrete IFN-γ in response to ΔMS95.5-infected JAWS II stimulators. Splenocytes from the same mice were restimulated *in vitro* and used as polyclonal effectors in a $^{51}Cr$ release assay. Both the total number and frequency of CD8s that responded in the ICS assays in A and B are shown.

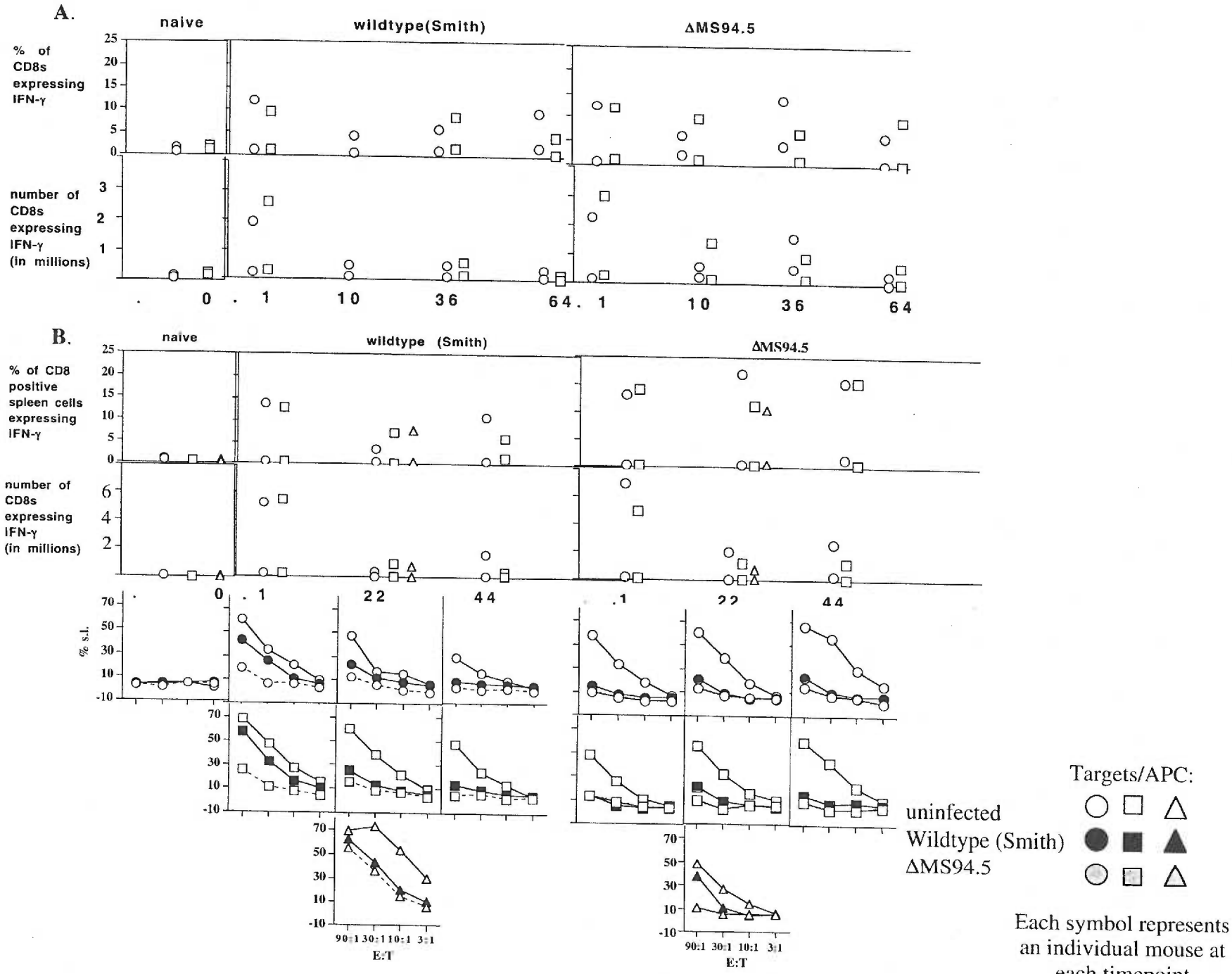
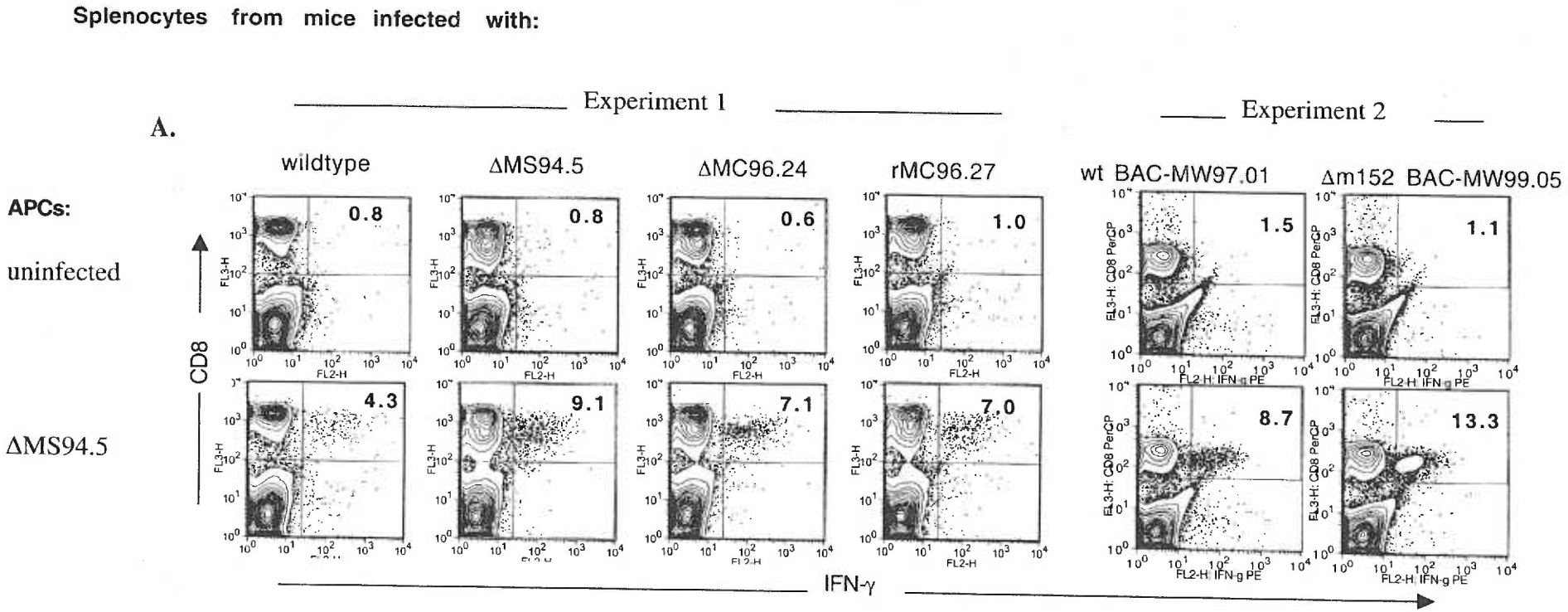

A.
naive
wildtype(Smith)
ΔMS94.5
% of CD8s expressing IFN-γ
number of CD8s expressing IFN-γ (in millions)
0
1
10
36
64
1
10
36
64
B.
naive
wildtype (Smith)
ΔMS94.5
% of CD8 positive spleen cells expressing IFN-γ
number of CD8s expressing IFN-γ (in millions)
0
1
22
44
1
22
44
% s.l.
E:T
90:1 30:1 10:1 3:1
Targets/APC:
uninfected
Wildtype (Smith)
ΔMS94.5
Each symbol represents an individual mouse at each timepoint

**Figure 13. *m152* does not alter the size of the MCMV-specific CD8 response. A.** Quantitation of antigen-specific CD8s from mice infected for 30 weeks with different MCMVΔm152 mutant viruses and the ir parental strains using the ICS assay. **B.** Histogram showing CD8 expression levels on the total CD8 population. **C.** Histogram showing the frequency of CD8s that are NKG2A+ **D.** Histogram showing the frequncy of antigen-specific CD8s that are NKG2A+.



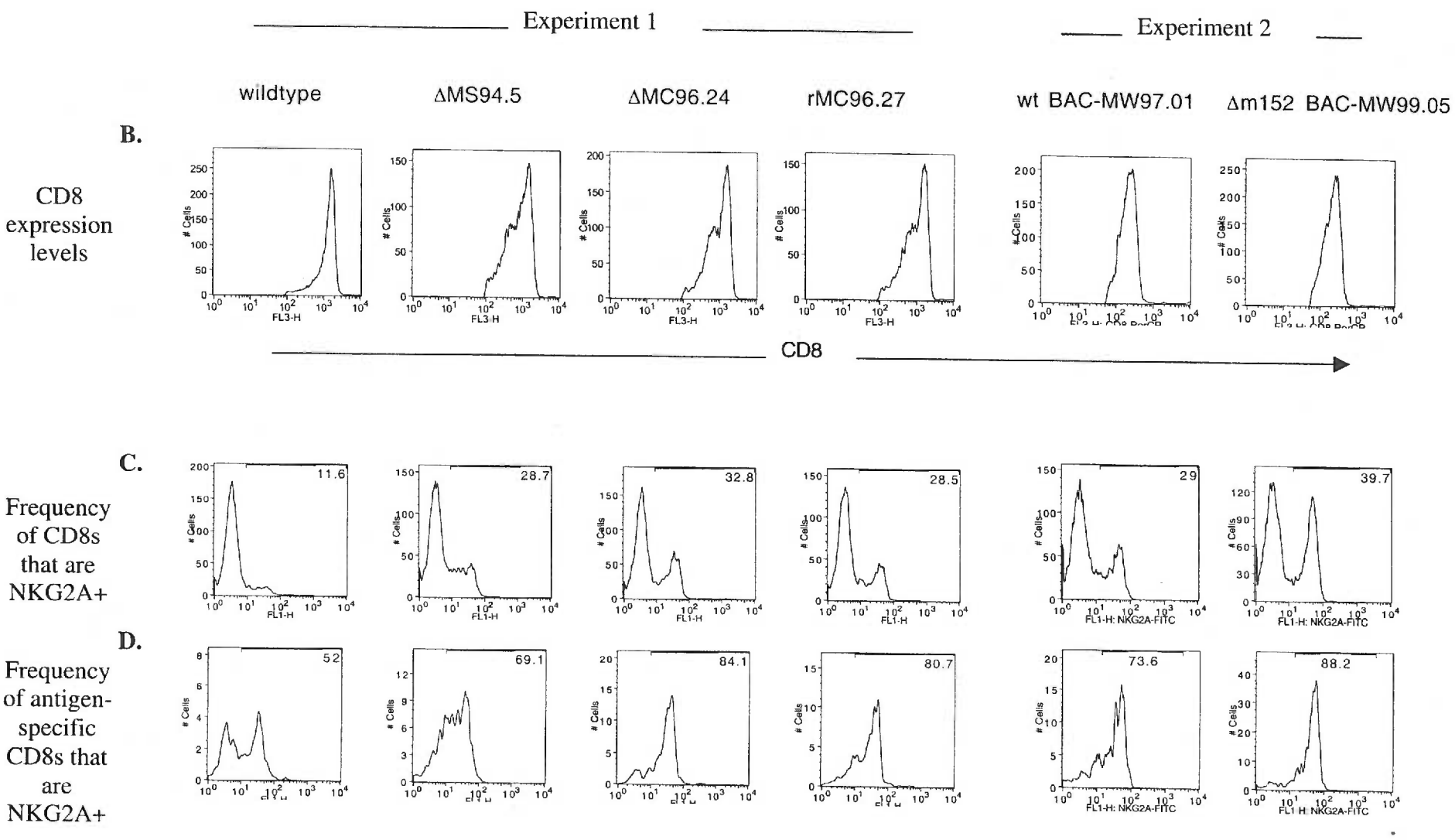

Experiment 1
Experiment 2
wildtype
ΔMS94.5
ΔMC96.24
rMC96.27
wt BAC-MW97.01
Δm152 BAC-MW99.05
B.
CD8 expression levels
# Cells
FL3-H
CD8
C.
Frequency of CD8s that are NKG2A+
11.6
28.7
32.8
28.5
29
39.7
FL1-H
FL1-H: NKG2A-FITC
D.
Frequency of antigen-specific CD8s that are NKG2A+
52
69.1
84.1
80.7
73.6
88.2

**Figure 14. MCMV-specific CD8s show signs of activation. A.** CD8 expression levels from representative naïve mice or mice infected with wildtype (Smith ) MCMVor mutant ΔMS94.5 for 1 or 22 weeks. **B.** Frequency of CD8s that express both Ly6C and IFN-γ. **C.** Frequency of all CD8s that express NKG2A. **D.** Frequency of MCMV-specific CD8s (these are all IFN-γ positive) that express NKG2A.

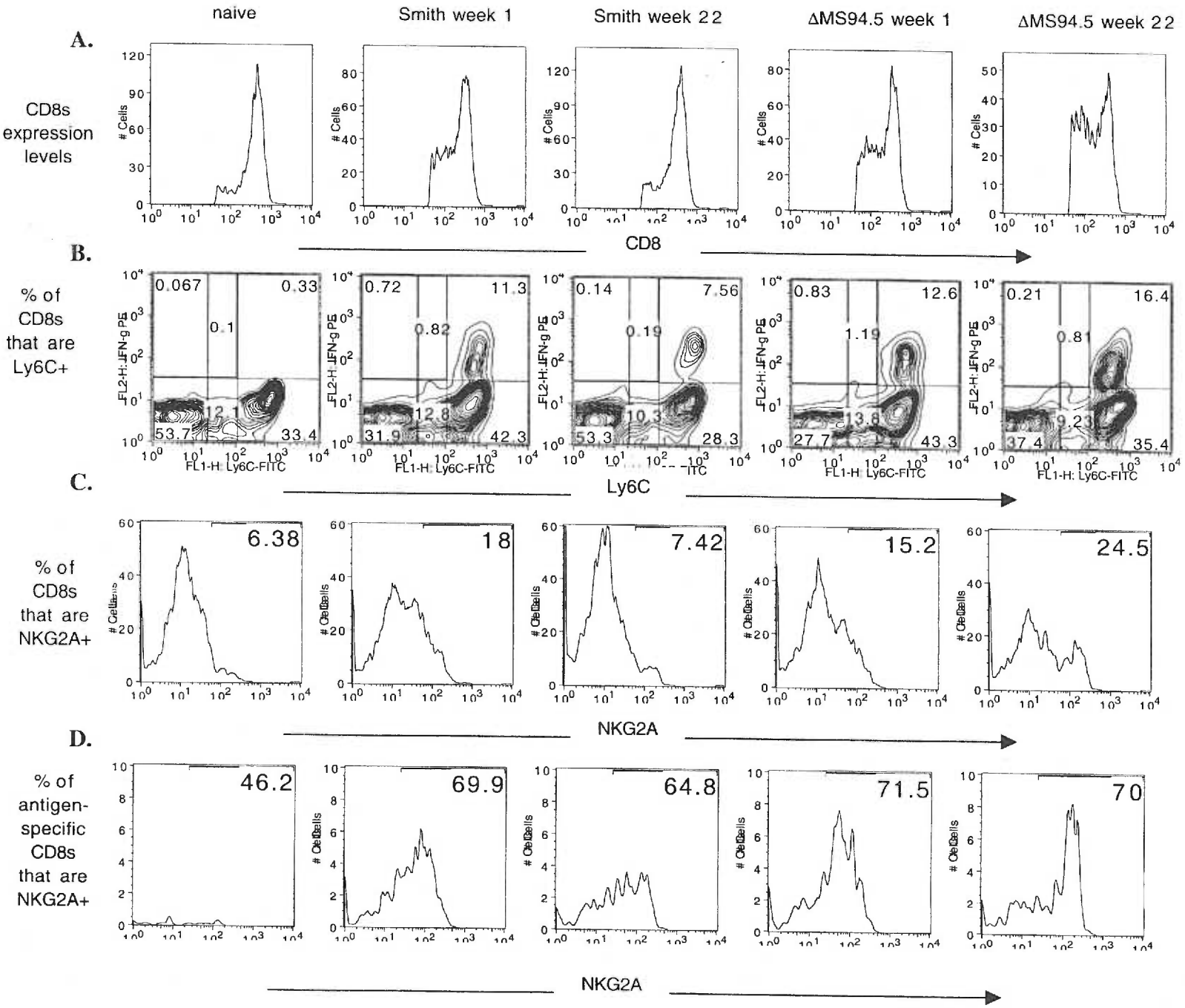

naive
Smith week 1
Smith week 22
ΔMS94.5 week 1
ΔMS94.5 week 22
A.
CD8s expression levels
# Cells
CD8
B.
% of CD8s that are Ly6C+
FL2-H: IFN-g PE
FL1-H: Ly6C-FITC
0.067
0.33
0.1
12.1
53.7
33.4
0.72
11.3
0.82
12.8
31.9
42.3
0.14
7.56
0.19
10.3
53.3
28.3
0.83
12.6
1.19
13.8
27.7
43.3
0.21
16.4
0.81
9.23
37.4
35.4
Ly6C
C.
% of CD8s that are NKG2A+
6.38
18
7.42
15.2
24.5
NKG2A
D.
% of antigen-specific CD8s that are NKG2A+
46.2
69.9
64.8
71.5
70
NKG2A

**Figure 15. CD8$^+$ splenocytes from mice chronically infected with MCMV did not undergo proliferation within the 6 day period prior to the ICS assay.** Mice infected for 1 week or 30 weeks were fed BrdU for 6 days prior to having their spleens removed. A naïve mouse was included as a negative control. CD8$^+$ splenocytes were tested for their ability to produce IFN-γ in the ICS assay, an additional nuclear permeabilization step was included to label DNA that had incorporated BrdU. Cells are gated on CD8.

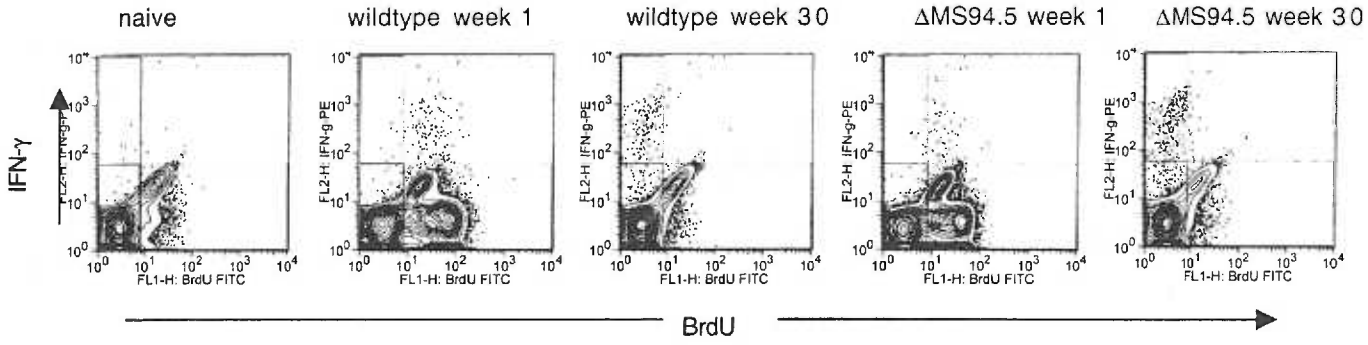


**Table 4- *m152* is not necessary for the establishment of latency**- Mice infected with either Smith or ΔMS94.5 for the times indicated were sacrificed. Organs were removed and DNA was isolated which was used to detect the presence of MCMV DNA from the IE1 gene using TaqMan PCR. Three different experiments were performed.

| **Experiment 1** | | Genome copies (average of triplicate samples) | |
|---|---|---|---|
| Virus used to infect mice | Week post infection | lung | liver |
| **uninfected** | 0 | 0.0 | 0.0 |
| | 0 | 0.0 | 0.0 |
| **Smith** | 1 | 4600.0 | 883.0 |
| | 1 | 1050.7 | 798.7 |
| | 22 | 15.0 | 7.7 |
| | 22 | 4.7 | 5.3 |
| **ΔMS94.5** | 1 | 5.3 | 230.0 |
| | 1 | 103.7 | 0.0 |
| | 22 | 32.3 | 43.0 |
| | 22 | 3.7 | 0.0 |

| **Experiment 2** | | Genome copies (average of triplicate samples) | |
|---|---|---|---|
| Virus used to infect mice | Week post infection | lung | liver |
| **uninfected** | 0 | 6.45 | 0 |
| | 0 | 0 | 0 |
| **Smith** | 1 | 195.5 | 0 |
| | 1 | 1756 | 0 |
| | 10 | 3649.5 | 15.5 |
| | 10 | 953 | 80 |
| | 36 | 10.5 | 19.5 |
| | 36 | 19.5 | 0.9 |
| | 64 | 4.5 | 0 |
| | 64 | 31.5 | 4.9 |
| **ΔMS94.5** | 1 | 1.8 | 0 |
| | 1 | 0 | 0 |
| | 10 | 25 | 4.75 |
| | 10 | 40 | 3.2 |
| | 36 | 0.05 | 1.95 |
| | 36 | 0.55 | 0 |
| | 64 | 5.2 | 2.5 |
| | 64 | 2.95 | 3.2 |

| Experiment 3 | | Genome copies (average of triplicate samples) | |
|---|---|---|---|
| Virus used to infect mice | Week post infection | lung | liver |
| **uninfected** | 0 | 9.8 | |
| | 0 | 0.0 | |
| | 0 | 8.4 | |
| **Smith** | 1 | 645 | 636 |
| | 1 | 3745 | 986 |
| | 22 | 14.3 | |
| | 22 | 22.3 | |
| | 22 | 11.0 | |
| | 44 | 27.7 | |
| | 44 | 8.2 | |
| | 118 | 13.3 | |
| **ΔMS94.5** | 1 | 28.7 | 4616 |
| | 1 | 19.3 | 1371 |
| | 22 | 3.0 | |
| | 22 | 30.3 | |
| | 22 | 0.0 | |
| | 44 | 15.4 | |
| | 44 | 11.7 | |
| | 102 | 0.0 | |
| | 102 | 0.0 | |

## Data chapter 2.3- The MCMV gene *M45* encodes a $D^b$-restricted CTL epitope.

### *Strategy to identify MCMV H-$2^b$ CTL epitopes.*

The strategy we used to identify MCMV H-$2^b$ epitopes is schematically represented in figure 16. To increase our chances of identifying MCMV H-$2^b$ epitopes, we generated a genomic MCMV library that was made up of random MCMV DNA fragments. Full gene expression is not required for the generation of peptide epitopes and we could therefore maximize expression by using DNA fragments. Furthermore, smaller DNA fragments would minimize the possibility of encountering stop codons. We used the eukaryotic expression vector pcDNA4His Max to express the MCMV DNA fragments. This vector has a CMV promoter, a sequence which allows enhanced expression from the inserted DNA sequence, and an SV-40 origin of replication, which can be used to enhance episomal replication in a cell expressing the SV-40 T antigen. Furthermore, three variants of the pcDNA4His Max vectors named A, B, and C were used. The three vectors are identical except that the each has a staggered start site such that a particular fragment can be read in one of the three open reading frames. We hoped this would enhance our ability to express peptides in the correct reading frame. Therefore, once MCMV DNA fragments were successfully ligated into the vectors, vector DNA could be transfected into cells to allow expression of the fragments and peptides could be detected using MCMV-specific CTL clones.

It was also necessary to optimize other steps. We first needed to test several cell lines for their ability to be transfected. Ultimately, we used K41 cells for several reasons. K41 cells are an SV-40 transformed H-$2^b$ fibroblast. As such they express both $K^b$ and $D^b$ and would therefore allow presentation of the library peptides in the proper context. Furthermore, K41s contain the T antigen which should allow episomal replication of the pcDNAHisMax vectors, and in theory, increase the expression of the inserted DNA.

More importantly though, K41s could be efficiently transfected. Up to 50% of the cells could be transfected with plasmid DNA as detected using EGFP expression (data not shown).

The next step we needed to optimize was the library screening. Once K41s were transfected we wanted to use MCMV-specific CTL clones to detect the presence of peptide antigens. The first reason for using the clones was that they had been isolated from MCMV-infected mice. Therefore, we knew the specificities they recognized were biologically relevant and furthermore, they might even be immunodominant. The second reason for using the clones is that they were a great technical asset. Large numbers of clone cells could be produced. This could be advantageous for screening peptides that might be present in very small quantities. Furthermore, we already knew the restricting element for each clone. Finally, assays to detect CTL function were available even if they still needed to be optimized for our purposes. Although we first optimized the IFN-γ ELISPOT, we ultimately used the TNF bioassay because we found it to be exquisitely sensitive. After all these preliminary requirements were met, we could then screen the library.

### *Identification of a $D^b$-restricted epitope from the MCMV gene M45.*

MCMV DNA was isolated as fragments from pUC-based plasmids encoding MCMV HindIII DNA fragments A through P with the exception of fragment E. Fragment E was not included for technical reasons, however, this was not of great concern because ΔMS94.5 is missing most of the HindIII E fragment and the clones we used to screen the library were primarily isolated from mice infected withΔMS94.5. The MCMV DNA fragments from each of the plasmids were purified from their vectors, gel purified, quantified by gel electrophoresis, and combined into two non-overlapping pools of DNA known as "A" and "B". DNA from both pools was digested to in an attempt to generate fragments of 200 to 400 bp with Bam HI compatible ends to maximize the production of

peptides without encountering a stop codon. However, with DNA pool "A", which was available first, we used of a combination of Bcl1, BglII and Bam HI enzymes, and this yielded average fragments of 1100bp in size. To obtain smaller DNA fragments from pool "B" we used the restriction enzyme Sau3AI and indeed, we obtained fragments of on average 530bp in size. DNA from pools A and B were then separately ligated into the Bam HI site of vectors pcDNAHisMax A, B, C. Confirmation that the fragmented MCMV DNA was ligated into a combination of the vectors was performed by analyzing the DNA isolated from individual bacterial clones from each library. Greater than 80% of the vectors contained insert and MCMV DNA was confirmed to be present from a random sample of individual bacterial clones. We calculated the number of potential clones present and arrayed them into pools of 50 to favor the expression of antigens while minimizing the number of samples to be screened. A total of 25,000 bacterial clones were prepared to be screened from each library.

In order to screen the library, we transfected the DNA isolated from each pool of 50 bacterial clones into K41 cells, allowed them to express for 48 hours and then added MCMV-specific CTL clones. CTL clones 3, 5,11,55, and 96 (Table 3 chapter 2.1) were used to screen the expression library. Using the TNF bioassay we identified a plasmid that both CTL clones 3 and 55 recognized. CTL clones 3 and 55 are $D^b$-restricted, isolated from mice infected with the MCMV mutant ΔMS94.5, and both specific for an E antigen (see figures 5 and 6 data chapter 2.1).The plasmid recognized by clones 3 and 55 encoded a fragment of the MCMV early gene *M45* gene (Rawlinson et al., 1996). Based on the published MCMV sequence (Rawlinson et al., 1996), and the corrected addition of a cytosine at nucleotide position 61918 of the MCMV genome (Brune et al., 2001), *M45* encodes a 1174 amino acid protein that is homologous to HCMV *UL45*, and to the large subunit of the class Ia murine ribonucleotide reductase gene (Rawlinson et al., 1996).

### *M45 encodes a $D^b$-restricted CTL epitope*

From the sequence of the expressed *M45* fragment, we predicted four potential $D^b$-binding peptides that were synthesized and used to sensitize RMA-S cells for lysis by clones 3 and 55 in a $^{51}Cr$ release assay. Figure 17 shows that both the 9-mer HGIRNASFI (aa 985-993) and the 10-mer KHGIRNASFI (aa 984-993) were equally able to sensitize targets for killing by clones 3 and 55. Both peptides sensitized targets down to $10^{-12}$ M. Mike Munks also performed a stabilization assay (Deres et al., 1993) which shows (Figure 18) that the 9-mer and the 10-mer were equally effective at stabilizing cell surface $D^b$. It is likely that the 9-mer represents the minimal epitope with the histidine residue occupying the "A" pocket of $D^b$ (Rammensee et al., 1993).

### *The M45-specific response makes up 20% of CD8s 8 days after infection of a Kb-/- mouse infected with a Δm152 virus*

We next determined whether we could detect HGIRNASFI-specific CD8 T cells directly *ex vivo*. Because of the low frequency of CD8s responding to previously identified E antigens in BALB/c mice (Holtappels et al., 2000; Holtappels et al., 2000), we optimized our chances of detecting (K)HGIRNASFI-specific CD8 T cells *in vivo* by looking at the peak of the response in mice whose only class I molecule is $D^b$. $K^{b-/-}$ mice were infected with the MCMV mutant ΔMS94.5. The intracellular cytokine staining (ICS) assay was used to quantify the frequency of M45-specific $CD8^+$ spleen cells from acutely infected mice. Over 20% of all $CD8^+$ splenocytes from $K^{b-/-}$ mice infected for 6 days with ΔMS94.5 were (K)HGIRNASFI-specific (Figure 19).

### *The M45-specific response makes up 2.5% of CD8s in a B6 mouse chronically infected with a Δm152 virus*

We were encouraged by the strength of this response to look at the CD8 T cell response in a chronically infected B6 mouse. In a mouse infected with MCMV-Δm152

for 1.5 years, 2.5% of $CD8^+$ splenocytes recognized the (K)HGIRNASFI peptides (Figure 19). Finally, no response to HGIRNASFI was detected in a naive B6 mouse while in the same assay, 4% of CD8s, from a mouse infected with ΔMS94.5 for twelve weeks, made IFN-γ in response to HGIRNASFI (Figure 20).

**Figure 16. Strategy used to identify H-2$^b$ MCMV CTL epitopes.**

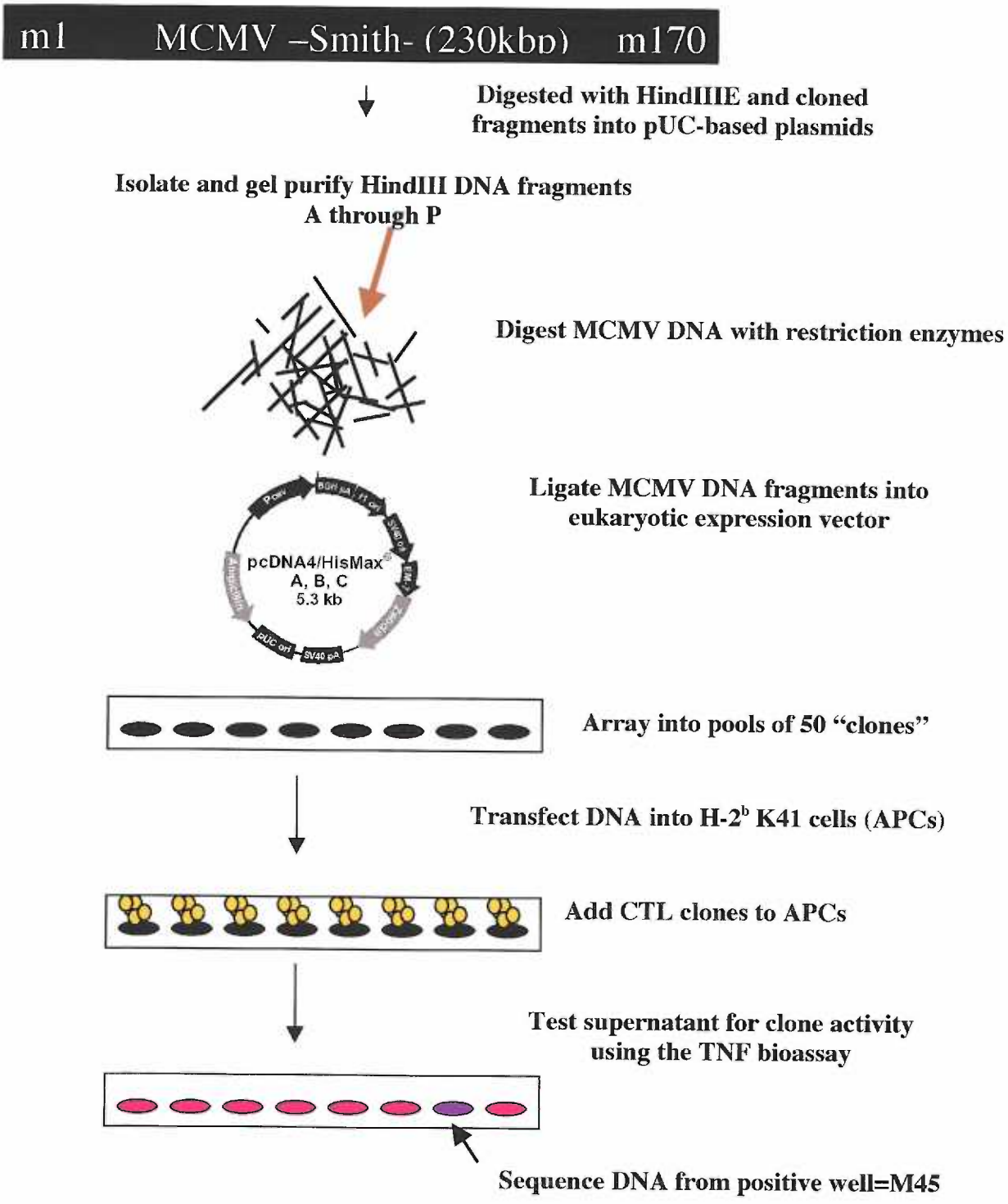


**Figure 17. M45 encodes a $D^b$-restricted CTL epitope. Clones 3 and 55 recognize HGIRNASFI and KHGIRNASFI peptides.** Clones 3 and 55 were used as effectors in a $^{51}Cr$ release assay using RMA-S target cells loaded with 4 peptides titrated from $10^{-7}$ to $10^{-13}$ M.

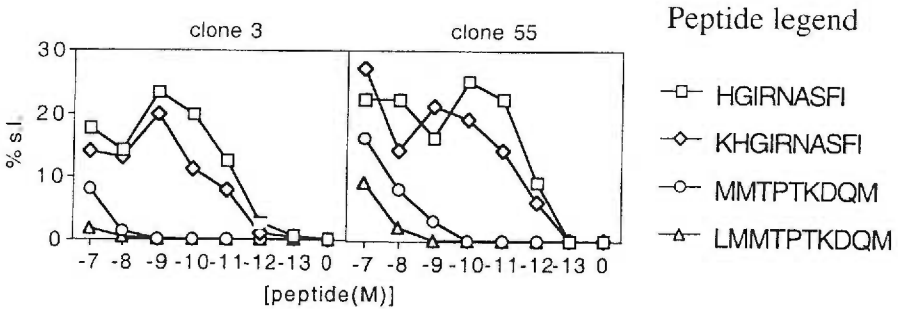



**Figure 18 . Both HGIRNASFI and KGHIRNASFI are equally able to stabilize the $D^b$ class I molecule.** Peptide stabilization of cell surface $D^b$ on RMA-S cells was analyzed by flow cytometry as described in materials and methods.

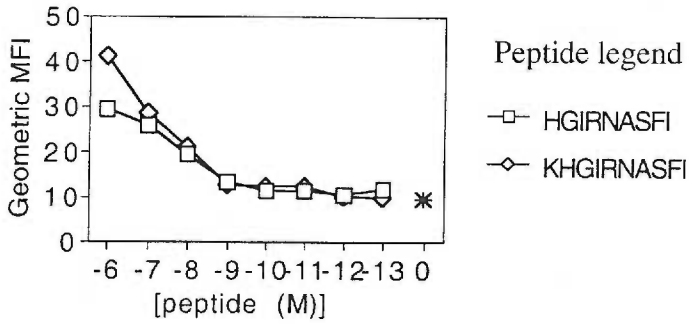


**Figure 19. Both acutely infected $K^{b-/-}$ mice and chronically infected B6 mice generate M45-specific responses.** Using the ICS assay, $CD8^+$ splenocytes from a $K^{b-/-}$ mouse infected with ΔMS94.5 for 6 days (left panel) or B6 mouse infected with Δm152-MW99.05 for 1.5 years (right panel) were tested for their ability to respond to M45 peptides. Peptides, titrated from $10^{-7}$ to $10^{-13}$ M, were incubated with *ex vivo* splenocytes in the presence of brefeldin A for 5 hours, and analyzed by ICS.

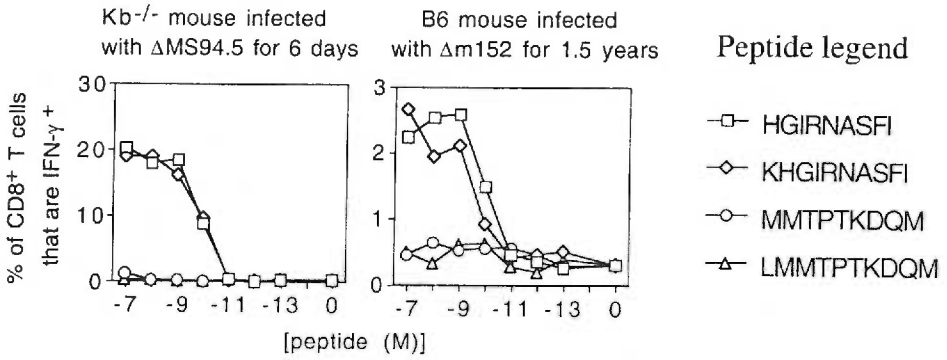
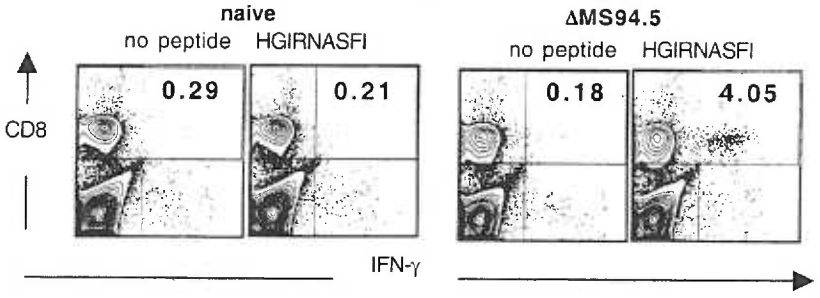



**Figure 20. Naive mice do not respond to HGIRNASFI.** Splenocytes from B6 mice infected with ΔMS94.5 for 12 weeks, or naive mice, were tested by ICS for their response to peptide ($10^{-7}$ M). The number in the upper right quadrant represents the % of CD8s that produced IFN-γ. Two mice were tested under each condition; one representative analysis is shown



## Data chapter 2.4- The murine cytomegalovirus immunomodulatory gene *m152* prevents lysis of infected cells by M45-specific CTL, but does not alter the immunodominance of the M45-specific CD8 T cell response *in vivo*.

### *Mice infected with wildtype MCMV generate a CD8 T cell response to the M45 antigen*

The effects of the immunomodulatory gene *m152* on M45 presentation *in vitro* are profound. Figure 21 shows that clones 3 and 55 were unable to kill fibroblasts infected with three different wildtype MCMV strains. However, this defect was reversed if *m152* was absent: clones 3 and 55 were able to kill fibroblasts infected with three different MCMV mutants lacking *m152*. Because *m152* appeared able to completely prevent presentation of M45 (at least as detected in $^{51}$Cr release assays *in vitro*), we hypothesized that mice infected with a wildtype (*m152*+) MCMV would not develop an M45-specific CTL response. To test this prediction, we infected $K^{b-/-}$ mice with wildtype MCMV (Smith), ΔMS94.5, ΔMC96.24 (Δm152), and the rescued virus rMC96.27 (ΔMC96.24 with *m152* restored). Figure 22 shows that, surprisingly, the percentage of CD8s recognizing M45 was very similar in each infection: between 12 and 13% of $CD8^+$ splenocytes on day 8 post infection were specific for M45.

### *m152 ablates killing of infected target cells by M45-specific CTL, but does not affect the immunodominance of M45 in vivo.*

Although mice infected with wildtype virus made a response to M45, we reasoned that *m152* might nevertheless affect the immunodominance of M45 within the hierarchy of the total CD8 response to MCMV. We were unable to test this hypothesis by comparing the M45 response to other specific peptides because no other H-$2^b$-restricted

epitopes have yet been identified. Instead, we calculated the ratio of the M45-specific response to the response we could detect to the whole virus, which was assessed using virus-infected APCs in the ICS assay. This time we used B6 mice, where $D^b$-HGIRNASFI also competes with $K^b$-restricted responses for immunodominance. Five mice were infected with the MCMV mutant Δm152 (ΔMC96.24) or the rescuant (rMC96.27) MCMV strains for 8 days. Figure 23 shows that CD8 T cell responses to MCMV (Δm152)-infected DCs ranged from 3 to 11%. No remarkable differences were seen in the CD8 T cell responses to virus-infected APCs after MCMV infection with or without *m152*. This indicated that *m152* did not affect the total numbers of MCMV-specific CD8 T cells that were generated *in vivo*. This assay also allowed a rough assessment of the immunodominance of M45 within the total MCMV-specific CD8 response. While the HGIRNASFI peptide stimulation probably detects all CD8s of this specificity, it is likely that simulation with virus-infected DCs underestimates the total number of MCMV specific CD8s. We therefore conclude that while a substantial number of CD8s recognized HGIRNASFI, other specificities exist. Finally, we compared the HGIRNASFI-specific response to the detectable response to whole virus after infection with MCMV with or without *m152*. Although there was mouse to mouse variation, the ratio of the peptide-specific response to the total MCMV-specific response did not differ significantly ($p=0.39$). The ratio of the M45-specific CD8 T cell response to the total MCMV-specific CD8 response averaged among 5 mice (expressed as a percentage) was 62.1% after MCMVΔm152 infection versus 72.8% after infection with the rescuant (Figure 23 and Table 5). These data again indicate that *m152* did not alter the immunodominance of the M45 antigen.

**Figure 21.*m152* prevents antigen recognition by clones 3 and 55.** Clones 3 and 55 were tested in a CTL assay with 3 different wildtype MCMV strains or with 3 mutants lacking *m152*; only the mutants were recognized.

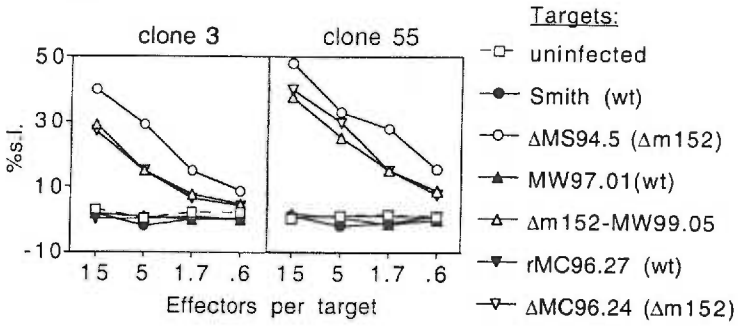



**Figure 22.*m152* does not affect the M45-specific response in acutely infected $K^{b-/-}$ mice.** Quantification by ICS assay of HGIRNASFI-specific $CD8^+$ splenocytes from $K^{b-/-}$ mice infected for 8 days with wildtype (Smith), ΔMS94.5, ΔMC96.24 (Δm152), and rMC96.27 (revertant) viruses. The number in the corner indicates the % of CD8 T cells specific for M45.

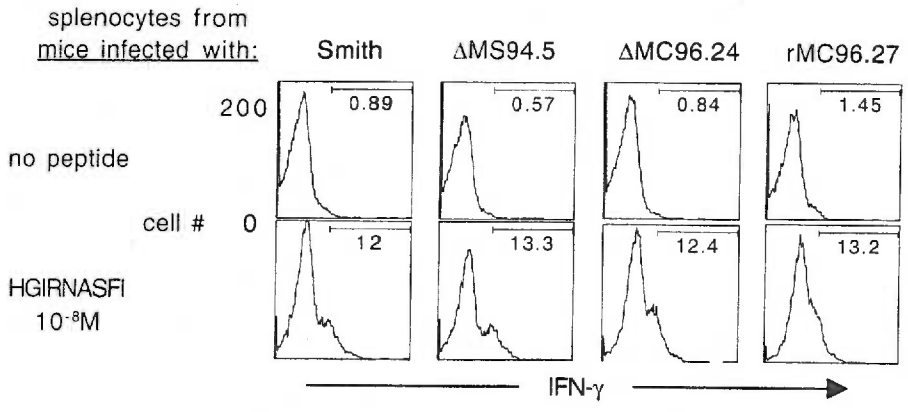


**Figure 23. *m152* does not affect the immunodominance of M45 in acutely infected B6 mice.** HGIRNASFI-specific and total MCMV-specific responses were compared by ICS assay. Splenocytes from B6 mice infected for 8 days with Δm152 (ΔMC96.24) or revertant (rMC96.27) were incubated for 6 hours either with $10^{-8}$ M HGIRNASFI peptide or with Δm152-infected JAWS II cells used as APCs.

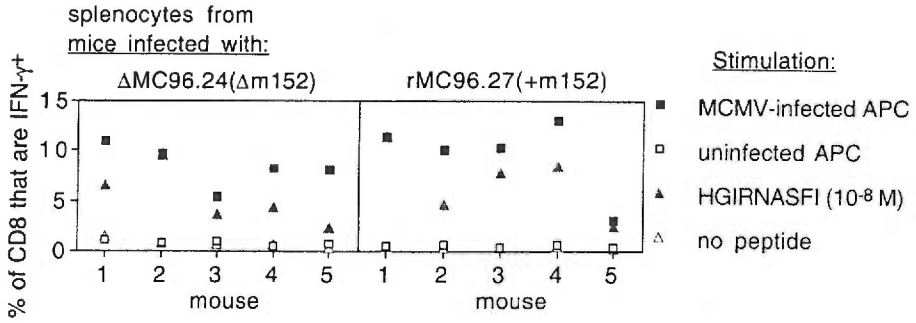


**Table 5. Calculated ratio of HGIRNASFI-specific response to the response to virus-infected APCs.** The frequency of M45-specific CD8s expressed as a % of the frequency of total MCMV-specific responses as assessed using virus-infected APCs using results from figure 17C.

| mouse | MCMV infection | |
|---|---|---|
| | ΔMC96.24 (Δm152) | rMC96.27 (wildtype) |
| 1 | 60.8 | 100 |
| 2 | 99.2 | 45.6 |
| 3 | 68.8 | 76.1 |
| 4 | 53.1 | 64.3 |
| 5 | 28.6 | 77.8 |
| Average +/- SD | 62.1+/-25.6 | 72.8+/19.9 |

## Discussion.

The goal of this thesis work was to study the role of *m4* and *m152* in antigen presentation *in vitro* and the effects of *m152* on the CD8 T cell response to MCMV *in vivo*. In the attempt to address these questions, we obtained some results that led us to some firm conclusions that I list below as a summary of results. However, we are still left with many questions to answer: specifically what is the relevance of *m152*, or other immunomodulatory genes, in MCMV infection *in vivo*? In the following section I discuss my results within the context of the current paradigms in immunology and the field of viral immune evasion.

**Summary of results.**

- both *m4* and *m152* inhibit antigen presentation in B6 fibroblasts *in vitro*.
- *m4* inhibits recognition by $K^b$ restricted CTL *in vitro*, but deleting *m4* is not sufficient to enable recognition by $D^b$ restricted CTL.
- The majority of MCMV-specific CTL are specific for E antigens in B6 mice.
- The MCMV-specific CD8 response chronically occupies 10% of the CD8 population.
- *m152* does not alter the size or kinetics of the MCMV-specific CTL response.
- *m152* is not necessary for the establishment of latency.
- MCMV *M45* encodes a $D^b$-restricted epitope that elicits a substantial proportion of the MCMV-specific CTL response.
- *m152* does not alter the immunodominance of the M45 antigen.

### 1. *Are MCMV-specific CTL clones a good representation of the polyclonal CTL response to MCMV?*

As described in chapter 2.1, restimulated polyclonal effectors from MCMV-infected mice allowed us to make limited but important conclusions about the CTL response to MCMV. First, restimulated polyclonal effectors showed that infection with

MCMV elicited a detectable CTL response in H-$2^b$ mice. This finding was later confirmed by the ICS assay in which we show that 10% of CD8s are specific for the virus. Nevertheless, while it was difficult to detect killing on wildtype-infected targets using restimulated polyclonal effectors, we were able to detect killing of cells infected with MCMV mutants lacking *m152*. MCMV mutant viruses lacking immunomodulatory genes have allowed us to identify antigen-specific CTL in MCMV and have allowed us to detect a greater frequency of antigen-specific CD8s. Furthermore, because we could better detect MCMV-specific CTL in targets lacking *m152*, we chose to screen CTL clones isolated from MCMV-infected mice using Δm152-infected targets. In HCMV studies, mutants lacking immunomodulatory genes have not been used to detect CTL and this may well explain the limited HCMV-specific CTL response that has been observed.

The second finding that was demonstrated with restimulated polyclonal effectors was that *m152* inhibited H-$2^b$ antigen presentation *in vitro*. The biggest drawback, however, in using *in vitro* restimulated effectors, was that experimental variation made data interpretation difficult. To overcome this, I isolated CD8 T cell clones, mainly from mice with ΔMS94.5 infections, for reasons that will be discussed below. The clones allowed us to confirm results obtained with the restimulated effectors: the clones reproducibly killed virus-infected targets lacking immunomodulatory genes well, they did not kill uninfected targets, and furthermore, they did not kill targets infected with wildtype MCMV. Therefore, the general phenotype seen in chromium release assays using restimulated polyclonal effectors was also seen using CTL clones. The biggest advantages of the clones was that they could be used first to identify CTL specificities and second to study immunomodulatory gene effects. The question of whether the CTL clones are a good representation of the polyclonal response became important when we wanted to use the clones to screen an MCMV genomic library to identify H-$2^b$-restricted epitopes. We wanted the clones to be good representatives of the total MCMV-specific CD8 response in order to identify immunodominant epitopes. This in fact proved to be a

good strategy since at least 25% of the CTL response to MCMV is specific for the M45 epitope (figure 20). As discussed below, the H-$2^b$-resticted MCMV-specific CTL clones I isolated have contributed to 1) a better understanding of immune evasion as a result of the functional characterization of *m4* as an immune evasion gene 2) the size of the M45-specific CTL response as a result of the M45 epitope identification and consequently 3) revised the thinking on the role of immune evasion genes in shaping immunodominant responses.

However, I would now like to highlight another aspect of the CTL response on which we have focused little attention but is worth noting: CTL exist that specifically kill wildtype (Smith)-infected targets; however, we have not successfully isolated clones of this phenotype. As shown in figure1 in chapter 2.1, CTL from Smith-infected mice usually killed Smith-infected targets poorly, but on occasion, we saw killing that appeared specific. This finding could have been an artifact of the *in vitro* restimulation. However, using the ICS assay, 1-2% of the CD8s from Smith-infected mice were able to specifically secrete IFN-γ in response to Smith-infected targets (figure 11 chapter 2.2). Therefore, some CD8s are capable of recognizing Smith-infected APCs. Further evidence that CD8s that recognize Smith-infected targets exist comes from clones isolated from Smith-infected mice that only survived a brief period *in vitro*; on occasion, newly isolated clones killed Smith-infected targets before they died (data not shown). Whether Smith-specific clones lose their ability to recognize wildtype virus after the *in vitro* stimulation is unknown. Perhaps clones of this phenotype are activated and undergo AICD more readily and do not survive the cloning process. Evidence supporting this hypothesis comes from the difficulty in isolating clones and the phenotype of clones from Smith-infected mice as will be discussed below. Are these CD8s of a different specificity from other MCMV-specific CD8s we've isolated? Can these CD8s better control virus *in vivo*? Ultimately, clones that can recognize wildtype virus remain to be isolated in order to answer such questions.

I would also like to note some interesting observations I made while isolating MCMV-specific CTL clones which perhaps lend credence to the idea that CTL from Smith-infected mice and those from ΔMS94.5-infected mice are different, a hypothesis which has not been demonstrated. The isolation of CTL clones from mice infected with Smith proved to be difficult while clones from ΔMS94.5-infected mice were more easily isolated. As shown in chapter 2.2, mice infected with the ΔMS94.5 virus have twice the number of MCMV-specific CD8s compared to mice infected with Smith. However, this two-fold difference cannot account for the 10-20 fold increase in cloning efficiency from ΔMS94.5-infected mice over the Smith-infected mice (data not shown). When cloning from Smith-infected mice, at least 10 fold higher numbers of precursors were plated per well. Furthermore, the cells did not grow as well, and those that were found to be specific often did not survive the subsequent *in vitro* restimulations. When performing a screening for specificity, a disproportionate number (>50%) of those from Smith-infected mice exhibited non-specific killing and some of these were found to be NK1.1 positive (unpublished data). Usually, only 1-2% of the surviving clones was able to kill Δm152-infected targets specifically. Ultimately, few of those cells survived and we are left with clone 96 as the sole survivor from Smith-infected mice.

In contrast, CD8s isolated from ΔMS94.5-infected mice showed little non-specific killing while over half of the cells in a screening showed specific killing. However, as with the Smith clones, many quickly lost all ability to kill after restimulation, and again many died during restimulations. This suggests that while clones isolated from both Smith and ΔMS94.5 infections appear to similarly undergo AICD, clones from Smith-infected mice are more likely to exhibit non-specific killing. Although these observations are non quantitative, they highlight differences among the CTL from mice infected with Smith versus ΔMS94.5 and could be useful in the interpretation of future experiments. What could be responsible for this indiscriminate killing by the Smith-derived clones? A perhaps far-fetched hypothesis is that Ly49H$^+$ NKs in the spleen of mice infected with

Smith are performing the apparent non-specific killing. This would be in contrast to ΔMS94.5 which is lacking 16 ORFs, and may be lacking the stimulatory ligand for Ly49H. As described in the introduction (p:8), MCMV infection in C57Bl/6 mice elicits the expansion of an Ly49H$^+$ NK subset that can specifically control MCMV in the spleen in acute MCMV infection. Whether Ly49H+ cells are even present or could control MCMV in the spleen during chronic infection is unknown.

***2. Δm4 is a CTL evasion gene that complements the function of m152.***

Restimulated polyclonal effectors from MCMV-infected mice clearly showed some reproducible intermediate level of killing on targets lacking *m4*. This was the first evidence that *m4* could inhibit antigen presentation to CTL. However, it was not until clones were isolated that the information from CTL assays combined with biochemical analyses by Daniel Kavangh allowed us to further refine our conclusions on the function of *m4*. As described in the chapter 2.1, only K$^b$-restricted CTL clones were inhibited by *m4* while the D$^b$-restricted clones were not. I obtained these results while Daniel Kavanagh in our lab discovered, through biochemical means, that the immunomodulatory gene *m152* retains almost all D$^b$ class I molecules while it only partially retains K$^b$ class I molecules (Kavanagh et al., 2001). Nevertheless, *m152* on its own was sufficient to interfere with presentation to both K$^b$ and D$^b$-restricted CTL, as seen in chromium release assays using a panel of CTL clones (chapter 2.1). *m152* therefore appears to globally retain class I molecules although different MHC class I alleles are affected by m152 to varying degrees. Nevertheless, functional evidence of the inability of *m152* to fully retain all K$^b$ class I molecules was revealed by the ability of K$^b$-restricted clones to recognize Δm4 mutants, even in the presence of *m152*. Therefore *m152* retains all newly synthesized D$^b$ class I molecules and this abrogates CTL recognition of all D$^b$-restricted CTL. Conversely, *m152* alone is unable to retain all K$^b$ class I molecules and therefore, *m4* is necessary to make-up for the incomplete retention of K$^b$ by *m152*. We therefore

concluded that *m152* and *m4* were not redundant but played cooperative and complementary roles in inhibiting antigen presentation; *m4* and *m152* apparently combine their efforts to cope with MHC class I polymorphism.

What makes *m4* so interesting is that is the only known CTL evasion gene that associates with class I at the cell surface; as such, *m4* is sure to offer new insights to the field of immune evasion. So far, no mechanism has been associated with the *m4* phenotype and whether m4/gp34 performs its evasion function as a result of its tight association with class I at the cell surface, or its more fragile association with class I in the ER is unknown. If *m4* were to function on the cell surface, m4/gp34 could either completely inhibit CTL from binding class I molecules through steric hindrance, or prevent the activation of CTL by allowing sub-optimal binding that could prevent CTL activation. In the ER, m4/gp34 could prevent antigen processing and presentation in more subtle ways: m4/gp34 could inhibit peptide binding in the ER, interfere with proper folding of class I, or act as a decoy chaperone. m4/gp34 could therefore, through non-mutually exclusive mechanisms, act at both the cell surface and in the ER. Resolving the mechanism by which *m4* inhibits antigen presentation could provide further insights into class I immune evasion mechanisms as well as T cell activation and antigen processing. Furthermore, the fact that *m4* is encoded by MCMV allows its biological relevance to be studied *in vivo*.

### *3a. The role of calreticulin in the class I assembly complex.*

Calreticulin is a soluble ER-resident protein that serves as a chaperone to glycoproteins including class I MHC. Calreticulin associates with class I once it has associated with the light chain $\beta_2$m and increases the efficiency of peptide loading (Gao et al. in press). However, the role of calreticulin in the class I loading complex is incompletely resolved. We were interested in addressing the role of calreticulin in antigen processing and presentation. We also wanted to know if calreticulin was required for the function of

immunomodulatory genes although this was not directly addressed in these experiments. As shown in data chapter 2.1 only MCMV-specific CTL clone 11 was able to kill ΔMS94.5-infected calreticulin$^{-/-}$ targets while clone 96 was not. Both of these clones are $K^b$-restricted indicating that the requirement for calreticulin is not simply class I allele-dependent. Furthermore, three other MCMV-specific clones were also unable to recognize calreticulin$^{-/-}$-infected targets (data not shown). The differential requirement for calreticulin has also been demonstrated with clones specific for $K^b$-SIINFEKL and for the $D^b$-restricted antigen from LCMV gp160 both delivered through recombinant vaccinia viruses. In those cases, B3Z T cell hybridomas specific for SIINFEKL were able to produce IL-2 in response to calreticulin $^{-/-}$ infected targets while the gp160-specific clones were not able to kill infected calreticulin $^{-/-}$ (Gao et al. in press). Furthermore, in cells lacking calreticulin, there is an increase in the amount of peptide-receptive class I molecules (Gao et al. in press). Therefore, calreticulin could affect antigen presentation in two manners which would both be reflected in the inability of some clones, but not others, to be impaired by the absence of calreticulin as illustrated above. The first is that calreticulin could affect loading of specific peptides; this might be the case if in fact calreticulin plays a role in peptide quality control in the ER. The second is that calreticulin affects peptide loading in a general manner and its absence results in decreased levels of surface antigen. Consequently, clones requiring higher levels of antigen would exhibit impaired recognition due to sub-optimal TCR activation.

***3b. The role of tapasin in the class I assembly complex.***

In chapter 2.1, I showed that while MCMV-specific CTL clones 3 and 94 could kill ΔMS94.5-infected B6 targets, the lack of tapasin prevented their ability to kill. The lack of killing of tapasin-/- cells was also seen with several other MCMV-specific clones (data not shown).The requirement for tapasin to act as a bridge between HC/β2m/calreticulin and TAP in the class I assembly complex is clear (Pamer and Cresswell, 1998). Tapasin

also stabilizes TAP thereby enhancing the rate of peptide translocation by TAP. Furthermore, in tapasin-negative cells, peptide-empty class I molecules leave the ER at an increased rate. Therefore, tapasin could act as a protective chaperone to retain class I molecules while they are peptide-empty. Ultimately, it is unclear if tapasin is simply acting as a bridge to facilitate class I association with TAP, and in this manner enables peptide loading, or if tapasin stabilization of TAP promotes enhanced quantitative and qualitative peptide loading. Unfortunately, we cannot conclude whether tapasin is required to bridge class I to TAP or if tapasin primarily exerts its function through stabilizing TAP because the cells used so far were completely lacking tapasin. We now have a mutant tapasin that is unable to bind to class I molecules but is still able to stabilize TAP. Further experiments with this mutant will allow us to address the separate roles tapasin might be playing in 1) bridging class I to TAP, 2) chaperoning peptide-empty class I molecules, 3) stabilizing TAP, and 4) providing enhanced peptide quality control.

***4. The CTL response to MCMV.***

Although studies performed prior to this thesis work showed that infection with MCMV elicited a CD8 T cell response in BALB/c mice that was protective, the size and kinetics of the MCMV-specific CTL response were undefined. In an effort to ask what effect immunomodulatory genes had on the size and the kinetics of the CTL response *in vivo*, we first began by assessing the size of the CD8 response to MCMV in C57Bl/6 mice using the ICS assay. As shown in chapter 2.2, close to 10% of $CD8^+$ splenocytes were shown to be MCMV-specific on day 7 post infection. Day 7 is the peak of the acute response to MCMV in B6 mice (data not shown). Because MCMV causes a systemic infection in mice, assessment of CD8 numbers from the spleen, as was performed here, are likely to reflect the overall response to MCMV. The peak of the acute CD8 response to MCMV, although small compared to that seen in LCMV infection, is on par with CTL

responses to other viruses such as MHV-68, HIV and SIV, and significantly larger than the responses to influenza or LM. However, as described in the introduction, what regulates the massive expansion, and ultimate peak size of the CD8 response in acute infection is undefined.

Although the size of the acute response to MCMV lacking *m152* did not differ from the response to wildtype virus (data not shown), the response to ΔMS94.5, the mutant lacking 15 genes including *m152*, was reproducibly higher and was often closer to 20% of the CD8s at the peak of the response. The fact that infection withΔMS94.5 elicited a larger CTL response was used to ask questions about CD8 control of virus as will be discussed below. Nevertheless, *m152* alone did not affect the size of the acute CD8 T cell response. This finding was surprising. We would predict that better antigen presentation would occur in an infection with a Δm152 mutant virus if indeed what we see in fibroblasts *in vitro* also occurs in the infection *in vivo*. Evidence that better antigen presentation occurs in Δm152-infected mice comes from Krmpotic et al. who showed that CD8s from wildtype MCMV-infected mice, when adoptively transferred, were better able to control an infection with a Δm152 virus compared to wildtype MCMV in BALB/c mice. If better antigen presentation is occurring, this might allow more CD8s to be recruited into the acute response and result in a larger CD8 expansion. Conversely, better antigen presentation might result in better clearance of antigen and be reflected in a reduced CD8 population if indeed less CD8s are recruited into the acute phase. Although the degree to which antigen presentation may be altered in MCMV infections lacking *m152* is unknown, we can conclude that whatever differences exist are insufficient to alter the size of the acute CD8 T cell response to MCMV.

The kinetics of the CTL response to MCMV were then followed for up to 64 weeks post infection. Mice infected with MCMV either with or without *m152* maintained a sizeable antigen-specific CD8 T cell response that made up close to 10% of the CD8s. These results support the idea that MCMV, like other persistent viruses, maintains CD8s

at a higher set point than viruses that do not persist, and therefore, contributes to the accumulation of data to support that trend. Furthermore, although the higher set point is presumably the result of repeated antigenic stimulation, it is not clear how this larger CD8 response is generated. One possibility is that continuous exposure to reactivating antigen may be responsible for raising and maintaining this higher set point. In the influenza system, re-exposure with a comparable influenza strain allowed both naïve and memory cells to expand (Turner et al., 2001). This indicates that if some CD8s are not originally activated by the first exposure to the virus, they might be upon re-challenge, or in the case of MCMV, reactivated virus. Therefore, repeated antigenic stimulation might be required for the recruitment of all antigen-specific precursors for any particular infection. Conversely, the die-off that normally occurs after the acute phase, may not be as severe in MCMV infection. Generally, the memory CD8 population is thought to make up 5% of the peak of the acute CD8 response. In fact, one notable difference in the size of the CD8 memory response to MCMV is that it actually reflects a number that is closer to 20% of the peak of the acute response. This appears to even be higher in infections with ΔMS94.5 where 25 to 30% of the acute peak numbers are sustained in the memory phase. Nevertheless, the increase in numbers seen with ΔMS94.5 infections is not the result of *m152* as infections with viruses singly deleted for Δm152 parallel the wildtype infection. We therefore conclude that *m152* does not alter the kinetics of the CTL response to MCMV.

Whether the higher set point in MCMV is therefore due to repeated antigenic stimulation or due to a less severe death phase therefore still remains to be determined. However, an observation I made on several occasions leads me to believe the latter of the two hypotheses appears less likely. While I do not have quantitative evidence for this observation, I found that CD8s from mice infected with MCMV for 14 days, the time when antigen-specific cells would be dying for lack of antigenic stimulation, were very labile. On occasion, as shown in figure 3 of chapter 2.1, CD8s were not even recovered.

This suggests that the death phase of the CD8 response to MCMV is severe and occurs as it does in responses to other viral infections, the extent of which remains to be quantified.

Does the memory CD8 response to MCMV mature over time? I am using the term maturation to describe either the focusing of the CTL response on fewer specificities, or the selection of CTL with higher affinity for peptide/MHC. As I began to perform CTL assays using splenocytes from mice chronically infected with MCMV, it appeared that CTL responses from mice which had been infected for longer periods of time were more effective in killing target cells. This observation led me to perform the experiments shown in chapter 2.1 figure 3, where CTL assays were performed using splenocytes from mice infected over a period of 40 weeks. The results from those experiments suggested that the CTL response to MCMV matured over time. The maturation of a CTL response could result from qualitative changes in the CD8 response, ie: affinity maturation or focusing of the repertoire, or quantitative changes ie: an increase in MCMV-specific CD8s over time. So far we have only addressed this question by quantifying the MCMV-specific CD8 response over time using the ICS assay. Results shown in chapter 2.2 figure 2 demonstrated no increase in the numbers of MCMV-specific CD8s over time. Furthermore, alongside with those same splenocytes as those used in the ICS assay, I performed a CTL assay ,which this time, did not reproduce the results seen with the first two timecourse CTL assays; only 2 out of the three kinetic CTL studies suggested maturation of the CTL response over time. Therefore, although the possibility of an increase in CTL numbers is unlikely unless they are undetectable using our assay, the question of a qualitative maturation of the CTL response is still unresolved.

The question of how antigen affects the CD8 population in chronic infections is unclear and furthermore, no definite consensus exists on whether antigen-specific CD8s that are present are of an effector or memory phenotype. Repeated antigenic stimulation might alter what we conventionally think of as memory CD8s and this could now be assessed in MCMV infection. To begin to address this, we looked at Ly6C expression.

Effector CD8s express Ly6C at an intermediate level and memory CD8s express high levels of Ly6C. Although reports (Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000) suggested that an Ly6C intermediate phenotype could be used to characterize CD8 effectors, we did not find it a useful marker for that phenotype as Ly6C was rapidly upregulated. By day 7 post infection, close to 90% of all MCMV-specific CD8s expressed an Ly6C hi phenotype and this marker remained expressed on antigen-specific CD8s in the chronic phase of infection (figure 14B chapter 2.2). Ly6C therefore could be used as a marker of antigen-experienced cells but did not allow us to differentiate effector from memory CD8s.

While performing ICS assays to quantify the CD8 response to MCMV, we began to notice that MCMV-specific CD8s displayed decreased levels of the CD8 marker. At the time, little was known about CD8 as an activation marker on CD8s. However, over the last few years several studies have shown that CD8 downregulation can be used as an indicator that CD8s have been "recently" activated. CD8 downregulation is most pronounced between days 7 and 14 after infections with LCMV, influenza, and LM (Busch et al., 1998; Flynn et al., 1998; Slifka and Whitton, 2000). However, CD8 levels are restored after 30 to 60 days. Only a very small fraction remains $CD8^{lo}$. I have included two examples from the literature to illustrate my point. In response to a primary infection to LM (Figure 24a), the levels of CD8s are restored by day 35 while by day 60 after infection with LCMV the number of CD8 with a CD8 low phenotype is very low (figure 24b). The difference after MCMV infection is that a large proportion of MCMV-specific CD8s remain $CD8^{lo}$ even 6 months after infection. This finding suggests that CD8s in MCMV infection display a phenotype that is suggestive of effector CD8s or memory CD8s that have been recently activated.

We therefore began to think of MCMV-specific CD8s as expressing an "effector memory" phenotype. However, the "effector memory" phenotype associated with the MCMV-specific CD8s did not correlate with increased cycling by those CD8s as

assessed using BrdU (chapter 2.2 figure 15). Whether the "effector memory" phenotype is the sign of repeated antigenic stimulation will need to be addressed by preventing reactivation of virus.

The further characterization of CD8s exhibiting this "effector memory" phenotype is interesting for another reason. CD8s of this phenotype, based on markers such as CD69, CD43 and CD62L, are increased at peripheral sites such as the lung, compared to the spleen, after localized non persistent infections, such as with influenza and Sendai (Hogan et al., 2001) . Although these CD8s would be considered to be memory cells based on the time after which the infection occurred, the increased expression of effector markers or ones associated with recent activation, call into question their exact phenotype. Furthermore, whether markers can be used to distinguish functionally different CD8s is also called into question by studies with vesicular stomatitis virus (VSV), a model for a nonpersistent virus, which like MCMV causes a systemic infection. Both the spleen and non-lymphoid organs of mice infected with VSV contain $CD62L^{lo}$ CD8s, a sign associated with recent activation. However, the CD8s from non-lymphoid sites, unlike the CD8s from the spleen, were able to perform direct *ex vivo* cytolytic functions (Masopust et al., 2001). Presumably, in those examples I highlighted, antigen is fully cleared and therefore the effector phenotype is not the result of recent exposure to antigen. Are the CD8s present at peripheral sites in localized infections similar to the MCMV-specific CD8s found in the spleen where MCMV replicates? Interestingly, LCMV also replicates in the spleen. However, after 30 days of infection, splenic LCMV-specific CD8s do not show signs of activation and are in fact used as the hallmark of typical memory cells. The lack of more defined phenotypic markers to better characterize CD8s makes the relevance of different CD8s found in different compartments unclear.

At some point in this work, we began a collaboration with David Raulet who studies NK receptors. He supplied us with an antibody to NKG2A which was known to

be expressed on NK cells. However, NKG2A expression on CD8s was undefined. NKG2A is now known to be present on approximately 5% of CD8s in a naïve mouse (McMahon and Raulet, 2001). When we looked at NKG2A expression on CD8s from mice that were infected with MCMV, we found that about 15% of CD8 from Smith-infected mice and up to 30% of the CD8s from ΔMS94.5-infected mice were NKG2A$^+$. Although some variation was seen between experiments, some conclusions could be made. The appearance of NKG2A+ CD8s exactly paralleled that of the antigen-specific cells and was first visible 5 days post infection. This finding has also been observed in influenza and LCMV-specific CD8 responses (McMahon and Raulet, 2001) as well as with vaccinia (MC Gold unpublished observation). Initially, therefore, it appeared NKG2A might be an early activation marker. However, the percent of antigen specific CD8s that were NKG2A+ did not change over time and 60 to 70% of antigen-specific CD8s in the memory phase remained NKG2A+. The finding that 70% of antigen-experienced cells express NKG2A has also been observed in LCMV infection (Chris McMahon personal communication). Therefore, NKG2A+ can be used associated with antigen-experienced CD8s. No function has been associated with NKG2A on CD8s. On NK cells, NKG2A acts as an inhibitory receptor when it binds its non classical class I ligand Qa-1. Perhaps NKG2A on CD8s serves a similar purpose and modulates the antigen-specific response.

Due to the fact the more CD8s expressed NKG2A than produced IFN-γ, and that on occasion, a larger proportion of CD8s also showed CD8 downmodulation, we questioned whether we were detecting all MCMV-specific CD8s using the ICS assay. The ICS procedure was initially described using peptide to stimulate antigen-specific responses, however, we needed to modify the stimulation procedure because no MCMV epitopes had yet been identified in H-2$^b$ mice. We started by using MEFs, then shifted to using DCs infected with a Δm152 virus as APC stimulators. Stimulation with these APCs allowed us to conclude that in mice infected with MCMV, 10% of the CD8s are specific

for the virus although we questioned whether all antigen-specific CD8s were being stimulated.

Using virus-infected APCs as stimulators may underrepresent MCMV-specific CD8s for several reasons. The first is that in MCMV-infected cells, gene regulation is complex and some antigens may be underrepresented either because of the timing of infection or because of the type of cell that is used as the APC. Differential antigen presentation by different cell types has been demonstrated and is illustrated in two examples I give below (Butz and Bevan, 1998; Skoberne et al., 2001). Butz et al. showed that LCMV-infected fibroblasts and dendritic cells, used as stimulators to drive CTL lines for three different $D^b$- restricted LCMV specificities, induced the selection of different CTL specificities. Because both cell types presented all three epitopes, as assessed by chromium release assays, the authors concluded that quantitative differences in antigen presentation between the two cell types were likely to account for the differential selection of CD8s (Butz and Bevan, 1998). The second example demonstrates that *in vivo* and *in vitro* presentation of antigens is likely to be different. Splenocytes from mice infected with LM were used as APCs directly *ex vivo*, compared to *in vitro*-infected APCs, to detect LM-specific responses in an ELISPOT assay. As was previously shown by others (Villanueva et al., 1995), *in vitro*-infected APCs elicited a minority of LLO-specific CD8s compared to subdominant-specific CD8s. Conversely, when *ex vivo* splenocytes were used as APCs, a different response was seen. A strikingly higher number of LLO-specific CD8s were now stimulated while the responses from the subdominant-specific CD8s were diminished. Furthermore, the pattern of recognition by the CD8s followed the pattern of immunodominant and subdominant CD8 responses seen *in vivo*. It would be interesting to determine if in fact the *in vivo* pattern of antigen presentation by splenocytes in LM infected mice affects the immunodominance hierarchy which Pamer and colleagues hypothesize is due to CTL precursor frequency (Busch et al., 1998). Nevertheless, both of these examples illustrate that the type of APC used to

stimulate antigen-specific CD8s may result in the detection of different numbers of CD8s and even preferentially favor the detection of some specificities over others. Furthermore, both examples suggest that different cell types exhibit quantitative differences in the amount of antigen they present. The ability of JAWS, compared to MEFs, to allow us to detect more MCMV-specific CD8s is yet another example. In theory therefore, we might be able to detect more antigen-specific CD8s, or a different profile of CD8 specificities using a different APC. Moreover, qualitative differences in co-stimulation may also be involved although this was not addressed directly in the examples I presented.

Another reason why using MCMV-infected APCs as stimulators may under-detect the number of MCMV-specific CD8s is because of MCMV encodes immunomodulatory genes which inhibit CD8 responses in *in vitro* assays (Del Val et al., 1989; Kavanagh et al., 2001). To overcome this problem and enhance our ability to detect MCMV-specific CD8s, APCs or targets were infected with MCMV mutants lacking *m152*. Of the three known immunomodulatory genes, *m152* appears dominant. However, we cannot rule out that some antigens are more affected by immunomodulatory genes *m4* and *m6*; as we use stimulator APCs that express these two genes, some specificities could be missed. Furthermore, whether *m152* , or *m4* and *m6*, function differently in different cell types is unclear. Hengel et al showed that *m152* and *m6* do not block presentation of the pp89 antigen in H-$2^d$ macrophages as they do in H-$2^d$ fibroblasts (Hengel et al., 2000). However, contradicting results from Diane LoPicolo in our lab show that *m152* indeed functions in H-$2^b$ bone marrow-derived macrophages. The reasons for these differences are unclear however, they could be attributed to differences in the cell type used, the H-2 background, or the antigen affected. Nevertheless, they highlight the point that immunomodulatory genes may act differently in different cell types. A final possibility exists that other MCMV immune evasion genes, which have not yet been identified, could prevent the detection of CD8s of other specificities. Although we have no evidence

for this, it would not be surprising if other MCMV immunomodulatory genes were discovered.

***5. MCMV gene m152does not prevent the establishment of latency.***

As I mentioned above, when we quantified the CD8 T cell response to MCMV, and compared it to the response to ΔMS94.5, we found that mice infected with ΔMS94.5 had twice as many MCMV-specific CD8s. At that time, we did not yet have information on the CD8 T cell response to single *m152* deleted viruses. We initially assumed that the enhanced CD8 response in ΔMS94.5-infected mice was due to the lack of *m152* and hypothesized that a potential role for *m152*, which presumably affects antigen presentation to CD8s *in vivo*, is to allow the establishment of latency through the generation of a less effective CD8 response. While this was an attractive hypothesis, other data suggested this was not the case. First, the CD8s from ΔMS94.5-infected mice appeared more activated than those from Smith-infected mice; a greater proportion of antigen-specific CD8s fromΔMS94.5-infected mice had downmodulated CD8 expression. This suggested that virus reactivation was stimulating CD8s. Second, the CTL response to ΔMS94.5, like the one to Smith, appeared to mature over time ( chapter 2.1 figure 3) once again suggestive of ongoing repeated antigenic stimulation. Therefore, to directly address whether *m152* prevented the establishment of latency, we performed PCR on organs from mice infected with Smith and ΔMS94.5. We detected MCMV DNA after both infections. Furthermore, we confirmed that *m152* does not prevent the establishment of latency by detecting MCMV DNA in mice infected with single *m152* deletion mutants. However, these experiments do not allow us to conclude whether the DNA we detected can result in viable virus production. To answer that question definitively, we will need to induce the virus to reactivate and detect virus production. Ultimately, whether CD8s are important in the establishment of latency remains to be determined. The quantitatively

larger CTL response to ΔMS94.5 also suggests that more CD8s do not prevent the establishment of latency, if in fact they play a role at all.

### *6. MCMV M45 encodes a $D^b$-restricted CTL epitope.*

In order to identify H-$2^b$-restricted MCMV CTL epitopes I generated an MCMV genomic library that would allow maximal representation of MCMV antigens and screened the library with CTL clones isolated from mice infected with MCMV. Although most of the clones were isolated from mice infected with ΔMS94.5, two other clones were included, one was from wildtype (Smith)-infected mice (clone 96) and the other from Δm152 (ΔMC96.24)-infected mice (clone 5). We used clones because of their reproducibility, their known specificity, and the fact they could be repeatedly propagated. Furthermore, we thought the clones would maximize the chances of detecting an antigen that 1) would have in fact elicited a CD8 response *in vivo* and 2) by virtue of cloning by limiting dilution, would more likely be representative of specificities that were present at a higher frequency within the total CD8 response and hopefully immunodominant. As described in chapter 2.3, clones 3 and 55 recognized a DNA fragment that encoded a portion of the MCMV M45 gene, and the epitope HGIRNASFI was identified. Clones 3 and 55 had been isolated from infections with ΔMS94.5 which had at least 25% of the MCMV-specific CTL specific for M45 (chapter 2.3 figures 19 and 20, chapter 2.4 figure 23). The strategy of screening the library with the clones was validated by this result.

The strategy I just described therefore allows the identification of antigens which are likely to be present at high frequencies and which may even be immunodominant. In order to use MCMV as a model to study CD8 T cell responses in a persistent infection, ideally, all immunodominant antigens need to be identified and conclusions about the CD8 response in MCMV should include specificities that comprise the majority of the CD8 population. Hence, more immunodominant antigens in B6 mice need to be identified. This is likely to also be the case in BALB/c mice. As I described in the

introduction, the response to the pp89 antigen in BALB/c mice has long been thought to be immunodominant and now m164 is also thought to elicit an immunodominant response. However, I would argue these conclusions may need to be reconsidered and that other immunodominant H-$2^d$ epitopes remain to be identified.

The methods by which H-$2^d$ antigens are being identified, and the methods used so far to assess CD8 responses in BALB/c mice call into question whether the total MCMV-specific H-$2^d$-restricted response (BALB/c) has been accurately assessed, and ultimately whether pp89 and m164 are in fact immunodominant antigens. Candidate MCMV-specific antigens in BALB/c mice were screened based on their potential to bind H-$2^d$ molecules. However, it is precisely because the requirements to generate immunodominant peptides are poorly understood that solely using the quality of peptide binding to class I molecules may miss the most important antigens in MCMV. As described in the introduction, the response to three H-$2^d$ antigens, m4, M83, and M84, identified in this manner, make up a minority of the CD8 response to MCMV (less than 1% combined).

Furthermore, the frequencies of CD8 responses to the peptides that are considered to be the immunodominant epitopes are suspiciously low. Using the more conventional and accepted ELISPOT method, Reddehasse and colleagues show that only 1-2 % of the CD8s are specific for the combined pp89 and m164 antigens in the chronic phase of MCMV infection (Holtappels et al., 2002). If, however, the magnitude of the CD8 T cell response in BALB/c mice is similar to the one in B6 mice, then close to 10% of total CD8s should be MCMV-specific as I showed in figure 12. If this assumption is true, then 90% of the MCMV-specific response in H-$2^d$ mice remains unaccounted for. Reddehasse suggests that pp89 and m164 are immunodominant because the frequency of CD8s to both antigens combined is the same frequency he believes is the total MCMV-specific response (1-2% in chronic infection) based on a redirected CD3 stimulation assay. As I described in the introduction, Reddehasse and colleagues use the redirected CD3

stimulation assay to estimate MCMV-specific CD8 numbers and they conclude that 1-2% of total CD8s are specific for MCMV in the chronic phase of infection. The αCD3 assay is not conventionally used to quantify antigen-specific responses and it is likely that a substantial proportion of MCMV-specific CD8s are not being detected. Furthermore, based on CD8 frequencies to other persistent viral infections, the 10% of CD8s specific for MCMV is likely to reflect a number closer to reality than the 1-2% detected by the αCD3 assay.

Interestingly, I once performed an ICS assay using peptide restimulation to detect the pp89-specific frequency from BALB/c mice. I found that close to 4% of H-$2^d$-restricted CD8s, from chronically infected BALB/c mice, were specific for the pp89 epitope (data not shown). This number is 4 times greater than the one obtained by ELISPOT used by Reddehasse and colleagues. It is therefore possible that the ELISPOT also underestimates the frequency of antigen-specific cells and that pp89 is in fact immunodominant.

Finally, using peptide restimulation in the ELISPOT assay, Reddehasse determined that about 2 % of the CD8s were specific for each of pp89 and m164 antigens during acute infection. Using this same method, he determines that in the chronic phase of infection, the frequencies of pp89 and m164-specific CD8s decrease to 0.7%. This suggests that 35% of CD8s from the peak of the response go on to form these CD8 memory populations, a trend similar to the one I described in B6 mice. Whether MCMV, as opposed to other viruses, allows an unusually high set point of memory CD8s remains to be determined.

### *7. m152 does not alter the immunodominance of the M45 antigen .*

The identification of M45 as an H-$2^b$ antigen finally allowed us to track the M45-specific CD8s and now allows us to ask a variety of questions about the acute and chronic M45-specific CD8 responses in MCMV infection. Importantly, we could now ask how the

CTL response to M45 would be altered by *m152* and specifically, whether *m152* could alter the immunodominance to an antigen it was known to affect in *in vitro* assays. MCMV-specific CTL clones 3 and 55, which had been used to identify M45, had been isolated from mice infected with ΔMS94.5. We therefore knew that presentation of M45 to clones 3 and 55 was abrogated in cells infected with wildtype virus; *m152* completely inhibited the ability of M45-specific CTL to kill infected fibroblasts. Moreover, although we knew that MCMV-specific CD8s were generated in mice infected with wildtype virus, we did not know if the CTL specificities would be the same as those generated in mice infected with a virus lacking *m152*. If *m152* abrogated presentation to M45 *in vivo* as it did *in vitro*, the resulting repertoire of CD8s in wildtype MCMV-infected mice could be altered. In fact, we found this was not the case. Strikingly, wildtype MCMV-infected mice made a response to the M45 antigen which was quantitatively similar to the M45-specific response in Δm152-infected mice. Furthermore, the M45-specific response was not altered within the total detectable MCMV-specific response and we therefore concluded that *m152* did not alter the immunodominance of the M45 antigen (figure 2 chapter 2.3). Both of these results were surprising and leave us to speculate on how M45-specific CD8s are elicited in wildtype MCMV-infected mice. Ultimately, the lack of evidence to support the hypothesis that *m152* alters immunodominance leaves us again with the question of what is *m152* doing *in vivo*? I will first propose several mechanisms that could account for the generation of an M45-specific CD8 response and finally speculate on the role of *m152 in vivo*.

One possibility for the generation of an M45-specific response in wildtype MCMV-infected mice is that *m152* does not function in the cells that prime the CTL response. It has been shown that MCMV infects dendritic cells *in vivo*, and presumably, these are important in priming the CD8 response to MCMV, as has been shown in other systems. The higher levels of class I expressed on dendritic cells, compared to fibroblasts, may be responsible for an inability of *m152* to retain enough class I ultimately resulting

in normal antigen presentation. If true, this scenario might suggest that the role of *m152* is not to prevent the priming or generation of CD8 responses but to act further downstream in the CD8 response.

Another mechanism by which M45-specific CD8s could be generated is through cross-priming. In this scenario, cells infected with MCMV, which presumably would be impaired in the class antigen presentation pathway, would be taken up by dendritic cells that could cross present MCMV peptides on their cell surface. Since no known MCMV immunomodulatory genes impair the generation of peptides, but only their presentation, the DCs should be able to cross present processed MCMV peptides. However, determining the importance of direct presentation versus cross presentation is difficult for two reasons. The first is that the pathway used for cross-presentation is not defined and preventing cross presentation is not currently possible. The second is because MCMV infects DCs; distinguishing whether M45 was directly presented or cross-presented *in vivo* is not as yet possible. Studies on the role of DCs in MCMV infection and the effects of immunomodulatory genes on antigen presentation in DCs should shed light on these matters.

Another issue I raised in the introduction regarding cross-presentation is the possible outcome of either a productive CD8 response or an ineffective CD8 response as a result of cross-priming. The advantages of cross-priming antigens that are affected by immunomodulatory genes could be seen in the ability of CD8s to control virus in cells where immunomodulatory genes are less or non-functional. The other possibility is that a CD8 response to an antigen, which is never expressed in the infected cells due to immunomodulatory gene function, is raised and ultimately ineffective. Antigens expressed at higher levels through cross-presenting DCs might preferentially induce the proliferation of CD8s which would not be as effective in controlling reactivating virus from latently-infected cells. In the case of M45-specific CD8s it remains to be determined if these are protective. Although it is likely that they are, it is possible that CD8s of other

specificities are more effective and the efficacy of M45-specific CD8s remains to be assessed and compared to MCMV-specific CD8s of other specificities.

### *What is m152 doing in vivo?*

*m152* is clearly a potent inhibitor of antigen presentation in MCMV-infected fibroblasts; by retaining MHC class I molecules in the ER cis-Golgi, *m152* interferes with CD8 T cell recognition *in vitro*. Although the role of *m152 in vitro* appears obvious, its role *in vivo* appears elusive. Because CD8s appear to be the targets of *m152* function, we have studied the CD8 T cell response to MCMV with and without *m152*. Aside from interfering modestly with CD8 control in neonatal BALB/c mice (Krmpotic et al., 1999), *m152* shows little effect *in vivo*. At this point we can rule out a role for *m152* in altering the size and kinetics of the MCMV-specific CD8 response, the establishment of latency, and in altering the M45-specific CD8 response in H-$2^b$ mice. However, if *m152* is in fact altering antigen presentation to CD8s *in vivo*, it could affect CD8s in ways which remain to be studied.

*m152* may prevent the presentation of antigens in some cell types but not others. Although it remains to be determined if immunomodulatory genes perform differently in different cells, the conflicting evidence I presented above suggests this may be the case. Because DCs are important in the priming of CD8 responses, I would begin testing the effects of *m152* on antigen presentation in DCs. The *in vitro* studies performed in MEFs could be repeated in DCs to determine if *m152* has an effect on class I presentation. Furthermore, the ability of *in vivo* -infected APCs to stimulate CD8s in *in vitro* assays could be tested using splenocytes from mice infected with MCMV with and without *m152*.

Nevertheless, evasion in a cell type at an immunoprivileged site could allow the production of virions without much interference by CD8s. This might occur in the

salivary glands where CD8s have been shown to be ineffective in controlling MCMV in the acute phase of infection; potentially *m152* functions effectively in salivary glands in the chronic phase of infection to allow the production and spread of infectious virions. Whether *m152* alters the ability of virus to spread could be tested by determining if pups fed by MCMV- infected mothers are less likely to become infected.

As mentioned in the introduction, other viruses which also cause chronic infections encode class I immunomodulatory genes. In this manner, *m152* in MCMV is primarily guilty by association with other viruses with a similar lifestyle. However, we have little data to support that *m152* is important in the chronic phase of infection. We have shown that *m152* does not appear to interfere with the priming of an MCMV-specific CD8 response. The total numbers of MCMV-specific CD8s appear similar at the peak of the response and the resulting number of CD8s which enter memory also appear the same. Furthermore, this trend is also seen with the M45-specific response which appear parallel in a Δm152 (ΔMC96.24) virus and its parental wildtype (rMC96.27). However, we have not yet looked at the quality of the responses to wildtype MCMV and Δm152 viruses. Experiments to test possible differences in CD8 affinity maturation, potential differences in CD8 effector functions, and focusing of the CD8 repertoire in mice infected with MCMV and MCMVΔm152 remain to be performed.

The lack of an apparent *m152* phenotype from our *in vivo* studies may be due to a requirement for *m152* to cooperate with *m4* and *m6* (and/or maybe other immunomodulatory genes) to completely prevent antigen presentation to CD8s *in vivo*.. MCMV is like other herpesviruses in that it encodes several genes which affect the class I pathway. In some cases it is unclear why so many genes are required and if they are simply redundant or if they cooperate. In MCMV, *m152* is necessary to prevent antigen presentation to CD8s *in vitro* , but is not sufficient to completely block $K^b$-restricted CD8 responses and *m4* is required to make up for this deficiency in *m152* function. In MCMV therefore, cooperation from at least two immunomodulatory genes appears required for

effective escape from CTL. Further studies using triple knockout Δm4,Δ6, Δm152 MCMV virus are required to address these questions.

## Summary and Conclusions.

MCMV provides a biologically relevant animal model to study the role of immune evasion genes *in vivo*. Our focus has been to determine how MCMV genes that interfere with class I MHC affect the CD8 T cell response *in vivo*. Although we have not been successful in determining the relevance of *m152 in vivo*, results from this thesis have contributed to both the field of immune evasion and CD8 T cell biology. Assumptions about the importance or impact of immune evasion genes must be reassessed. Although we confirmed in H-$2^b$ mice that *in vitro*, *m152* potently inhibits class I, the effects of *m152 in vivo* as examined by assessing the CD8 T cell response appear modest at best. Furthermore, assumptions that class I immune evasion genes would alter the size of the CD8 T cell response or alter the CD8 response to immunodominant antigens also need to be reevaluated.

However, the study of the CD8 response to MCMV has provided us with some interesting results. The CD8 response to MCMV, like CD8 responses to other viruses that cause chronic infections, is maintained at a higher set point than the response to viruses that are cleared. The ability of the immune response to expand and contract allows many different pathogen-specific responses to be elicited. However, if a host is infected with several different herpesviruses, each of which elicits 10% of the CD8 response, does this not alter the CD8 compartment? And if so how?

Furthermore, the CD8s responding to MCMV exhibit an activated phenotype. It remains to be determined if the repeated antigenic stimulation in MCMV infection provides the high set point of CD8s, as well as the activated CD8 phenotype; experiments to prevent reactivation are planned0 to address this. However, CD8s with an activated

phenotype are also seen in response to other infections, some of which are non persistent. Therefore whether a CD8 activated phenotype can be used to determine if cells have been recently triggered by antigen is unclear.

**Figure 24. Two examples from the literature are used to demonstrate that CD8 expression levels are restored 60 days after the clearance of nonpersistent infections.**

# Coordinate Regulation of Complex T Cell Populations Responding to Bacterial Infection

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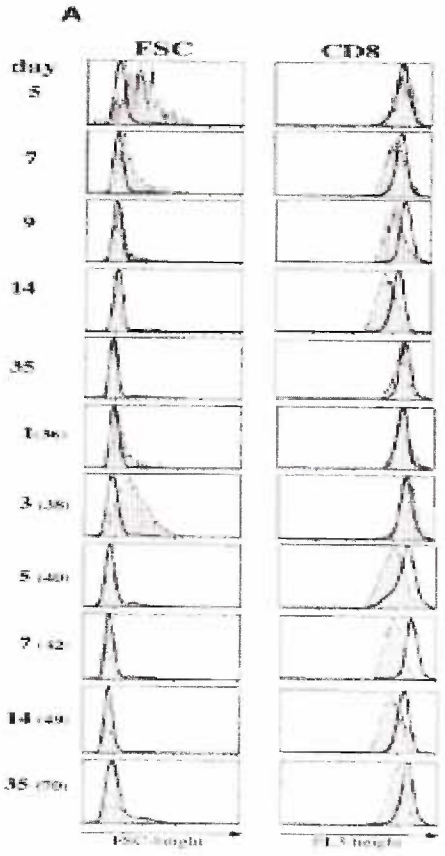
Figure 6. Phenotypic Shifts of Distinct T Cell Subpopulations Occur Synchronously during Primary and Recall Response to *L. monocytogenes* Infection

BALB/c mice were infected with *Listeria*, and epitope-specific CD8$_1$ T cells were analyzed on sequential days following infection. CD8$_1$-enriched splenocytes were stained for CD8, CD62L, and each of the four *L. monocytogenes* epitopes containing H2-K$_d$ tetramers.

(A) Histograms demonstrate the FSC profiles of CD8$_1$, CD62L$_{low}$, and H2-K$_d$/LLO$_{91-99}$ tetramer-positive T cells (filled areas) and CD8$_1$, CD62L$_{high}$, and H2-K$_d$ tetramer-negative T cells (black lines) on sequential days following primary and reinfection with *L. monocytogenes*. The level of CD8 expression is also indicated for the same T cell populations in the right column of histograms. FSC, linear scale (0–250); FL3, four log-phases.





# Activated and Memory CD81 T Cells Can Be Distinguished byTheir Cytokine Profiles and Phenotypic Markers1

**Mark K. Slifka2 and J. Lindsay Whitton**

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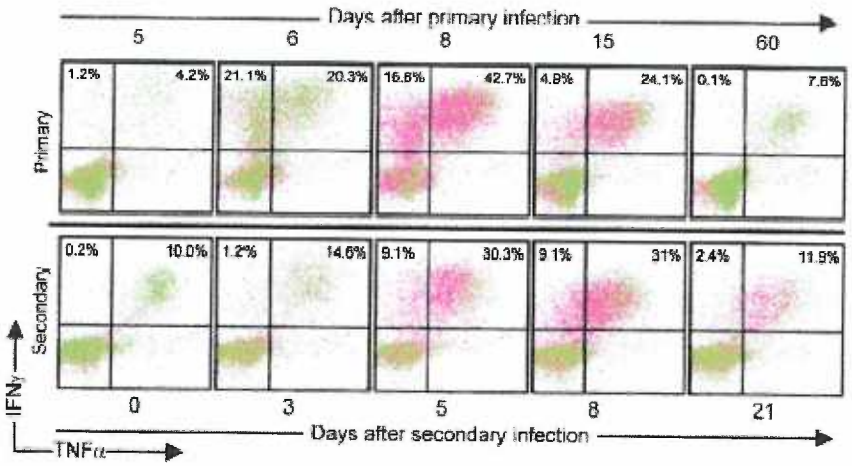


**FIGURE 5.** Cytokine profiles and CD8 levels during primary and secondary responses to viral infection. Naive BALB/c mice were infected with 2 $310_5$ PFU LCMV at staggered time points to allow $CD8_1$ T cell-mediated cytokine responses to be assayed on the same day. LCMV-immune mice wereinfected with 2 3 $10_6$ PFU LCMV-Armstrong at staggered time points from 114 to 132 days after primary infection to assay them together at 135 dayspostprimary infection. Total spleen cells were stimulated directly ex vivo with peptide for 6 h and analyzed by intracellular cytokine staining for IFN-g and TNF-a. The flow cytometry plots were gated on $CD8_1$ T cells using a large gate (fluorescence range, 120-3400). The number in the *upper left quadrant*of each dot plot represents the percentage of $CD8_1$ T cells that are IFN-$g_1$TNF-$a_2$, and the number in the *upper right quadrant* shows the percentage thatare IFN-$g_1$TNF-$a_1$. The events in the *lower right quadrants* were present also in unstimulated cultures and have not been subjected to further analysis.
To distinguish $CD8_{high}$ cells from $CD8_{low}$ cells, the events are colored either green (representing the high CD8 levels (fluorescence range, 930-3400)observed in naive T cell controls) or red (representing T cells with down-regulated levels of CD8 (fluorescence range, 120–930)). The MFI of CD8-negativespleen cells was ,50. Each plot shown is representative of two to four individual samples.

## Materials and methods.

### Generation of mutant MCMVs (For a list of the viruses see Table 2 in chapter 2.1).

Generation and characterization of recombinants ΔMS94.5 (with a deletion of ORFs *m150* to *165*), ΔMC96.24 (with a deletion of ORF *m152*) and rMC96.27 (revertant for ΔMC96.24) were described previously (Krmpotic et al., 1999; Thale et al., 1995).

The recombinant Δm4-MC95.33, with an insertion of the *lacZ* gene in place of the *m4* ORF, was generated by insertional mutagenesis in eukaryotic cells as described previously (Crnkovic-Mertens et al., 1998), using the plasmid construct pm4. The homologous recombining region of pm4 was produced by flanking the *lacZ* gene with MCMV genomic sequences adjacent to the 5' (nt 2739 to 3250 left flank) and 3' (nt 4041 to 4737 right flank) ends of the ORF. Plasmid DNA (pHindIIIA) (Ebeling et al., 1983) serving as MCMV genomic template and primer pairs for the left flanking sequence [sense (5'-AA**CTCGAG**CATCACGGTGAACGATACCA), anti sense (5'-TT**GGATCC**TGGAACAACGAATGAGACAGA)] and right flanking sequence [sense (5'-AT**GCGGCCGC**TCGAACTTCAAACCGCTTAAGAG), anti sense (5'-AA**CCGCGG**ACTTATCGACGTACAATCCTGT)] were used in separate PCR reactions to produce fragments with convenient restriction sites to ligate to the *lacZ* gene (XhoI, BamHI and NotI and SacII, respectively in bold). These fragments were inserted into corresponding sites within the plasmid pIC4, which contains the *lacZ* gene under control of the Rous sarcoma virus (RSV) promoter, SV40 poly(A), and flanking loxP sites (Crnkovic-Mertens et al., 1998). Thirty fmol of linearized pm4 plasmid DNA was cotransfected with wt MCMV DNA (1.5 $\mu$g) into NIH3T3 fibroblasts by calcium phosphate precipitation to generate the recombinant virus Δm4-MC95.33. Recombinant virus was isolated and plaque-purified as described previously (Crnkovic-Mertens et

al., 1998). Correct recombinatorial mutagenesis within the genome of Δm4-MC95.33 was confirmed by restriction enzyme analysis (data not shown).

The MCMV genome was recently cloned as an infectious bacterial artificial chromososme (BAC ) in *E. coli* (Messerle et al., 1997). The MCMV-BAC plasmid pSM3fr contains the complete MCMV genome and was transfected into permissive eukaryotic cells to reconstitute the virus MW97.01 (wt) (Wagner et al., 1999). MW97.01 (wt), which contains the complete MCMV genome without any BAC sequence, has wildtype properties both *in vitro* and *in vivo*, indicating that the MCMV genome can be passaged in *E. coli* without altering the properties of the reconstituted viruses.

Recombinant MCMVs Δm4-MW99.03, Δm152-MW99.05, and Δm4+m152-MW99.04 were generated in the lab of Ulrich Koszinowski by transfection of the MCMV BAC plasmids pΔm4, pΔm152, and pΔm4+m152, respectively, into primary MEFs by calcium phosphate precipitation technique as described previously (Messerle et al., 1997). The MCMV BAC plasmid pΔm4, which encodes an exact deletion of the *m4* ORF (nt 3270 to 4067) by insertion of the prokaryotic kanamycin resistance marker ($kan^r$), was constructed using contiguous m4-kan sequence primer pairs: sense (5'-TAATGATCTAGACGGCAATTTCTGTCTCATTCGTTGTTCCAGAGCGACGGATG GTACAAG) and antisense (5'-TACTCAGAACACCGGAAAATGGTTTACTCAAGGGGATTTTTATTTAGGGG GTTAGTTACT). The plasmid pACYC177 (New England Biolabs) served as template for the kanamycin resistance marker. A linear DNA fragment containing flanking homologies of 55 bp to the *m4* gene (nt 3215 to 3269 and nt 4068 to 4123 in the MCMV genome) and the $kan^r$ was generated by PCR amplification. This fragment was inserted into the wt MCMV BAC plasmid pSM3fr (Wagner et al., 1999) by homologous recombination in *E. coli* to generate the MCMV BAC plasmid pΔm4. The MCMV BAC plasmid pΔm4+m152 was generated using contiguous *m152*-zeocin primer pair PCR

amplification. The fragment containing flanking homologies of 60 bp to the *m152* gene (nt 210184 to 210243 and nt 210378 to 210437) and the zeocin resistance gene was generated using sense (5'-GCTCGAGCGAGAGCACCCGACGATCTGACATTGTCCAGTGTGCCGGTCGCAC GAACATCAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAACGTTTACA ATTTCGCCTGATGCG) and antisense (5'-TCACAAGCCGTGTCACCGCTCCACGTTTCACCGTCGTCGGTCTCCCGATCGCT AGCCTGAACAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTGAAGTTTT AGCACGTGTCAGTCCT) primer pairs and the plasmid pZero1 (Invitrogen) as template. This fragment was inserted into the MCMV BAC plasmid pΔm4 by homologous recombination in *E. coli*, generating plasmid pΔm4+m152. Plasmid pΔm4+m152 thus carries exact deletions of the *m4* and *m152* ORFs and insertions of the kanamycin resistance marker (in the case of *m4*) and the zeocin resistance marker (in the case of *m152*) instead. Plasmid pΔm152 was generated by homologous recombination between pSM3fr and the *m152*-zeocin fragment. Correct mutagenesis was confirmed by restriction enzyme and southern blot analysis (data not shown).

Recombinant MCMVs m4-Tn3514, m4Tn3516 and m4TnP (with Tn1721 transposon insertions within the *m4* gene or putative promoter, at nt 3514, nt 3516 and nt 3099 respectively) were reconstituted from recombinant MCMV-BAC plasmids generated by direct transposon mutagenesis as described previously (Brune et al., 1999) (Hobom et al., 2000). The site of mutagenesis was confirmed by restriction enzyme analysis and sequencing (data not shown).

Loss of m4/gp34 expression in the BAC-derived recombinants was confirmed by Western blot analysis of cell lysates from infected NIH3T3 cells with the antiserum m04-3 that detects m4/gp34 (Kavanagh et al., 2001).

## Virus Stocks.

Virus stocks were generated by infecting subconfluent MEFs with low-passage seed stock at an MOI of 0.001. Cells were then switched to DMEM + 10% normal calf serum until the monolayer became 100% infected. Stocks were harvested by scraping and sonication of cells. Titer of plaque forming units (PFU) was determined by serial dilution and agarose overlay on Balb-3T3s.

## Experimental Animals.

B6 mice were purchased from Simenson, Swiss Webster mice from Charles River, and B10.A5R, and B10.A2R from Jackson Labs. $K^{b\,-/-}$ mice (Thale et al., 1995) (Krmpotic et al., 1999) were a gift from Francois Lemmonnier.

## Cells.

MEFs were grown from trypsin-digested day 12-14 mouse embryos, from C57Bl/6 mice or congenic mouse stains, and used between passage 3 and 6. MEFs were maintained in DMEM supplemented with 10% fetal calf serum. NIH 3T3s (CRL-1658) and Balb3T3s (CCL-163) were obtained from ATCC and maintained in DMEM supplemented with 10% fetal (for NIH3T3s) or newborn (for Balb3T3s) calf serum. JAWSII cells were obtained from ATCC (CRL-11904) and maintained in αMEM supplemented with 20% FBS, sodium pyruvate, non essential amino acids, and 5ng/ml of GMCSF.

## Polyclonal CTL.

B6 mice were infected intraperitoneally with $5 \times 10^4$ PFU MCMV. For restimulation, spleens were harvested and 10% of splenocytes were incubated with MCMV (of the same strain with which mice were infected) and returned to culture with the remaining splenocytes. For polyclonal effectors, cultures were used 5 days later in $^{51}Cr$ release assays.

## CTL cloning.

### *Initial restimulation.*

As with polyclonal effectors, one tenth of the splenocytes from at least two infected mice were restimulated by infecting the cells for 1.5 hrs with the same virus that the mice had originally infected with. The cells were washed twice before adding them to remaining splenocytes. Cultures were resuspended at $4X10^6$ cells/ml in RPMI-C and incubated at 37C for 3-4 days.

### *Cloning by limiting dilution.*

The restimulated effectors were centrifuged and resuspended in RPMI-C. Cells were counted and diluted to achieve 10, 3 and 1 lymphocytes/well in 50ul in a round bottom well of a 96 well plate. 100ul of irradiated feeders + 2ug/ml of concavalin A was added to the cells and incubated for 3 days at 37C at which point 100ul clone food** was added per well and cultures were incubate until the screening on day 12.

Irradiated feeders:

Equal numbers of spleens from a combination of naïve Swiss-Webster mice and C57Bl/6 mice were made into a single cell suspension in DMEM (or HBSS) + 5% FBS. The cells were irradiated with 3000 rads with $^{137}Cs$. The cells were washed twice in DMEM-5% FBS, counted and resuspended in RPMI-C +2ug/ml concavalin A at $2X10^6$ cells /ml.

**Clone food:

RPMI+10% FBS +10% Rat Con A supernatant (50ml) (supernatant from naïve rat splenocytes incubated in RPMI-C with 2ug/ml conA for 48hrs) + PSG+ 2-ME ($5 X10^{-5}$M)+ 5 ml of 100X IL-2 stock (prepared fromX63.653 cells)

To produce IL-2 sup stock:

$10^5$ X63.653 cells /ml (these are transfected with a plasmid encoding the IL-2 gene (Karasuyama and Melchers, 1988) are incubated in RPMI-C for 5 days. The supernatant

was collected by centrifuging the cells and filtering the supernatant. The potency of the supernatant can be assessed using the MTT assay (Bioassay to test the ability of the sup to maintain an IL-2 dependent cell line) or tested in a thymidine incorporation assay for the ability of con A blasts to proliferate

***Screening.***

Clones from wells which showed positive growth (see notes below)were screened for anti-viral function 12 days post cloning. The ability to specifically kill IFNγ-boosted ΔMS94.5-infected MEFs was compared to uninfected MEFs in a typical cytolytic assay. 1/2 of each positive clone well (100ul) was transfered to individual wells of a sterile 96 well (plated out in the same order as they were tested on plates with labeled targets). The remaining clones were saved and fed as soon as possible with clone food and incubated at 37C.

***Notes:*** For each MCMV CTL cloning, at least 2 spleens from MCMV-infected mice were used. Positive wells are those which have definite T cell growth. This usually appears as a dense white button when the plates are observed from the bottom; positive wells should be confirmed by microscopy. Based on the Poisson distribution there should be about 30 positive wells/ plate at 1 cell per well. If there are close to or more than 30/plate plan to subclone from killer clones obtained from that plate as they may be contaminated with CD4s or irrelevant CD8s that may be better growers. Subcloning is performed by the same limiting dilution method used to derive the clones initially and is followed by the screening procedure described above.

***In vitro clone restimulations.***

In order to propagate the clones *in vitro*, they were restimulated every 10 to 12 days. Clone cells were divided into 2 to 3 new wells of a 24-well plate for a 1 to 2 or a1 to 3 split respectively, and mixed allogeneic feeders were added to the clone cells. After 3

days, the clones were fed with clone food. Every 3 days the medium was replaced with fresh clone food.

### *Freezing and thawing the clones.*

Clones were generally frozen around 5 days after an *in vitro* restimulation when they would most likely be in the growth phase. Cells were centrifuged and concentrated to $1X10^6$/ml and frozen in 90% FBS + 10% DMSO. They were placed in a freezer box at –80C and then shifted to liquid nitrogen for long-term storage. To thaw the clones, vials of cells were rapidly thawed at 37C, resuspended in RPMI-C and washed twice. The clone cells were then resuspended into 1 or 2 wells of a 24 well plate in the presence of 1ml of irradiated feeder cells. The cells were then maintained as described above for the in vitro restimulated clones.

Notes: At some point the clones became mycoplasma contaminated. We continue to propagate them on mycoplasma removal agent. If the clones lose their specificity, they are discarded and newly thawed cells are grown.

## Assays.

### *Cytolytic T cell assays.*

MEF target cells were plated into 96-well plates at 5,000 cells/well and treated with recombinant mouse IFNγ (50 U/ml, Sigma) for 24 hours, infected with MCMV at an MOI of 30, unless otherwise indicated, and labeled with $^{51}$Cr (NEN) overnight, in the presence of 0.3 mg/ml phosphonoacetic acid (PAA, Sigma) to prevent expression of viral late genes. Effectors were added at the indicated effector-to-target ratios for five hours, after which supernatants were harvested and assayed for γ-irradiation with a Topcount scintillation counter (Packard). Background $^{51}$Cr-release was determined by incubating targets with medium alone, and total $^{51}$Cr release was achieved by lysing targets with medium containing 2% Triton X-100. The percent specific lysis was calculated as

(experimental cpm-background cpm)/(total cpm-background cpm). Each data point represents the mean of triplicate wells.

***Selective expression of IE and IE and E genes.***

IE only or IE and E gene expression were enhanced as previously described (Del Val et al., 1989) . Briefly, to selectively enhance IE gene expression, B6 targets were infected with MCMV in the presence of cycloheximide for 3 hours (50ug/ml, Sigma) followed by a 3-hour incubation with actimomycin D (5ug/ml, Sigma). For IE and E gene expression, cells were infected in the presence of cycloheximide alone for 3 hours followed by a 3-hour incubation without drugs.

***Extraction and purification of peptides from MCMV-infected cells.***

48-hour-IFN-γ-pretreated B6 MEFs were infected with ΔMS94.5 in the presence of PAA or left uninfected. The next day, cells were scraped in 0.1%trifluoroacetic acid (TFA) and then lyophilized. The pellet was resuspended in 1ml 0.1% TFA and centrifuged through a Centricon 10,000 filter to exclude proteins>10,000 MW. The fraction was HPLC - purified using a C-18 column on a 0-60% acetonitrile gradient. One ml fractions were collected every minute.

***The ICS assay.***

We initially used MEFs as stimulators in the ICS assay. In those cases, MEFs were pretreated with 50U/ml IFN-γ for 24 hours and then infected with MCMV at an m.o.i.of 50 for 2 hrs after which the medium was replaced and cells were incubated overnight with PAA. On the day of the assay, cells were trypsinized, washed, counted and resuspend at $2.5X10^6$/well in a 24 well plate in RPMI-C. Effectors, either clone cells or ex vivo splenocytes, were counted and washed twice before being resuspend to $2X10^7$ cells/ml. $1X10^7$ effectors in 0.5 ml were added to the APCs. BrefeldinA was added and

cells were mixed and incubate for 6 hrs at 37C. A PMA/ionomycin-treated sample was used as a positive stimulation control.

When using JAWS as stimulators, JAWS II cells were resupended to $1X10^6$ cells/ml in JAWS complete medium. No increase in antigen presentation was seen with IFN-γ pretreatment, therefore this step was not included (MC Gold unpublished data).100ul of cells were added per well in a 96 well plate and the JAWSII cells were infected with MCMC using an m.o.i. of 50 for 2 hours after which the medium was replaced and the cells were incubated O/N with PAA. The next day, effectors were prepared as described above in MEF protocol except they were resuspended to $1X10^6$ cell/ml in RPMI-C. The 96-well plate containing infected or uninfected JAWS cells was centrifuged before flicking out the medium and 100ul ($1X10^5$) of effector cells with Brefeldin A are added to each well; this gave an optimized ratio of 1 APC per effector (MC Gold unpublished data). The cells were incubated for 6 hrs before the staining procedure.

The staining took place in 2 steps starting with the surface staining cells for CD8 and other surface molecules such as NKG2A followed by the intracellular cytokine staining (such as IFN-γ) after fixing and permeabilizing cells as described in the PharMingen fix/perm protocol. A variation on this ICS protocol was included for BrdU staining. The BrdU PharMingen kit was used for this purpose and the manufacturer's protocol was used.

## MCMV genomic library

### Construction of the MCMV DNA expression libraries.

Two non-overlapping MCMV expression libraries representing the MCMV genome were constructed using MCMV DNA originally cloned as HindIII fragments into pUC-based plasmids (known as plasmids A through P). Library "A" was constructed using equimolar amounts of MCMV HindIII fragments A, G, H, I, J, K, M, N, O, and P (equivalent to

99,563bp) purified by agarose gel electrophoresis of the respective HindIII digested plasmids. Similarly, library "B" (equivalent to 106,408 bp) was constructed using equimolar amounts of a HindIII purified fragments B, C, D, F, and L. HindIII E DNA (22,749bp) was not included in the libraries. Purified HindIII fragments (1066bp to 33141bp) were fragmented by incomplete restriction enzyme digestion with a combination of Bcl I, Bgl II, Bam HI (library I) or Sau3AI (library II) to generate BamHI compatible ends, and ligated to BamHI cut pcDNA4 HisMax (Invitrogen, Carlsbad, CA). The pcDNA4 HisMax A, B, C vectors allow expression of DNA in each reading frame by staggered insertion of genomic fragments downstream of a QBI SP163 translational enhancer. Characterization of a subset of clones showed that insert frequencies were 92% (I) and 83% (II). The average insert sizes were 1130bp (I) and 530bp (II). Approximately 30,000 independent clones for library I and 25,000 independent clones for library II were arrayed in pools of 50 clones/pool. DNA for transfections was prepared using Qiagen 96 Turbo-Prep plates.

**Library Screening.**

For transfections, 100ng (per pool) of library DNA was mixed with OptiMem (Invitrogen) and 1ul of the transfection reagent Fugene-6 (Roche, Basel, Switzerland). The libraries were screened using K41 cells, an SV-40 transformed H-$2^b$ fibroblast line (gift of Marek Michalek), that were seeded at 5000 cells/ well in a 96-well plate 24 hours prior to transfection. Wells were supplemented with medium 2 hours after transfection. Two days later, 2,000 T cells clones were added to the transfected K41s in the presence of clone food*. After 8 hours at 37°C, supernatants from the cultures were collected and assayed for TNF-by bioassay using the TNF-sensitive indicator cell line WEHI164/Clone20, derived (SP Fling unpublished data) from WEHI164 (ATCC # CRL-1751), that is similar to that described by Khabar et al (Khabar et al., 1995)

**Detection of TNF by bioassay.**

$3X10^4$ WEHI 164 cells were plated onto each well of a 96-well pate in RPMI-C in the presence of 2ug/ml Actinomycin D and 25mM $LiCl_2$ and combined with supernatants obtained from the library screening. Cells were incubated for 20hours after which the Alomar Blue dye was added to detect the presence of living cells which can convert the blue dye to a pink color. After an additional 24 hour incubation, wells that remain blue suggest that TNF present in the supernatant was responsible for WEHI cell death. Controls should include medium only control and as wells as a TNF titration curve in each assay.

**Epitope identification.**

One pool of 50 DNA clones from library B was confirmed to elicit a positive response by two $D^b$-restricted MCMV-specific CTL clones (clones 3 and 55). From the positive pool, individual bacterial clones were derived and DNA was prepared and screened as described above. Individual bacterial clones that stimulated the CTL clones were sequenced. DNA was confirmed to be MCMV sequence. Peptides were synthesized based on an algorithm of optimal $D^b$ peptide-binding motifs (http://bimas.dcrt.nih.gov/molbio/hla_bind).

## RMA-S Stabilization Assay.

HGIRNASFI and KHGIRNASFI peptides were titrated from $10^{-6}$ M to $10^{-13}$ M in RPMI supplemented with 10% FBS, added to RMA-S cells, and incubated overnight at 23°C. The next day, the cells were washed 4 times in PBS pH 7.4 at 23° C to remove excess peptide, and incubated at 37°C for 4 hours. Only peptide-loaded class I MHC is stable at the cell surface at this temperature (Krmpotic et al., 1999; Thale et al., 1995). Cells were washed, then surface stained using the $D^b$-specific mAb B22.249 (ATCC) followed by FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Labs, Gaithersburg, MD), and analyzed by flow cytometry as above.

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