Inhibition of CD8 T cell effector functions by murine cytomegalovirus immune evasion genes

by

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A DISSERTATION

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AIDS	Acquired Immune Deficiency
APC	Antigen Presenting Cell
BAC	Bacterial Artificial Chromosomes
BFA	Brefeldin A
BMT	Bone Marrow Transplant
BMMΦ	Bone Marrow Macrophage
CTL	Cytotoxic (cytolytic) T Lymphocyte
CMV	Cytomegalovirus
CPE	Cytopathic Effect
DNA	deoxyribonucleic acid
DC	Dendritic cell
EBV	Epstein- Barr Virus
ELISPOT	enzyme-linked immunospot
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate compartment
FACS	fluorescence-activated cell sorter
HAART	highly active antiretroviral therapy
HCMV	Human Cytomegalovirus
HSV	Herpes Simplex Virus
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
KSHV	Kaposi's Sarcoma related Herpes Virus
MCMV	Murine Cytomegalovirus
MHV-68	Mouse Herpesvirus 68
MHC	Major Histocompatibilty Complex
MOI	Multiplicity of Infection
MULT-1	murine UL-16 binding protein-like transcript 1
NK cell	natural killer cell
PAA	Phosphonoacetic Acid
PFU	Plaque Forming Unit
RAE-1	retinoic acid early inducible genes-1
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
TAP	transporter associated with antigen processing
TNF	tumor necrosis factor
VIPR	viral protein interfering with antigen presentation
VV	Vaccinia Virus

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Abstract

Murine cytomegalovirus (MCMV) has been used for many years as a model pathogen for studying persistent virus infections. The virus has devised several mechanisms for immune evasion, one of the best characterized, is the encoding of genes that interfere with MHC class I. Three immune evasion genes encoded by MCMV (*m04, m06* and *m152*) all function by interfering with MHC class I presentation of peptides to CD8 T cells. While there is no evidence that these immune evasion genes are responsible for viral persistence, they have been conserved within the MCMV genome for millions of years. This dissertation will describe work done to characterize the effectiveness of the individual immune evasion genes at blocking functional antigen-specific CD8 T cell responses.

The studies performed in this dissertation will demonstrate that the coordinated function of MCMV's three immune evasion genes results in a powerful inhibition of lysis of infected cells by CD8 T cells. Removal of any one immune evasion gene enables lysis by at least some CD8 T cells. We have shown that there is no preferential downregulation of epitopes bound to H2-K^b or H2-D^b isoforms by *m06* and *m152*. *m04's* effects on inhibiting CTL killing are epitope specific and *m04* is not involved in the global downregulation of MHC class I caused by *m06* and *m152*. The NKG2D ligand RAE-1 can function as a costimulatory on antigen presenting cells and is downregulated by *m152*. We have shown that the impact of NKG2D ligand downregulation on CD8 T cell effector functions is modest, and does not account for the dramatic effect of *m152* on inhibition of CD8 T cell killing.

Finally the immune evasion genes are able to inhibit CD8 T cell killing even when cognate peptide can be detected on the surface of the MCMV infected cell. CD8 T cells can kill cells infected with a mutant MCMV, missing all three immune evasion genes (TKO), pulsed

with cognate peptide. However, TAP-/- cells, which have reduced the total MHC class I on the surface, infected with TKO are not killed by antigen-specific CD8 T cells. Restoration of total cell surface MHC class I, with the addition of non-cognate peptide to stabilize the MHC class I on the TAP-/- cells, results in the killing of TAP-/- TKO-infected cells. We have also shown that CD8 T cells take longer to kill virally infected cells as compared to uninfected cells expressing equal amounts of cognate peptide MHC. We believe the reason CD8 T cells cannot kill MCMV infected cells is due a reduction in total MHC class I levels, which leads to a weaker interaction between the CD8 T cell and the infect cells. This weaker interaction prevents the T cell from killing the virally infected cell either by reducing the conjugation time between the T cell and the infected cells or by altering the synapse structure so the lytic granules are not effectively targeted to the infected cell.

Chapter 1

Background and Introduction

Cytomegaloviruses (CMV) are ubiquitous host-specific viruses that persist within a host for the lifetime of the organism. CMVs encode multiple different types of immune evasion genes that interact with and counteract the host immune response. Within this dissertation the CMV immune evasion genes referred to are the genes that function by interfering with the major histocompatability complex (MHC) class I. The diverse molecular mechanisms employed by CMV immune evasion genes to alter MHC class I loading and transport have intrigued scientists for over a decade. While the immune evasion genes are usually assumed to be important for CMV to evade host immunity and persist within the host, actual functional studies of the significance of immune evasion genes are rare. The work discussed in this dissertation examines CMV's strategies for evading the host immune response and uses killing and cytokine production as functional readouts for determining the significance of the immune evasion genes.

History

Both human CMV (HCMV) and murine CMV (MCMV) encode immune evasion genes, but inevitably the "burden of proof" of the biological significance of immune evasion gene function must lie with the murine model. From the outset skeptics have raised the question: if immune evasion genes are so effective, how is the host able to mount such a large CD8 T cell response to CMV, which was shown decades ago to be protective in both the human and murine models? On the other hand, if immune evasion genes are not functionally important, why do the viruses encode them at all? CMV immune evasion genes have been studied by multiple groups; work on these genes has helped elucidate both mechanisms of CMV immune evasion and cellular processes of antigen processing and presentation by MHC class I molecules.

Since the discovery, over thirty years ago, that MHC was used by CD8 T cells to recognize infected cells, we have been working to understand how CD8 T cells interact with MHC class I and how this interaction induces a functional response. We now know a great deal more about MHC class I, and its ability to present self and non-self peptides to CD8 T cells. The antigenic peptides bound to MHC class I molecules signals the presence of an infected cell to a CD8 T cell. We are beginning to understand the steps involved in CD8 T cell activation following the recognition of antigenic-peptides on the MHC class I molecule. These earlier studies have dramatically advanced our understanding of CD8 T cell responses to antigen, however most of these initial studies have looked at T cell activation in non-physiological conditions, and not in response to infection.

Infection alters many cellular processes and the response required by a CD8 T cell to effectively eliminate the infected cell may be different than the responses examined in the initial studies of CD8 T cell stimulation. Viruses encode many different inhibitors that affect CD8 T cell effector responses, including inhibitors of cytokines, T cell activation, and apoptosis. How these viral inhibitors affect the CD8 T cell response has not been studied in the context of a viral infection and the threshold CD8 T cell effector response require to kill an infected cell has not been measured. CMV provides an excellent model for studying CD8 T cell recognition of MHC class I. CMV immune evasion genes target MHC class I, thereby altering how the CD8 T cell perceives the infected cell. Although CD8 T cells are able to respond to CMV, the strength and efficacy of the response have not been measured. We believe that by understanding how the CD8 T cells respond to CMV, we can develop therapies to improve the efficacy of the CD8 T cell response, which may lead to clearance of CMV from persistently infected individuals.

CMV

CMV is a member of the Herpes virus family and it is part of the β-herpesvirus subfamily. Like other members of the Herpes virus family, it causes a life-long infection of its host. HCMV is a large double stranded DNA (dsDNA) virus, with a genome encoding over 200 open reading frames, and over 170 for MCMV. The virus structure is multilayered, with a dsDNA core, surrounded by a capsid, followed by the tegument composed of phosphoproteins and finally a lipid bilayer envelope that contains the glycoprotein receptors. Like all herpes viruses CMV replication is an ordered process that follows a distinct pattern of gene expression.

Replication of CMV can be observed in multiple different cell types, however it is commonly seen in cells of glandular origin, particularly in the salivary glands and the kidneys. Replication of CMV is a coordinated multi-step process. After CMV enters the host cell, the viral DNA is uncoated and transported to the nucleus. After the viral DNA enters the nucleus transcription of immediate-early (IE) genes begin. IE gene transcription is driven by the IE promoter/enhancer, which is activated by cellular transcription factors. The IE genes encode for the regulatory proteins involved in transactivating the next group of genes. Following expression of IE genes, proteins encoded by early (E) genes are expressed. E genes encode most of the essential proteins for viral replication and survival within the cell. Early genes also encode the proteins involved in viral DNA synthesis. Only after viral DNA synthesis begins can late (L) gene transcription be initiated. L genes encode the viral structural proteins. After late gene expression begins the virus begins to assemble new progeny within the host cell. There is high viral replication during the acute phase of infection and virus can be detected in multiple organs. Following the acute phase of infection the amount of CMV replication decreases and the virus load within the host is reduced. CMV then establishes a latent infection within its host. While

the site of CMV latency of is still unknown, CMV behaves as a typical herpes virus persisting for the lifetime of the host while remaining largely undetected. Unable to be cleared by the CMV-specific immune response.

HCMV is found all over the world, and between 50% and 80% of adults in the United States are infected. Although HCMV is found everywhere there is an increased incidence of disease in poor and developing countries. Following infection healthy individuals often exhibit mild infectious mononucoleosis-like symptoms with a slight fever. At the end of the acute phase of disease HCMV persists in a latent state within its host. In healthy individuals infected with HCMV there is no overt disease after the virus has established latency. However, HCMV can cause disease in unborn babies and in people with a weakened immune system. It is the most common virus transmitted to a pregnant woman's unborn child. The poor immune systems in newborn babies makes them highly susceptible to permanent disabilities due to HCMV infection acquired before or at birth. HCMV infections are also a significant complication among bone marrow transplant (BMT) recipients, and also in solid organ transplantation. Viral reactivation of HCMV in is commonly seen in BMT patients and serious infections can result due to the immune compromised state of the patient.

HCMV infection also has serious implications for individuals infected with HIV. During HIV infection, HCMV acts as an opportunistic infection, taking advantage of the immune compromised state of the infected individual. HCMV disease impacts approximately 40% of HIV-infected patients during their life-time (Bowen et al., 1996), and can causes retinitis or enteritis in individuals infected with both viruses. Prior to the introduction of the highly active anti-retroviral therapy (HAART) treatment, the median survival after diagnosis of HCMV disease was 4–9 months (Harb et al., 1991). HAART treatment allows for the re-establishment

of the immune system, which has led to a reduced incidence of HCMV disease. However even with the advent of HAART therapy, HCMV still poses a high risk to HIV patients. HCMV-seropositive individuals progress 2.5 times more rapidly to AIDS and death than those who are HCMV seronegative (Sabin et al., 1995). In most poor and developing countries the access to expensive therapies such as HAART is limited, and HCMV is still a major cause death in HIV infected individuals. The direct link between a healthy immune system and reduced incidence of HCMV disease makes us question why this effective immune response can only control and not clear the virus.

MHC class I assembly and peptide loading

Before discussing how the MCMV immune evasion genes interfere with antigen presentation we must first briefly review the steps of antigen processing and presentation. These processes have been reviewed in great detail by a number of groups. Below is a brief summary of the literature (Cresswell et al., 1999; Pamer and Cresswell, 1998; Yewdell, 2005). The importance of MHC class I was clearly shown over thirty years ago when Zinkernagel and Doherty demonstrated that CD8 T cells recognized virally infected cells via MHC class I (Zinkernagel and Doherty, 1974). Since this initial observation a great deal of work has been done to understand how CD8 T cell recognition of MHC class I signals to the T cell that the MHC class I expressing cell is infected.

We now know that MHC class I is assembled and loaded with both self and non-peptides in the endoplasmic reticulum (ER). Newly synthesized MHC class I heavy chain (HC) is translocated into the ER where it associates with the chaperone calnexin and binds β-2-microglobulin. The HC-β-2 microglobulin complex dissociates from calnexin and becomes

associated with a second chaperone calreticulin. This complex then associates with the transporter associated with antigen processing (TAP) and the chaperones tapasin and Erp57. The HC-β-2 microglobulin complex is then loaded with short peptide sequences with the aid of the associated chaperones. The peptides have been generated by the degradation of self or viral proteins in the cytosol by the proteasome. The resulting peptides are then transported into the ER by TAP. MHC class I that has bound peptide is subsequently transported out of the ER through the Golgi, to the cell surface, where it can prime a CD8 T cell response.

Following recognition of the antigenic peptide, CD8 T cells undergo marked proliferation, and kill the cells that express their target antigen, through granzyme and perforin effector molecules, and interaction between Fas and Fas ligand on the CD8 T cell. CMV immune evasion genes affect the presentation of antigenic peptides via MHC class I, presumably with the goal of interfering with the potent anti-viral effector function of CD8 T cells. Most infections are resolved or controlled by the combined efforts of several arms of the immune system including NK cells, CD4 and CD8 T cells and B cells. A small percentage of the antigen-specific CD8 T cells that were initially generated are then retained as memory cells, which can respond rapidly to a second encounter with their antigen. However, in the case of CMV, the virus persists within the host, even in the presence of an effective immune response.

HCMV Immune Evasion Genes

CMVs are highly species-specific, having co-evolved with their hosts since the mammalian radiation. Thus, even though all CMVs encode immune evasion genes, the immune evasion genes found in each species have developed specialized mechanisms to interfere with the MHC class I pathway of their particular host. The study of the CD8 T cell immunity to CMV is

fascinating both because of the unique features of the response that are shared between different species, and because of the diversity of mechanisms that the immune evasion genes employ to evade it. In the acute phase of infection, CMV elicits a strong immune response; however this response fails to completely clear the virus. Instead, the CMV persists in the host, often in a latent state, and recurrent infections may be observed if the host becomes immunocompromised. Although there is no direct evidence, it has been proposed that CMV persistence is due to its ability to encode immune evasion genes to modulate the immune response toward the virus.

HCMV encodes four immune evasion genes: US2, US3, US6 and US11. US3 is expressed in the IE phase, but the other 3 HCMV immune evasion genes are all expressed in the E phase of infection. Structurally, they are all type I membrane glycoproteins. The immune evasion genes all interfere with the cell surface expression of MHC class I molecules. US2 and US11 are both involved in redirecting nascent MHC class I proteins from the ER into the cytosol, where they are destroyed by ubiquitin-dependent proteasomal degradation (Wiertz et al., 1996a; Wiertz et al., 1996b). The US3 gene product prevents egress of MHC class I proteins from the endoplasmic reticulum (ER) to the Golgi apparatus (Ahn et al., 1996; Jones et al., 1996). US6 binds TAP at its ER luminal side, preventing peptide loading of MHC class I (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997).

Although the characterization of the molecular mechanism employed by each of the HCMV immune evasion genes to downregulate MHC class I has been described in great detail, there has been little study of the functional efficacy, meaning their ability to prevent lysis of infected cells by CMV-specific CTL, of the HCMV immune evasion genes. These genes were all identified by their ability to decrease the cell surface expression of MHC class I (Jones et al., 1996). The initial identification of HCMV immune evasion genes was made possible by the generation of a

mutant virus that lacked all the HCMV open reading frames from IRS1 to US9 and US11, but was nevertheless able to replicate normally in tissue culture (Jones et al., 1996). Because the main role of MHC class I is to present peptide antigens to CD8 T cells, it is presumed that immune evasion genes impair this function. However, since an activated CTL needs only about 3 MHC-peptide complexes in order to exert effector functions (Krogsgaard et al., 2003; Purbhoo et al., 2004), reduction of total cell surface class I is only a crude assessment of the putative functional effect of the immune evasion genes. The following papers are some of the published studies of HCMV immune evasion genes effect on CD8 T cell function, and all are artificial to varying degrees. In one of the first papers describing HCMV immune evasion gene activity, Warren et al showed that HCMV infection of fibroblasts reduced their sensitivity to lysis by alloreactive CTL (Warren et al., 1994). In their initial description of US6, Lehner et al showed that transfection of an influenza-infected cell with US6 inhibited the recognition of the infected cells by influenza specific CD8 T cells (Lehner et al., 1997). More recently, US3 and US11 were expressed from retroviral vectors in human fibroblast cell lines that were then infected with HCMV. In this case the additional US3 and US11 expression over and above the expression from HCMV itself decreased CD8 T cell recognition of HCMV antigens (Berger et al., 2000). However, the most obvious experiment- comparing lysis of wild-type HCMV with the mutant lacking US2-11- has not been reported.

The best characterized HCMV antigens- pp65 and IE1- were both identified using HCMV-infected fibroblasts (Nowak et al., 1984; Stinski et al., 1983). These antigens must therefore be at least somewhat resistant to the effects of the immune evasion genes, or else they would not have been identified. IE1 is expressed immediately upon infection, before the immune evasion genes, which are mostly E genes, are functional. pp65 is a virion protein that can be processed and

presented without any new virus gene expression, and hence is also presented before full immune evasion gene activity. It has often been suggested that these antigens are immunodominant precisely because they are relatively resistant to the effects of immune evasion genes. Of course, a test of the in vivo effect of HCMV immune evasion genes in humans is not possible. However, interestingly, recent studies have shown that despite the presence of immune evasion genes, there is a detectible CD8 T cell response to a large variety of antigens expressed during IE, E, and L phases of infection (Elkington et al., 2003; Manley et al., 2004). Since the CMV immune evasion genes are expressed during the IE and E phase of infection proteins expressed during this time should be less immunogenic. This broad epitope specific response to HCMV brings into question the presumed role of the immune evasion genes in blocking antigen presentation *in vivo*.

MCMV

MCMV has long been used as a model to study the biology HCMV infections. MCMV is similar to HCMV with regard to virion structure, genome organization, and gene expression. MCMV has a linear genome of 230 kb and encodes more than 170 genes. Because MCMV can infect mice, the effects of genetic manipulation of the viral genome can be observed during the course of infection. Infection of mice by MCMV resembles HCMV with respect to pathogenesis, establishment of latency, and reactivation after immunosuppression, transfusion, or transplantation. MCMV also has a similar tissue tropism to HCMV and after entry into the host, the virus spreads through the blood to various organs and infects many different cell types. The MCMV model allows the integration of questions about both molecular mechanisms and biological significance of the CMV immune evasion genes.

The fact that there are few studies examining the functional efficacy of the immune evasion genes illustrates the difficulty of studying HCMV both *in vitro* and *in vivo*. Even if *in vitro* functionality of HCMV immune evasion genes were established, *in vivo* experiments would still be impossible. For this reason MCMV is used in animal models for the study of β-herpesvirus virology and impact of CMV infection on its host. Surprisingly, there is no sequence homology between immune evasion genes encoded by MCMV and HCMV. The immune evasion genes encoded by MCMV, *m04*, *m06,* and *m152*, all function to inhibit antigen presentation to CD8 T cells, but by different mechanisms than HCMV.

Identification of *m152*

Early experiments looking at CD8 T cell control of MCMV infection revealed that in fibroblasts the recognition of the MCMV antigen pp89 was reduced once E genes were expressed (Reddehase et al., 1986). This initial observation inspired other investigators to explore the mechanisms by which this reduced recognition was achieved. A significant reduction in cell surface MHC class I expression was seen following MCMV infection (Campbell et al., 1992; del Val et al., 1992), which was later shown to be due to a block in the transport of MHC class I in a pre-Golgi compartment (del Val et al., 1992). Interestingly, antigenic peptides were properly loaded onto MHC class I molecules within the ER but the MHC class I did not reach the Golgi in the presence of MCMV E genes.

The location of genomic region of MCMV responsible for the observed inhibition of MHC class I antigen presentation was established by Thale et al. Restriction enzyme digest of the MCMV genome with Hind III resulted in 16 fragments A-P (Figure 1). Microinjection of each fragment into tissue culture cells implicated the Hind III E region as responsible for the retention

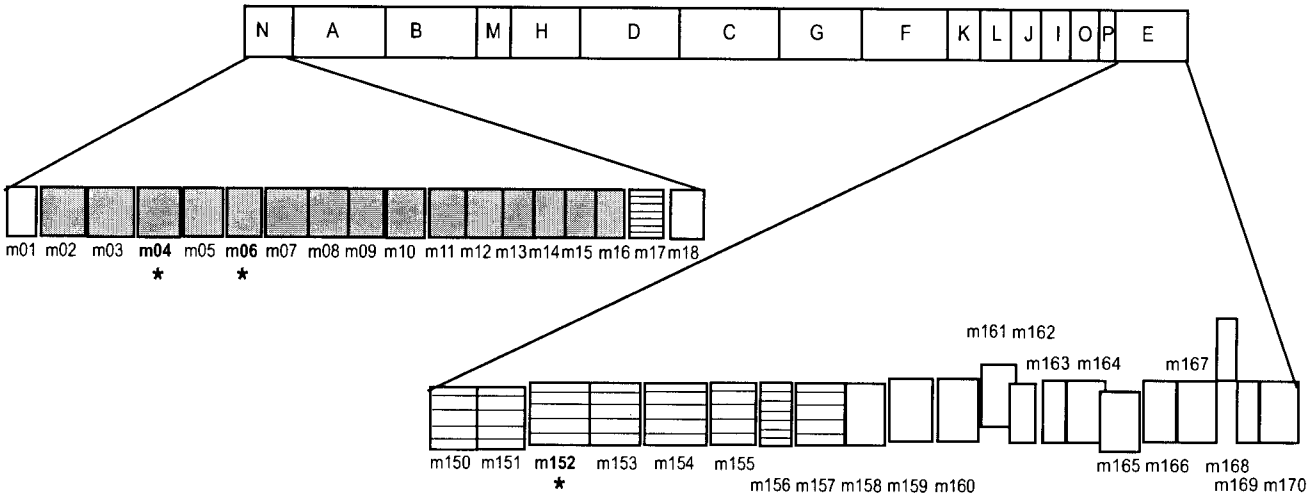

Figure 1:
HindIII cleavage map of the MCMV genome with the HindIII-A and E regions expanded to show the m02 and m145 gene families, which contain the MCMV immune evasion genes. The HindIII-A region contains the m02 family members: m02, m03, m04, m05, m06, m07, m08, m09, m10, m11, m12, m13, m14, m15, and m16, shown in the gray boxes. The HindIII A region also contains the m145 family member: m17 shown in stripes. The enlarged HindIII-E contains members of the m145 family: m150, m151, m152, m153, m154, m155, m156, m157, and m158 shown in stripes.

of MHC class I molecules in a pre-Golgi compartment (Thale et al., 1995). When cells were infected with a MCMV mutant virus lacking the Hind III E region, MHC class I expression was maintained during the first hours of E gene expression (Thale et al., 1995). The retention of MHC class I molecules correlated with the observed inhibition of antigen recognition by pp89-specific CD8 T cells. Additional restriction enzyme digestion of the Hind III E fragment identified *m152* (m for murine/152 gene position) as the open reading frame responsible for MHC class I downregulation during E gene expression (Ziegler et al., 1997). However the investigators noted that in the absence of *m152,* MHC class I was still lost later in the infection, indicating another gene expressed later than *m152* also downregulates MHC class I (Thale et al., 1995). That gene was later identified as *m06* (see below).

m152 encodes a 40kDa type I glycoprotein (gp40) of 378 aa (Rawlinson et al., 1996; Ziegler et al., 1997). *m152* is a member of the m145 gene family within MCMV, all members of which encode predicted type I glycoproteins with no significant sequence homology to any other known genes, including HCMV genes (Rawlinson et al., 1996; Ziegler et al., 1997). However, secondary structure predictions reveal that *m152*, along with some other m145 family members, may have structural homology to MHC class I proteins (Smith et al., 2002). Although *m152* is classified as an early gene, transcription starts very early after infection, overlapping with transcription of some IE genes: transcripts are detected 2 hours post infection and protein expression begins at 3-4 hours, peaking at 5-6 hours and declining significantly thereafter (Holtappels et al., 2000; Ziegler et al., 1997). The expression kinetics of *m152* can readily explain the early inhibition of antigen presentation, but later effects would seem to require the expression of other genes.

Molecular studies of *m152*

One of the most striking findings about *m152*'s molecular mechanism is that in spite of clear evidence for a specific effect of *m152* on MHC class I transport, no direct biochemical interaction between *m152*/gp40 and MHC class I has ever been found. In fact, Ziegler et al. showed that during MCMV infection MHC class I is retained in the ERGIC with an extended half-life, while *m152* has a much shorter half-life and is rapidly degraded (Ziegler et al., 2000). To understand how *m152* functions, a closer examination of where *m152* interferes with the progress of MHC class I molecules loaded with peptide was carried out. Actinomycin D and cyclohexamide were used to limit MCMV gene express to IE genes only, or IE + E genes. When only IE genes were expressed, an immunodominant peptide from pp89 was loaded onto the MHC class I molecule H2-L^d, and correctly transported, allowing recognition by pp89-specific CTL (del Val et al., 1992). When E genes were also expressed, there was no longer any recognition by CTL, and H2-L^d molecules were retained in the ERGIC. However, when H2-L^d molecules were extracted from cells expressing IE and E genes or from cells expressing only IE genes, it was found that the same amount of pp89 peptide was bound to H2-L^d in each case. This led to the conclusion that MCMV peptides are correctly processed and the proper MHC class I trimolecular complex is formed after MCMV infection, which indicates that *m152* does not interfere with antigen loading and processing (del Val et al., 1992). Pulse-chase experiments also demonstrated that MCMV infection does not alter the rate of degradation of MHC class I molecules (Campbell and Slater, 1994). Endo-H sensitivity indicated that *m152* prevents MHC class I molecules from reaching the medial Golgi (del Val et al., 1992). This inhibition of MHC class I maturation has a profound effect on cell surface MHC class I expression, while the expression of other surface glycoproteins remains unaffected (Campbell and Slater, 1994; del

Val et al., 1992). However, *m152* does not affect all MHC class I alleles equally: for instance, H2-D^b is much more profoundly affected than H2-K^b (Kavanagh et al., 2001a; Wagner et al., 2002). In general, D locus alleles are more profoundly affected than K locus alleles (Wagner et al., 2002). Later studies determined that the lumenal domain of *m152* is responsible for the retention of MHC class I molecules, and that the retained molecules are localized to the ER–Golgi intermediate compartment (ERGIC) (Ziegler et al., 2000; Ziegler et al., 1997). Despite the identification of where class I export is blocked, how *m152* is able to block MHC class I transport is still an open question. It is possible that *m152* functions either by inhibiting other molecules required for the transport of MHC class I, or that it interacts directly with MHC class I through a weak association. Even if *m152* interacts directly with MHC class I, how *m152* is able to cause MHC class I to remain in the ERGIC while *m152* itself goes on to the lysosome for destruction is still a mystery. Recent studies have shown that *m152* is also capable of downregulating the NKG2D ligand Rae-1(Krmpotic et al., 2002; Lodoen et al., 2003), which bears structural homology to class I in the extracellular domain. Whether *m152* also blocks the export of Rae-1 in a similar manner has not yet been addressed. If *m152* blocks the transport of both molecules similarly, this might point to *m152* functioning to inhibit a common transport receptor for both molecules.

Phenotypic and Functional effects of *m152*

m152's ability to inhibit antigen presentation was studied using recombinant Vaccinia viruses (recVV) as well as MCMV mutant viruses. Ziegler et al used two recVVs, one expressing *m152* and the other expressing the IE antigen pp89, to look at how *m152* functions in the absence of other MCMV gene products. They observed that CD8 T cell recognition of the recVV-pp89 is

impaired in the presence of a recVV-*m152* (Ziegler et al., 1997). Looking at the function of *m152* in the context of the whole virus, Gold et al observed that H2-D^b-restricted CD8 T cell clones specific for the antigen *M45* were able to recognize cells infected with a virus lacking *m152* (Δ*m152*), but not wildtype virus-infected cells (Gold et al., 2002). This suggested that *m152* has a more profound effect on T cell recognition of H2-D^b-restricted peptides than H2-K^b-restricted peptides, in keeping with the allelic differences seen in biochemical studies (Kavanagh et al., 2001a; Wagner et al., 2002). The first purpose of the experiments described in this thesis was to determine whether this principle would hold true when the susceptibility to inhibition of presentation of 16 different Kb and Db-restricted epitopes was tested.

The studies investigating *m152*'s effect on CD8 T cell recognition described above were all conducted *in vitro*. The results from the *in vivo* studies have shown that the behavior of the immune evasion genes *in vivo* is more complicated than we would have predicted. When compared with wildtype MCMV, Δ*m152* is modestly impaired in its growth *in vivo*: so far, a CD8 T cell dependent phenotype for *m152* in vivo has only been reported in mice that are immunocompromised in one way or another. In newborn BALB/c mice, infection with Δ*m152* results in lower virus titers by 1-2 logs at day 5 post infection, and reduced mortality compared to wildtype virus (Krmpotic et al., 1999). This effect was attributed to CD8 T cells, because Δ*m152* and a revertant virus grew to the same titers in animals deficient in CD8 T cells.

More recently, adoptively transferred M45-specific CD8 T cells into lethally irradiated, bone marrow transplanted mice that were infected simultaneously with wildtype and Δ*m152* MCMV: the adoptively transferred CD8 T cells were able to control Δ*m152* but not wildtype virus (Holtappels et al., 2004). This latter experiment showed convincingly that *m152* could impair the ability of CD8 T cells to recognize and control virus infection *in vivo*. These authors believe

that *m152*'s ability to impair CD8 T control is only functionally significant for some epitopes, such as H2-D^b-restricted M45. This may be in keeping with the differential effect of *m152* on different CD8 T cell epitopes observed in ^{51}Cr release assays *in vitro*, as described above. Holtappels and Reddehase have shown that CD8 T cells specific for several H-2^d-restricted epitopes are able to control wild-type MCMV *in vivo,* in spite of the action of *m152*(Holtappels et al., 2002a). We suspect that even for cells of these specificities, CD8 T cell control of *Δm152* will prove more efficient than control of wild-type virus. However, this remains to be tested experimentally: to date, H2-D^b-restricted M45 is the only epitope for which a direct comparison of the effect of *m152* on the efficacy of CD8 T cell control has been performed. More studies *in vivo* are needed to gain a clearer picture.

In vitro assays and the *in vivo* studies indicate that *m152* profoundly impairs antigen recognition. If directly infected cells are responsible for priming the CD8 T cell response, *m152* should have a profound effect on the generation and maintenance of an antigen-specific CD8 T cell response *in vivo.* However, infection of BALB/c mice with wild-type MCMV results in a measurable pp89-specific CD8 T cell response that increases over time (Karrer et al., 2003), and the percentage of M45-specific CD8 T cells in C57BL/6 mice is similar after infection with either wild type MCMV or Δ*m152* (Gold et al., 2002). Thus, priming of the CD8 T cell response appears to be unaffected by the impact of *m152* on antigen presentation. We have suggested that cross-presentation of MCMV antigens could explain the lack of impact of *m152* on the CD8 T cell response. Alternatively, the immune evasion genes could be altering T cell effector functions such that T cells are able to be primed in response to viral epitopes however the ability of the T cells to kill the infected targets is reduced due to the presence of the immune evasion genes.

In summary, *m152* retains newly synthesized class I in the ERGIC by an unknown

mechanism. This results in a profound impairment of the ability of CD8 T cells of atleast some specificities to detect infected cells, which has been demonstrated both in vitro and in vivo. However, *m152* does not appear to affect the priming of CD8 T cells, possibly because CD8 T cells can be efficiently primed by cross-presented antigen.

m06: a second MCMV immune evasion gene

During the identification of *m152,* another protein encoded by MCMV was observed to downregulate MHC class I molecules at a later time during the E phase of infection (Thale et al., 1995). Believing that this immune evasion gene formed a tight association with MHC class I molecules, Reusch et al, used MHC class I complexes from MCMV-infected cells to immunize BALB/c mice. The mice then generated an antibody response specific for a MCMV molecule that associated with MHC class I. These antibodies were then used to identify a protein encoded by the MCMV gene *m06* (Reusch et al., 1999).

m06 is a member of the m02 gene family and encodes a 48 kDa type 1 transmembrane glycoprotein, gp48 (Rawlinson et al., 1996). Transcription of *m06* reaches its maximum at 3 to 6 h post infection, and the protein is then produced at high levels throughout the replication cycle. Unlike *m152*, *m06* forms a tight association with MHC class I molecules in the ER. After passing through the Golgi, the MHC class I–*m06* complex enters the endocytic route and reaches the lysosomal compartment, where both *m06* and MHC class I undergo rapid proteolysis (Reusch et al., 1999). *m06* redirects the MHC class I complex to the lysosome through a targeting sequence, composed of a di-leucine motif, located in its cytosolic domain (Reusch et al., 1999).

Phenotypic and Functional effects of *m06*

The phenotypic outcome of *m06* expression is a dramatic reduction in cell surface MHC class I expression (Hengel et al., 1999; Wagner et al., 2002). As with *m152,* differences in inhibition of surface expression of different MHC class I surface isoforms were observed with *m06*. In general, downregulation of the K locus alleles, namely H2-K^d and H2-K^b, was stronger than the downregulation of D locus alleles, H2-D^d and H2-D^b. There were also allele-specific differences, with the downregulation of H2-D^b greater than that of H2-D^d (Wagner et al., 2002). The allelic preferences of *m06* suggest that it functions to complement the effects of *m152*: *m152* has a stronger effect on H2-D and *m06* has a greater impact on H2-K. However, overall, *m06* had a more profound effect on cell surface expression of class I than did *m152* (Wagner et al., 2002). This maybe due to the kinetics of *m06* expression: *m06* remains detectible throughout MCMV infection, unlike *m152*, which tapers off during the latter phase of E gene expression.

A functional phenotype for *m06* has been shown *in vitro*: CTL clones of at least 3 H-$2D^b$-restricted specificities lysed targets infected with a virus *m06* (Δ*m06*), whereas cells infected with wildtype virus were not lysed. Like *m152*, *m06* can function in the absence of the other MCMV genes: cells transfected with *m06* were infected with a recVV expressing the MCMV antigen pp89 were less well recognized by pp89-specific-CD8 T cells than vector-transfected cells (Hengel et al., 1999). However, *m06* functioning in isolation did not completely inhibit CD8 T cell recognition. Also, unlike *m152, m06* has not yet been shown to have a detectible phenotype *in vivo*.

m04: the third MCMV immune evasion gene

In experiments similar to those described for *m06*, the third MCMV immune evasion gene, *m04,* was identified because it is able to form a detergent-stable association with MHC class I molecules. Unlike *m06* and *m152*, *m04* does not block the transport of MHC class I to the cell-surface. Instead, it forms a tight association with the MHC class I molecules and is exported to the cell surface. *m04* was isolated by the precipitation of MHC class I molecules from metabolically labeled MCMV-infected fibroblasts to look for MCMV products that remained associated with the MHC class I molecules. By comparing infected and uninfected cells, an MCMV glycoprotein of 34 kDa apparent molecular weight was identified. Further analysis revealed that gp34 was encoded by the MCMV gene *m04* (Kleijnen et al., 1997). Like *m06, m04* is part of the m02 gene family and encodes a type 1 transmembrane glycoprotein (Rawlinson et al., 1996).

Molecular studies of *m04*

Within the ER *m04* forms a detergent stable association with MHC class I complexes containing B2m in the presence of TAP (Kavanagh et al., 2001b; Kleijnen et al., 1997). The complex is exported from the ER through the Golgi to the cell surface. The stochiometry between *m04* and MHC class I both within the ER and at the cell surface is still unknown. Within the ER there is a large excess of *m04* compared with MHC class I and only when associated with MHC class I can *m04* leave the ER to traffic to the cell surface (Kavanagh et al., 2001b; Kleijnen et al., 1997). Unassociated *m04* molecules are retained in the ER until they are degraded. *m04* forms complexes with all MHC class I alleles tested; it was previously reported that *m04* did not associate with H-2K^d, but recent studies have been able to detect an

association between H-2K^d and *m04* (Xiuju Lu unpublished observation). In addition to the detergent-stable *m04*-class I complex described above, within the ER a weaker NP40 labile complex with MHC-class I can also be detected (Kavanagh et al., 2001b). Why *m04* forms two separate types of association, and why *m04* is the only immune evasion gene to remain associated with MHC class I at the cell surface, are presently unknown.

m04 does not decrease cell surface expression of MHC class I. In fact, for some MHC class I alleles, it may counteract the action of *m152* and lead to a net increase in cell surface class I. However, in functional assays, *m04* has been shown to inhibit antigen presentation to CTL. Some clonal CTL lines were able to recognize cells infected with a virus lacking *m04* (Δ*m04*), but were unable to recognize wild-type infected cells (LoPiccolo et al., 2003). In the original study, only H2-K^b, but no H2-D^b, restricted clones recognized Δ*m04* (Kavanagh et al., 2001a). These studies have recently been extended using polyclonal CTL lines specific for 16 different H2-K^b or H2-D^b -restricted epitopes. These studies suggest that (a) *m04*'s effect is much weaker than that of *m152* or *m06* (b) *m04's* main effect is seen when it acts in combination with another immune evasion gene and (c) *m04* can affect H2-D^b as well as H2-K^b-restricted presentation (Pinto et al., 2006). Recent work reported by Holtappels et al. has provided some evidence for *m04* antagonizm of *m152*'s effect on MHC class I downregulation (Holtappels et al., 2006). Using a panel of mutant viruses similar to the ones we used in Chapter 2, this group demonstrated that in the absence of *m06*, the expression of *m04* prevented *m152* inhibition of killing. These results contradict some of the results reported in Chapter 2 on this thesis, and interpretations of these differences will be addressed in the discussion section.

m04 is the most enigmatic of MCMV's three immune evasion genes. It is expressed in very high quantities in the ER, and is also found in at the cell surface, bound to MHC class I

(Kavanagh et al., 2001b; Kleijnen et al., 1997). It has some ability to inhibit antigen presentation, although the mechanism for this is unknown. It has also been proposed that *m04* could counteract the loss of cell-surface MHC class I caused by *m06* and *m152,* thus preventing the 'missing self' activation of NK cells. However, in C57BL/6J mice, Δ*m04* grew to the same titer as wild-type virus at day 3 post infection, suggesting that *m04* does not affect NK function, at least in that mouse strain (A. Pinto, unpublished data).

MCMV and NK function

The potential importance of NK cells in controlling MCMV is illustrated by the different sensitivity of BALB/c and C57BL/6 mice to MCMV infection. The NK resistance of C57BL/6 mice maps to the cmv^r locus on chromosome 6, which encodes an NK cell activatory receptor, Ly49H (Daniels et al., 2001; Lee et al., 2001; Scalzo et al., 1990). Ly49H directly interacts with the m157 gene product of MCMV, enabling efficient NK recognition of virus infected cells and control of virus replication. However, the Ly49H/m157 pairing is apparently an artifact of laboratory infection, and in wild mice a susceptibility phenotype is probably more common (Scalzo et al., 2005). In susceptible BALB/c mice, depletion of NK cells during MCMV infection has little if any effect on the course of the virus infection (Farrell et al., 1997). The immune evasion genes' ability to down regulate MHC class I molecules should make infected cells in BALB/c mice more "visible" to NK cells ("missing self"). However, this does not appear to be the case, suggesting that MCMV in susceptible mouse strains has evolved a way to prevent NK cell activation. One explanation for this observation is the ability of MCMV to downregulate ligands for the NK cell activatory receptor, NKG2D.

NKG2D is found on the vast majority of murine NK cells, and in the mouse is thought to

be the most important activatory receptor. There are several known murine ligands for NKG2D, which are expressed on a large number of different cell types and show some homology with MHC class I proteins. Ligands for murine NKG2D include a family of retinoic acid early inducible 1 gene (RAE-1) products (RAE-1α, RAE-1β, RAE-1δ, RAE-1γ, and RAE-1ε) (Cerwenka et al., 2000), a minor histocompatibility antigen (H-60) (Diefenbach et al., 2000; Malarkannan et al., 1998), and murine UL16-binding protein-like transcript-1 glycoprotein (MULT-1) (Carayannopoulos et al., 2002). The expression of these ligands is usually low in normal cells, but is upregulated in certain NK susceptible tumors. Importantly, virus infections including MCMV, upregulate transcription of these NKG2D ligands leading to the activation of NK cells (Diefenbach and Raulet, 2001; Diefenbach et al., 2002; Groh et al., 2001).

Although MCMV infection upregulates transcription of NKG2D ligands, MCMV-infected cells in general do not show an increase in their cell surface expression of these ligands. A series of papers has revealed that MCMV specifically inhibits the expression of each of the NKG2D ligands at a post-translational level. *m152* inhibits the expression of the RAE-1 family (Lodoen et al., 2003). *m155* inhibits H-60 (Lodoen et al., 2004), and *m145* inhibits MULT-I (Krmpotic et al., 2005). The importance of *m152* in preventing NK cell activation was shown by the fact that BALB/c mice infected with Δ*m152* had lower titers at day 3 in the spleen than mice infected with wild-type virus. The difference was due to NK cell control of Δ*m152* (Krmpotic et al., 2002). Similar studies were performed to show that inhibition of H-60 by m145 also impaired NK cell control of MCMV (Hasan et al., 2005; Krmpotic et al., 2005).

In addition to its role in NK cell activation, expression of the NKG2D receptor is induced by activation on CD8 T cells, where it can function as a co-stimulatory molecule (Jamieson et al., 2002): ligation the NKG2D receptor on CD8 T cells lowers the threshold of stimulation required

by the CD8 T cell for effector function. As has been found for other infections, all MCMV-specific activated CD8 T cells express NKG2D (A. Pinto, submitted). Thus, *m152*'s ability to inhibit CD8 T cells may come from two routes: its role as an immune evasion gene (i.e. inhibiting antigen presentation) and its impairment of costimulation through NKG2D. In situations where the activation of a CD8 T cell requires a strong costimulatory signal in combination with antigen recognition, the effect of *m152* on NKG2D ligand should be considered.

Combined effects of the Immune evasion genes

As we have described, to date there are three MCMV genes that are know to interfere with MHC class I-mediated antigen presentation (figure 2):

- *m152* blocks MHC class I from exiting the ERGIC, and also inhibits expression of the RAE-1 family of NKG2D ligands
- *m06* binds MHC class I and redirects it to the lysosome to be degraded
- *m04* associates with MHC class I in the ER and follows it to the cell surface.

One question that has fascinated scientists since viral interference with antigen processing was first discovered is: why do CMVs, both human and mouse, need to encode multiple immune evasion genes with different mechanisms? There are at present in the literature several observations or theories that might shed light on this issue:

1. Individual MHC class I alleles are differentially affected by the immune evasion genes (Kavanagh et al., 2001a; LoPiccolo et al., 2003; Pinto et al., 2006; Wagner et al., 2002). We have described above the evidence for this: when the effects on cell surface

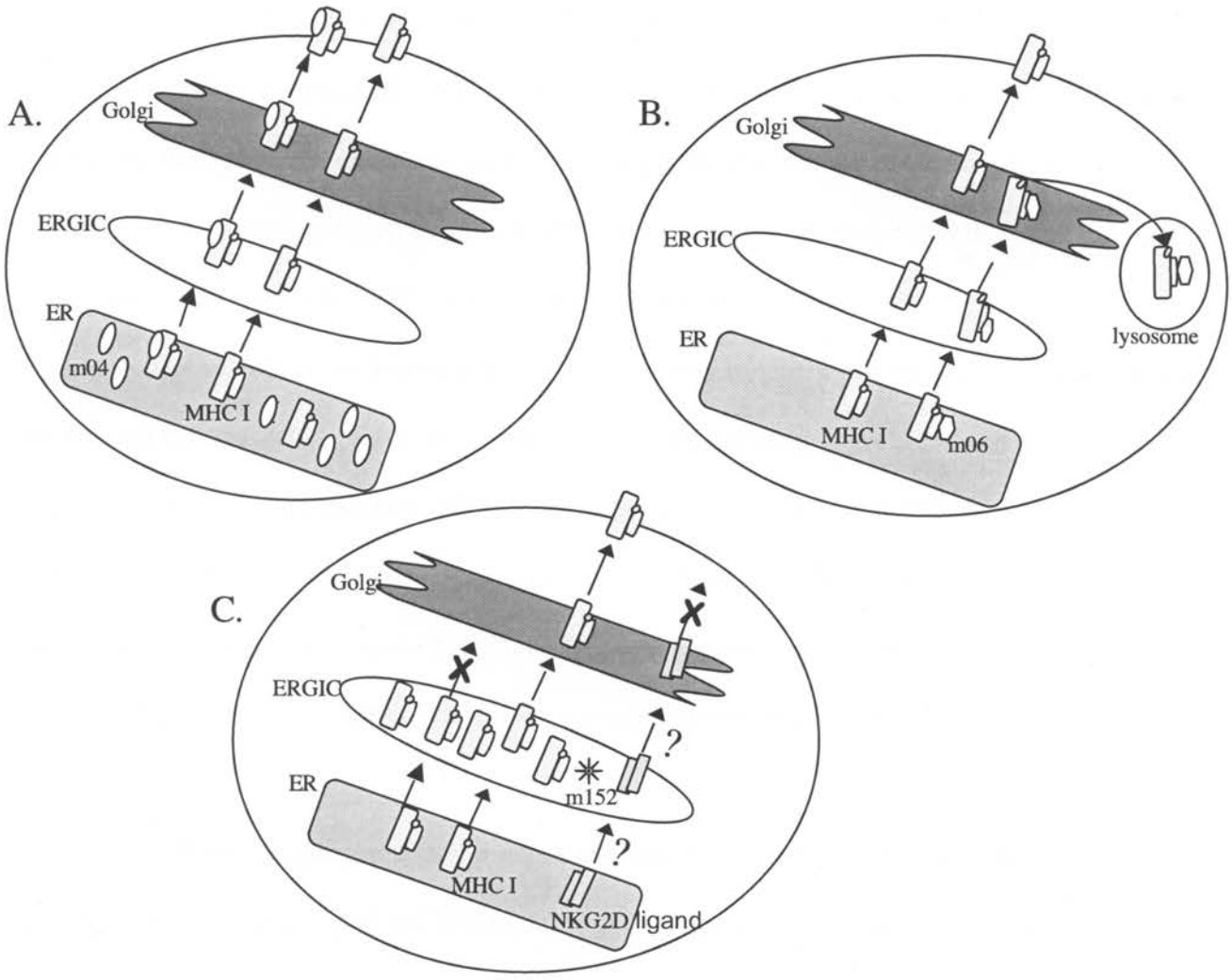

Figure 2:
Function impact of the immune evasion genes on MHC class I and NKG2D ligand. A. m04 associates with MHC class I in the ER and remains associated with the MHC class I complex as it traffics to the cell surface. B. m06 binds to class I in the ER and redirects it to the lysosome for degradation. C. m152 blocks MHC class from leaving the ERGIC. m152 also blocks NKG2D ligand expression at the cell surface through an as yet unknown mechanism.

expression or on transport of the bulk population of MHC molecules, without regard for peptide cargo, is assessed, *m152* has a stronger effect on D locus alleles and *m06* has a stronger effect on K locus alleles. This explanation has evolutionary appeal: since one of the evolutionary pressures that drives MHC polymorphisms may be the effect of immune evasion genes, it seems reasonable that CMV has countered MHC polymorphism with multiple mechanisms of its own. We have revisited this question using a much broader panel of T cell epitopes than was previously available: these results are described in Chapter 2.

2. The effects of the immune evasion genes may be more or less dramatic depending on the cell type infected. For example, MCMV mutants lacking single immune evasion genes were more strongly recognized in infected macrophages than in infected fibroblasts (LoPiccolo et al., 2003). Whether this is because macrophages are better APCs, and hence the removal of individual immune evasion genes more readily unmasked antigen presentation, or because of actual cell type specific effects of the immune evasion genes, remains to be determined.

3. Individual immune evasion genes can interact co-operatively or competitively. A major advance in the study of MCMV immune evasion genes occurred when Wagner et al., used BAC technology to create a panel of six MCMV mutants that expressed all possible combinations of MCMV's three known immune evasion genes (Wagner et al., 2002). This enabled the functional interactions of the individual immune evasion genes to be examined in the physiologically relevant setting: in an infected cell, with all the other MCMV genes expressed. The cell surface expression of five MHC class I alleles (K^d, D^d, L^d, K^b, D^b) was examined following infection with the panel of MCMV mutants (Wagner et al., 2002). *m152* and *m06* tended to act co-operatively to reduce cell surface expression; viruses expressing both genes had

lower levels of class I than a virus expressing only one. In contrast, *m04* was observed to actually antagonize the effect of *m152* for all the class I alleles (Wagner et al., 2002). In other words, a virus containing both *m04* and *m152* but not *m06* (*Δm06)* had a higher level of class I expression than a virus containing only *m152* (*Δm06+m04*). It is interesting to note that this "competitive" effect was only observed in the absence of *m06:* if *m06* was present, the presence of *m04* did not affect the extent to which *m152* downregulated MHC class I. It is worth noting that this study was performed entirely in transformed cell lines: MCMV powerfully stimulates the synthesis of MHC class I molecules in primary fibroblasts, which is not observed in transformed fibroblasts, causing the effects of immune evasion genes on cell surface expression of class I to vary much more with time of infection and between experiments (Pinto et al., 2006).

The detailed phenotypic characterization of the MCMV immune evasion genes in combination performed by Wagner et al, is a good foundation before beginning the functional characterization, which we performed using the same panel of mutant viruses. The results paint a picture that is much less clear-cut than that suggested by the studies of cell surface expression (Pinto et al., 2006). To understand how these immune evasion genes interact functionally to impact antigen presentation will ultimately involve understanding impact of the differential timing of the expression of the immune evasion genes, as well as the peptide cargo of the MHC class I populations affected by each immune evasion gene, and of the protein providing the peptide epitope.

CD8 T cell recognition of virally infected cells

After reviewing what is known about the CMV immune evasion genes, we now switch the focus to understanding how a CD8 T cell response is generated. There are several general

characteristics of CD8 T cell recognition of virally infected targets as well as some specific differences seen in CD8 T cell recognition of MCMV infected targets. We will start with the necessary background information of how CD8 T cells recognize and respond to antigen presenting cells (APC). This will be followed by a section on synapse formation and finally a section on the effector responses generated by activated CD8 T cells.

CD8 T cells are stimulated to release cytokines and kill using cytolytic molecules when the CD8 T cell receptor (TCR) recognizes it's cognate peptide MHC (pMHC) on the surface of infected cells. Each CD8 T cell expresses only one TCR and so it is able to respond only to its cognate peptide presented on the MHC class I molecule. In a naïve mouse the frequency of a single TCR is highly variable but in one study it was estimated that there is about 1 in $2x10^5$ CD8 T cells specific for the LCMV epitope gp33 in a naïve mouse (Blattman et al., 2002). This extremely low number in a naïve mouse suggests that a CD8 T cell has to sample a lot of pMHCs before it finds a match. Once the TCR recognizes its cognate antigen the T cell relies on the strength of the TCR-pMHC interaction as well as co-receptors to maintain contact with its APC. Surrounding the CD8 TCR are multiple co-receptors, which are all important for maintaining the interaction of the pMHC class I with the TCR and directing the CD8 T cell to responding appropriately.

The structure of the CD8 TCR is critical for the recognition of the peptide presented on the MHC class I molecule. The structure of the CD8 TCR is similar to that of immunoglobulins. Like antibodies the TCR is generated from the rearrangement of gene segments to create a diverse repertoire of TCRs to respond to the vast number of different peptide epitopes presented on MHC class I. The TCR consists of a variable and a constant region, as well as a transmembrane domain and a short cytoplasmic tail. The variable regions TCR Vα and TCR Vβ

are found within the two heterodimeric subsets, TCRα and TCRβ. The Vα and the Vβ domains contain three hyper-variable compliment determining regions (CDRs) each. The CDRs are termed CDR1, CDR2 and CDR3, it is these regions within the variable domains that are believed to interact with the peptide on the MHC class I molecule. Several years ago Garboczi et al proposed that the TCR α/β heterodimer lies diagonally across the pMHC complex, and this orientation was shared between the TCRs of both CD8 and CD4 T cells (Garboczi et al., 1996). Further experiment by Garcia et al determined that the Vα region lies over the N-terminal of the epitope peptide, whereas Vβ is located over the carboxylterminal portion of the epitope (Garcia et al., 1996). X-ray crystal structures by multiple groups have indicated that the CDR3 and CDR1 regions interact with the peptide and the MHC to determine the antigen specificity and the CDR2 region binds to conserved regions on the MHC class I molecule (Hennecke and Wiley, 2001). The TCR has been crystallized both alone and in contact with pMHC. Differences in the conformation of the TCR between the two states has lead many group to propose the induced fit hypothesis, where the TCR structure changes to accommodate the peptide bound to the MHC molecule (Garcia et al., 1999). The induced fit model does explain how the TCR recognizes its cognate pMHC class I molecules, however it does not explain how the T cell becomes activated following recognition. This initial step in T cell activation is still under some debate, however it is clear that accessory molecules are important in both pMHC recognition and for the signaling events that follow.

The TCR noncovalently associates with the accessory molecules CD3ε and ζ (Weiss, 1991). CD3ε and ζ are involved in translating the TCR recognition of cognate peptide MHC into a signaling event, which stimulates CD8 T cell effector responses. Along with the CD3ε and ζ, other costimulatory molecules on the surface of the T cell bind to their ligand on the APC

to enhance the CD8 T cell signaling. These costimulatory molecules and their ligands include: LFA-1/ICAM-1, CD28/B7-1 or 2, and CD40/CD40L.

Unique among the accessory molecules present on CD8 T cells is the CD8 α/β heterodimer. The CD8 co-receptor binding is required for CD8 T cell activation and effector responses (Kupfer and Singer, 1989). The CD8 co-receptor binds to a non-polymorphic region of the MHC class I molecules. The CD8 N-terminal domain of the α-chain binds the α-3 domain of MHC class I molecules (Norment et al., 1988; Salter et al., 1990). The binding of the CD8 heterodimer to MHC helps enhance the specificity and stability of the interaction between the TCR and the cognate peptide, as well as increases the recruitment of protein kinase Lck and through interactions with the CD8α chain enhancing T cell activation.

The recognition of the pMHC by the TCR and stabilization of these molecules with the CD8$\alpha\beta$ heterodimer leads to activation of CD3 complex, which is the start of a complex signaling cascade. The initial step is the trans-phosporylation of the immune tyrosine-based activation motifs (ITAM) located on the CD3 complex by Src family kinases such as Lck and Fyn. Upon activation of Src family kinases there is phosphorylation of the SH2 domains of Zap70, generating the enzymatically active form of this enzyme. Although the exact series of events during initial activation remains to be elucidated, the localized initial cascade leads to the formation of large signaling complexes. The signaling complexes function to activate diverse cellular processes such as enhanced transcription and translation, actin polymerization, cytosketetal rearrangement and chromatin remodeling. The timing needed to set some of these events into motion is very brief while other processes take place over a period of hours after the T cell has disengaged from the pMHC (reviewed in (Jones et al., 1998)). While the release of lytic granules from an activated effector cell can be measured in as little as 5 minutes after

antigen exposure (Purbhoo et al., 2004), interferon gamma (IFN-γ) secretion can be detected about 1 hour after antigen exposure. However maximal cytokine production does not occur in most activated effector cells for 6-18 hours after antigen exposure (Harty et al., 2000; Lalvani et al., 1997; Raue et al., 2004). The events of pMHC recognition alter a naïve CD8 T cell permanently, decreasing the threshold required for T cell activation. The activated T cell can respond much more readily to future antigen encounters.

Many groups have shown that only a short stimulation is needed to induce an effector response from an activated CD8 T cell (Huppa and Davis, 2003; Valitutti et al., 1996). The term serial killer has often been applied to activated CD8 T cells because they are able to quickly kill a target cell and then move on to kill a new target. Live microscopic images by Poenie et al and others have shown that CD8 T cells are capable of releasing lytic granules in less then 10 minutes and that these T cells are then able to move on to kill other target cells in the same area within minutes of its previous attack (Matter, 1979; Poenie et al., 1987; Rothstein et al., 1978; Zagury et al., 1975). When all of these groups look at the duration of interaction between a CD8 T cell and its target APC, the target APC is either plate bound pMHC class I or uninfected APCs pulsed with exogenous antigen. The problem with these studies is that the biologically relevant targets for CD8 T cells are either tumor cells or virally infected cells. These traditional targets of CD8 T cells have devised multiple and unique mechanisms to reduce the efficacy of the CD8 T cell response and are therefore much harder to kill then the APCs used in the studies described above. Each of these traditional CD8 T cell targets will most likely require a different duration of contact and amount of lytic granules released to overcome the unique protective mechanisms devised by the virus or tumor to stay alive. We must therefore revise our thinking about "serial

killer" CD8 T cells to include the duration and strength of signal required to kill multiple types of targets.

Immunological Synapse

As CD8 T cells interact with an APC, the CD8 T cells reorganize their surface membrane proteins to form an immunological synapse (Stinchcombe et al., 2001b). The organized structures that occur at the immunological synapse were first described for CD4 T cell-APC interactions (Grakoui et al., 1999; Lee et al., 2002; Monks et al., 1998), but similar patterns of surface protein reorganization has been observed following CD8 T cell-APC interactions (Stinchcombe et al., 2001b) (reviewed in (Davis and van der Merwe, 2001)). During synapse formation T cell surface ligands and costimulatory molecules begin by localizing to distinct regions at the interface between the T cell and the APC. These organized clusters of proteins are called the supramolecular activation complexes (SMACs). This organized rearrangement of surface proteins can be studied using fluorescently tagged proteins and antibodies and has become a useful tool for visualizing initial events surrounding T cell responses.

Upon initial contact between a CD8 T cell and an APC, T cell surface proteins begin to rearrange and concentrate at the contact site; LFA-1 and talin form a ring around the TCR, Lck, and PKC- θ . The outer ring containing talin and LFA is referred to as the peripheral SMAC or pSMAC. The centrally located TCR, LCK and PKC- θ form the cSMAC (Stinchcombe et al., 2001b). As the synapse is organized on the T cell surface, the microtubule organizing center (MTOC) is rapidly polarized and begins to reorient itself within the cell so the lysosomes containing lytic granules can associate with the synapse (Greijer et al., 2000; Kupfer and Singer, 1989; Yannelli et al., 1986). This is accomplished in a stepwise manner, where the MTOC

moves toward the synapse reorienting the cytoskeleton and finishes moving when it is located beneath the synapse. The small GTP-binding protein, Rab27 aids in the movement of the lytic granules into the area of the cSMAC along the microtubules (Stinchcombe et al., 2001a). The movement of the lytic granules close to the cSMAC occurs rapidly after TCR stimulation. Within the synapse there is a space or cleft between the T cell and the APC cell membranes (Batista et al., 2001). The TCR and other T cell signaling molecules are located in close proximity to the cleft within the pSMAC. The entire synaptic area is sealed by a tight ring of adhesion molecules, which bind the two membranes together during the interaction. It is interesting to note that Stinchcombe et al were able to observe the formation of an immunological synapse before the release of lytic granules, and that TCR-signaling contacts were maintained during granule release (Stinchcombe et al., 2001b). The observation that T cells need to form a stable interaction with the APC while granule release is occurring points to the need for a strong recognition of the target APC before killing occurs.

However, while Stinchombe et al have noted that some form of synapse does occur prior to lytic granule release, Purbhoo et al have shown that a mature synapse is not required for the release of lytic granules (Purbhoo et al., 2004). These seemingly contradictory results may be due to differences in model systems. Stinchchombe et al use allogeneic T cells to study synapse formation. Allogeneic recognition may require a more stable interaction with the target APC in order for the CD8 T cells to kill. However Purbhoo et al group were assessing the minimal amount of peptide antigen required to stimulate a T cell to kill, and in this case there was no need for a stable synapse. An early form of an immunological synapse was observed, this less ordered structure contained both TCR and CD8, but a tight seal between the membranes and mature synapse were not observed. As discussed above, these studies do provide us with some insight

into early activation events, however they have less biological relevance in the context of an ongoing immune response to virally infected cells. CMV infection alters the amounts of surface molecules that may be required for T cell activation. It takes very little stimulation for an activated CD8 T cell to kill an uninfected peptide-pulsed or allogeneic target. However the threshold stimulus required to kill an infected target cell is presumably much higher and may require a more stable interaction and possibly a mature synapse.

MHC class I levels on the surface cells

To signal the presence of a viral infection, MHC class I molecules can present viral peptides to CD8 T cells. The 8-13 aa peptide binds in the groove formed from the N-terminal domain of the heavy chain of the MHC molecule (Fremont et al., 1992; Fremont et al., 1995). As reviewed above, viral peptides are loaded onto class I molecules in the ER and the peptide loaded MHC class I (pMHC) can present viral antigens to CD8 T cells. T cells use the peptides presented on MHC class I molecules to survey the health of cells. Cells presenting endogenous self-peptides are presumed to be healthy and do not stimulate a T cell response. Cells presenting foreign peptides derived from pathogens signal a potential infection and, if "licensed' by signals from the innate immune system, stimulate a T cell response. This basic tenet of CD8 T cell recognition of MHC class I, has helped clarify our understanding of the immune response and opened the door to closer investigations of how CD8 T cells respond to MHC class I.

TCR stimulation is based on the presence of the appropriate TCR with an affinity for the pMHC and the amount of cognate peptide on the cell surface (Sykulev et al., 1995). Recently there have been several studies looking at the amount of cognate peptide MHC that is required to stimulate a CD8 T cell. Purhboo et al have shown that it takes only one pMHC expressing the

cognate antigen to induce a CD8 T cell to interact with the MHC class I, but it requires between 3 and 10 pMHC molecules to stimulate a T cell to release calcium in response to its cognate antigen (Purbhoo et al., 2004). The fact that such a small amount of pMHC is required to stimulate a T cell, leads us to question why there is such an abundance of MHC on the surface of cells?

In a B16 tumor model system Wells et al demonstrated that transfection of a B16 tumor cell line with HSP72 led to an increase in the amount of MHC class on the surface of the infected cells (Wells et al., 1998). Normally these tumor cells are non immunogenic, however if the cells were transfected with HSP72 and the class I levels increased, the tumor cell line was then susceptible to CTL lysis by LCMV specific T cells following infection of the cells. This result was not seen when TAP-/- RMAs cells were transfected with HSP72 and infected with LCMV. The authors attribute the improved killing to more peptide loaded MHC class I on the surface of the infected cells in the presence of HSP72. However this result could not be distinguish from an increase in overall MHC class I contributing to the enhanced immunogenicity of the tumor cell line.

Several studies have also pointed to the importance of the CD8 co-receptor and its interaction with MHC- class I in relation to T cell activation. Unlike the CD4 molecule, the interaction of the CD8 molecule with MHC class I is required for T cell activation (Anikeeva et al., 2006; Cho et al., 2001; Davis and van der Merwe, 2001). Highlighting the importance to CD8-MHC interactions, Cho et al measured the affinity of chip bound TCR and compared it to the affinity of T cell clones. They found that the T cell clones had an increased affinity compared to the plate-bound TCR, and this increase was due to the interaction of CD8 with MHC (Cho et al., 2001). A recent study by Anikeeva et al noted that total MHC class I levels

were important for achieving T cell activation (Anikeeva et al., 2006). In this study a quantum dot core (QDs) was loaded with different concentrations of cognate and non-cognate pMHC, and then incubated with antigen specific T cell clones. After incubating the T cells with the QD-MHC complexes the group noted that the total amount of MHC on the surface of the QD was important to achieve T cell activation. This study went on to demonstrate that the CD8 co-receptor interaction with MHC was required for T cell activation. The CD8 co-receptor did bind to both cognate and non-cognate pMHC on the QD to activate the T cell. Their conclusion was that non-cognate pMHC could cooperate with cognate pMHC on APCs to help stimulate the T cell response (Anikeeva et al., 2006). This result has great relevance when examining the CD8 T cell response to MCMV. Because the immune evasion genes downregulate total MHC class I from the cell surface the virus could prevent the CD8 co-receptor interactions with both non-cognate and cognate pMHC. Without this interaction CD8 T cells may not be able to respond effectively to MCMV infected cells.

CD8 T cell effector responses

CD8 T cells are known to be stimulated by peptide bound MHC class I molecules, leading to multiple responses, which can be broken down into three dominant types; proliferation, cytokine release, direct target lysis. The expansion of antigen specific CD8 T cells by proliferation is an essential component of controlling the spread of virally infected cells throughout a host. These antigen specific cells control virus spread through cytokine release, such as IFN-γ, and the lysis of the APCs mediated by granzymes and perforin. Although T cells are known to kill cells through additional mechanisms such as fas-fas ligand mediated interaction, the perforin-granzyme pathway is the primary defense mechanism against many

different viral pathogens (Kagi et al., 1996). For this reason, this section on CD8 T cell mediated killing will focus primarily of the perforin-granzyme pathway. It is the effector CD8 T cell responses, cytokine and lytic granule release, that virally infected cells must avoid or inhibit in order to persist within its host because if the CD8 T cells can deliver their complete arsenal of weapons uninhibited onto the infected cell the virus would be killed.

CD8 T cell cytokine response

A cytokine can be defined loosely as a small secreted protein that can affect the behavior or properties of the cell itself or another cell (Janeway, 2005). Cytokines have long been known to be a potent arm of the effector immune response for controlling viral infections. Cytokines are secreted during both the innate and adaptive immune responses and their role as antivirals has been well documented. In 1957, Isaacs and Lindenman identified a protein substance called interferon, which, when added to normal cells in culture, protects them from viral infection (Isaacs and Lindenmann, 1957; Isaacs et al., 1957). Since this initial discovery, many different cytokines have been identified. There has been a great deal of work looking at the role of cytokines during both the innate and adaptive immune response (reviewed in (Salazar-Mather and Hokeness, 2006)). This dissertation is focusing primarily on CD8 T cell effector responses so the impact of cytokines during the innate immune response to viral infections will not be discussed. However it is important to consider cytokines secreted during innate responses when examining the role of cytokines in controlling viral infections.

During the adaptive immune response CD8 T cells are one of the most prolific producers of cytokines. Following antigen stimulation CD8 T cells release a number of cytokines, the most studied of which are IFN-γ, and tumor necrosis factor alpha (TNF-α). These effector cytokines

enhance the inflammatory environment at the site of foreign antigen and aid in the recruitment of cells as well as stimulate the maturation of professional antigen presenting cells. Because most cytokines need to bind to receptors on target cells in order to function their activity during an inflammatory response can be directed. Some cytokines do have a less directed focus and can act systemically to induce cell recruitment and maturation.

IFN-γ functions to upregulate MHC class I and class II thereby enhancing the amount of antigen being presented at the site of inflammation. IFN-γ also induces other changes within the cells to allow them to more efficiently process and present peptides on MHC class I, including upregulation of the interferon inducible genes. Genes that encode components of the immunoproteasome are induced by IFN-γ allowing peptides that bind to MHC class I molecules to be more efficiently generated (Strehl et al., 2005).

TNF-α is also a potent anti-viral cytokine. TNF-α produced by CD8 T cells upregulates ICAM-1, and VCAM-1. As discussed above ICAM binding to LFA-1 on the CD8 T cell is important for mediating CD8 T cell recognition of cognate pMHC. At the same time ICAM-1 and VCAM-1 upregulation on endothelial cells helps to recruit more T cells to the sight of inflammation.

Studies completed to determine the threshold needed to activate a CD8 T cell have shown there are different thresholds required to stimulate a CD8 T cell to kill versus release cytokines. In most situations the threshold required to stimulate a CD8 T cell to kill is lower then that required to induce cytokine secretion (Gervois et al., 1996; Hollsberg et al., 1995; Otten and Germain, 1991; Slifka et al., 1999; Valitutti et al., 1996). This means that at low antigen doses and reduced amount of costimulation, as is present on non-professional antigen presenting cells, a CD8 T cell is more likely to release its lytic granules then it is to secrete cytokines. This

response of the CD8 T cells makes sense in the context of a minor immune stimulus. Cytokine secretion increases vascularization of tissues, and upregulation of chemokines leading to an inflammatory environment, which could result in nonspecific pathology. The release of lytic granules is targeted and confined. If a cell is inappropriately targeted by a CD8 T cell for lytic granule release, the net loss to the organism is a single cell, as compared to multiple cells that could be affected by cytokine secretion.

Because cytokines have such diverse modes of action both in recruiting inflammatory cells and directly blocking viral infection, CMV has devised several strategies for dealing with cytokines. Recently it was discovered that MCMV does encode some genes to block some of the effects of individual cytokines. The MCMV protein pM27 binds to the downstream signaling molecule STAT-2 and induces its ubiquitin and proteasome-dependent degradation (Zimmermann et al., 2005). Blocking STAT-2 signaling prevents the activation of multiple anti-viral genes. The CMV proteins IE1 and IE2 were shown to suppress the apoptotic anti-viral response induced by TNF α (Zhu et al., 1995). CMV encodes multiple genes to inhibit cytokine effector functions, highlighting the importance of cytokines as potent anti-virals.

Cytolytic Activation and the release of Granzyme B and Perforin

In conjunction with cytokine secretion activated T cells are stimulated through their TCR to secrete lysosomes containing prepackaged lytic granules directly at the APC. There are many different molecules found within these secreted lysosomes as shown in Table 1 below. The most extensively studied components of lytic granules are granzymes A and B and perforin. The combined actions of perforin and granzymes induce cellular apoptosis and work as a potent effector mechanism to control viral infection.

Table 1 Lytic Granules

Protein	Function
Perforin	Forms pores in membranes, exact function unknown
Granulysin	Lyses cells and bacteria
Granzyme A	Initiates apoptosis upon intracellular access to target cell substrates.
Granzyme B	Initiates apoptosis upon intracellular access to target cell substrates.
Granzyme H	Unknown
Granzyme K	Initiates apoptosis upon intracellular access to unknown substrates.
Granzyme M	Unknown
Granzymes C, D, E, F, and G	Unknown
Sulfated proteoglycan	May bind granzymes and perforin.
Calreticulin	Unknown
Lysosomal glycosidases and cathepsins	Unknown

The role of perforin in mediating cytolytic effector functions has long been a subject of controversy. Although perforin is essential for the induction of apoptosis mediated by granzymes, perforin alone does not normally cause cell death. At very high concentrations perforin by itself can induce necrosis, but there has been no evidence to show that perforin can induce apoptosis at physiological concentrations in the absence of granzymes. Early studies have conclusively demonstrated that granzymes require the presence of perforin in order to induce apoptosis of the infected cell. It has however been difficult to determine the role perforin plays in the induction of apoptosis. A clue to perforin's mode of action is its functional and structural homology to complement. Due to the similarities to complement perforin was long thought to facilitate the movement of granzyme into the cell via pores generated in the membrane. However it has since been shown that the pores generated by perforin are only big enough to allow a molecule smaller then 10 kDa to pass through; granzyme molecules are much

bigger then this and therefore cannot pass through the pores generated by perforin (Shi et al., 1992). More recently it has been demonstrated by Keefe et al that physiological concentrations of perforin can induce membrane repair mechanisms within the cell (Keefe et al., 2005). It is now believed that the induction of these repair mechanisms causes the receptor-bound granzymes to be engulfed and brought into the cytoplasm by a yet unknown mechanism. Although it is still unclear as to how perforin functions, many groups have shown that perforin-mediated killing is important in controlling many different viral infections (Kagi et al., 1996; Mullbacher et al., 1999; Tay and Welsh, 1997; Loh et al., 2005).

Granzymes are one of the main effector molecules in the perforin-granzyme mediated killing pathway. Granzymes are a family of serine proteases, they are synthesized as pro-enzymes that become proteolytically active following cleavage of signal peptides. The active granzymes are then stored in the lytic granule and upon release into an infected cell they cleave proteins at aspartic residues. As discussed above, it is now clear that granzymes enter the cell in a perforin-dependent manner with the help of cellular receptor mediated endocytosis (Froelich et al., 1996; Pinkoski et al., 1998; Shi et al., 1997; Trapani et al., 1996). One of the receptors though to be involved in granzyme endocytosis is the mannose 6-phosphate receptor (MPR). MPR has been shown to bind to granzyme B and is believed to be involved in granzyme-B receptor mediated endocytosis (Motyka et al., 2000).

Granzymes resemble caspases in their structure and mode of action. For example granzyme B is capable of cleaving and activating multiple caspases and some caspase substrates. A more detailed explanation of the caspase cascade and its involvement in apoptosis is described below. For the purposes of this section, it is important to know that the cleavage of a pro-caspase into an active caspase is essential for the induction of apoptosis. After granzyme B

enters the cell it targets multiple pro-caspases, including caspases -3, -6, -7, -8, and -10, cleaving them and initiating the caspase cascade (Russell and Ley, 2002). Granzyme B can also interact with pro-apoptotic Bcl-2 family members leading to the initiation a mitochondrial apoptotic cascade (described below) (Heibein et al., 2000). Unlike perforin there is a great deal of redundancy in the functions of granzymes. Because of this the loss of a single granzyme does not have a dramatic effect on viral replication. The only observable defects due to the loss of a single granzyme is a slight delay in cell death. However work with ectomelia virus has shown that a loss of both granzyme A and B leads to a reduction in apoptosis and an increase susceptibility of mice to the virus (Mullbacher et al., 1999). It is the combined functions of the granzymes that initiate apoptosis leading to the death of the cell, thereby controlling viral infection.

Apoptosis

Apoptosis is an essential process of controlled cell death used to remove old and damaged cells while avoiding bystander cell damage. Apoptosis is also used by the immune system to remove virally infected cells without triggering a strong inflammatory process. Apoptotic cells undergo an orderly process, which is characterized by an energy-dependent enzymatic breakdown of the cell. The characteristics of apoptosis are: DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of small membrane-enclosed vesicles that may be phagocytosed and reused (Majno and Joris, 1995). The essential mediators of apoptosis are caspases (cysteinyl aspartate proteinases) (Cohen, 1997; Salvesen and Dixit, 1997; Salvesen and Dixit, 1999; Thornberry and Lazebnik, 1998).

The first caspase (Caspase 1) was identified, not because it was involved in apoptosis, but

because it was able to cleave Interleukin 1b. Caspase-1 was originally called interleukin 1b converting enzyme (ICE) (Cerretti et al., 1992; Thornberry et al., 1992). Soon after similarities were noted between ICE and CED-3, a gene identified in Caenorhabditis elegans that was know to be responsible for programmed cell death. With this observation a link was made between the CED-3-mediated programmed cell death that occurs in the worm and a similar pattern of cell death in mammalian cells. Shortly thereafter multiple proteins similar to ICE and CED-3 were identified (Vaux et al., 1992; Yuan et al., 1993a; Yuan et al., 1993b).

Caspases are zymogens existing as pro-caspases within healthy cells. They are composed of 3 domains: the N-terminal pro-domain, and a p20 and a p10 domain (Earnshaw et al., 1999). Upon activation from an apoptotic signal, some caspases can undergo proteolytic cleavage. The pro-caspase form is cleaved at an Asp residue to generate 2 subunits creating an active caspase (Salvesen and Dixit, 1997). The active caspase is a heterodimer containing two p10/20 heterodimers with two active sites (Earnshaw et al., 1999). There are two types of caspases found within cells, the initiator caspases (-2, -8, -9, and -10) and the effector caspases (-3, -6, and -7). Initiator caspases are the first caspases to be cleaved. Following their cleavage the active caspases can then cleave other inactive pro-caspases in a hierarchical manner causing the cascade of activation. Since active caspases can cleave pro-caspases after the induction of apoptosis the amplification of the caspase cascade leads the cell inexorably toward death by apoptosis. The initiator caspases contain two types of domains, caspases 2 and 9 contain a caspase recruitment domain (CARD), and caspases 8 and 10 have a pair of death effector domains (DEDs). Unlike initiator caspases, effector caspases lack the ability to self-activate and they require initiator caspases to cleave them into their active form (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). The effector caspases target multiple proteins within the cell

leading to the cleavage of cytoskeletal proteins, induction of DNA damage and loss of cell adhesion.

There are two main apoptotic pathways known in mammalian cells, the mitochondrial death pathway, which is also know as the intrinsic pathway, and the death receptor mediated apoptosis pathway or extrinsic pathway. These two main pathways are initiated by different events but eventually converge leading to the characteristic phenotype of apoptosis.

The intrinsic pathway can be activated by internal cell stresses and by granzymes following a CD8 T cell effector response. The Bcl-2 family members regulate the intrinsic pathway, and the pro-apoptotic family members include Bax, Bad, and Bak (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). Both pro-apoptotic and anti-apoptotic Bcl-2 family members share one or more conserved regions known as Bcl-2 homology (BH) domains (Yin et al., 1994). Despite a large body of literature on the Bcl-2 family members, it remains unclear as to how these proteins function in the prevention and initiation of apoptosis events. It is clear that activation of the pro-apototic Bcl-2 family members Bax/Bak causes the permeablization of the mitochondrial membrane and the release of cytochrome c (Zamzami and Kroemer, 2001). Cytochrome c binds the adaptor protein Apaf-1 and facilitates the formation of the septameric apoptosome that recruits and activates caspase-9 (Li et al., 1997). The caspase-9 complex then acts to cleave the effector caspases including caspase-3, and 7. Caspase-3 cleaves the inhibitor of caspase activated DNase (lCAD), resulting in the release and activation of CAD. CAD then translocates to the nucleus where it mediates DNA fragmentation (Enari et al., 1998). Wyllie demonstrated that CAD cuts the genomic DNA between nucleosomes, to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs (Wyllie, 1980; Wyllie et al., 1980). This observed DNA ladder is a distinguishing characteristic

of apoptosis and has been used as a marker for apoptotic cell death.

The Fas receptor, which is also known as CD95, is a transmembrane glycoprotein that binds to Fas ligand to trigger the death-receptor, or extrinsic apoptosis pathway. Binding of Fas ligand to Fas induces receptor clustering and formation of a death inducing signaling complex. The complex engages the adaptor molecule, Fas-associated death domain protein (FADD) (Medema et al., 1997) and multiple procaspase-8 molecules. Procaspase-8 is recruited to the death-inducing signaling complex (DISC) via binding to the adaptor protein FADD (Kang et al., 2004; Muzio et al., 1996; Varfolomeev et al., 1998). Caspase-8 cleavage may be due to its close proximity to FADD or autolytic cleavage may occur due to the high concentration of pro-caspase-8. Inhibition of caspase-8 cleavage can occur if c-FLIP, a degenerate caspase, is recruited into the complex with FADD (Irmler et al., 1997) suggesting that FADD is in someway involved in the generation of active caspase-8. After its cleavage active-caspase-8 then cleaves procaspase-3. After the cleavage of caspase-3, many different proteins are targeted for destruction and the phenotypic characteristics of apoptosis begin to appear. Caspase-8 also cleaves the Bcl-2 family member Bid. The cleavage of Bid connects the extrinsic and intrinsic pathways and enhances the rate of apoptosis. Bid translocates to the mitochondria where it promotes the release if cytochrome c which leads to the eventual fragmentation of DNA (Cohen, 1997).

Viral inhibitors of apoptosis

Cells often use apoptosis as a defense mechanism against infection, and because caspases can target so many different sites and enhance their own activation, stopping apoptosis from occurring after T cells release perforin and granzyme into the target APC seems insurmountable.

However cells have several methods to inhibit apoptosis, and viruses have subverted these anti-apoptotic pathways for their own survival. There are many instances where the death of a cell, such as a neuron, is not beneficial for the survival of the organism. In these cases, inhibitors of apoptosis proteins (IAPs), act to protect the cells from death. Cells can inhibit apoptosis at many steps in the process; they can inhibit cleavage of initiator caspases, as well as protecting targets of caspases from cleavage thereby stopping the programmed cell death.

Viruses have an obvious need to encode inhibitors of apoptosis. Virus infection of a cell can cause great stress to the cell, and as the virus takes over the cellular machinery to create progeny, most cells are not equipped to handle the dramatic increase in transcription and translation of viral protein, and respond by initiating apoptosis. The first caspase inhibitor, the cytokine response modifier A (CrmA), was identified in a virus in the same year as the first caspase. CrmA was first shown to inhibit ICE activity (Ray et al., 1992). CrmA was initially isolated from cowpox virus, however when expressed in mammalian cells, it inhibited induced apoptosis by inhibiting caspase 8 and 10 (Callus and Vaux, 2007; Miura et al., 1993). Soon after the identification of CrmA, other viruses were examined and shown to encode inhibitors of apoptosis; many of these viral inhibitors were later shown to have cellular homologues. These inhibitors were identified by their ability to prolong cell viability under conditions that would normal cause cells to die by apoptosis. Herpes simplex virus 1 (HSV-1) encodes two anti-apoptotic genes, Us3 and Us5, both inhibit apoptosis, however their mechanism of action is unknown (Jerome et al., 1999). It is interesting to note that there is the large diversity of sites of apoptosis that are targeted by viruses (reviewed in (Benedict et al., 2002; Boya et al., 2004; Callus and Vaux, 2007)) suggesting that inhibition of apoptosis is a common survival strategy shared by multiple viral families.

CMV, perhaps because it is such a large virus and has a relatively slow replication cycle, has an apparent need to encode multiple anti-apoptotic proteins. CMV's ability to inhibit apoptosis was recognized well before the proteins involved in the inhibition were identified (Kosugi et al., 1998). CMV genes have been shown to inhibit both the intrinsic and the extrinsic pathway of apoptosis. The gene product of UL36 is the viral inhibitor of caspase-8 activation (vICA) inhibits the cleavage of pro-caspase-8, thereby blocking activation of the extrinsic apoptosis pathway. vICA accomplishes this by blocking the recruitment of pro-caspase-8 to the DISC (Skaletskaya et al., 2001). The MCMV homologue of UL36, M36 (vMIA) is also able to inhibit apoptosis in a manner similar to that observed for vICA (McCormick et al., 2003). CMVs have also developed ways to block the intrinsic pathway, by upregulating the anti-apoptotic members of the Bcl-2 family (Andoniou et al., 2004; Billstrom Schroeder et al., 2002). The upregulation of the anti-apoptotic Bcl-2 family members appears to protect the mitochondria from permeablization by the pro-apoptotic Bcl-2 family members (Billstrom Schroeder et al., 2002).

There is also a group of genes encoded by CMVs that have been shown to inhibit apoptosis but their mechanism of action has not yet been defined. M45 of MCMV has been shown to inhibit apoptosis in endothelial calls (Brune et al., 2001), however the significance of this remains unknown. It has a sequence homology to ribonucluotide reductase genes but it does not appear to function as an active enzyme (Lembo et al., 2004; Lembo et al., 2000). The MCMV-encoded gene m41 has also been shown to have anti-apoptotic activities (Brune et al., 2003). Brune et al identified m41 from a library of transposon mutants. When cells were infected with a virus containing a mutated m41 gene the cells underwent a premature death. m41 is known to localize to the mitochondria but its mode of action is still unknown (Brune et al.,

2003). Many of the genes involved in the inhibition of apoptosis by CMV have not been identified. For example, the TNFR has been shown to be downregulated by both HCMV and MCMV. Although the mechanism for this downregulation is still unknown, the immune evasion genes have been shown not to be involved (Baillie et al., 2003; Popkin and Virgin, 2003).

All viruses encode anti-apoptotic proteins, and it is interesting to note the diverse locations in the apoptosis pathway that are targeted. Viruses can alter their gene expression to block apoptosis when it is a benefit to the virus, and induce apoptosis when replication is complete. While most viruses encode genes to block apoptosis induction it seems very few viruses want to keep the cell alive forever. Viruses more often encode anti-apoptotic genes that prolong the life of the cell just long enough to allow the virus to replication. Then the virus shuts off the anti-apoptotic gene or allows the apoptosis signals to overwhelm the cell. This eventual death of the cell can then be used as a tool for spreading the virus by either phagocytosis of the apoptotic cell by a new host cell or through lytic release of infectious virus. As with many other host cell mechanisms viruses have exploited the apoptosis pathway to its benefit and to the detriment of its host.

Conclusions

Since the discovery of CMV immune evasion genes over ten years ago, much has been learned about their fascinating molecular mechanisms, although there is still a great deal that has yet to be determined. Much less is known about their functional significance. For HCMV, we even lack evidence that they actually impair antigen presentation in virus-infected cells. For MCMV, we have good evidence that immune evasion genes do impair antigen presentation *in vitro*, and even *in vivo,* at least for one epitope. However, so far we have not discovered any real

impact of this function on virus replication in normal animals.

CMVs have evolved with their hosts since mammalian radiation. This evolution with its host has allowed the virus and the immune response to become intimately related. As the mammalian immune response has developed so have the CMVs strategies to evade it. After acute infection, it is only when the immune response breaks down that we see any evidence of CMV infection. The immune system is able to tolerate CMV without much pathology or longterm effects and this controlled response is probably regulated by both the virus and host. The immune system devotes a great deal of attention to CMV, the high frequency of antigen specific CD8 T cells probably keeps viral reactivation in check. While CMV for its part, encodes immune evasion genes and other immune resistance genes that can impact the effectiveness of the response. The combination of the immune evasion and other immune resistance genes allows the virus to tolerate the immune response just as the high frequency of CMV specific cells allows the host to tolerate the CMV infection.

These results discussed within this dissertation begin to address the question of why MCMV encodes multiple immune evasion genes. In Chapter 2 we have systematically measured the CD8 T cell effector responses generated against 16 different H2-b epitopes of MCMV. From these experiments the primary conclusion that we have drawn is that all three of the MCMV immune evasion genes are required to completely inhibit CD8 T cell killing of MCMV infected cells, because the absence of any one immune evasion gene leads to killing by some antigen specific CD8 T cells. By analysis the effects of the specific immune evasion genes on CD8 T cell killing we have demonstrated that *m06* and *m152* appear to affect CD8 T cell killing equally in most of the cell types examined. However, *m04* may have more cell type specific effects, which are discussed in Chapter 2. In Chapter 3 we studied the impact of *m152*'s downregulation

of the NKG2D ligands on CD8 T cell killing. We determined that *m152* downregulation of NKG2D ligands did impact CTL recognition. However the inhibition of CTL killing was ineffective and only observed for some epitopes. Chapter 4 focuses more closely on why CD8 T cells cannot kill wild-type MCMV infected cells. We determined that CD8 T cell are able to respond to cognate peptide on the surface of infected cells. Wild-type infected cells are able to overcome the response by the CD8 T cells while uninfected cells die when a similar amount of granzyme B is released. The immune evasion genes, *m06* and *m152* downregulate the total amount of cell surface MHC class I, and it is this downregulation of total cell surface MHC class I that prevents the CD8 T cells from killing the wild-type infected cell.

This project has helped define the roles of the immune evasion genes in protecting the infected cell from CD8 T cell mediated killing. I have defined a novel mechanism of protection against death which involves both the ability of the virus to increase the infected cell's resistance to death and to downregulate MHC class I molecules, which leads to a reduction in the CD8 T cell effector response.

Chapter 2

Coordinated function of murine cytomegalovirus genes completely inhibits CTL lysis

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Abstract

Murine cytomegalovirus (MCMV) encodes three viral genes that interfere with antigen presentation (VIPRs) to CD8 T cells, *m04, m06*, and *m152*. Because the functional impact of these genes during normal infection of C57BL/6 mice is surprisingly modest, we wanted to determine whether the VIPRs are equally effective against the entire spectrum of H-2b-restricted CD8 T cell epitopes. We also wanted to understand how the VIPRs interact at a functional level. To address these questions we used a panel of MCMV mutants lacking each VIPR in all possible combinations, and CTL specific for fifteen H-2b-restricted MCMV epitopes. Only expression of all three MCMV VIPRs completely inhibited killing by CTL specific for all fifteen epitopes, but removal of any one VIPR enabled lysis by at least some CTL. The dominant interaction between the VIPRs was co-operation: *m06* increased the inhibition of lysis achieved by either *m152* or *m04*. However, for one of 15 epitopes *m04* functionally antagonized *m152*. There was little differential impact of any of the VIPRs on K^b versus D^b, but a surprising degree of differential impact of the three VIPRs for different epitopes. These epitope-specific differences did not correlate with functional avidity, or with timing of VIPR expression in relation to antigen expression in the virus replication cycle. While questions remain about the molecular mechanism and in vivo role of these genes, we conclude that the co-ordinated function of MCMV's three VIPRs results in a powerful inhibition of lysis of infected cells by CD8 T cells.

Introduction

Cytomegaloviruses (CMVs) are ubiquitous species-specific viruses that persist within a host for its entire life. CMVs encode a group of proteins called VIPRs: viral genes that inhibit antigen presentation to CD8 T cells (Yewdell and Hill, 2002). Even though all CMVs encode VIPRs, the VIPRs found in each species have developed specialized mechanisms to interfere with the MHC class I pathway of their particular host.

Murine CMV (MCMV) has three VIPRs, *m04*, *m06,* and *m152,* which encode the glycoproteins m04/gp34, m06/gp48 and m152/gp40. All three VIPRs function to inhibit CD8 T cell recognition of infected cells, but each VIPR employs a unique strategy to accomplish this task. *m152* primarily functions by blocking MHC class I transport from the Endoplasmic reticulum Golgi intermediate compartment (ERGIC) to the Golgi, resulting in an accumulation of peptide-loaded class I molecules in the ERGIC, and a reduction in cell surface class I expression (del Val et al., 1992; Thale et al., 1995; Ziegler et al., 1997). Interestingly, although *m152* has a pronounced effect on MHC class I transport, no direct biochemical interaction between m152/gp40 and MHC class I has ever been demonstrated. In contrast, m06/gp48 forms a tight association with MHC class I molecules in the endoplasmic reticulum (ER). A di-leucine motif in the cytoplasmic tail of m06/gp48 targets the MHC class I–m06/gp48 complex to a lysosomal compartment, where both proteins undergo rapid proteolysis (Reusch et al., 1999), causing a dramatic reduction in cell surface MHC class I expression (Hengel et al., 1999; Wagner et al., 2002). MCMV's third VIPR, m04/gp34, is primarily ER resident. A small portion of m04/gp34 forms a stable association with MHC class I molecules in the ER (Kavanagh et al., 2001b; Kleijnen et al., 1997). These complexes are exported to the cell surface, where they

remain for several hours. However, the exact mechanism by which m04/gp34 inhibits CD8 T cell recognition remains to be determined.

The fact that multiple VIPRs are encoded by both HCMV and MCMV is intriguing, and the advantage to the virus of this multiplicity is still not clear. For MCMV, the possibility that these genes were redundant was soon excluded: removing any one of MCMV's three VIPRs enabled recognition of infected cells by at least some CTL clones (Kavanagh et al., 2001a; LoPiccolo et al., 2003). Evidence has been obtained for co-operativity- i.e. that the combined actions of two or more VIPRs more efficiently inhibited antigen presentation than any VIPR acting alone (Kavanagh et al., 2001a). There is also evidence that different VIPRs may play a greater role in some cell types than others. For example, we observed that *m04* appeared to play a more prominent role in macrophages than in fibroblasts (LoPiccolo et al., 2003).

We also suggested that MCMV's VIPRs acted in complementary fashion; with efficient action of one VIPR against some MHC class I isoforms being complemented by a more efficient action of another VIPR against other isoforms (Kavanagh et al., 2001a). Metabolic labeling and pulse chase analysis revealed that *m152* inhibited the transport of H-2D^b more efficiently than H-2K^b. Although m04/gp34 co-precipitated with both K^b and D^b, we observed that three K^b-restricted MCMV-specific CTL clones could lyse cells infected with a MCMV lacking *m04* (Δ*m04*), whereas two D^b-restricted clones could not. We thus suggested a contribution from *m04* was required to completely inhibit K^b-restricted antigen presentation, because of *m152*'s relatively weak activity against K^b. In contrast, because D^b transport was much more efficiently inhibited by *m152*, we suggested that a contribution from *m04* was not necessary to inhibit D^b-restricted antigen presentation.

A more detailed study of the interaction of MCMV's VIPRs was made possible when

Wagner et al. used BAC technology to create a panel of seven MCMV mutants that expressed all possible combinations of MCMV's three known VIPRs (Wagner et al., 2002). These mutants were used to examine the impact of the VIPRs on total cell surface MHC class I expression levels by FACS analysis of infected transformed fibroblasts. These experiments revealed that cells infected with a virus lacking all three VIPRs (*Δm04+m06+m152*) expressed equivalent cell surface MHC class I to uninfected cells, indicating that MCMV contains no other genes that can downregulate cell surface class I. These experiment also demonstrated preferential action of the individual VIPRs against different class I isoforms: for example, they confirmed that *m152* affected D^b more strongly than K^b, and *m06* caused greater downregulation of the K locus alleles (K^b and K^d), than did *m152*. Co-operativity was also observed between *m152* and *m06*, with their combined impact being greater than the impact of either alone. A fascinating result of that study was that *m04* could antagonize the impact of *m152*, a feature that was observed only in mutants that did not express *m06*. The authors suggested that MHC class I is initially retained in the ER by m152/gp40, but that thereafter m06/gp48 and m04/gp34 compete for the MHC class I molecules. They postulated that when all three VIPRs are present, m06/gp48 generally wins this competition, escorting class I to lysosomes for destruction. However, if *m06* is absent, *m04* can rescue some class I from *m152*-mediated retention, escorting it to the cell surface and leading to an overall greater cell surface level of class I.

Measuring the impact of the VIPRs on total cell surface class I is a fairly crude surrogate for assessing their impact on antigen presentation. An activated CTL requires only about 10 MHC-peptide complexes in order to exert effector functions (Krogsgaard et al., 2003). The VIPRs cannot completely suppress cell surface class I expression in fibroblasts, and in fact 30-50% of total class I is still present on the cell surface during infection (Kavanagh et al., 2001b;

Wagner et al., 2002). The question arises: how well does this level of cell surface class I reduction correlate with inhibition of CTL function?

Previous functional studies of the impact of MCMV's VIPRs have been carried out with CTL clones specific for a limited number of epitopes. For example, the conclusion that *m04* would be required to inhibit K^b- but not D^b-restricted antigen presentation was based on data from three K^b-restricted and two D^b-restricted clones. Our recent identification of the antigens recognized by these clones (Munks et al., 2006) revealed that, while the three K^b-restricted clones recognized three different antigens, both D^b-restricted clones were specific for the same epitope from M45. We have now identified sixteen K^b-restricted and ten D^b-restricted CD8 T cell epitopes. It therefore seemed timely to revisit the questions and hypotheses concerning the interplay between MCMV's VIPRS.

Materials and Methods

Cells and Mice IC-21, a SV40-transformed macrophage cell line from C57BL/6 mice (Cavanaugh et al., 1996), a gift from Ann Campbell Eastern Virginia Medical School, were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4.5g/L Glucose, and1.5g/L sodium bicarbonate and antibiotics. BALB/c 3T3 (ATCC), L929 (ATCC), K41 and K42 (SV-40-transformed H-2^b fibroblasts, a gift from Marek Michalak University of Alberta) and B16-FL (Driessen et al., 1999) (a gift from Glen Dranoff, Harvard Medical School) were cultured in DMEM supplemented with 10% FBS and antibiotics. L929 supernatant, a source of macrophage colony-stimulating factor (M-CSF), was harvested from L929 cells grown for 10 days after reaching confluence. Primary bone marrow macrophages (BMMΦ) were isolated by the procedure described by Bouwer et al (Bouwer et al., 1997). Briefly, bone marrow was cultured on non-tissue culture treated Petri dishes in DMEM supplemented with 10% FBS, 30% M-CSF from L929 supernatant and antibiotics. Six to ten days later adherent cells were isolated. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME), were maintained in our animal facilities at Oregon Health and Science University (Portland, OR) and were used in experiments no earlier than 6 wk postbirth.

Viruses Wild-type BAC-derived MCMV strain MW97.01 (Wagner et al., 1999), Δ*m04*, Δ*m06*, Δ*m152* Δ*m04+m06*, Δ*m04+m152*, Δ*m06+m152*, and Δ*m04+m06+m152* (Wagner et al., 2002) were grown on C57BL/6 Mouse embryo fibroblasts (MEF), then purified by pelleting over a 15% sucrose cushion (Brune, 2005). Each virus stock was titered without centrifugal enhancement on BALB-3T3 cells. The mean of three virus titrations was used to calculate titers for use in these assays.

T cell lines Female C57BL/6 (B6) mice were purchased from NCI Fredrick (Baltimore, MD) or The Jackson Laboratory (Bar Harbor, ME), and infected with either $1x10^6$ or $5x10^6$ pfu of MCMV. Spleens were harvested from mice that had been infected at least 11 weeks previously. As a source of DC-enriched splenoctyes to stimulate CTL lines, we used spleens from mice that had been infected 14 days previously with the Flt-3 ligand-secreting tumor, B16FL. Splenocytes from B16FL-injected mice were γ-irradiated and pulsed with peptide at 10^{-8}M, and cultured with splenocytes from MCMV-infected mice in RPMI supplemented with 10% FBS for 3 days, after which 10U/ml recombinant IL-2 (eBioscience) was added. After 10 days, the percentage of CD8 T cells responding to the simulating peptide epitopes was assessed by intracellular cytokine staining (ICCS), and the cells used in ^{51}Cr release assays.

Antibodies and tetramers α-gB and α-gH, (a gift from Lambert Loh (Loh et al., 1988; Loh and Qualtiere, 1988; Rapp et al., 1994)). α -K^b (Y3) and α -D^b (B22-249) (ATCC), α-pp89 (Del Val et al., 1988) were purified on Protein A , G (Sigma/Aldrich) columns and conjugated to FITC (Molecular Probes), PE, or APC (Cyanotech) according to published protocols (Hardy, 1986). α–IFN-γ (XMG1.2) and α -CD8 (53-6.7) were purchased from eBioscience.

FACS analysis IC-21, BMMΦ or K41 cells were infected overnight with the panel of mutant viruses at an MOI of 20 in the presence of 0.3 mg/ml phosphonoacetic acid (PAA, Sigma/Aldrich). For intranuclear staining, cells were stained with cell surface antibodies then fixed with CytoFix/CytoPerm (BD bioscience) then permeablized by incubation for 5 minutes with 0.1% Triton X in PBS. The cells were then stained for 30min with anti-pp89 in the presence of 0.1% Triton X. The cells were washed 3x in 0.1% Triton X then washed 1x in FACS buffer

before analysis. All cells were analyzed using a FACSCaliber flow cytometer (BD Bioscience, Franklin Lakes, N.J.) in conjunction with Cell Quest (BD Bioscience). All further analyses were performed using FlowJo software (Treestar, San Carlos, Calif.).

Assay for cell-mediated cytotoxicity 10^4 target cells per well were plated in 96 well plates, infected with the indicated viruses at an MOI of 20 and labeled with 100μCi ^{51}Cr (NEN) in the presence of 0.3 mg/ml of PAA for 12 hr. For peptide-pulsed targets, ^{51}Cr-labeled cells were incubated with 1 μM peptide for 1 h at 37°C and then washed three times. Effector cells were then added at the indicated effector to target (E:T) ratios, incubated for 6 h, and supernatants were harvested and assayed with a Topcount scintillation counter (Packard Instruments, Meriden, Conn.). Background Cr release was determined by incubating targets with medium alone, and total Cr release by lysing targets with medium containing 1% Nonidet P40 (USB, Cleveland OH). Percent specific lysis was calculated as follows: (experimental cpm - background cpm)/(total cpm - background cpm).

Real-time PCR $2x10^6$ IC-21s were infected with wt MCMV at an MOI of 20 in the presence of PAA (0.3mg/ml). RNA was extracted from cells harvested at 0, 1, 2, 3, 4, 6, 8, 12, 18, and 24hrs post infection using the Sigma GenElute Total Mammalian RNA kit (Sigma/Aldrich), quantified and stored at -80°C. Samples were then DNase treated at 1unit/μg of RNA (Fermentas), cDNA was then generated using the Invitrogen SuperScript III First-Strand Synthesis SuperMix. To ensure that there was no DNA contamination, a portion of each sample was treated identically but without addition of reverse transcriptase (no RT controls). cDNA was stored at -20°C until needed. Quantitative PCR was performed using Platinum SYBR green qPCR SuperMix UGD

with ROX using the primers at 250nM. The samples were run on an ABI PRISM® 7700 Sequence Detection System. The program settings used were according to company specifications (Invitrogen). No product was detected from the no-RT controls. Relative gene expression was determined by normalizing each gene to β-actin as the control, and comparing the gene expression relative to cells at 0 h. The calculations were made following the method described in the User bulletin #2: ABI Prism 7700 sequence detection system. Subject: Relative Quantitation of Gene Expression, Applied Biosystems.

Primers The primers were tested by PCR and shown not to amplify nonspecific cellular genes. Primer sequences are available upon request.

Validation of Primers All primers were validated using the β-actin template as the control, using the method described by Applied Biosystems Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR.

Statistics Statistical significance was determined using Students T test. A paired two-tailed T test was used and all comparisons were determined to be of equal variance.

Results

The combined action of MCMV's VIPRs effectively inhibits CTL specific for sixteen H-2b restricted epitopes.

To assess the impact of MCMV's VIPRs we generated polyclonal CD8 T cell lines for use in ^{51}Cr release assays. CTL lines specific for sixteen of the identified C57BL/6 MCMV epitopes (Table 1) were generated by peptide restimulation of splenocytes from MCMV-infected mice, and were used after 7 to 14 days of culture. The macrophage cell line, IC-21, was used as a target because macrophages support the full virus replication cycle, and are important for viral dissemination *in vivo* (Pollock et al., 1997). Infection was carried out in the presence of PAA, which inhibits viral DNA replication and hence late gene expression. We have previously shown that PAA treatment does not alter the pattern of CTL killing, nor the impact of the VIPRs ((LoPiccolo et al., 2003), and A.K.P., unpublished data). IC-21s were infected with wild-type MCMV or a virus lacking all three VIPRs (Δ*m04+m06+m152*) and tested by ^{51}Cr release assay for lysis by each of the epitope-specific CTL lines (Figure 1A and 1B). In every case, the CTL readily lysed targets infected with Δ*m04+m06+m152* but failed to lyse wild-type virus-infected and uninfected targets. We concluded that the combination of all three VIPRs is highly effective at inhibiting antigen specific CD8 T cell function for all epitopes tested.

Table 1

Gene	Amino Acids	Peptide Sequence	Function [a]	Kinetic class [a]	EC50 [b]	HCMV Homologue	Ref
M31	297-305	VAPDFGVRM	Unknown	Unknown	ND	UL31	(Kattenhorn et al., 2004; Rawlinson et al., 1996)
M33	44-57	GGPMNFVVL	GPCR	3-4H	$4x10^{-9}$M	UL33	(Davis-Poynter et al., 1997; Melnychuk et al., 2005; Rawlinson et al., 1996)
M36	213-221	GTVINLTSV	Anti-apoptotic factor M v-ICA	Immediate Early	$3x10^{-9}$M	UL36	(McCormick et al., 2003; Menard et al., 2003)
M38 (M38.5)	316-325	SSPPMFRV	vMIA	Unknown	$2x10^{-7}$M	UL38 (UL37)	(McCormick et al., 2005)
M44	130-138	ACVHNQDII	Polymerase Processivity factor	Delayed Early	$5x10^{-9}$M	UL44	(Loh et al., 1999; Loh et al., 2000)
M45	985-993	HGIRNASFI	Non function ribonucleotide reductase	12-24H	$6x10^{-8}$M	UL45	(Brune et al., 2001; Gold et al., 2002; Lembo et al., 2000)
M57	816-824	SCLEFWQRV	Major DNA binding protein	Early	ND	UL57	(Messerle et al., 1992b)
M77	474-482	GCVKNFEFM	Unknown	Unknown	ND	UL77	(Rawlinson et al., 1996)
M78	8-15	VDYSYPEV	GPCR	Early	$4x10^{-11}$M	UL78	(Oliveira and Shenk, 2001; Rawlinson et al., 1996)
M86	1062-1070	SQNINTVEM	Major Capsid Protein	Unknown	$3X10^{-11}$M	UL86(MCP)	(Kucic et al., 2005; Morello et al., 2000)
M97	210-217	IISPFPGL	Protein Kinase	Early	$9X10^{-7}$M	UL97(PK)	(Michel and Mertens, 2004; Rawlinson et al., 1997; Scott et al., 2005; Wagner et al., 2000)
M100	72-79	RIIDFDNM	gM	Late	$6X10^{-8}$M	UL100(gM)	(Scalzo et al., 1995)
M102	446-455	SIVDLTFAVL	Helicase Primase	Late	ND	UL102	(Rawlinson et al., 1996)
M112	171-179	AAVQSATSM	E1 (transcription factor)	Early	ND	UL112	(Rawlinson et al., 1996)
M122	416-423	RALEYKNL	IE3 (transcription factor)	Immediate Early	ND	UL122 (IE2)	(Angulo et al., 2000; Keil et al., 1987; Koszinowski et al., 1987; Manning and Mocarski, 1988; Martinotti et al., 1993; Messerle et al., 1992a; Sandford et al., 1993)
m139	419-426	TVYGFCLL	Replication in Macrophages	Early	$2X10^{-9}$M	US22 (GF2)	(Hanson et al., 1999a; Hanson et al., 2001; Hanson et al., 1999b; Karabekian et al., 2005; Menard et al., 2003)
m141	16-23	VIDAFSRL	Replication in Macrophages	Early	$1X10^{-9}$M	US24 (GF2)	(Hanson et al., 1999a; Hanson et al., 2001; Hanson et al., 1999b; Karabekian et al., 2005; Menard et al., 2003)
m164	D^b 267-275 K^b 283-290	WAVNNQAIV GTTDFLWM	Putative membrane glycoprotein	Early	D^b $1X10^{-9}$M K^b ND		(Holtappels et al., 2002a; Holtappels et al., 2002b; Pahl-Seibert et al., 2005)

Table 1. MCMV antigens and epitopes used in this paper. (a). Gene function and kinetic class are based on the referenced publications. In some instances (e.g. M100) these differ from the apparent behavior in this study (Figure 5), possibly due to differences in cell type infected or to the moi. (b). EC50 indicates the concentration of peptide at which half maximal lysis was observed in ^{51}Cr assay using IC-21s as target cells.

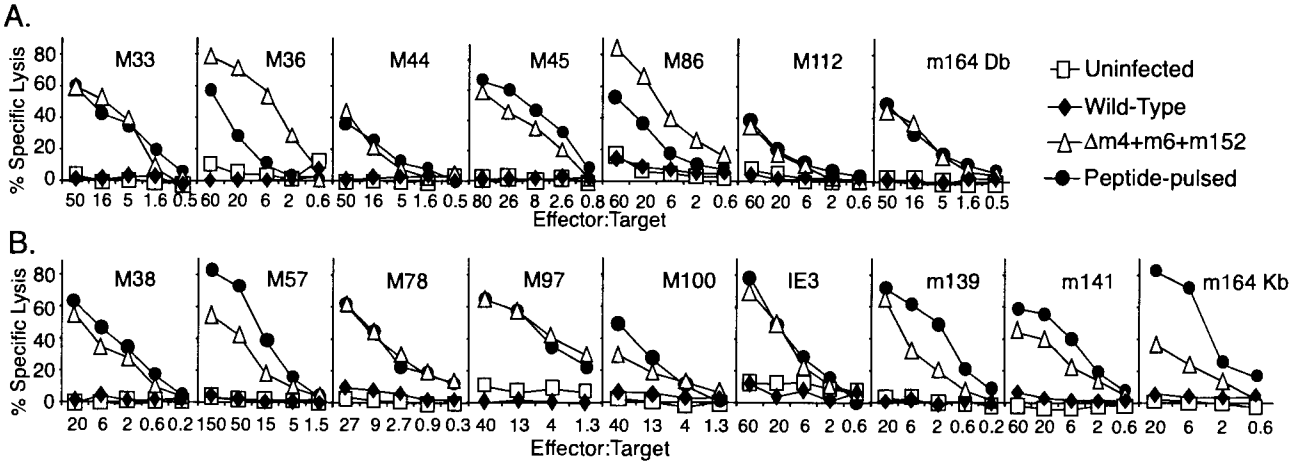

Figure 1:
Lysis of Δm04+m06+m152 but not Wild-Type MCMV by 16 epitope-specific CD8 T cell lines. 51Cr release assay on uninfected, infected, and peptide pulsed IC-21s. T cell lines were generated from splenocytes from C57BL/6 mice that had been infected with MCMV for > 11 weeks. Splenocytes were pulsed with MCMV peptide at 10-8M and cultured for 14 days prior to being used in the 51Cr release assay. A. Db restricted epitopes. B. Kb restricted epitopes.

Effects of Individual VIPRs on total cell surface K^b and D^b in macrophages and fibroblasts

The complete inhibition of CTL lysis when all three MCMV VIPRs are present is remarkably efficient. To address the relative contribution of the individual VIPRs to this inhibition, we used the panel of BAC-derived mutant viruses lacking each VIPR alone and in combination (Wagner et al., 2002) (Table 2). In order to correlate cell surface class I levels with the functional killing assays, we first assessed the impact of the VIPRs on cell surface class I levels on IC-21s using the same conditions that we used for ^{51}Cr release assays. Flow cytometry was performed 16 hours after infection in the presence of PAA (Figure 2). Infection with wild-type MCMV caused a marked downregulation of both H-$2K^b$ and H-$2D^b$ as compared to uninfected cells (figure 2A). However, cells infected with *Δm04+m06+m152* showed no reduction of cell surface class I compared to uninfected cells. This confirmed the conclusion of Wagner et al, that *m04*, *m06* and *m152* are the only MCMV genes that affect cell surface MHC class I (Wagner et al., 2002).

Table 2 Panel of mutant viruses.

Virus	VIPRs Expressed		
	m04	*m06*	*m152*
Wild-type	+	+	+
Δm04	-	+	+
Δm06	+	-	+
Δm152	+	+	-
Δm04+m06	-	-	+
Δm04+m152	-	+	-
Δm06+m152	+	-	-
Δm04+m06+m152	-	-	-

Table 2. Panel of mutant viruses, indicating which of the three identified MCMV VIPRs are expressed in each mutant.

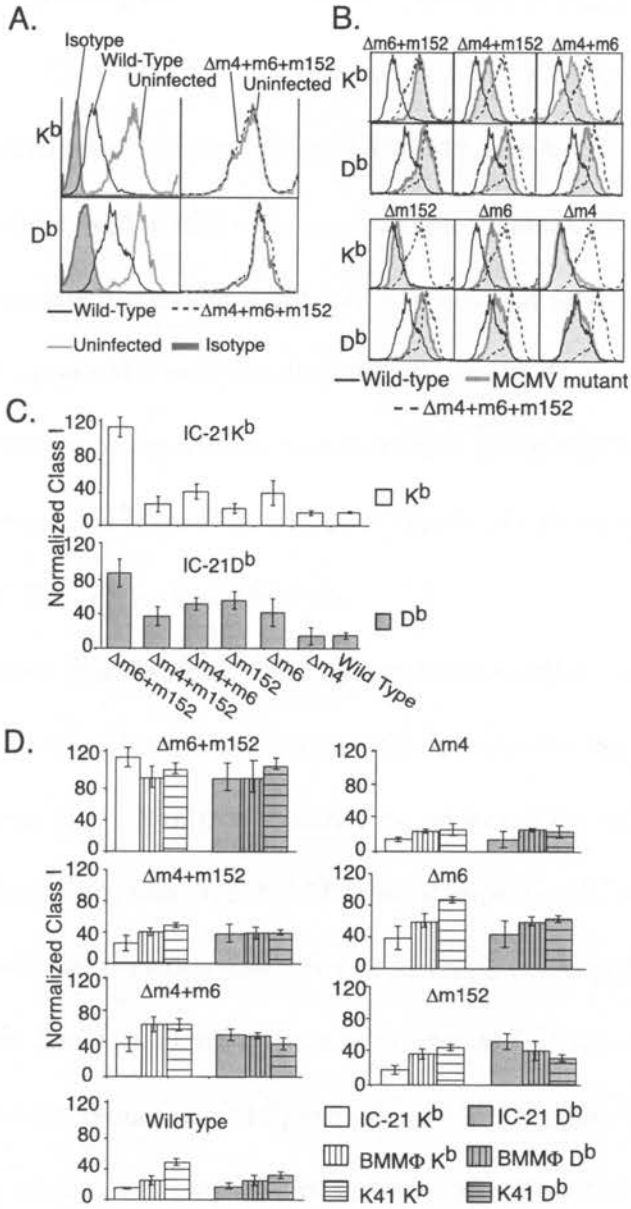

Figure 2. Impact of MCMV VIPRs on MHC class I cell surface expression. MCMV VIPR effects on MHC class I cell surface expression. A. Cell surface staining of the MHC class I on IC-21s. Uninfected = grey line, wild-type MCMV =solid black lines and Δm04+m06+m152 = dotted line. B. Cell surface class I expression in IC-21s comparing the effects of the individual VIPRs. The mutant virus infections = grey filled in histograms, wild-type MCMV=solid black line and Δm04+m06+m152 = dotted line. C. Average mean fluorescent intensity (MFI) of class I surface expression normalize to Δm04+m06+m152. Normalized results of 3 independent experiments the bars =the mean values of the percentage of class I expression relative to the class I expression Δm04+m06+m152. D. Similar to C Normalized results of 3 independent experiments the bars =the mean values of the percentage of class I expression relative to the class I expression Δm04+m06+m152 for IC-21s, BMMΦ and K41s (transformed fibroblasts).

We next assessed the impact of each of the individual VIPRs on cell surface expression of K^b and D^b, by comparing the results for the panel of mutants to that of wild-type and *Δm04+m06+m152* infected IC-21s (Figure 2B). These experiments were also performed in bone marrow derived primary macrophages (BMMΦ), and K41s. K41s (SV40 transformed H-2b+ fibroblasts) were used in order to compare these results with the previous study, which was performed in transformed fibroblasts after 12 hours of infection without PAA (Wagner et al., 2002). To facilitate this analysis, the mean fluorescence intensity (MFI) of K^b and D^b for cells infected with each of the mutants was expressed as a percentage of the MFI in cells infected with *Δm04+m06+m152* (Figure 2C). Figure 2D shows the mean +/- SD of three such normalized assays for IC-21s, K41s, and BMMΦ.

We note first that the overall pattern between all the cell types tested was very similar. The current study of fibroblasts (Figure 2D) gave similar results to the previously published report (Wagner et al., 2002). In general, the impact of the VIPRs in infected macrophages was similar to fibroblasts, although the differential impact on K^b versus D^b was less striking. In agreement with the previous results we observed in macrophages that (1) The impact of *m152* alone, seen in *Δm04+m06* infection, was greater on D^b than on K^b (49 % reduction of D^b compared to 66% reduction of K^b) and (2) m*06* had a greater impact on K^b than did *m152* (82% reduction by *m06* alone compared to 63% reduction by *m152* alone). *m04* had little impact on cell surface class I in all cell types tested. In the previous (Wagner et al., 2002) study of fibroblasts, it was observed that that *m04* antagonized the impact of *m152*. However, little evidence for such antagonism was observed under the conditions used in the current study.

The VIPR mutant panel in ^{51}Cr release assays

To examine the functional impact of the VIPRs, polyclonal T cell lines were used in ^{51}Cr release assays against IC-21s infected with each of the mutant viruses listed in table 2; an example is shown in Figure 3A. Each epitope specificity was tested at least three times. To integrate the data from multiple assays, the results for each assay were normalized, with lysis of each of the mutants expressed as a percentage of the lysis in cells infected with *Δm04+m06+m152* using an effector:target (E:T) ratio that was below the plateau of maximum killing. The mean and standard deviation of multiple assays were then calculated, as shown in Figure 3B.

Figure 4 shows the results of this analysis for each of the epitopes listed in Table 1. The data are grouped by lysis of individual mutant viruses, and are arranged to show the effect of the loss of a single VIPR in the left hand column, and the impact of the VIPR alone on the right. For example, for *m04*, the panel on the left shows *Δm04*, and on the right *Δm06+m152*. To facilitate comparison, the normalized cell surface staining for K^b and D^b from Figure 2 is shown alongside the normalized specific lysis. These experiments were performed over a period of 12 months using CTL lines specific for the same epitopes but derived from different animals. Nevertheless, the pattern of lysis of the individual mutants was consistent for each epitope, allowing statistically valid comparisons to be made. The asterisks indicate a significant increase in lysis compared to wild-type infection, and the crosses indicate a significant inhibition of lysis compared to *Δm04+m06 +m152*.

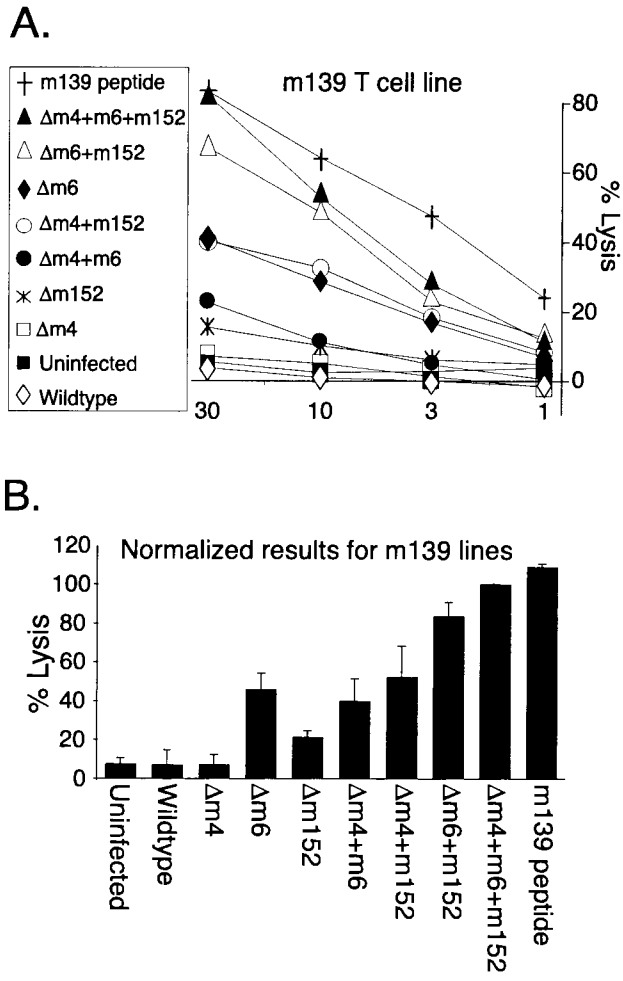

Figure 3. Experimental setup for 51Cr assay analysis. A. Representative ^{51}Cr assay using IC-21 as the target cell line. The highest effector to target ratio is identified. B. The normalized results of the ^{51}Cr assay with percent lysis of Δm04+m06+m152 at the highest effector to target ratio set to 100% and the percent killing of the other mutant viruses normalized to Δm04+m06+m152.

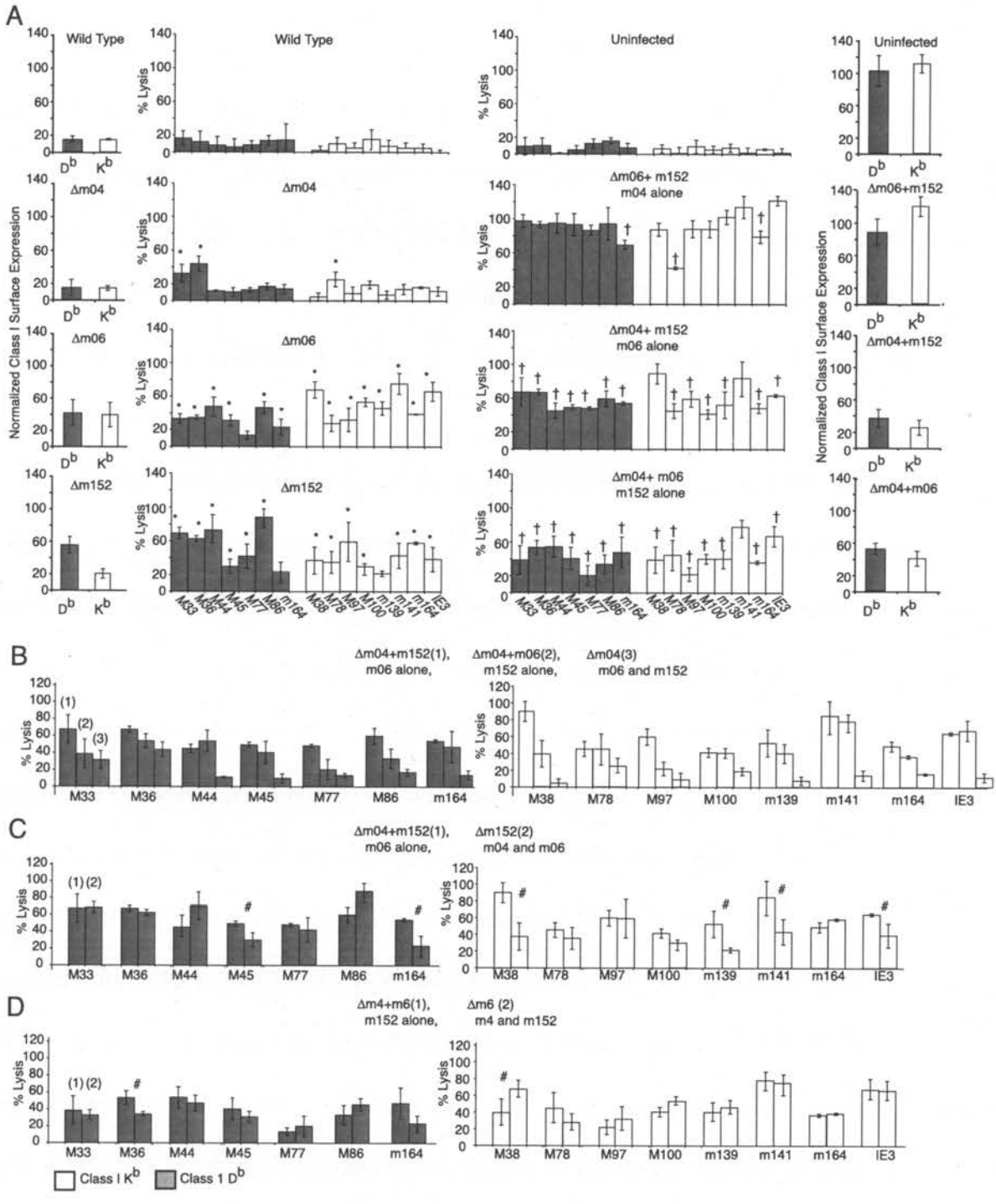

Figure 4 Comparison of the impact of the VIPRs alone and in combination on lysis by CTL specific for 15 epitopes. The results are normalized to Δm04+m06+m152, which was set at 100% lysis. The grey bars are the Db restricted peptide epitopes and the indicates a significant inhibition of recognition compared to Δm04+m06+m152 and # is a significant difference between the mutant viruses. A. The effects of the VIPRs on class I cell surface expression and CTL recognition. B. Normalized ^{51}Cr assays comparing the contributions of m06 and m152 alone and together. C. Comparing the addition of m04 to m06 at inhibiting CTL recognition. D. Normalized ^{51}Cr results m152 comparing to m152 with m04. Error bars represent SD.

This analysis has enabled us to draw several conclusions regarding the interactions of MCMV's three VIPRs:

(1) *All three VIPRs were needed to completely prevent lysis of wild-type infected cells by CTL specific for all fifteen epitopes.* While most antigens could be inhibited by the combined actions of *m06* and *m152*, there were three epitopes, M33, M36 and M78 that required the presence of *m04* for complete inhibition (i.e. Δ*m04*-infected cells were lysed significantly better then wild-type infected cells).

(2) *There was significant difference in efficacy of the individual VIPRs for different epitopes.* For example, for most epitopes, *m04* alone had no impact, evidenced by lysis of Δ*m06+m152* that was close to that of Δ*m04+m06+m152*. However, M78-specific CTL seemed peculiarly sensitive to the actions of *m04*: lysis by M78-specific cells was significantly impaired by *m04* alone, and M78-specific CTL could also lyse Δ*m04*-infected cells. In fact, for M78-specific CTL, the impact of *m04* alone was similar to that of *m06* alone, and only slightly less than that of *m152* alone. All other epitopes were more affected by *m06* and/or *m152* than by *m04*, with *m152* generally having the greatest impact. However, there was also differential susceptibility to these two VIPRs. For example, compare the ability of different D^b-restricted CTL to lyse cells infected with Δ*m152*. In addition, epitopes such as K^b-M100 were equally impacted by *m152* alone (60% inhibition) and *m06* alone (60% inhibition), whereas others such as K^b-M38 were much more affected by *m152* alone (60% inhibition) than *m06* alone (10% inhibition).

(3) Inhibition of killing was not directly proportional to downregulation of cell surface class I levels by m06 and m152. Overall, the two VIPRs that downregulate cell surface class I, *m06* and *m152*, also had the greatest impact on CTL lysis. However, for these two VIPRs, the

degree of downregulation of K^b and D^b did not directly predict their impact on CTL lysis. For instance, *m06* alone had a slightly greater impact on cell surface levels of K^b than did *m152* alone (82% inhibition in Δ*m04+m152* versus 65% inhibition in Δ*m04+m06*), yet *m152* alone was generally more potent at inhibiting killing by K^b-restricted CTL. Similarly, cell surface D^b was equally downregulated by *m06* and *m152*, yet *m152* more potently affected D^b-restricted killing.

(4) There was little or no differential impact of the individual VIPRs on K^b-restricted versus D^b-restricted CTL lysis. Our previous hypothesis that *m04* would play a role in K^b-restricted but not D^b-restricted killing is clearly incorrect: CTL specific for one K^b-restricted epitope (M78) and two D^b-restricted epitopes (M33 and M36) were able to lyse cells infected with Δ*m04*. In addition, the major difference in the impact of *m152* on K^b versus D^b that had been predicted based on pulse chase analysis (LoPiccolo et al., 2003) and cell surface staining (Wagner et al., 2002) was not observed.

Functional interactions between the VIPRs.

These assays have also enabled us to assess the extent to which the VIPRs act cooperatively- i.e. add to each other's impact, and also to ask whether any evidence for antagonism between *m152* and *m04* is seen at the functional level, as was predicted from surface MHC I expression levels (6). To facilitate this analysis we have displayed the data from Figure 4A again in Figures 4B, 4C and 4D; showing for each epitope the impact of an individual VIPR, either *m06* or *m152,* with the addition of *m04*. Several conclusions can be drawn:

1) *Strong co-operation was seen between m06 and m152 (figure 4B)*, which together inhibited lysis by most of the epitope-specific CTL. As described above, the necessity of a contribution

from *m04*, evidenced by lysis of cells infect with Δ*m04*, was only seen for M78-K^b, M33-D^b and M36-D^b.

2) m04 acted co-operatively with m06 (Figure 4C). Although *m04* alone had little impact, *m04* was observed to add to the ability of *m06* to inhibit lysis. When we compared the impact of *m06* alone (lysis of Δ*m04+m152*) to the impact of *m04* in combination with *m06* (Δ*m152*), it was clear that *m04* enhanced the ability of *m06* to inhibit CTL lysis. This was seen for two D^b-restricted epitopes and four K^b-restricted epitopes.

3) Interactions between m04 and m152 (Figure 4D). The addition of *m04* to *m152* generally had little impact. For only one epitope (M36), addition of *m04* added to the inhibition observed with *m152* alone ($p=0.05$). The opposite effect (antagonism) was observed for M38 ($p=0.005$).

_m04_, but not _m06_ or _m152_, acts more strongly in primary than in transformed macrophages.

We have previously observed that Δ*m04*-infected cells were readily lysed by several K^b-restricted CTL clones (Kavanagh et al., 2001a; LoPiccolo et al., 2003). Lysis of Δ*m04*-infected cells was particularly strong in primary BMMΦ. In the light of those previous results, we were surprised to find that Δ*m04*-infected IC-21s were not lysed by most of the epitope-specific CTL lines examined in this study, including those specific for m141 and M97. There were two major differences between the previous study and that reported here: (a) the previous study used CTL clones rather than short term peptide-driven polyclonal lines and (b) the previous study used primary macrophages rather than the transformed IC-21 cells used here. We wanted to reconcile the two studies. The CTL clones used in the previous study are no longer available, so we compared the ability of polyclonal CTL lines to lyse primary BMMΦ or IC-21s infected with

wild-type MCMV or mutant viruses. Figure 5 shows that polyclonal CTL lines specific for M86, m141 and M38 lysed BMMΦ infected with Δ*m04* whereas they failed to lyse IC-21s infected with the same virus. Thus a requirement for *m04* to completely inhibit CTL lysis is seen more strongly in primary BMMΦ than in transformed macrophages. This increased requirement for VIPR function in primary BMMΦ seemed specific for *m04*, because in the same assays, lysis of cells infected with either Δ*m06* or Δ*m152* was similar for primary and transformed macrophages. We also note that even in primary BMMΦ, *m04* displayed epitope-selectivity: *M45*-specific CTL failed to kill Δ*m04*-infected BMMΦ, consistent with our previous results using CTL clones in both macrophages and fibroblasts.

Timing of expression of MCMV antigens

One obvious explanation for the differential impact of the VIPRs on different epitopes could be the relative timing of expression of the viral antigens and the VIPRs. In primary fibroblasts, low levels of *m06* transcripts were observed from the beginning of infection (Reddehase et al., 1987). High levels of transcription were first seen for *m152*, followed by *m04* and then *m06*. Whereas *m04* and *m06* expression continued throughout the infectious cycle, *m152* expression decreased at later time points. We therefore postulated that *m152* might have a greater impact on the earliest expressed genes, and *m06* on those expressed later. In order to correlate gene expression with the results reported here, we examined gene expression by quantitative RT-PCR in IC-21s that were infected in identical conditions to those used for the ^{51}Cr release assays (Figure 6).

The expression kinetics of most genes examined here was rather similar, with expression beginning at 3 or 4 hours post infection, consistent with their expected early (E) kinetics. An

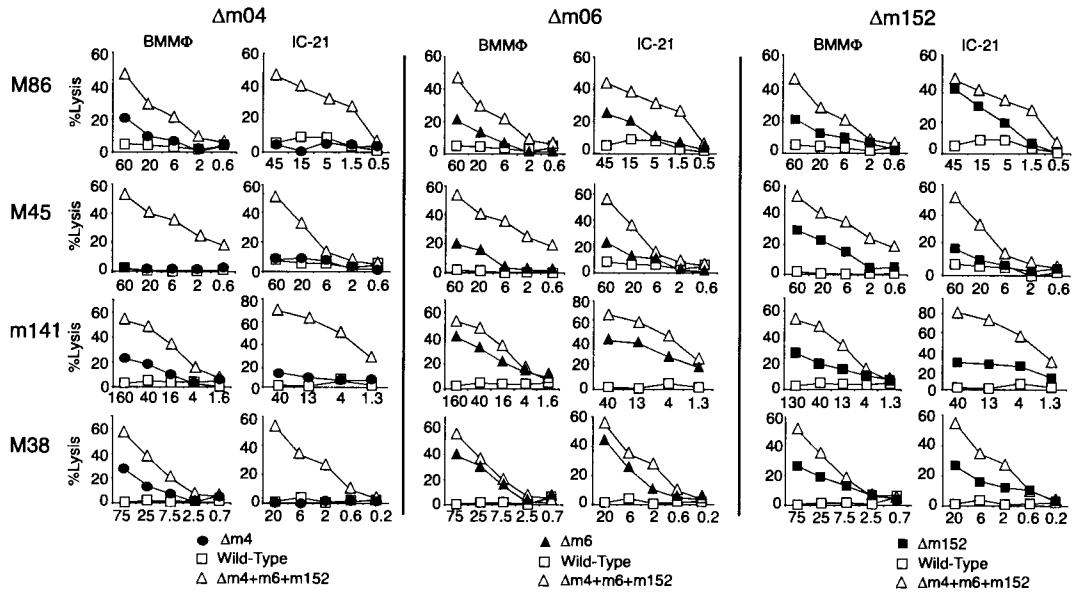

Figure 5. Comparison of the impact of the VIPRs in Primary and transformed macrophages The targets were either infected BMMΦ or infected IC-21s. The circles with the dashed lines = TKO targets, squares with the dashed lines =wild-type infected targets, the triangles=Δm04 infected targets, diamonds =Δm06 infected targets, and the stars=Δm152 targets. Error bars represent SD.

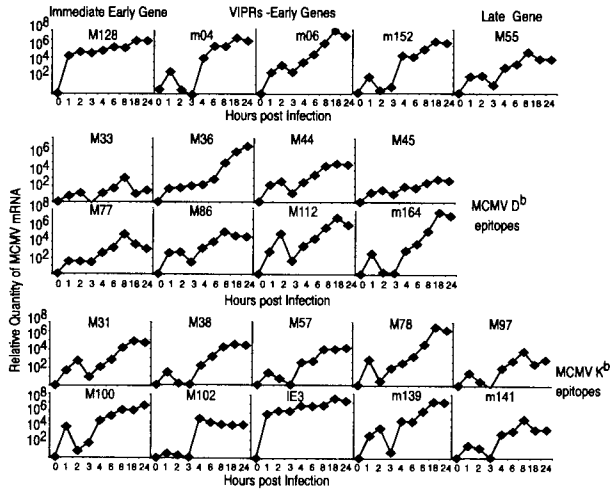

Figure 6. Kinetics of transcription of the VIPRs and antigens. IC21s were infected as above, and transcript levels assessed by realtime PCR. The relative gene expression of each of the MCMV genes was determined after each time point was normalized to a β-actin control.

unexpected small early peak of transcription for many genes at one hour post-infection was observed in repeated assays. Because of the high moi used here, this is probably due to virion-associated transcripts, as have been described for other herpesviruses (Bresnahan and Shenk, 2000; Sciortino et al., 2001; Terhune et al., 2004). A higher level of transcription of the known IE genes, *M122*, *m123* and *m128*, was detected at all time points. *m04* and *m152* were expressed with similar E kinetics, with transcription clearly detected at 4 hours post infection and then increasing slightly throughout the 24 hour period. Some transcription of *m06* was detected at all time points, but maximal transcription of *m06* occurred later than *m04* and *m152*, very similar to the pattern described for fibroblasts (Holtappels et al., 2002a).

We were unable to identify a correlation between the time that transcription of a gene was detected and the ability of the VIPRs to inhibit presentation of its encoded epitope. For example, the *IE3* transcript (M122) was one of only three transcripts we could identify that appeared earlier than both *m04* and *m152*, yet lysis of cells by IE3-specific CTL was efficiently inhibited by combined actions of *m06* and *m152* (figure 4). Similarly, there was nothing unusual about the transcription kinetics of M78 and *m04* that might explain the unique susceptibility of K^b-M78 to inhibition by *m04*.

Discussion

There have been almost no studies addressing the impact of VIPR multiplicity at a functional level. Studies with one or two epitopes can generate inaccurate over-generalizations, as we discovered with *m04* {(Kavanagh et al., 2001a) and Figure 4 above}. The fact that the CD8 T cell response of C57BL/6 mice to MCMV is remarkably broad- encompassing at least 26 epitopes identified to date- suggested that we now had a large enough range of epitope specificities to more accurately test some of these ideas.

These experiments confirmed that the VIPRs act as a single entity to inhibit CTL lysis. In fact, the three VIPRs acting together are remarkably efficient at inhibiting lysis of infected macrophages by CTL specific for all of the H-2^b-restricted epitopes tested. Thus, the surprisingly modest impact of the VIPRs in vivo in C57BL/6 mice (Gold et al., 2004) is not likely to be explained by there being major populations of CD8 T cells that are unaffected by the VIPRs. The results in Figure 1 show that the three VIPRs cooperatively have a powerful impact on CTL efficacy, which is likely to translate into at least a quantitative impact on CTL efficacy in vivo, as has been demonstrated for the M45 epitope (Holtappels et al., 2004). Furthermore, a profound impact was seen for all fifteen epitopes tested, which cover the majority of the CTL response in C57BL/6 mice (Munks et al., 2006). This highly efficient inhibition of lysis is slightly at odds with the results obtained by the Reddehase and Koszinowski groups in the BALB/c system, where the VIPRs sometimes fail to completely inhibit lysis in ^{51}Cr release assays. Since we (Lu et al., 2006b)and the Reddehase group (Holtappels et al., 2004) have at least partially confirmed each other's results, it seems likely that these are genuine mouse strain differences, rather than differences in experimental methodology. Further work is needed to uncover the basis of these

strain differences. More importantly, the paradox between the profound inhibition of lysis observed in ^{51}Cr release assays in vitro, and the fact that the VIPRs do not have a major impact on virus survival or the CTL response in vivo ((Gold et al., 2002; Gold et al., 2004; Holtappels et al., 2004) and M. Munks et al, ms in print), remains to be resolved.

Our previous results suggested that m04/gp34 plays a greater role in macrophages than in fibroblasts (LoPiccolo et al., 2003). The results in the current paper provide an interesting twist to this story, demonstrating a greater need for a contribution from m04/gp34 to inhibit antigen presentation in primary macrophages than in transformed macrophages. This was particularly intriguing because the impact of *m06* and *m152* was similar in primary and transformed macrophages. Transcription of MCMV genes could also be different in the two cell types. We would predict that the altered gene expression profile is responsible for the differential impact of m04/gp34. However, because the mechanism of m04/gp34's inhibition of antigen presentation remains unknown, it is difficult to speculate on the mechanism of the difference between cell types.

There is strong evidence in the literature that different VIPRs act preferentially on different class I isoforms (Kavanagh et al., 2001a; Machold et al., 1997; Wagner et al., 2002; Wiertz et al., 1996a). Since a differential impact of MCMV VIPRs on K^b versus D^b was clearly observed in previous studies (Kavanagh et al., 2001a; Wagner et al., 2002), we fully expected to see preferential activity of *m06* and *m152* against K^b versus D^b-restricted presentation. Surprisingly, we found no consistent difference in the impact of any VIPR on these two class I isoforms. Instead, a difference in individual epitopes' susceptibility to the VIPRs was much more striking than any overall difference between the class I isoforms. Such epitope-selectivity

was not readily explained by the timing of gene expression of individual antigens relative to the different VIPRs.

This study also revealed that the impact of individual VIPRs on total cell surface class I levels did not correlate directly with their impact on CTL lysis. In particular, *m152*'s impact on CTL lysis was disproportionately greater than its impact on cell surface class I. The explanation that this disproportionate impact would be explained by *m152*'s impact on NKG2D ligands was appealing, but experimental data suggests that NKG2D inhibition contributes only very modestly to *m152*'s overall impact on CTL lysis (ms in preparation A.K.P.).

There was no obvious correlation between the sequence of the peptide epitope, nor its functional avidity (Table 1), and susceptibility to individual VIPRs. In this study we have quantified antigen transcript levels and the final outcome of antigen presentation (peptide titration in ^{51}Cr release assays in Δ*m04+m06+m152*-infected cells, Table 1). However, the entire intervening sequence of events- protein synthesis, proteasomal degradation, TAP transport, loading onto MHC class I, and stability of the resultant complexes- have not been quantified, and it is quite possible that quantitative differences in these processes will affect the relative impact of the VIPRs. The contribution to epitope-selectivity of such quantitative differences, and qualitative considerations such as the site and nature of the MHC class I cargo targeted by each VIPR, remains to be determined.

Since the discovery of viral genes that inhibit antigen presentation (VIPRs) to CD8 T cells, it has been intriguing that many herpesviruses encode multiple genes with this function. KSHV encodes at least two (*K3* and *K5*) (Coscoy and Ganem, 2000; Ishido et al., 2000; Stevenson et al., 2000), HCMV at least 4 (*US2*, *US3*, *US6* and *US11*) (Reviewed in (Loenen et al., 2001)), and rhesus CMV encodes homologs of all 4 HCMV VIPRs and also has an additional

locus that prevents class I heavy chain synthesis (Colin Powers and K. Frueh, ms in preparation). As described above, MCMV encodes three identified VIPRs. Several hypotheses have been proposed to explain the advantage of multiple VIPRs. Ahn et al noted that HCMV's VIPRs are expressed sequentially (Ahn et al., 1996). US3, which retains class I in the ER, is expressed first, and these authors proposed that it may serve to "set up" class I for more efficient destruction by the later expression of US2 and US11. Whether different VIPRs act synergistically in this way or merely additively, the comprehensive study reported here demonstrates that the VIPRs have evolved to function in concert to completely inhibit CD8 T cell lysis.

CMVs have been co-evolving with their hosts' immune systems since before the mammalian radiation 60-80 million years ago. Because MCMV is a natural pathogen of the laboratory mouse (*Mus musculus)*, most of the multiple layers of intricate immune modulation, including those that are highly species specific, are likely to be fully functional in this model. Such layers of immune modulation may help to explain the paradox that the VIPRs act with exquisite co-ordiantion to inhibit CTL lysis in vitro but have a rather minor impact on viral pathogenesis in intact mice in vivo.

Footnotes:

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Chapter 3

The role of NKG2D signaling in inhibition of CTL lysis of murine cytomegalovirus infected cells by *m152*/gp40

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Abstract.

Three genes of murine cytomegalovirus (MCMV)- *m04*, *m06*, and *m152*, act together to powerfully impact the ability of primed cytotoxic CD8 T lymphocytes (CTL) to kill virus-infected cells. Of these three, the impact of *m152* on CTL lysis appears greater than would be expected based on its impact on cell surface MHC class I. It has therefore been proposed that *m152's* ability to impair CTL function involves molecular targets other than MHC class I. In addition to MHC class I, *m152* targets the Rae-1 family of ligands for NKG2D. NKG2D functions as a costimulatory molecule on CD8 T cells, and in cases where MHC I is limiting, has been shown to facilitate CD8 T cell effector functions. We therefore tested the extent to which *m152*'s ability to inhibit CTL lysis of MCMV-infected cells could be accounted for by its inhibition of NKG2D signaling. As predicted by the literature, only cells infected with MCMV lacking *m152* displayed measurable levels of NKG2D ligands. To determine whether NKG2D signaling contributed to the ability of CTL to lyse these cells, we used a blocking anti-NKG2D antibody. Blocking NKG2D signaling affected the killing of MCMV-infected cells for only a small minority of epitopes. Even for those epitopes, the impact of *m152* on CTL lysis was much greater than the impact of inhibition of NKG2D signaling. We conclude that downregulation of NKG2D ligands by MCMV makes only a small contribution to the impact of *m152* on CTL lysis, and only for a small subset of CTL.

Introduction

All Cytomegaloviruses interfere with the MHC class I pathway of antigen presentation. Although the biological ramifications of this interference remain unclear, the variety of molecular mechanisms employed by different CMVs to attack MHC class I is impressive. Murine Cytomegalovirus (MCMV) encodes three viral genes that interfere with antigen presentation to CD8 T cells (VIPRs), *m04, m06, and m152* (Yewdell and Hill, 2002). This interference has been demonstrated by their ability to impair CTL lysis of infected targets.

m06 binds class I and diverts it to lysosomes for destruction (Reusch et al., 1999). *m152* causes nascent class I to be retained in the ER cis Golgi intermediate compartment (del Val et al., 1992; Thale et al., 1995; Ziegler et al., 1997). *m04* is largely ER resident but binds to some class I and escorts it to the cell surface: in different circumstances *m04* can either increase or decrease susceptibility to CTL lysis (Holtappels et al., 2006; Kavanagh et al., 2001b; LoPiccolo et al., 2003; Pinto et al., 2006). Both *m06* and *m152* reduce cell surface expression of class I, whereas *m04* either has no impact on cell surface class I or slightly elevates the levels (Kleijnen et al., 1997; Pinto et al., 2006; Wagner et al., 2002). We recently assessed the relative impact of the three VIPRs on the ability of CTL specific for 15 H-2^b-restricted epitopes to lyse infected cells (Pinto et al., 2006). This study revealed that the three VIPRs act powerfully together to impair CTL lysis of infected cells. We used a panel of mutant viruses generated by Wagner et al (Wagner et al., 2002), in which the three VIPRs are deleted either alone or in combination; This revealed that *m06* and *m152* made the major contribution to this inhibition, with a contribution from *m04* being needed only for certain epitopes. Thus, the two VIPRs that downregulate cell surface class I also had the greatest impact on CTL lysis.

However, the degree of cell surface class I downregulation did not correlate precisely with inhibition of CTL lysis. This was most marked in the case of *m152*, which has only a moderate impact on ER export of nascent class I and on cell surface class I levels, but had a profound impact CTL lysis. The fact that *m152*'s impact on CTL lysis was greater than would be expected from its impact on cell surface class I was also noted by Wagner et al in the original description of their panel of VIPR-deficient viruses. These authors postulated that the discrepancy might be explained by a recently uncovered second function of *m152*, namely its ability to prevent expression of the RAE-1 family of ligands for NKG2D (Krmpotic et al., 2002; Lodoen et al., 2003).

NKG2D is an activating receptor found on NK cells and on antigen-experienced CD8 T cells. Most NKG2D ligands are not constitutively expressed but are induced by transformation or stress, notably by viral infection (Bauer et al., 1999; Gasser et al., 2005). In the mouse there are three groups of known NKG2D ligands: RAE-1 (retinoic acid induced and expressed) molecules, H-60 and MULT-1. These molecules are a major target of CMV immune evasion: four MCMV genes (m138, m145, m152 and m155) are involved in preventing their expression on infected cells (Hasan et al., 2005; Krmpotic et al., 2002; Krmpotic et al., 2005; Lenac et al., 2006; Lodoen et al., 2004).

m152 prevents expression of the RAE-1 family of ligands for NKG2D. Thus, *m152* has at least two targets- MHC class I and RAE-1 molecules. NKG2D can function as a costimulatory molecule on CD8 T cells (Jamieson et al., 2002). In fact, for two HCMV-specific CTL clones, costimulation through NKG2D was necessary to enable lysis of infected targets once the HCMV VIPRs had downregulated MHC class I (Groh et al., 2001). We therefore wondered whether the apparently disproportionate impact of *m152* on CTL lysis could be

explained by its ability to inhibit RAE-1 expression, depriving the CTL of NKG2D-mediated costimulation.

In the current study we investigated expression of NKG2D on MCMV-specific CD8 T cells, its role in augmenting CTL lysis, and the role that RAE-1 downregulation plays in *m152*'s ability to inhibit CTL lysis. We found that all MCMV-specific CD8 T cells come to express NKG2D over time. However, costimulation through NKG2D played only a small role in promoting CTL lysis, and only for a subset of epitope specificities. For these specificities, inhibition of NKG2D signaling contributed to *m152*'s impact on CTL lysis.

Materials and Methods

Cells IC-21, a SV40-transformed macrophage cell line from C57BL/6 mice (Cavanaugh et al., 1996), a gift from Ann Campbell Eastern Virginia Medical School, were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4.5g/L Glucose, and1.5g/L sodium bicarbonate and antibiotics. K42 (SV-40-transformed H-2^b fibroblasts, a gift from Marek Michalak University of Alberta) and B16-FL (Driessen et al., 1999) (a gift from Glen Dranoff, Harvard Medical School) were cultured in DMEM supplemented with 10% FBS and antibiotics. Lymphokine activated killer (LAK) cells were prepared by incubating C57BL/6 mice splenocytes for 4 days in RPMI complete medium containing 1 μg/ml IL-2.

Viruses Wild-type BAC-derived MCMV strain MW97.01 (Wagner et al., 1999), D*m04*, Δ*m06*, Δ*m152* Δ*m04+m06*, Δ*m04+m152*, Δ*m06+m152*, and Δ*m04+m06+m152* (Wagner et al., 2002) were grown on C57BL/6 Mouse embryo fibroblasts (MEF), then purified by pelleting over a 15% sucrose cushion (Brune, 2005). Each virus stock was titered without centrifugal enhancement on BALB-3T3 cells. The mean of three virus titrations was used to calculate titers for use in these assays.

T cell lines Female C57BL/6 (B6) mice were purchased from NCI Fredrick (Baltimore, MD) or The Jackson Laboratory (Bar Harbor, ME), and infected with either $1x10^6$ or $5x10^6$ pfu of MCMV. Spleens were harvested from mice that had been infected at least 11 weeks previously.

As a source of DC-enriched splenocytes to stimulate CTL lines, we used spleens from mice that had been injected 14 days previously with the Flt-3 ligand-secreting tumor, B16FL. Splenocytes from B16FL-injected mice were γ-irradiated and pulsed with peptide at 10^{-8}M, and cultured with splenocytes from MCMV-infected mice in RPMI supplemented with 10% FBS for 3 days, after which 10U/ml recombinant IL-2 (eBioscience) was added. After 10 days, the percentage of CD8 T cells responding to the simulating peptide epitopes was assessed by intracellular cytokine staining (ICS), and the cells used in ^{51}Cr release assays.

Antibodies and tetramers anti-gB and anti-gH, (a gift from Lambert Loh (Loh et al., 1988; Loh and Qualtiere, 1988; Rapp et al., 1994)). Anti-NKG2D (MI-6)(Jamieson et al., 2002) anti-pp89 (Del Val et al., 1988) were purified on Protein A, G (Sigma/Aldrich) columns and conjugated to FITC (Molecular Probes), PE, or APC (Cyanotech) according to published protocols (Hardy, 1986). anti-IFN-g (XMG1.2) and anti-CD8 (53-6.7) were purchased from eBioscience. Staining with NKG2D tetramers was as previously described (Diefenbach et al., 2000). Staining with MCMV tetramers were generated using MCMV peptides (Munks et al., 2006), following the previously described protocols (Altman et al., 1996) and coupled to streptavidin-PE.

FACS analysis IC-21 cells were infected overnight with the panel of mutant viruses at an MOI of 20 in the presence of 0.3 mg/ml phosphonoacetic acid (PAA, Sigma/Aldrich). For intranuclear staining, cells were stained with tetramer specific for anti-NKG2D ligands then fixed with CytoFix/CytoPerm (BD bioscience) then permeablized by incubation for 5 minutes with 0.1% Triton X in PBS. The cells were then stained for 30min with anti-pp89 in the presence of 0.1% Triton X. The cells were washed 3x in 0.1% Triton X then washed 1x in FACS buffer before analysis. Intracellular cytokine staining was used to demonstrate the antigen specificity. For

intracellular cytokine staining, T cell lines were incubated with their appropriate peptide at 10^{-6}M in the presence of brefeldin A (Golgi-plug, BD Bioscience) for 6 hours, stained with anti-CD8 and anti-NKG2D, then fixed with CytoFix/CytoPerm (BD bioscience) pemeabilized with PermWash (BD bioscience) and stained with anti-IFN-γ. All cells were analyzed using a FACS Caliber flow cytometer (BD Bioscience) in conjunction with Cell Quest (BD Bioscience). All further analyses were performed using FlowJo software (Treestar).

Assay for cell-mediated cytotoxicity 10^4 IC-21 or K42 target cells per well were plated in 96 well plates, infected with the indicated viruses at an MOI of 20 and labeled with 100μCi ^{51}Cr (NEN) in the presence of 0.3 mg/ml of PAA for 12 hr. For peptide-pulsed targets, ^{51}Cr-labeled cells were incubated with 1 μM peptide for 1 h at 37°C and then washed three times. Effector T cells were incubated with appropriate concentration of anti-NKG2D (MI-6) for 1 h then the effector cells were added at the indicated effector to target (E: T) ratios. The cells were incubated for 6 h, and supernatants were harvested and assayed with a Topcount scintillation counter (Packard Instruments, Meriden, Conn.). Background ^{51}Cr release was determined by incubating targets with medium alone, and total ^{51}Cr release by lysing targets with medium containing 1% Nonidet P40 (USB, Cleveland OH). Percent specific lysis was calculated as follows: (experimental cpm - background cpm)/(total cpm - background cpm).

Statistics Statistical significance was determined using Students T test. A paired two-tailed T test was used and all comparisons were determined to be of equal variance.

Results

NKG2D is expressed on MCMV-specific CD8 T cells in vivo and in vitro

According to the literature, NKG2D is expressed on murine CD8 T cells within several days of activation. Because CMV-specific memory CD8 T cells have an unusual phenotype, and usually fail to express the costimulatory molecules CD28 and CD27, we first assessed whether MCMV-specific CD8 T cells express NKG2D. C57BL/6 mice were infected with MCMV then sacrificed at various times post infection. MCMV specific responses were assessed by intracellular cytokine staining (ICS) for IFN-γ, and NKG2D expression determined by co-staining with the NKG2D specific antibody. Figure 1 shows that the rate of acquisition of NKG2D differed for different epitope-specific responses. CD8 T cells specific for m139 and m141 were mostly NKG2D positive from day 7-post infection, whereas it took 30 days before the majority of CD8 T cells specific for M38 and M45 expressed NKG2D. However, most cells continued to express NKG2D at 123 days post infection.

The phenomenon of *m152*'s disproportionate impact on CTL lysis has been described using in vitro CTL lines. To confirm that NKG2D expression was maintained upon culture, we generated short term polyclonal CTL lines specific for four epitopes and assessed NKG2D expression. Figure 1c shows that the majority of cells in these lines continued to express NKG2D.

Impact of *m04*, *m06* and *m152* on expression of NKG2D ligands in infected macrophages.

MCMV downregulation of NKG2D ligands has been described in infected fibroblasts. As described above, *m152* has been shown to downregulate the RAE-1 family of NKG2D ligands. Neither *m04* nor *m06* has been reported to have any impact on NKG2D ligands.

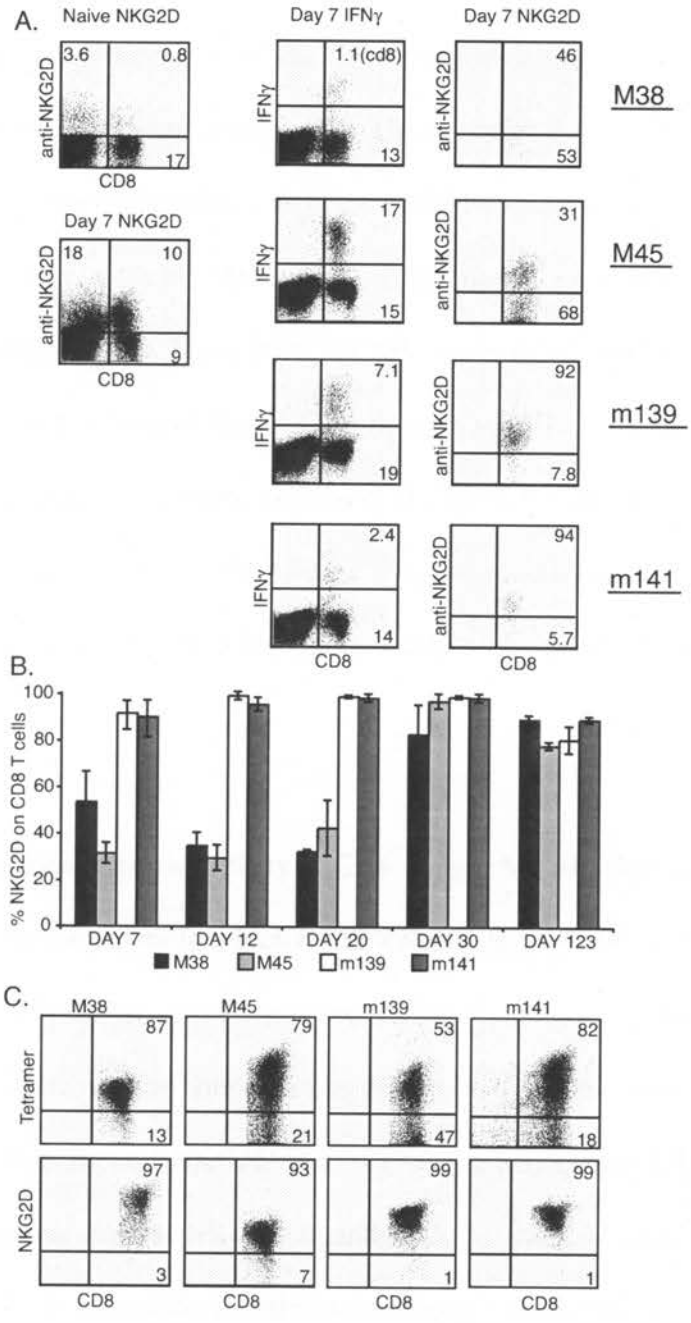

Figure 1.
NKG2D expression on MCMV specific CD8 T cells. A. The total NKG2D expression on lymphocytes from days 0 and 7 post infection of B6 mice. Followed by a representative example, from a single mouse at day 7 post infection, of the IFN- expression used to identify antigen specific CD8 T cells. Then the NKG2D expression on CD8+ IFN-γ+ antigen specific T cells from the second column. B. Normalize totals of antigen specific CD8+ NKG2D+ from splenocytes of 5 mice on days 7, 12, 20, 30, and 123 post infection. C. FACs plots of antigen specific CD8 T cell lines gated on CD8+ tetramer+ population, and below is the NKG2D expression on the tetramer+ population.

Because our recent characterization of the impact of the VIPRs on CTL lysis was performed using the macrophage cell line IC-21, we wanted to confirm that *m152* impacted RAE-1 expression in this cell line. Staining with NKG2D tetramers demonstrated that IC-21s constitutively express NKG2D ligands (figure 2). Upon infection with MCMV, expression was downregulated, and this downregulation was not relieved by infection with viruses lacking *m04* or *m06*. However, infection with MCMV lacking *m152* allowed expression of NKG2D at almost the same level as uninfected cells. Three other MCMV genes m145, m155 and m138 are known to downregulate either one or both of the NKG2D ligands MULT-1 and H60 (Hasan et al., 2005; Krmpotic et al., 2005; Lenac et al., 2006; Lodoen et al., 2004) but in the case of IC-21s, infection with a virus lacking *m152* restores NKG2D ligand expression to a level similar to that observed in uninfected cells, suggesting that IC-21s predominantly express only the RAE-1 NKG2D ligand.

Impact of NKG2D blockade on the ability of CTL to lyse MCMV-infected cells.

In order to assess the extent to which *m152*'s ability to inhibit CTL lysis can be attributed to its downregulation of NKG2D ligands, we used the blocking anti-NKG2D antibody to inhibit CTL lysis. We first determined the concentration of anti-NKG2D that could completely inhibit NKG2D signaling by titrating anti-NKG2D in a ^{51}Cr release assay using LAK cells against two cell lines that constitutively express NKG2D ligands: IC-21s and K42 cells. Figure 3 shows that maximal inhibition of lysis was achieved using an antibody concentration of 50 micrograms/ml. We note that this concentration did not completely inhibit LAK lysis of IC-21s, presumably because other non-NKG2D-mediated LAK receptors are involved in the killing of that cell line

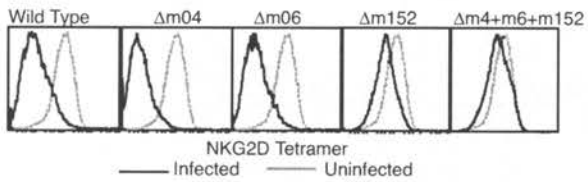

Figure 2
NKG2D ligand expression on IC-21s. FACs plots with grey line is the NKG2D ligand expression level on uninfected IC-21s. The black line is the NKG2D ligand expression level on infected IC-21s with the panel of mutant MCMV viruses.

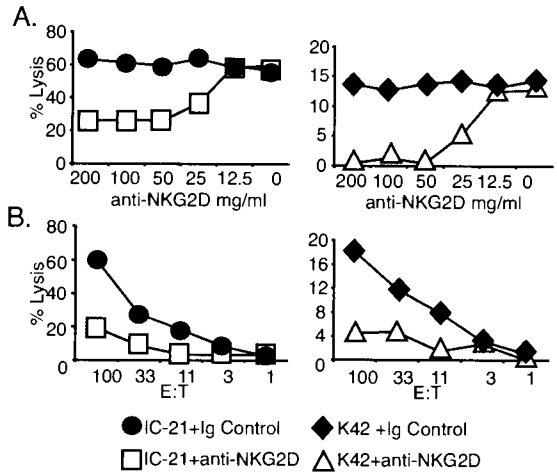

Figure 3
Inhibition of LAK cell killing by anti-NKG2D. A. Titration of the NKG2D blocking antibody on LAK cells using IC-21s and K42 cells as targets. B. Titration of the LAK cells using a concentration of 50mg/ml of the anti-NKG2D blocking antibody with IC-21s and K42 cells as targets.

(Jamieson et al., 2002). We concluded that anti-NKG2D at a concentration of 50μg/ml could be used to block NKG2D signaling.

We then tested the impact of NKG2D blockade on the ability of MCMV-specific CTL to lyse IC-21 cells infected with the panel of VIPR-deficient MCMV viruses created by Wagner et al (Wagner et al., 2002). Short-term polyclonal CTL lines were tested in ^{51}Cr release assays against targets incubated with anti-NKG2D or rat IgG at 50 μg/ml. A typical assay is shown in figure 4a. We tested CTL lines against 8 different epitope specificities, and each epitope-specificity was tested at least three times. To integrate the data from multiple assays, the results for each assay were normalized, with lysis of each of the mutants, with or without anti-NKG2D, expressed as a percentage of the lysis in cells infected with *Δm04+m06+m152* in the presence of rat IgG. An effector:target (E: T) ratio that was below the plateau of maximum killing was used for the calculation. The mean and standard of multiple assays were then calculated.

Figure 4B shows the results of these normalized assays for eight CTL specificities. In some cases, NKG2D blockade resulted in reduced CTL lysis: significant reduction of lysis by the addition of NKG2D is indicated with an asterix. The impact of NKG2D blockade was most clearly seen for M97-specific CTL. Interestingly, NKG2D blockade reduced lysis of targets infected with viruses that lacked *m152*, but not of targets infected with viruses in which *m152* was present. This result is consistent with the above observation that IC-21 cells infected with a virus containing *m152* do not express ligands capable of engaging NKG2D.

Several other CTL specificites, M38, m139 and m141, and also showed some impact of NKG2D blockade, although not for all viruses containing *m152*. However, for the majority of CTL, NKG2D blockade caused little or no reduction of target lysis.

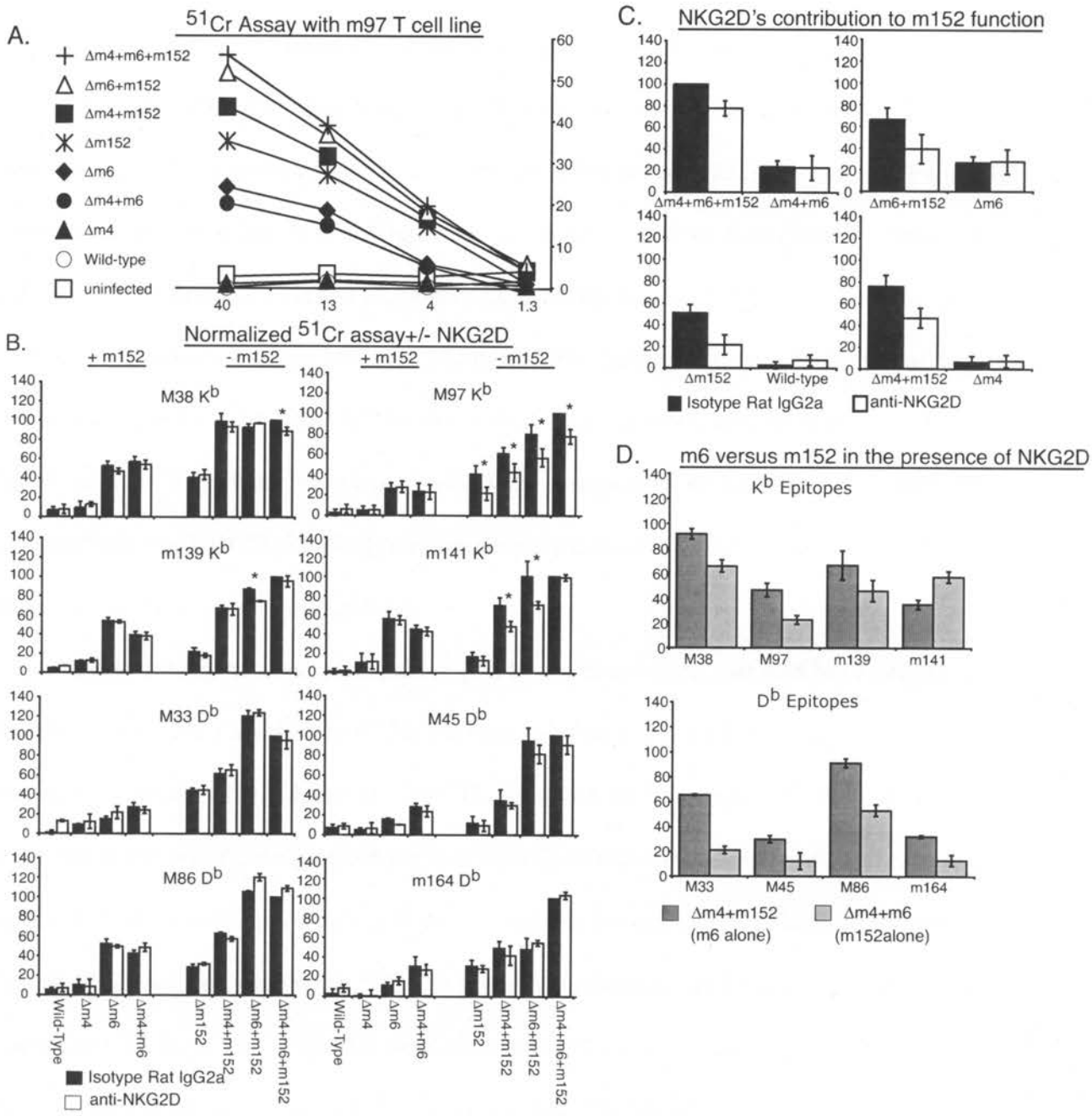

Figure 4
The effect of NKG2D on CD8 T cell killing. A. Representative 51Cr release assay in the presence of 50μg/ml of the anti-NKG2D. Each symbol is representative of a different mutant virus. B. Normalized results of the 51Cr assays with anti-NKG2D (White bar) or the control IgG (Black bar). At least 3 51Cr assays were completed for each epitope and the results were normalized and pooled together. The error is the SEM between the assays. C. The effect of anti-NKG2D on inhibiting the killing by the M97 T cell line in targets lacking m152. D. Comparison of the effect of m152 to m06 alone in the presence of anti-NKG2D.

Contribution of NKG2D blockade to *m152*'s impact on CTL lysis.

For those CTL that were affected by NKG2D blockade, we were interested to determine how much of *m152*'s impact could be attributed to its impact on NKG2D signaling. Figure 4C shows the normalized results from Figure 4B, re-displayed in order to contrast the impact of *m152* with the impact of NKG2D blockade. Even for the specificites that were most impacted by NKG2D blockade, such as M97, the addition of *m152* caused a greater reduction of target cell lysis than was achieved by NKG2D blockade alone (e.g. for M97, 85% lysis of Δ*m04+m06+m152* in the presence of anti-NKG2D, compared with 25% lysis of Δ*m04+m06*). We conclude that NKG2D blockade contributes only modestly to *m152*'s impact on CTL lysis, and then only for a minority of epitopes.

This investigation was prompted in part by the observation that *m06* has a greater impact on cell surface class I levels than *m152*, but generally has less impact on CTL lysis. In order to compare the impact of *m152* and *m06* on CTL lysis with *m152*'s impact on NKG2D signaling taken out of the picture, we graphed the lysis of cells infected with Δ*m04+m06* (*m152* acting alone) and Δ*m04+m152* (*m06* acting alone), both in the presence of anti-NKG2D antibody. Figure 4D shows that, even when NKG2D signaling is blocked, *m152* generally had a greater impact on CTL lysis than *m06*. We conclude that *m152*'s disproportionate impact on CTL lysis cannot solely be explained by its ability to inhibit NKG2D ligands.

The impact of NKG2D blockade is not determined by peptide-MHC density.

We were intrigued by the finding that CTL specific for different MCMV epitopes differed in their susceptibility to NKG2D blockade. Co-stimulation via NKG2D might be required when the target cell presents a low avidity target for the TCR. We postulated that those

MCMV-specific CTL that were affected by NKG2D might be specific for epitopes that are poorly presented by infected cells, resulting in a suboptimal number of peptide MHC complexes at the cell surface. To test this idea, we performed a CTL assay in the presence or absence of anti-NKG2D using IC-21 targets incubated with a range of peptide concentrations. If NKG2D costimulation contributes to T cell activation when peptide MHC density is low, we expected that anti-NKG2D would inhibit lysis at lower peptide concentrations. However, Figure 5 shows that anti-NKG2D did not inhibit CTL lysis at any concentration of peptide on uninfected IC-21 cells for any of the epitopes tested.

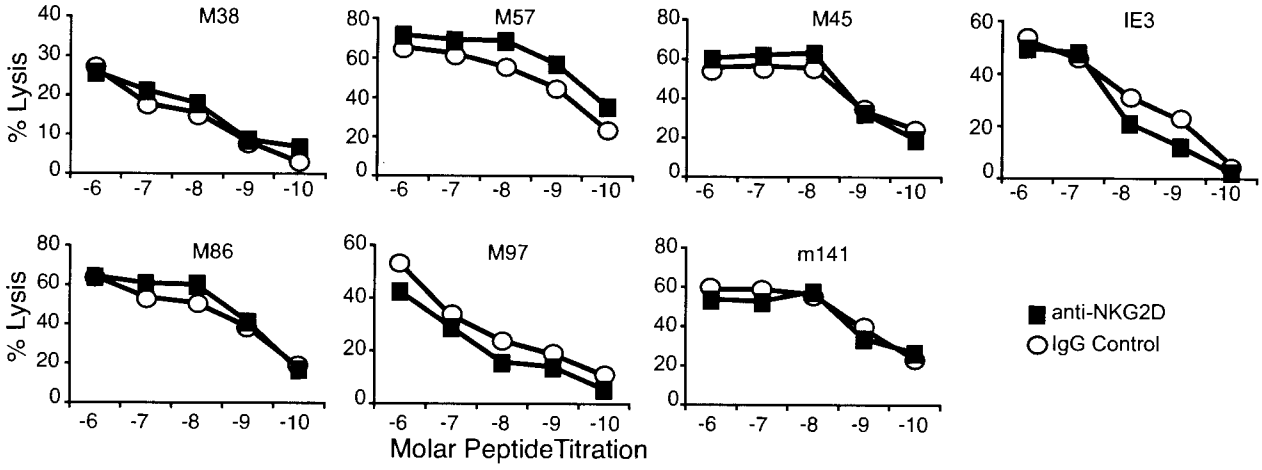

Figure 5
The effect of peptide density on anti-NKG2D inhibition of CTL killing. 51Cr Assay peptide titration the closed squares are treated + anti-NKG2D and the open circles are treated with IgG control with the MCMV specific T cell lines shown.

Discussion

The fact that *m152* efficacy in inhibiting CD8 T cell function seems greater than its impact on MHC class I levels has been noted by several investigators (Holtappels et al., 2004; Kavanagh et al., 2001a; Pinto et al., 2006; Wagner et al., 2002). A simple explanation for this discrepancy seemed to be offered by the finding that, in addition to targeting MHC I, *m152* could also target the RAE-1 family of NKG2D ligands. Since activated CD8 T cells express NKG2D, inhibition of NKG2D's costimulatory activity seemed a likely explanation for *m152's* disproportionate impact. This explanation was rendered even more plausible by the clear demonstration of the importance of NKG2D costimulation in enabling lysis of HCMV-infected targets by two pp65-specific CTL clones (Groh et al., 2001). However, the data presented here provide little support for this hypothesis. NKG2D inhibition had no impact on CTL lysis by polyclonal CTL lines specific for most of the MCMV epitopes tested here (Figure 3B). NKG2D inhibition did, however, impact lysis by CTL specific for 4 epitopes: M97, M38, m141 and m139. Notably, this inhibition was only seen for targets infected with viruses lacking *m152*, confirming that *m152* effectively inhibits the majority of NKG2D ligand expression in the IC-21 cells used in this assay. However, even for the epitope specificites for which NKG2D blockade did inhibit lysis, NKG2D blockade had less of an impact on target lysis than did adding *m152* to the genes expressed by MCMV (Figure 3C). When we directly compared the efficacy of *m152* and *m06* at inhibiting lysis in the presence of NKG2D blockade, it was still evident that *m152's* impact on CTL lysis was greater than that of *m06* (Figure 3D); despite the fact that *m06* has a greater impact on cell surface class I levels (Pinto et al., 2006; Wagner et al., 2002). *m152's* disproportionate impact must therefore have another explanation. One possibility would be that, acting at the peptide-loading complex, *m152* has some level of peptide cargo discrimination,

perhaps more effectively impacting higher affinity epitopes that are likely to become immunodominant. Another possibility would be that *m152* has yet further cellular targets that impact CTL efficacy. It is becoming apparent that some MCMV immune evasion genes do indeed have multiple cellular targets: for example, *m152* targets MHC class I and RAE-1. The mechanism of *m152's* disproportionate impact thus requires further investigation.

It was surprising to note that NKG2D inhibitions impacted CTL lysis by lines specific for only a subset of epitopes. The reason for this selectivity remains unclear. NKG2D seems to play a costimulatory role when TCR stimulation might be sub-optimal, such as in tumor or autoimmune epitopes. The initial observation that NKG2D provided necessary costimulation for HCMV-infected targets seemed in keeping with this idea, since HCMV profoundly downregulates MHC class I. Hence, we suspected that those MCMV epitopes affected might be those that presented lower avidity ligands for CTL, either because they were present at low epitope density on the surface of infected targets or because they presented low affinity ligands for the TCR. We have not directly assessed the affinity of peptide-MHC for TCR. However, we note that the epitopes that were affected by NKG2D were not obviously of low affinity for TCR, as assessed by peptide titration (Figure 4). Furthermore, peptide titration on IC-21 cells in the presence or absence of NKG2D blockade provided no evidence for the hypothesis that NKG2D costimulation would prove crucial when peptide MHC density is limiting; at least for the target cells used in this assay. Hence, the reason for NKG2D's impacting only a minority of epitopes remains unclear.

Finally, our studies of NKG2D expression on MCMV-specific CD8 T cells revealed an unexpected feature. NKG2D was expressed on the majority of memory CD8 T cells by three weeks post infection. However, the rate at which T cells specific for different epitopes acquired

NKG2D was quite different: m139 and m141-specific CD8 T cells were almost entirely NKG2D positive by day 7 post infection, whereas CD8 T cells specific for M38 and M45 took much longer to mostly become positive. This difference does not correlate with continued activation during the chronic phase of infection. Both M38 and m139 undergo pronounced "memory inflation", whereas both M45 and m141 contact severely in the chronic phase. m139 and m141 are both members of the same gene family and appear to play an important role in macrophage tropisms. Whereas M45 is involved in endothelial cell tropism and the role of M38 is not known. Hence, one possible explanation for these epitope specific differences could be the types of target cells *in vivo* that most express the antigens. This speculation obviously requires further investigation. We do note, however, that the slow rate of acquisition of NKG2D postivity by M45 and M38-specific CD8 T cells is the exception to the rapid acquisition of NKG2D that has been more commonly described in the literature.

The importance of NKG2D in CMV biology can be deduced from the number of genes that both MCMV and HCMV devote to downregulating its ligands. Inhibitions of NKG2D signaling has a major impact on the efficacy of murine NK cells, resulting in significantly higher virus titers in the first few days of infection. Our data suggest that NKG2D signaling in NK cells is likely the major focus of the viral genes that inhibit NKG2D ligands, and that targeting NKD2D on CD8 T cells has only a minor impact on CTL function.

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Chapter 4

The downregulation of total MHC class I by the MCMV immune evasion genes *m06* and *m152* reduces the stimulation of CD8 T cells preventing them from killing the infected cells.

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Introduction:

Cytomegaloviruses (CMV) are ubiquitous, host-specific viruses that can persist within a host for the lifetime of the organism. CMVs encode many unique genes that allow the virus to persist and avoid clearance by the host immune response. CMV's immune evasion genes referred to within this chapter are the genes that function by affecting the process of presentation of antigenic peptides to CD8 T cells. Both human CMV (HCMV) and murine CMV (MCMV) encode immune evasion genes, but their functional significance remains a mystery. During an acute infection the host is able to mount a large CD8 T cell response to CMV, which was shown decades ago to be protective in both the human and murine models. What then are the immune evasion genes doing if not protecting the virus from the CD8 T cell response? We have begun to address this question by looking at the impact of the immune evasion genes on CD8 T cell effector functions. Previously we noted that the immune evasion genes were able to completely inhibit CD8 T cell killing of infected cells while we could detect some IFN-γ secretion from the responding CD8 T cells {(Holtappels et al., 2006; Pinto et al., 2006), Doom CM unpublished observation}.

m152, *m06,* and *m04* are all immune evasion genes encoded by MCMV that function to inhibit antigen presentation to CD8 T cells, but do so by different mechanisms. *m152* inhibits MHC class I maturation past the endoplasmic reticulum Golgi intermediate compartment (ERGIC), this leads to a profound effect on cell surface MHC class I expression (Campbell and Slater, 1994; del Val et al., 1992; Ziegler et al., 2000). It is now clear that *m152*'s blocking MHC class I surface expression results in a profound impairment of the ability of CD8 T cells to lyse infected cells in vitro and to control virus replication in vivo (Gold et al., 2002; Gold et al., 2004; Kavanagh et al., 2001a; Krmpotic et al., 2002; Krmpotic et al., 1999; LoPiccolo et al., 2003;

Pinto et al., 2006; Wagner et al., 2002; Ziegler et al., 1997). Unlike other identified MCMV immune evasion genes *m152* appears to have another function apart from its ability to downregulate MHC class I. Recent studies have shown that *m152* also downregulates the NKG2D ligand Rae-1 (Krmpotic et al., 2002; Lodoen et al., 2003), whose extracellular domain bears structural homology to MHC class I. The importance of *m152*'s downregulation of NKG2D ligands in inhibiting NK cell effector functions has been demonstrated by several groups (Krmpotic et al., 2002; Lodoen et al., 2003), however the impact of NKG2D ligand downregulation on CD8 T cell effector functions has only recently been examined (Pinto et al manuscript in preparation; chapter 3). Similar to *m152*, *m06* also blocks MHC class I from reaching the surface of infected cells by redirecting the MHC class I complex to the lysosome (Hengel et al., 1999; Reusch et al., 1999; Wagner et al., 2002). Unlike *m06* and *m152*, *m04* does not block the transport of MHC class I to the cell-surface, but forms a tight association with the MHC class I molecule and co-traffics to the cell surface with MHC class I (Kavanagh et al., 2001b; Kleijnen et al., 1997). The mechanism of *m04* inhibition of CD8 T cell killing is still unclear, and the effects of *m04* are far less dramatic then those of *m152* and *m06* (Pinto et al., 2006; Holtappels et al., 2006; Wagner et al., 2002). When the three immune evasion genes are expressed together they are a potent force for inhibiting the killing by all antigen-specific CD8 T cell responses tested (Holtappels et al., 2006; Pinto et al., 2006).

Since the discovery over thirty years ago that MHC was used by CD8 T cells to recognize infected cells, we have been working to understand how CD8 T cells interact with MHC class I and how this interaction induces a functional response. It is the presence of the antigenic peptides bound to MHC class I molecules that signals the presence of an infected cell to a CD8 T cell. Following recognition of antigenic peptide, CD8 T cells undergo marked proliferation,

produce effector cytokines and kill the cells that express their target antigen. Each CD8 T cell expresses only one T cell receptor (TCR) and so it is able to respond only to its cognate peptide presented on the MHC class I. Surrounding the CD8 TCR are co-receptors, and costimulatory molecules important for maintaining the interaction of the pMHC class I with the TCR and directing the CD8 T cell to responding appropriately. These activated antigen-specific CD8 T cells, or cytolytic T lymphocytes (CTLs), control virus spread through the release of cytokines, such as interferon gamma (IFN-γ), and the lysis of the antigen presenting cells (APCs) mediated by granzymes and perforin. IFN-γ secretion controls viral replication and recruits effector cells important to the inflammatory response. The combined action of perforin and granzymes induces cells to undergo apoptosis, and this pathway is the primary defense mechanism against MCMV, and many other viral pathogens (Kagi et al., 1996; Loh et al., 2005; Mullbacher et al., 1999; Tay and Welsh, 1997). It is these effector CD8 T cell responses that virally infected cells must avoid or inhibit in order to persist within its host.

To signal the presence of a viral infection, MHC class I molecules can present viral peptides to CD8 T cells (Fremont et al., 1992; Fremont et al., 1995). Viral and self-peptides are loaded onto class I molecules in the ER and the peptide-loaded MHC class I (pMHC) is recognized by CD8 T cells. CD8 T cells use the peptides presented on MHC class I molecules to survey the health of cells. Cells presenting endogenous self-peptides are presumed to be healthy, and do not stimulate a T cell response. Cells presenting foreign peptides derived from pathogens signal a potential infection and stimulate a T cell response. TCR stimulation is based on the presence of the TCR with an appropriate affinity for the pMHC and the amount of cognate peptide on the cell surface (Sykulev et al., 1995). Avoiding this TCR stimulation was believed to be the primary reason MCMV encoded immune evasion genes.

Recently there have been several studies looking at the amount of cognate peptide MHC that is required to stimulate a CD8 T cell. It takes only one pMHC expressing the cognate pMHC to induce a CD8 T cell to interact with the MHC class I, but it requires between 3 and 10 pMHC molecules to stimulate a T cell to release calcium in response to its cognate antigen (Purbhoo et al., 2004). This observation has encouraged us to question further our MCMV model of immune evasion. Can the virus reduce enough of the pMHC class I from the surface to prevent between 3-10 peptide-specific MHC molecules from being presented? We know that between 30 and 50 % of the MHC found on the surface is associated with *m04* (Kavanagh et al., 2001b), however it is not clear whether *m04* association with MHC inhibits CD8 T cells activation. Even if MHC class I association with *m04* does inhibit antigen presentation, there is still a substantial amount of MHC available on the surface of a cell. It is hard to imagine that MCMV immune evasion genes are so completely effective at inhibiting presentation, that 3 epitope specific-MHC molecules never make it to the surface of the infected cell to stimulate CD8 T cells. As mentioned above, a protective CD8 T cell response is generated against wild-type MCMV in mice, so at least some peptides do make it to the surface and stimulate a CD8 T cell response. This leaves us with a conundrum: antigen-specific CD8 T cells can be generated against MCMV but these antigen-specific CD8 T cells cannot kill MCMV infected cells in vitro (Pinto et al., 2006).

To probe this conundrum further, we infected cells under multiple conditions in the presence or absence of surface molecules important for CD8 T cell mediated killing. We demonstrated by ^{51}Cr assays that CD8 T cells cannot kill wild-type MCMV infected cells, even in the presence of exogenously added cognate peptide. However, if all three immune evasion genes are removed from the virus (TKO), T cells can then kill the infected cells. Granzyme B

transfection assays established that uninfected cells are more sensitive to CD8 T cell killing then infected cells, and that wild-type- and TKO-infected cells are equally resistant to death induced by granzyme B. Finally, with the aid of TAP-/- cells, we were able to demonstrate that total MHC class I levels are important for enabling lysis of MCMV-infected cells. We propose that wt-MCMV-infected cells fail to die because the T cells are not getting a strong enough signal to kill the target due to a lack of total MHC class I levels, rather than a lack of cognate peptide. This weak signal results in a diminished T cell response with lower amount of granzymes released, which can be blocked by the anti-apoptotic defenses set up by the virus during the infection.

Materials and Methods:

Viruses Wild-type BAC-derived MCMV strain MW97.01 (Wagner et al., 1999), Δ*m04*, Δ*m06*, Δ*m152*, Δ*m06+m152*, and Δ*m04+m06+m152* (Wagner et al., 2002) were grown on C57BL/6 Mouse embryo fibroblasts (MEF), then purified by pelleting over a 15% sucrose cushion (Brune, 2005). Each virus stock was titered without centrifugal enhancement on BALB-3T3 cells. The mean of three virus titrations was used to calculate titers for use in these assays.

Cells and Mice IC-21, a SV40-transformed macrophage cell line from C57BL/6 mice (Cavanaugh et al., 1996), a gift from Ann Campbell Eastern Virginia Medical School, were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4.5g/L Glucose, and1.5g/L sodium bicarbonate and antibiotics. BALB/c 3T3 (ATCC), L929 (ATCC), K41 (SV-40-transformed H-2^b fibroblast, a gift from Marek Michalak University of Alberta), Fas -/- MEFs (isolated from LPR mice) C57BL/6 MEFs (isolated from C57BL/6 mice) and B16-FL (Driessen et al., 1999) (a gift from Glen Dranoff, Harvard Medical School) were cultured in DMEM supplemented with 10% FBS and antibiotics. L929 supernatant, a source of macrophage colony-stimulating factor (M-CSF), was harvested from L929 cells grown for 10 days after reaching confluence. Primary bone marrow macrophages (BMMΦ) were isolated by the procedure described by Bouwer et al (Bouwer et al., 1997). Briefly, bone marrow was cultured on non-tissue culture treated Petri dishes in DMEM supplemented with 10% FBS, 30% M-CSF from L929 supernatant and antibiotics. Six to ten days later adherent cells were isolated. Primary bone marrow dendritic cells (BMDCs) were generated by slightly modifying the procedure described by Lutz et al (Lutz et al., 1999). Briefly, femurs were harvested from C57BL/6 female mice and the bone marrow was flushed out

onto non-tc treated 150mm dishes. The DCs were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 200μM β-mercapthoethanol, 4.5g/L Glucose, and 25ng/ml of GM-CSF. The BMDCs were grown for 8-10 days before harvesting. The phenotype of the DCs was checked by FACS. C57BL/6, OT-1, and the perforin knockout mice were purchased from Jackson Laboratories (Bar Harbor, ME), were maintained in our animal facilities at Oregon Health and Science University (Portland, OR) and were used in experiments no earlier than 6 wk postbirth.

T cell lines Female C57BL/6 (B6) mice were purchased from NCI Fredrick (Baltimore, MD) or The Jackson Laboratory (Bar Harbor, ME), and infected with either $1x10^6$ or $5x10^6$ pfu of MCMV. Spleens were harvested from mice that had been infected at least 11 weeks previously. As a source of DC-enriched splenoctyes to stimulate CTL lines, we used spleens from mice that had been infected 14 days previously with the Flt-3 ligand-secreting tumor, B16FL. Splenocytes from B16FL-injected mice were γ-irradiated and pulsed with peptide at 10^{-8}M, and cultured with splenocytes from MCMV-infected mice in RPMI supplemented with 10% FBS for 3 days, after which 10U/ml recombinant IL-2 (eBioscience) was added. After 10 days, the percentage of CD8 T cells responding to the simulating peptide epitopes was assessed by intracellular cytokine staining (ICCS), and the cells used in ^{51}Cr release assays. SIINFEKL specific T cell lines were generated from the spleens of naïve OT-1 mice. The spleens were harvested and the splenocytes were pulsed with 10^{-8}M of SIINFEKL peptide. The splenocytes were cultured in the presence of RPMI supplemented with 10% FBS for 3 days, after which 10U/ml recombinant IL-2. The cells were harvested after 6-12 days and their antigen-specificity and activity was confirmed by FACs, before being used in the described assays.

Antibodies and tetramers α-gB and α-gH, (a gift from Lambert Loh (Loh et al., 1988; Loh and Qualtiere, 1988; Rapp et al., 1994)). α -K^b (Y3) and α -D^b (B22-249) (ATCC), α-pp89 (Del Val et al., 1988) were purified on Protein A , G (Sigma/Aldrich) columns and conjugated to FITC (Molecular Probes), PE, or APC(Cyanotech) according to published protocols (Hardy, 1986). α–IFN-γ (XMG1.2) and α -CD8 (53-6.7) were purchased from eBioscience.

FACS analysis K41 cells pretreated with IFN-γ for 24 h prior to the start of the assay then they were infected overnight with the panel of mutant viruses at an MOI of 20. For intranuclear staining, cells were stained with cell surface antibodies then fixed with CytoFix/CytoPerm(BD Bioscience) then permeablized by incubation for 5 minutes with 0.1% Triton X in PBS. The cells were then stained for 30min with anti-pp89 in the presence of 0.1% Triton X. The cells were washed 3x in 0.1% Triton X then washed 1x in FACS buffer before analysis. All cells were analyzed using a FACSCaliber flow cytometer (BD Bioscience.) in conjunction with Cell Quest (BD Bioscience). All further analyses were performed using FlowJo software (Treestar)

Assay for cell-mediated cytotoxicity 10^4 target cells per well were plated in 96 well plates, infected with the indicated viruses at an MOI of 20 and/or pulsed with cognate peptide and labeled with 100μCi ^{51}Cr (NEN) for the indicated times. When K41 or MEF cells were used in the assays they were first pretreated with IFN-γ for 24 h prior to the infection. Effector cells were then added at the indicated effector to target (E:T) ratios, incubated for 6 h, and supernatants were harvested and assayed with a Topcount scintillation counter (Packard Instruments). Background ^{51}Cr release was determined by incubating targets with medium alone, and total ^{51}Cr

release by lysing targets with medium containing 1% Nonidet P40 (USB). Percent specific lysis was calculated as follows: (experimental cpm - background cpm)/(total cpm - background cpm). For the PP2 experiments, 30μM of PP2(Calbiochem) was added at 0, 5, 20, 40, 60, 80, 100, 120, 240, and 360 minutes after the addition of the T cells. The assay was then allowed to complete the 6 hour incubation before harvesting.

Granzyme B and IFN-γ ELISPOT: MSIP ELISPOT plates (Milipore) were coated with the appropriate capture antibody, either IFN-γ (XMG1.2, eBioscience) or anti-granzyme B (RnD) overnight, then washed and blocked with ELISPOT blocking buffer (eBioscience). 10^4 target cells per were added to the appropriate wells. The cells were then infected with the indicated viruses at an MOI of 20 and/or pulsed with cognate peptide and incubated overnight. The plates were then washed 3x, to mimic the washes done in the ^{51}Cr assays. $5x10^3$ effector cells were then added incubated for 6 h or overnight. The cells were then washed off and the appropriate detection antibody was added, either IFN-γ (R4-6A2-biotinylated, eBioscience) or anti-granzyme B-biotinylated(RnD), for 2 h. The detection antibody was removed and SA-HRP (Vector) was used to detect the bound biotinylated antibodies. The spots were developed with and AEC kit(Vector) and the plates were read on a AID ELIspot Reader and the data was analyzed with Microsoft Excel. For the PP2 experiments, 30μM of PP2(Calbiochem) was added at 0, 5, 20, 40, 60, 80, 100, 120, 240, and 360 minutes after the addition of the T cells. The assay was then allowed to complete the 6 hour incubation before staining.

Granzyme B transfection: The transfection of Granzyme B was done according to the protocol described in Lu et al (Lu et al., 2006a). Briefly Pro-Ject protein transfection reagent kit (Pierce)

was prepared and aliquoted in methanol, according to the manufacturer's instruction. The recombinant mouse granzyme B(Sigma) was diluted to listed concentrations in HBSS and incubated with appropriate volume of pro-Ject and left at room temperature for 5 min. The wild-type, TKO, and uninfected cells were harvested and counted then plated at a concentration of $1x10^5$ cells per well in a 24 well plate. The pro-Ject containing the Granzyme B was added to the appropriate wells in the 24 well plate. The infected and uninfected cells were incubated with the pro-Ject/ granzyme B transfection complex in 200 µl of HBSS at 37°C for 4 h. After this point 10% serum was added to the wells and the cells were incubated overnight at 37°C. The cells were then harvested and immediately stained with Annexin V and 7AAD. The cells were then run on a FACs Calibur (BD Bioscience) and the results were analyzed using Flo-Jo software (Treestar).

Results:

Wild-type MCMV-infected cells are not lysed even when loaded with exogenous peptide

We have sought to understand why the MCMV immune evasion genes are so effective at inhibiting cytotoxicity but can still stimulate cytokine production {(Pinto et al., 2006); chapter 2 and Doom, CM unpublished observation}. As has been previously published (Pinto et al., 2006; Wagner et al., 2002), we observed a clear down regulation of both H2-K^b and H2-D^b MHC molecules in cells infected with wild-type-MCMV, as compared to uninfected and TKO-infected controls (Figure 1a). However this downregulation was not complete, there was still some measurable MHC class I on wild-type-infected cells as compared to isotype control stained cells. We wondered whether this remaining level of MHC on the surface of the wild-type infected cell could be enough to stimulate a CD8 T cell response. Put another way: have the immune evasion genes decreased the amount of cognate peptide MHC sufficiently to prevent TCR recognition?

To bypass the need for endogenous MCMV-peptides to be present on the surface MHC molecules, cognate peptide was exogenously loaded onto IFN-γ treated transformed fibroblasts, which had been infected with either wild-type MCMV, or TKO, or left uninfected. We then incubated these cells with cognate peptide-specific CD8 T cell lines, and asked how well these CD8 T cells could kill the infected target cells loaded with cognate peptide. Figure 1b shows a typical assay using T cells specific for 4 MCMV epitopes. CD8 T cell killing was measured by ^{51}Cr release assay. In confirmation of previous results, we were able to demonstrate that MCMV immune evasion genes effectively inhibited CD8 T cell killing. Interestingly, wild-type infected cells were not killed by the epitope-specific CD8 T cells even in the presence of exogenously loaded peptide. We repeated this experiment with multiple CD8 T cell lines specific for 3 H2-D^b

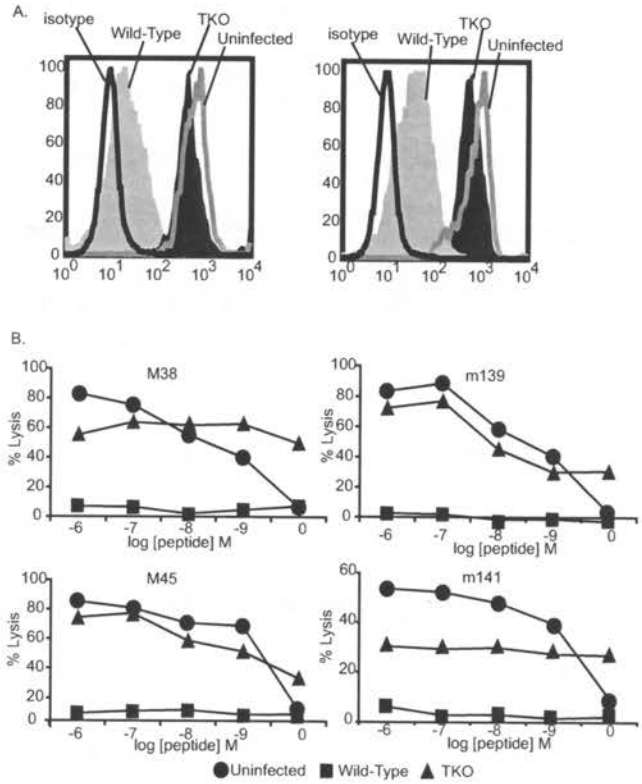

Figure 1. Inhibition of CTL lysis by MCMV. (A) FACs surface staining with H2-Kb (Y3) and H2-Db (B22.249) or IgG2b or IgG2a isotype controls respectively of uninfected, wild-type, and (Δm04+m06+m152) TKO K41 cells infected overnight. (B) M38, m139, M45 and m141 Peptide driven polyclonal T cell lines were used in 51Cr release assays with K41 targets that are pulsed with different concentrations of peptide at the same time as the cells were infected with wt- or TKO- MCMV, or left uninfected. Representative example of 3 experiments.

and 3 H2-K^b epitopes. In all cases under these conditions, we did not see killing of wild-type infected cells with exogenously added cognate peptide.

Time post infection at which resistance to killing is acquired

The ^{51}Cr experiments were repeated altering the time the target cells were infected and loaded with peptide prior to incubation with the T cells to demonstrate that the immune evasion genes were involved in blocking cytolytic CD8 T lymphocyte (CTL) killing. Target cells were infected either overnight or for three hours, prior to the addition of antigen-specific CD8 T cells. Without exogenous peptide, cells infected with wild-type MCMV were not killed by antigen specific CD8 T cells, however cells infected with TKO were killed under both infection conditions (Figure 2a). When cognate peptide was added to the cells at the same time as infection, again TKO-infected cells were killed by antigen-specific CD8 T cells under both conditions. As previously observed in Figure 1b, target cells infected with wild-type MCMV plus peptide overnight were not killed, (Figure 2b). However when cells were pulsed with peptide and infected with wild-type MCMV for only three hours prior to the start of the assay, CTL mediated killing was observed. Cells infected for three hours can make enough endogenous protein to be processed and presented, as evidenced by the killing of TKO infected cells after three hours, Figure 2a. Nevertheless, infection of cells with wild-type MCMV, which contains all the immune evasion genes, avoids CTL killing in response to endogenous antigen at 3 hours post infection, even though the peptide pulsing experiment demonstrates that the cells not yet completely resistant to lysis (Figure 2b). If the infection is allowed to continue, the immune evasion genes can inhibit the CTL killing even in the presence of exogenously added cognate peptide. This key result clearly demonstrates the importance of the immune evasion

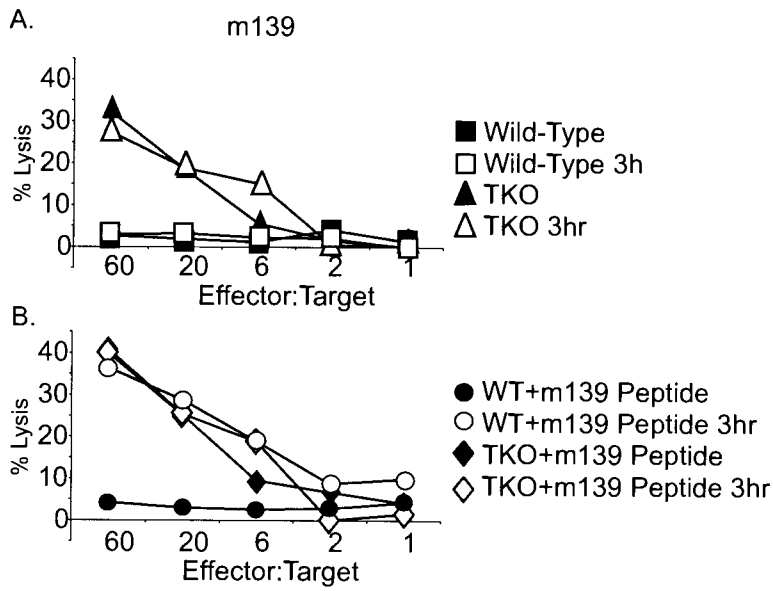

Figure 2. Timing of immune evasion gene inhibition of CTL killing. (A) 51Cr release assays with K41 targets infected with either wild-type or TKO, overnight or 3 h prior to the start of the assay. (B) Target cells were pulsed with10-6M cognate m139 peptide at the same time as the virus infection, either overnight or 3h, prior to the start of the assay. Representative example of 2 experiments. The effector cells shown in A and B are the m139 T cell line, the assays were repeated with 3 other T cell specificities, 2 times each.

genes for inhibiting CTL killing throughout the course of infection. Early on in infection, the immune evasion genes function to block antigen presentation of endogenously processed peptides, and as the infection progresses they are also able to prevent CD8 T cell killing of target cells loaded with exogenous peptides.

MCMV-immune evasion genes also inhibit killing by alloreactive CTL

We next generated allo-reactive T cells to determine if the inhibition of killing of wild-type infected cells was due to the lack of recognition of cognate pMHC. Allo-reactive T cells recognize non-self MHC, this recognition is a very potent stimulus to kill the non-self target. The allo-reactive T cells were generated from either C57BL/6 or BALB/c mice against their reciprocal target. BALB/c or C57BL/6 target cells were infected with wild-type MCMV, TKO or left uninfected, and tested for susceptibility to alloreactive CTL in a ^{51}Cr release assay (Figure 3). We used several different cell types as targets to determine if the immune evasion genes were equally potent in different cell types, including bone marrow dendritic cells (BMDC), bone marrow macrophages (BMMϕ), transformed Mϕ, mouse embryo fibroblasts (MEFs), and transformed fibroblasts (some data not shown). By removing the requirement for the recognition of cognate pMHC to stimulate the T cells to kill the infected cells, we can determine if the block in CD8 T cell is related to blocking T cell stimulus received by the recognition of cognate peptide. Interestingly, for most of the cell types we tested, infection with wild-type MCMV inhibited allo-reactive CTL mediated killing. Given the strength of the allogeneic killing signal, we were very surprised by this result. Wild-type infection of BMMϕ, and transformed Mϕ lead to the complete inhibition of allo-reactive CD8 T cell killing. Only in BMDC was infection with wild-type MCMV not able to inhibit CTL killing. This maybe due to a poor infection of the

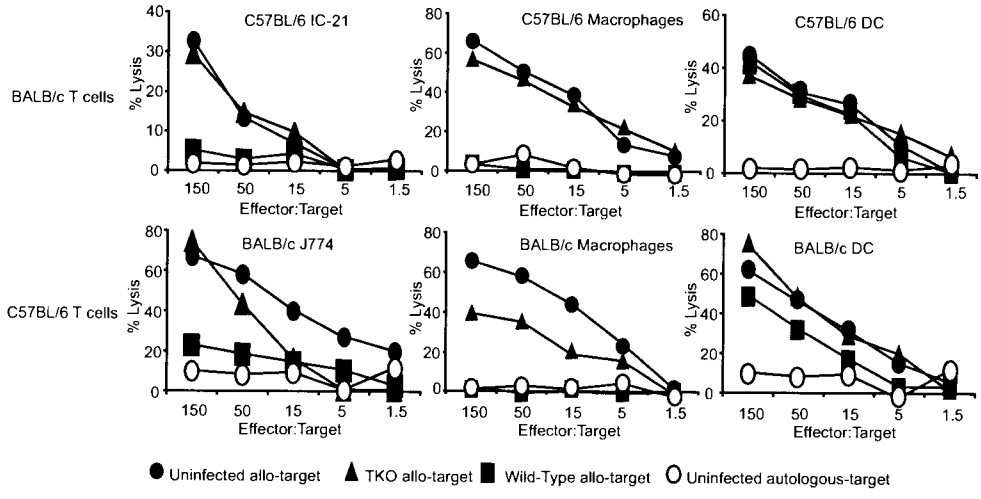

Figure 3. Allo-reactive T cell killing is inhibited by wild-type MCMV infection. 51Cr release assays with allogeneic targets uninfected or infected overnight with either wild-type or TKO. Lysis of infected an uninfected macrophage cell lines (IC-21, J774), primary BMMϕ, or BMDC by BALB anti B6 and B6 anti BALB alloreactive CTL. Representative example of 2 experiments.

BMDC or to other factors related to the ability of DCs to be highly qualified APCs. This observation remains to be investigated. These results did show us that MCMV was not only able to inhibit killing of cells expressing viral antigens, but was able to block the killing of infected cells non-specifically.

Role of costimulation and adhesion in resistance to killing

The inhibition of killing we observed was so dramatic, and does not require blocking the recognition of cognate peptide antigen, we felt that one possible explanation for this result was that the immune evasion genes are downregulating other cell surface molecules involved in CD8 T cell killing. In chapter 3 we began to look at the effects of NKG2D ligand down regulation. NKG2D receptor on T cells has been shown to act as a costimulatory molecule (Cerwenka et al., 2002; Diefenbach et al., 2002; Groh et al., 2001; Jamieson et al., 2002) and we wondered whether *m152*'s downregulation of the NKG2D ligands in the RAE-1 family was the explanation for the inhibition of killing. As discussed in Chapter 3 we were able to show that NKG2D ligand downregulation did have an effect on CD8 T cells killing, but this effect was not strong enough to explain the complete block in killing of wild-type infected cells (Pinto A.K., manuscript in preparation, Chapter 3). We then began a preliminary search of other molecules that could be downregulated from the surface of infected cells and in the inhibit CD8 T cell killing. We focused our attention on ICAM-1 because the interaction of ICAM-1 with its receptor LFA-1 is necessary for T cell killing. Also, previously published reports have shown that another herpes virus, Kaposi sarcoma herpes virus (KSHV) is able to down regulate ICAM-1 (Coscoy and Ganem, 2001), and the immune evasion gene responsible of ICAM downregulation, K5, also downregulates MHC class I. However, when we compared ICAM-1 expression on uninfected

cells and cell infected with wild-type or TKO we saw no downregulation of ICAM-1 (Figure 4). In fact, ICAM-1 expression was upregulated on the surface of cells infected with both wild-type and TKO-MCMV. We concluded that ICAM-1 downregulation was not responsible of the inhibition of CTL-mediated killing that we observed following infection with wild-type MCMV.

Inhibition of killing by OVA-specific CTL even though exogenous SIINFEKL peptide is loaded.

We adopted the OVA-SIINFEKL system to determine if MCMV was inhibiting the binding of exogenously loaded cognate peptide. The benefit of using the SIINFEKL system was that it is a well-established model with many available reagents. One of the most powerful reagents is the SIINFEKL-specific antibody 25D1.16 (Porgador et al., 1997). 25D1.16 is able to recognize the SIINFEKL peptide bound to the H-2K^b class I molecule. With this reagent we were able to determine how much peptide was bound to MHC class I molecules. To do this experiment we pulsed a transformed fibroblast cell line, K41s, with SIINFEKL at the same time as the infection and determined how much SIINFEKL peptide was bound to the surface of the infected cells (Figure 5a). We noted that the cells infected with TKO had the highest amount of peptide bound to the MHC class I, followed by wild-type infected cells, and then uninfected cells. We were surprised that uninfected cells had low amounts of SIINFEKL bound MHC, but total MHC class I surface staining done at the same time demonstrated that uninfected cells expressed an equal amount of class I to TKO. Wild-type infected cells, on the other hand, had dramatically reduced MHC class I levels (Figure 5b). Short term incubations of SIINFEKL with uninfected cells showed that the MHC on the surface of the cells could bind much larger amounts of SIINFEKL than was observed after overnight incubations (data not shown). We believe that the reduced amount of SIINFEKL bound to MHC class I on uninfected cells is due

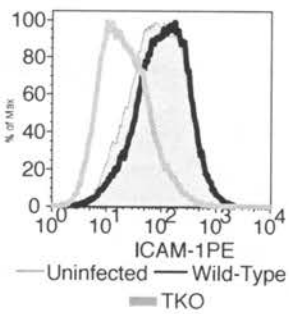

Figure 4. ICAM-1 does not explain resistance to lysis FACs analysis of ICAM-1 staining on infected and uninfected K41s

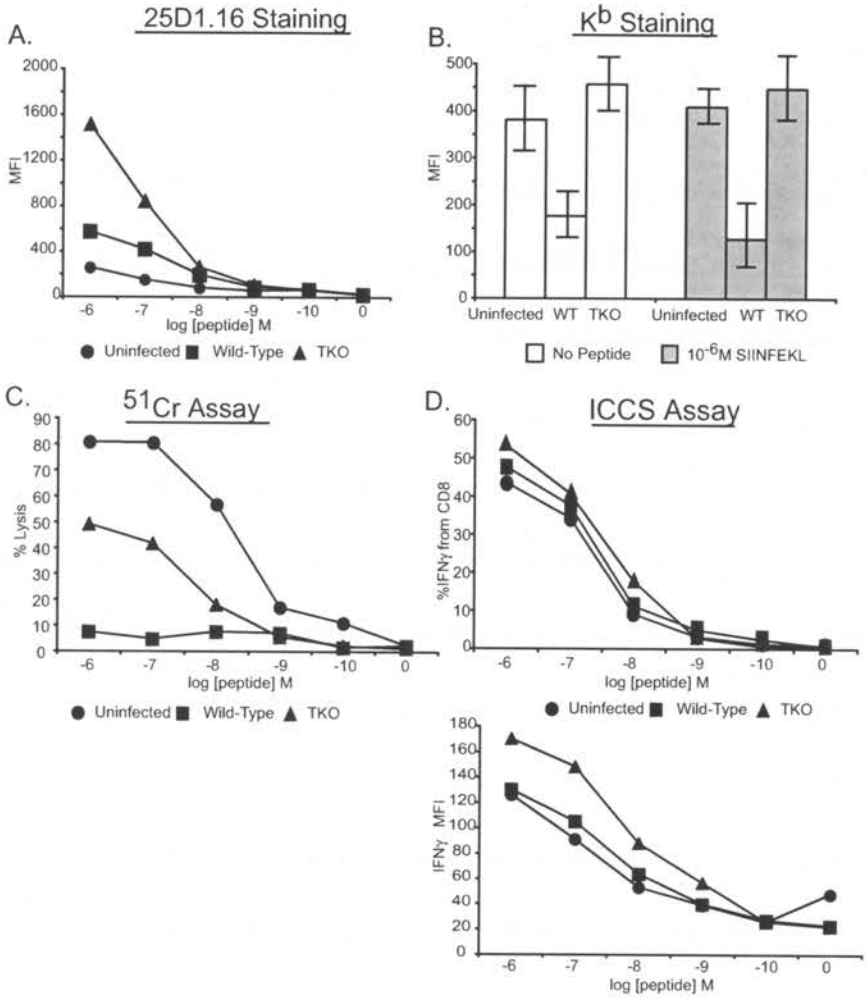

Figure 5. Direct comparison of antigen presentation and cytolysis using OT1 T cells and SIINFEKL. (A) K41s were left uninfected, or infected with wild-type, or TKO and pulsed with SIINFEKL peptide titration at the same time as infection, after overnight incubation the K41s were stained with 25D1.16. (B) FACs surface staining with H2-Kb (Y3) of K41 uninfected, wild-type, and TKO K41 cells with or without SIINFEKL infected overnight. (C) K41s prepared as in A and used in a 51Cr assay with an OT-1 T cell line used as the effector cells and the effector:target ratio at 20:1. (D) K41s prepared as in A and used in an intracellular cytokine stain (ICCS). OT-1 T cell lines were used and the T cells were gated on CD8 to determine % IFN-γ. A, B, C, and D are representative examples of 6 independently completed experiments.

to higher turnover rate of the class I and not due to poor binding of SIINFEKL to uninfected cells. Figure 5a indicated that when wild-type infected cells were pulsed with 10^{-6}M peptide the amount of pMHC on the surface was approximately equally to that of TKO-infected cells pulsed at $10^{-7.5}$M. So, when the total amount of pMHC was equal, we wanted to know if the wild-type infected cells could be killed as well as TKO infected cells (Figure 5c). Again we saw that wild-type infected cells were not killed by antigen-specific CD8 T cells, and this inhibition of killing was independent of the amount of cognate peptide bound to the MHC molecules. When TKO-infected cells were pulsed with SIINFEKL, the killing titrated with the amount of exogenous peptide added. These results demonstrate that when the amount of cognate pMHC was equal between wild-type and TKO infected cells, we did not observe killing of wild-type infected cells. In contrast to the results with infected cells, uninfected cells, which had the lowest amount of cognate pMHC on their cell surface, were efficiently killed by CD8 T cells. Interestingly, when we measured the IFN-γ response to SIINFEKL pulsed cells we saw no difference between the infection conditions in the amount of IFN-γ produced at any peptide concentration (Figure 5D). However we did see a slight reduction in the mean fluorescent intensity (MFI) of IFN-γ in response to uninfected and wild-type infected cells, indicating that less IFN-γ was being made on a per cell basis in response to less peptide on the surface of these cells (Figure 5D). OT-1 T cells produced IFN-γ in response to wild-type infected peptide-pulsed cells but no killing of the wild-type infected cells was observed. Based on these findings we suggest that MCMV infection alters either the effector functions of the responding CD8 T cells or the virus infection causes the cells to be more resistant to lytic granule release.

Obligate use of granule exocytosis in killing of fibroblast targets

We then wondered if the CD8 T cells were receiving a stimulus to kill the wild-type infected cells. We proposed that the immune evasion genes were blocking signals required by the CD8 T cells to kill infected cells. To address this question we first needed to determine how the CD8 T cell lines were killing infected target cells. CD8 T cell mediated killing may be performed by interactions of Fas ligand with Fas, leading to the induction of the extrinsic pathway of apoptosis. Alternatively, CD8 T cell lines could be killing cells through the release of lytic granules by CD8 T cells causing the activation of pro-apoptotic Bcl-2 proteins and the induction of the intrinsic apoptosis pathway. To determine which pathway the CD8 T cells were using to kill the uninfected and TKO infected cells we generated MCMV specific CD8 T cell lines from either normal C57BL/6 immune mice or from perforin -/- mice and determined if they could kill MEFs generated from naïve C57BL/6 mice or Fas -/- mice (Figure 6). We confirmed that the T cells generated from the both C57BL/6 and perforin -/- immune mice were antigen specific, by intracellular cytokine staining (ICCS) and tetramer staining (data not shown). CD8 T cells generated from perforin -/- mice were not able to kill TKO-infected or peptide pulsed uninfected cells for all the epitopes tested. However, CD8 T cells generated from immune mice were able to kill TKO-infected or peptide pulsed uninfected Fas-/- targets. We concluded from this result that the perforin-granzyme pathway way was the primary pathway used by the CD8 T cells to mediate killing. We did notice some effect of the loss of Fas on the CTL killing of uninfected cells. However this effect was not as dramatic as the loss of perforin and it was not observed in the CTL killing of the TKO infected cells.

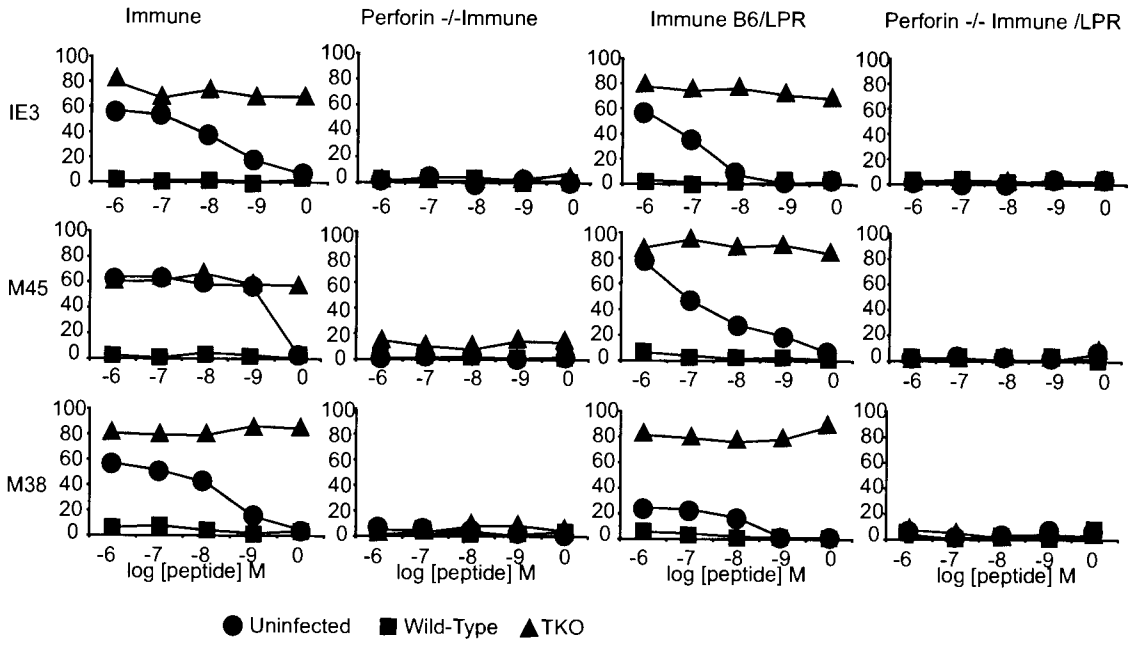

Figure 6. The perforin-granzyme pathway mediates killing in 51Cr assays. MEFs generated from either C 57BL/6 or LPR (Fas-/-) mice were left uninfected or infected with wild-type or TKO and pulsed with the cognate peptide overnight and used as targets in the 51Cr assay. IE3, M45 or M38 T cell lines generated from MCMV immune C57BL/6 or Perforin -/- mice were used as effector cells. The effector:target ratio was 50:1 for all of the assays shown.

CD8 T cells release granzyme in response to wild-type-infected cells.

After determining that the perforin-granzyme pathway was utilized by CD8 T cells to kill virally infected cells, we wanted to know if the CD8 T cells were being stimulated to secrete granzyme in response to the cognate antigen on wild-type infected cells. A granzyme B ELISPOT was used to determine lytic granule release and the results and compared with an IFN-γ ELISPOT. SIINFEKL-pulsed wild-type infected cells did stimulate granzyme secretion in antigen-specific cells as measured by granzyme B spot number (Figure 7a). The amount of granzyme released in response to wild-type infected cells was less then that seen in response to TKO, but greater than that observed in response to uninfected. This result mimics what we have seen for the cell surface SIINFEKL staining in Figure 5a. We also noted that the IFN-γ response was approximately equal for all infection conditions (Figure 7b), indicating that the amount of cognate peptide on the surface of the infected cell is not as important in stimulating an IFN-γ response. Also shown in Figure 7 is the intensity of the spots for granzyme B and IFN-γ. The results for spot intensity were similar to that observed for the number of spots. Individual CD8 T cells did not release as much granzyme B in response to wild-type infected cells as they did to TKO infected cells pulsed with SIIINFEKL. The measure of intensity of the spots for the IFN-γ was difficult to assess due to high background levels within the assay. However, these results suggest that the intensity of the IFN-γ spots was equal for all conditions. This result is different from the ICCS data, which suggests that the CD8 T cells made less IFN-γ in response to less cognate antigen on wild-type and uninfected cells. The differences are most like due to the difference in the sensitivities of the two assays. From the results of the ELISPOTS we concluded from these results: 1.) The number of cells releasing granzyme B is proportional to the amount of cognate pMHC on the surface of the target cell. 2.) The number of cells releasing

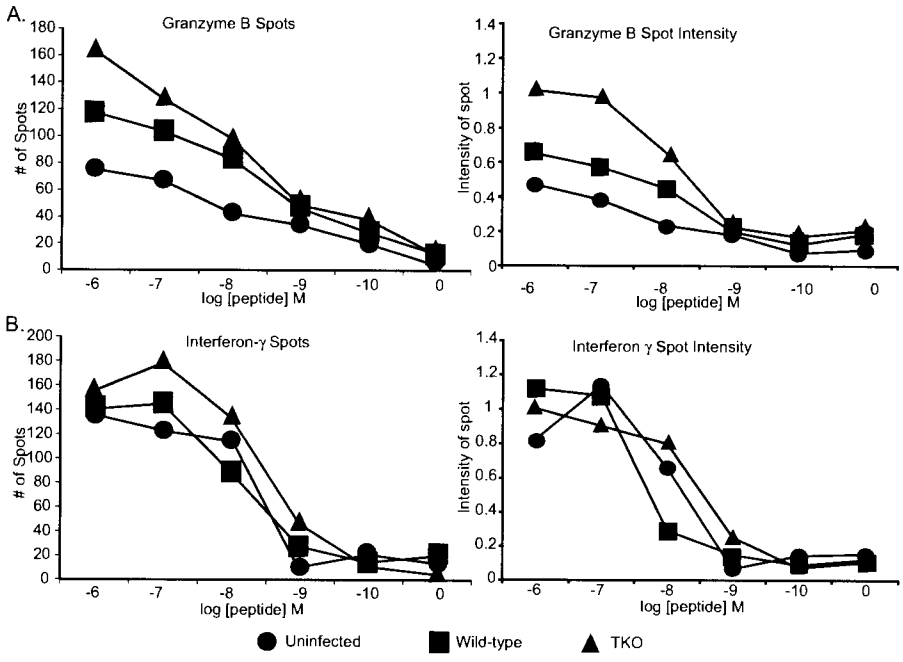

Figure 7. Granule exocytosis and cytokine secretion measured by ELISPOT. (A) ELISPOT for Granzyme B with IFN-γ pretreated K41s left uninfected, or infected with wild-type, or TKO and pulsed with SIINFEKL peptide titration at the same time as infection, after overnight incubation the OT-1 T cells were added and the granzyme B secretion was determined after 8 h. (B) The assay was setup as in A and IFN-γ secretion was measured. A and B are representative examples of 3 experiments.

IFN-γ, as measured by ELISPOT, is proportional to the cognate pMHC levels on the targets. 3.) The amount of granzyme B release/per cell is also proportional to the amount of cognate pMHC. 4.) It takes less granzyme B released to kill an uninfected cells as compared to a TKO infected cell. The last conclusion is based on comparing the granzyme B ELISPOT results with the results of the ^{51}Cr assays. Based on the previous findings we can now demonstrate that CTL mediated killing is primarily mediated by perforin and granzyme and that uninfected cells are very susceptible to CTL killing. We have also shown that the CD8 T cells are functional and able to respond to wild-type infected cells by releasing granzyme B.

Infected cells require a much longer duration of TCR signaling than uninfected cells in order to be killed.

To explore the observation that uninfected cells stimulated much less granzyme B release than TKO and yet were killed equally well, we wanted to determine if the time it takes to kill uninfected and infected cells. Our hypothesis was that it is much easier to kill uninfected cells than infected cells, so the time required to kill an infected cell would need to be longer then the time it would take to kill an uninfected cell. The *src* kinase inhibitor PP2 was used to determine if it takes longer to kill an infected cell than it does to kill an uninfected cell. PP2 has been shown to block CTL functions by stopping the signaling events that occur after CD8 T cell activation (Faroudi et al., 2003). After its addition to a ^{51}Cr assay, any further stimulatory signal received by CD8 T cell from the CD8 T cell–APC interaction should be blocked. We performed a ^{51}Cr assay using uninfected and TKO infected targets pulsed with SIINFEKL, similar to the experiment described previously. In this experiment, we added PP2 at different intervals after the addition of the CD8 T cells, to determine how much time CD8 T cells need to interact with

their targets in order to kill the target cell (Figure 8a). We observed killing of uninfected cells after 40 minutes of incubation with the CD8 T cells. Surprisingly, we did not observe killing of TKO infected cells until after 2 hours, with killing reaching its maximum after 4 hours of incubation with CD8 T cells. In contrast to CTL killing, it took 4 hours to see any IFN-γ being secreted from T cells in response to the target cells and it took 6 hours to see the maximum IFN-γ produced by the T cells (Figure 8b). There was no difference in the sensitivity of the T cells to produce IFN-γ in response to TKO infected cells in comparison to uninfected cells. Based on these findings we now believe that the wild-type infected cells are not being killed by the antigen-specific CD8 T cells because the CD8 T cells are not able to overcome the increased threshold require to kill an infected cell. We are unable to perform the PP2 experiment with wild-type infected cells because they are resistant to CTL mediated killing in this assay. However we know from the granzyme B ELISPOT that there was a small amount of granzyme B released in response to wild-type infected cells pulsed with peptide. In contrast there was a large amount of granzyme B released in response to TKO infected cells. We believe that it is this larger amount of granzyme B that is required to kill a TKO infected cell and that wild-type infected cells are not dying because they are receiving a much smaller dose of granzyme B.

Comparison of the amount of granzyme B needed to kill infected and uninfected cells.

To demonstrate that wild-type and TKO infected cells are equally sensitive to granzyme B-mediated death we transfected granzyme B into wild-type- and TKO-infected. Cells were infected with TKO or wild-type MCMV and their sensitivity to granzyme was compared with uninfected controls. The cells were transfected with different amounts of granzyme B using a protein transfection reagent that had been previously shown enable granzyme M to enter cells

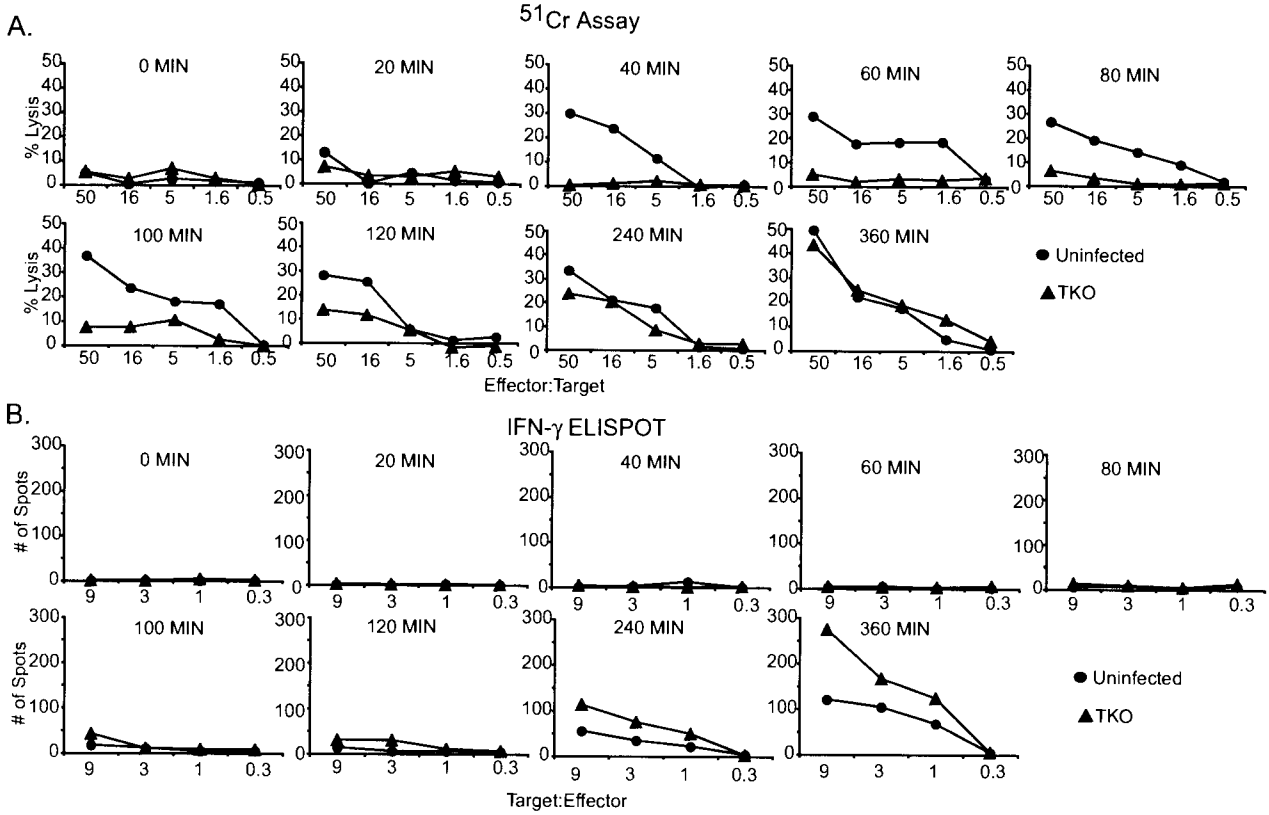

Figure 8. Duration of T cell stimulation required to kill. (A) K41s left uninfected, or infected with wild-type, or TKO and pulsed with SIINFEKL peptide titration at the same time as infection, after overnight incubation the OT-1 T cells were added for a standard 51Cr assay. PP2 was added at the times listed and the assay was allowed to incubate for the full 6 h before 51Cr release was measured. (B) IFN-γ ELISPOT was preformed under the same conditions as described in A. The PP2 was added at the times indicated and the assay was allowed to incubate for 8 h prior to detection of IFN-γ secretion.

(Lu et al., 2006a). 16 hours later, we determined the viability of the cells based on staining with annexin V (Figure 9a). We stained cells for both annexin V and 7AAD to determine viability, cells stained positive for 7AAD and/or annexin V were considered to be dead or dying, and cells that were negative for both were categorized as viable. By transfecting cells with different concentrations of granzyme B, we were able to demonstrate that TKO and wild-type were equally sensitive to apoptosis induced by granzyme B, but that they were markedly less sensitive then uninfected cells (Figure 9b). From these results we concluded that MCMV infected cells are more resistant to CTL mediated cell death then uninfected cells. This resistance to death cannot be attributed to the effects of the individual immune evasion genes as TKO and wild-type infected cells are equally sensitive to death.

Which immune evasion genes are involved in resistance to killing?

All of the results thus far have dealt with comparing TKO with wild-type to show that wild-type infected cells can not be killed when the immune evasion genes are present. To determine which of the immune evasion genes is mediating the resistance to killing we preformed ^{51}Cr assays similar to the ones described above using mutant viruses missing individual immune evasion genes (Figure 10). Both the infection with virus missing *m06* (*Δm06*) and the virus missing *m152* (*Δm152*) resulted in the restoration of killing of SIINFEKL pulsed cells. However, the loss of *m04* (*Δm04*) had no effect on the killing of the peptide pulsed cells. We concluded that the loss of either *m152* or *m06* allowed the CD8 T cells to kill the virally infected cells.

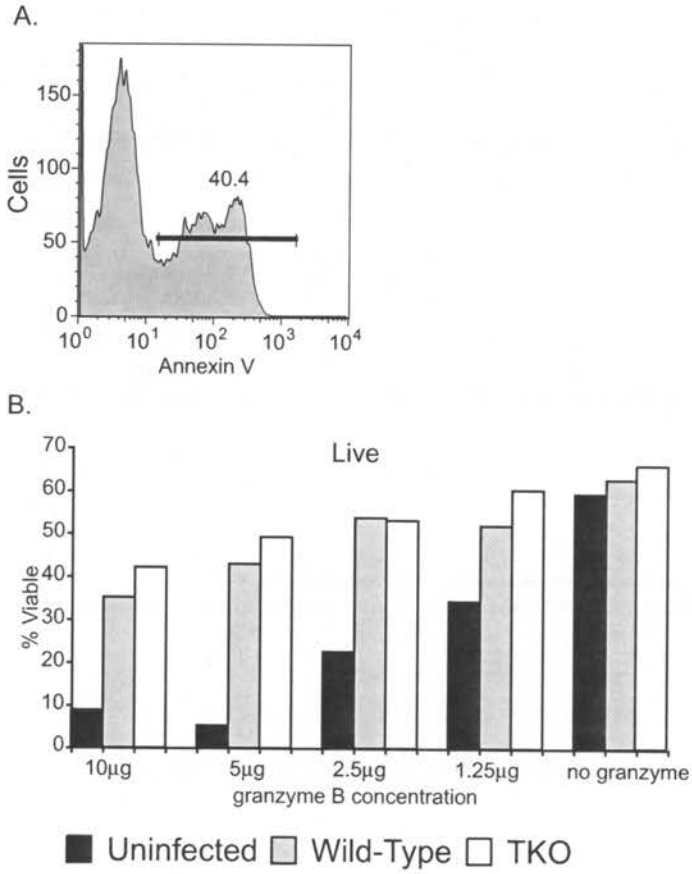

Figure 9. Wild-type and TKO infected cells are equally resistant to granzyme B mediated death.
(A) Representative FACs plot of K41s stained with annexin V to determine viability after granzyme B transfection.
(B) Compiled results of annexin V and 7AAD FACs staining of K41 cells left uninfected, or infected with wild-type, or TKO overnight then transfected with titrating amount of granzyme B. Cells were incubated overnight after granzyme B transfection then stained with 7AAD and annexin V. A and B are representative examples of 2 experiments.

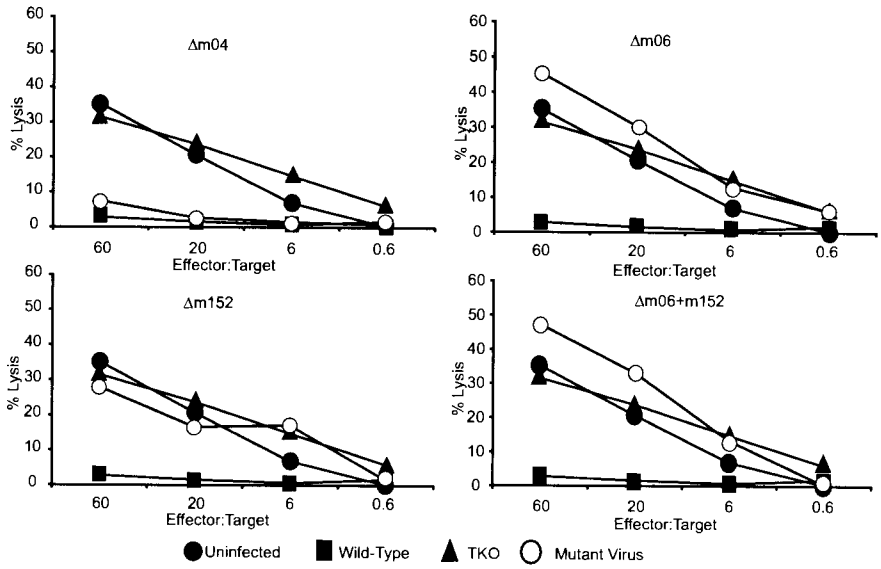

Figure 10. m152 and m06 but not m04 contribute to resistance to lysis. K41 cells infected with MCMV mutants shown, pulsed with 10-6M SIINFEKL were tested for lysis by OTI T cells. Shown is a representative example of 3 experiments.

Non-cognate MHC I contributes to killing of infected targets.

Since we had previously shown that the immune evasion genes did not impact the resistance to apoptosis (Figure 9) we wondered how both *m06* and *m152* could equally inhibit CD8 T cell killing. Although we have not formally ruled out the ability of both *m06* and *m152* to downregulate other surface class I molecules involved in CD8 T cell killing, the only known common function of these two immune evasion genes is the downregulation of MHC class I. In the experiments where we demonstrated that even when the total amount of cognate pMHC was equal between wild-type and TKO infected cells that wild-type infection still inhibited killing (Figure 5), there was a difference in the total MHC class I levels on the surface of the infected cells. We wonder whether this difference in total surface class I was important for the inhibition of killing. To address this question we used a transformed TAP-/- cell line, where the total amount of MHC on the surface of the infected cells is greatly reduced compared to the K41 controls (Figure 11a). The cells were also stained with the 25D.1.16 antibody to determine the total amount of SIINFEKL bound to the surface following the peptide pulse (Figure 11b). The TAP-/- and K41 cells were infected with the viruses or left uninfected and pulsed with SIINFEKL and the standard ^{51}Cr assay was performed (Figure 11c). We compared the ^{51}Cr assay result using TAP -/- cells with results from an assay run with K41 cells, which were used as a transformed fibroblast control. While uninfected TAP-/- cells were killed by the CD8 T cells, TAP-/- cells infected with TKO and pulsed with SIINFEKL were not killed by the antigen-specific CD8 T cells. We concluded from this result that the total amount of MHC on the surface of the infected cells was important in mediating killing of virally infected cells. In wild-type infected cells where the MHC levels are reduced due to the effects of *m06* and *m152*, CD8 T cells are not able to generate a strong enough effector response to kill.

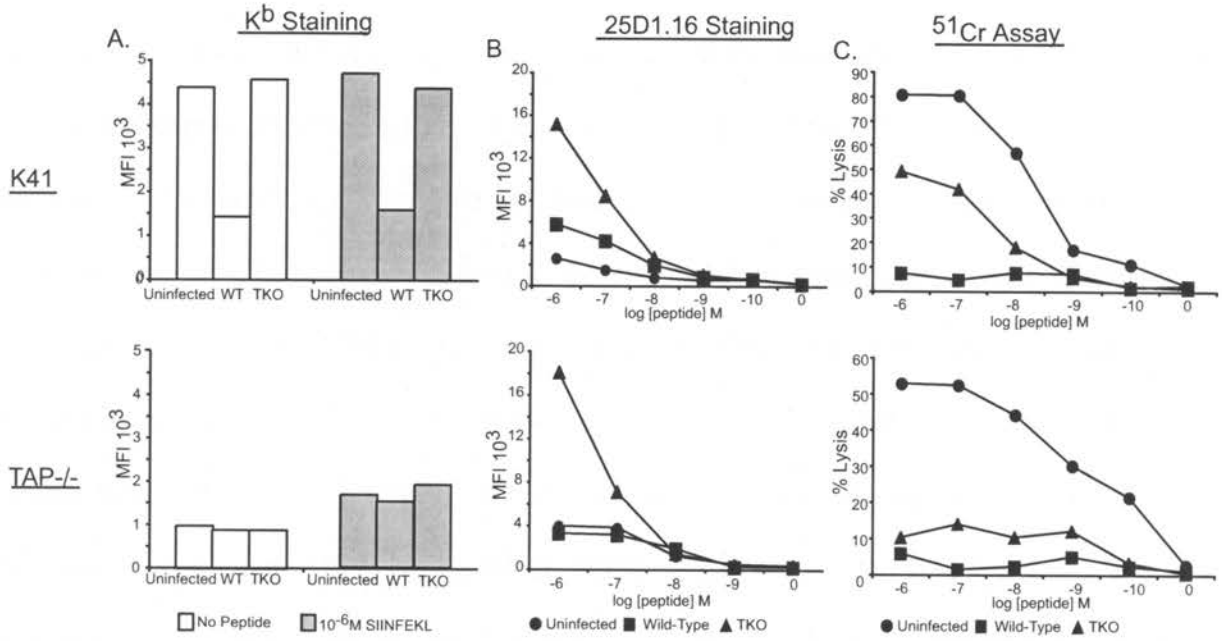

Figure 11. Total MHC class I is important for CTL mediated killing. TAP-/- and K41 cells were infected with wild-type, TKO or uninfected and pulsed with SIINFEKL peptide titration at the same time as infection (A) FACs surface staining with H2-Kb (Y3) with or without 10-6M SIINFEKL infected overnight. (B) SIINFEKL titration of peptide on cells at the same time as infection and stained with 25D1.16. (C) Cells prepared as in B and used in a 51Cr assay with an OT-1 T cell line used as the effector cells and the effector:target ratio at 30:1. A-C are representative examples of 5 experiments.

We then attempted to restore the class I levels on TAP-/- cells by the addition of exogenous noncognate peptide, to confirm that total amounts of MHC class I are important for mediating killing. For these experiments we added either H2-K^b (m141) or H2-D^b (M45)-binding peptides at different concentrations at the same time as we added the SIINFEKL peptide, and infected the cells. We then stained the cells for their total H2-K^b and H2-D^b class I cell surface expression (Figure 12a). We compared the H2-K^b and H2-D^b MHC surface expression of the TAP -/- cells pulsed with both cognate and noncognate with K41s pulsed with both peptides and TAP -/- pulsed only with the SIINFEKL titration. The addition of the m141 non-cognate peptide at 10^{-7}M increased the total H2-K^b above that of the TAP-/- pulsed with SIINFEKL alone, however when we added only 10^{-9}M m141 the H2-K^b cell surface staining was similar to that of TAP-/- with SIINFEKL alone. The H2-D^b peptide also did not increase the H2-K^b staining above the TAP -/- alone with the SIINFEKL titration. The H2-D^b staining was also as predicted, increasing the concentration of M45 did increase the H2-D^b staining as compared to the TAP-/- without any H2-D^b peptide added. Unfortunately even at the highest concentration of exogenously added noncognate peptide, we were unable to increase the H2-K^b or H2-D^b levels on the TAP-/- cells to that of the K41s. However since we did see an effect of the addition peptide, we can therefore determine if an increase in total MHC levels causes killing of infected cells. We also determined what affect the addition of noncognate peptide had on the amount of SIINFEKL bound (Figure 12b). In most cases the addition of noncognate peptide had no effect on SIINFEKL binding; only at the highest concentration of m141 was the binding of SIINFEKL inhibited (data not shown). These results suggest that the addition of noncognate peptide can upregulate the total MHC class I levels with little interference of SIINFEKL binding. The results of the ^{51}Cr assays allowed us then to determine if the increase in total MHC class I levels

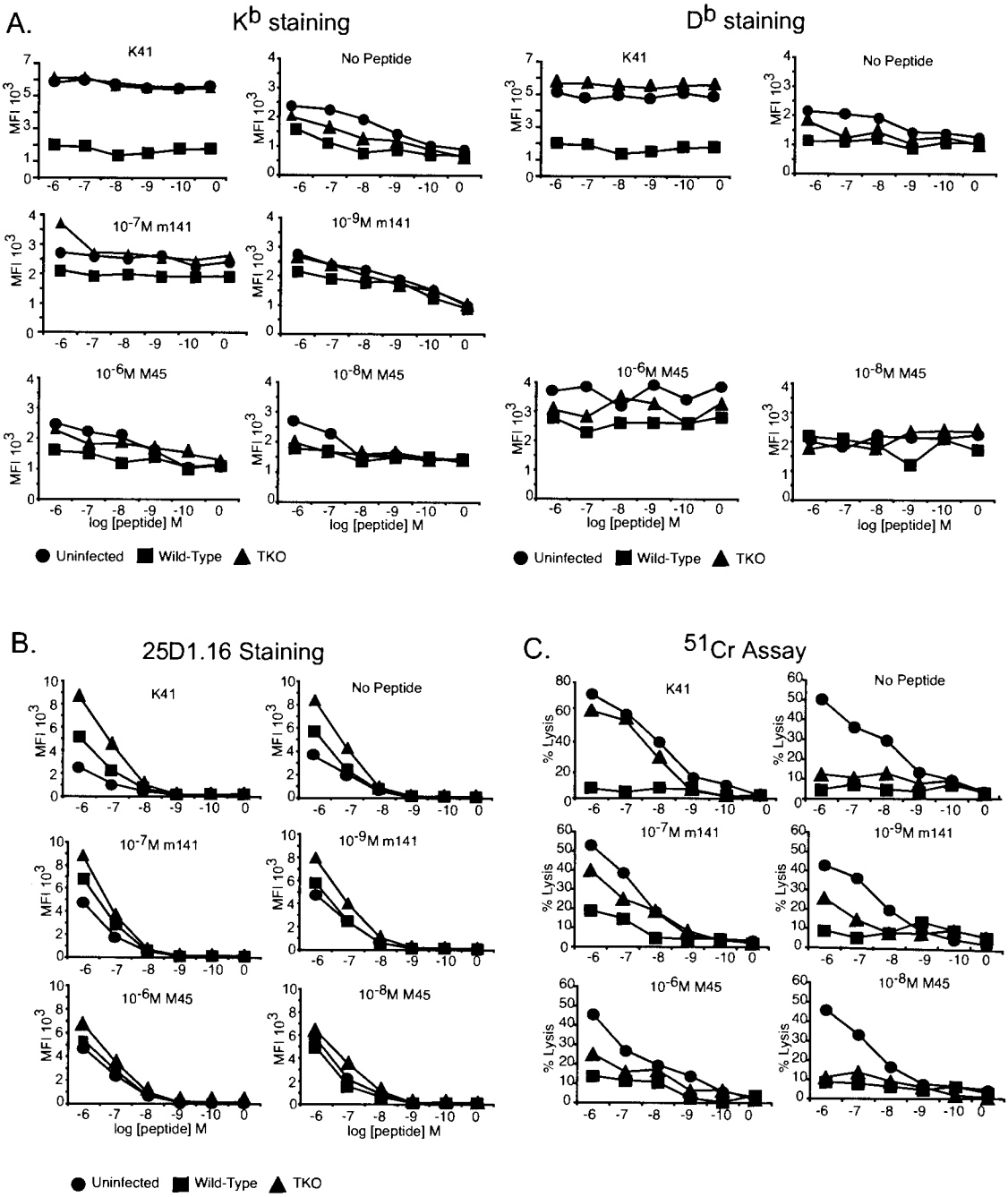

Figure 12. Restoration of MHC class I restores CTL killing of infected cells TAP-/- cells For all experiments shown the cells were infected with wild-type, TKO or uninfected and pulsed with SIINFEKL peptide titration shown on the X axis. At the same time the cells were pulsed with either m141 or M45 peptides at the concentrations shown.
(A) FACs surface staining with eithe H2-Kb (Y3) or H2-Db (B22.249)(B) Cells were stained with 25D1.16.
(C) 51Cr assay with an OT-1 T cell line used as the effector cells and the effector:target ratio at 45:1.

was enough to induce killing of the virally infected TAP-/- cells (Figure 12c). These results of the MHC and SIINFEKL surface staining corresponded with the results of the ^{51}Cr assay. At higher concentrations of noncognate peptide MHC the total amount of MHC class I is increased and the killing of TKO is restored. We noted that the effect of class I restoration on improved killing was greater with H2-K^b as compared to H2-D^b. This may be because SIINFEKL binds to H2-K^b class and the threshold for stimulating a killing response favors similar MHC class I molecules. Alternatively, the results we obtained may be based on the peptides chosen for this experiment. If killing is improved by the weak recognition of some noncognate pMHC, then altering the noncognate peptide may lead to variations in the results we have obtained. This possibility remains to be tested. Consistent with the 25D1.16 staining at the highest concentration of m141 peptide we did not see as much killing as we did at lower m141 concentration levels (data not shown). We believe this was due to competition for the MHC by both H2-K^b binding peptides, leading to a reduced total amount of SIINFEKL binding as observed in the 25D1.16 staining, causing the observed reduction in killing. Interestingly we did restore the killing of wild-type infected TAP -/- at high concentrations of m141, although the levels of killing were much lower then that observed for TKO. We can account for this result because the immune evasion genes are still functioning in the TAP-/- cells and, as shown by the class I staining, this leads to a reduced amount of MHC class I for the exogenous peptide to bind and stimulate a CD8 T cell response.

Discussion

The TCR's ability to recognize noncognate peptide MHC has been appreciated in many areas of immunology. The recognition of noncognate peptide MHC plays an important role in thymic selection. The weak interaction of the TCR with MHC presenting self-peptides results in positive selection (Fink and Bevan, 1978). TCR interactions with MHC in the absence of cognate antigen has also been shown to be important for the maintenance of memory CD8 T cells (Tanchot et al., 1997). More recently work has also been done looking at the role of noncognate peptide MHC in stimulating both the CD4 and CD8 T cell responses in the presence of cognate peptide (Anikeeva et al., 2006; Wulfing et al., 2002). Although the importance of noncognate loaded MHC has been demonstrated in many systems its relevance in an effector T cell response to a viral infection has not been explored. Studies examining the effector CD8 T cell response suggest that only a very small amount of MHC loaded with cognate peptide is needed to stimulate a CD8 T cell response (Purbhoo et al., 2004). If an effector T cell response can be generated with only a small amount of cognate antigen, there appears to be very little need for noncognate antigen. However, we have shown that the total amount of MHC present on the surface of an infected cell is critical for the generation of an effective CTL response.

We report here that the response stimulated by low amounts of cognate pMHC is not enough to kill MCMV infected cells. As part of the MCMV immune evasion strategy the virus downregulates the total MHC class I level on the surface of the infected cell, thereby reducing the total amount of MHC available to stimulate a T cell response. When we loaded equal amounts of exogenous cognate peptide onto cells infected with either wild-type or TKO-MCMV, we observed no killing of the wild-type infected cells while the TKO infected cells were efficiently killed. We were able to attribute the inhibition of killing to the reduction in total

MHC class I levels, because if we used a TAP-/- fibroblast cell line to mimic the phenotype of a wild-type virus infected cell we could inhibiting the lysis of TKO infected cells. The restoration of the MHC levels by the addition of exogenous noncognate peptide restored the killing of the TKO infected cells. The reduced MHC on the wild-type infected cell is protecting the infected cell from a lethal hit from the activated CD8 T cell.

As shown in Figure 7a, CD8 T cells released the least amount of granzyme B in response to uninfected cells pulsed with SIINFEKL. However the uninfected targets pulsed with peptide were highly sensitive to CTL-mediated killing as shown in Figures 2 and 5. To reconcile these findings, we now propose that it takes longer for CTLs to kill infected cells as compared to uninfected cells (Figure 8), because infected cells are more resistant to granzyme B (Figure 9). Although CD8 T cells are only releasing a small amount of granzyme B in response to uninfected cells, it is enough to kill the cells. However, killing of wild-type MCMV infected cells by CD8 T cells does not occur even though the amount of granzyme B released is greater than that released in response to peptide on uninfected cells. These differences are most likely due to the expression of anti-apoptotic genes encoded by MCMV.

MCMV's ability to inhibit apoptosis was recognized well before the proteins involved in the inhibition were identified (Kosugi et al., 1998). The MCMV gene, M36, also known as the murine viral inhibitor of caspase-8 activation (vMIA), inhibits the cleavage of pro-caspase-8, thereby blocking activation of the apoptosis (McCormick et al., 2003; Skaletskaya et al., 2001). MCMV also upregulates cellular anti-apoptotic members of the Bcl-2 family (Andoniou et al., 2004; Billstrom Schroeder et al., 2002), protecting the mitochondria from permeablization by the pro-apoptotic Bcl-2 family members (Billstrom Schroeder et al., 2002). There is also a group of genes encoded by CMVs that have been shown to inhibit apoptosis but their mechanism of

action has not yet been defined, these genes include M45 and m41 (Brune et al., 2001; Brune et al., 2003; Lembo et al., 2004; Lembo et al., 2000). We believe that these anti-apoptotic mechanisms combine to increase the resistance of an infected cell to death by CD8 T cell effector mechanisms.

The class I downregulation that occurs following MCMV infection is attributed to the actions of both *m06* and *m152* and not to *m04*. The effects of *m04* are antigen specific and are probably part of a unique immune evasion strategy that is unrelated to *m06* and *m152*. The downregulation of class I has a global effect on inhibiting the killing of the infected cells. The downregulation by *m06* and *m152* is only one part to the resistance of the infected cell to lytic granule. The MCMV genes involved in the blocking of CTL mediated death play an essential role in the immune evasion from CTL killing. By discovering the mechanisms involved in MCMV resistance to CTL mediated killing we can now begin to focus our attention on the impact of this resistance in vivo. The results reported here only take us one step closer to answering the question; why does MCMV encode immune evasion genes? Working from this new foundation we can focus on improving the functional effectiveness of the CD8 T cell response. The CD8 T cell response to MCMV is unsuccessful because of the cooperation between the MCMV anti-apoptotic and immune evasion genes; altering their interactions could lead to a more effective CTL response.

Chapter 5

Discussion and Conclusions

Summary

This project has been driven by one of the questions still outstanding in herpes biology: Why do herpes viruses encode immune evasion genes? The proteins encoded by the immune evasion genes are well known for their effects on MHC class I molecules. However, the CD8 T cell response to CMV dominates the immune response over time, suggesting that the immune evasion genes are inept at helping CMV to evade the immune response. Yet for a virus that is so highly evolved, it seems illogical that it would retain genes that do not serve a purpose, so we are still left to wonder: Why do herpes viruses encode immune evasion genes if not to prevent the immune response?

Studies looking at sequence variations within wild mouse viral isolates of MCMV noted that the three immune evasion genes are not conserved equally. The *m152* gene sequence is highly conserved within viral isolates, but multiple *m06* sequence variations were identified with different wild isolates. However, the critically important di-luecine motif of *m06,* which targets it and its MHC class I cargo to lysosomes, is conserved within the wild MCMV isolates (Smith et al., 2006). Interestingly, *m04*'s sequence within wild isolates is highly variable, and divergent from the laboratory isolate used to determine *m04*'s function (Corbett et al., 2007; Smith et al., 2006). It is tempting to postulate that the sequences important for the effector functions of *m06* and *m152* are more highly conserved then that of *m04*, because *m06* and *m152* have a greater impact on immune evasion than *m04* (Gold et al., 2002; Gold et al., 2004; Holtappels et al., 2006; Kavanagh et al., 2001a; LoPiccolo et al., 2003; Pinto et al., 2006; Wagner et al., 2002). The poor *m04* sequence conservation may be due to its weak overall impact on immune evasion. However, we still do not know how *m04* functions, so it is a bit premature to discount its impact

on immune evasion. We do know that all three of the immune evasion genes were identified because they could impact MHC class I molecules.

Chapter 2 clearly illustrates that understanding MCMV immune evasion is complicated, and the inhibitory actions of the individual immune evasion genes do not follow a discernable pattern. The combined actions of the immune evasion genes to inhibit CD8 T cell killing is absolute for the infection of H-2b cell lines. Prior to the studies described in chapter 2 we knew the immune evasion genes targeted individual MHC isoforms; *m06* was better at retaining H2-K^b MHC molecules, and *m152* had a more profound impact on H2-D^b (Kavanagh et al., 2001a; Wagner et al., 2002). We believed this specific targeting of MHC isoforms would translate into individual immune evasion genes more profoundly impacting presentation of MCMV epitopes associated with the individual isoforms. As the studies in chapter 2 indicate, MCMV immune evasion genes do not function in such a simple manner.

The initial studies looking at the impact of immune evasion genes on different class I isoforms also left us with many questions, that we had hoped to address in chapter 2. 1) Is the expression of any one immune evasion gene completely sufficient in inhibiting CD8 T cell killing of one epitope-specificity? 2.) Is the inhibition of K^b binding epitopes completely dependent on *m06* and conversely are D^b epitopes inhibited only by *m152*? 3) What is the impact of *m04*? 4) Does *m04* antagonize *m152* and does this antagonzation lead to killing of some epitope specificites that could be blocked by *m152* expression alone? 5) The immune evasion genes are expressed at early times post infection, so how well do they impact epitopes encoded during the IE phase of gene expression?

As we set out to answer some of these questions, we quickly realized our basic assumption that the immune evasion genes impact on down regulation of MHC class I isoforms

would translate into an impact of killing on different epitope specificities was incorrect. However we were still able to address many of the questions we had proposed, and develop new theories on immune evasion. While the studies preformed in chapter 2 and 3 provided us with a background understanding of how the individual immune evasion genes block epitope specific CTL killing, the work discussed in Chapter 4 focuses more closely on the why CD8 T cells cannot kill wild-type MCMV infected cells.

Through the use of a panel of mutant viruses developed by Wagner et al (Wagner et al., 2002) and multiple CD8 T cell epitopes defined by Michael Munks (Munks et al., 2006), I was able to show that no one immune evasion gene was completely sufficient at inhibiting antigen presentation of a signal epitope to its antigen specific CD8 T cell. This result highlights the importance of encoding multiple immune evasion genes for the evasion of immune responses. Studies on the immune evasion genes' impact on MHC surface levels had hinted that there needed to be more than one immune evasion gene present to down regulate the multiple MHC isoforms. These earlier studies did not indicate how the downregulation of class I affected CTL killing, and what if any impact *m04* was having on CTL effector functions. With the panels of epitopes and mutant viruses we were able to comprehensively test many of our earlier assumptions, and not surprisingly most of our assumptions were flawed.

Cell types used to examine immune evasion gene function

MCMV infects many different cell types including fibroblast, hepatocyte, endothelial, monocyte and macrophage cells (Podlech et al., 1998). Macrophages play a prominent role in CMV infection, therefore we used IC-21s, a transformed macrophage cell line, to do most of our studies in chapters 2 and 3. This choice of cells has shaped our understanding of how the

immune evasion genes function to inhibit effector functions. However, we have repeated most of the results obtained in macrophages in multiple other cell types and in most cases we have obtained similar results. I have noted the situations where we have seen a difference in the efficiency, or effects of the immune evasion genes. Differences between the results I have obtained and those of other groups may be explained by the choice in cell types and infection conditions. I have tried to reconcile some these differences. However, without completely repeating experiments under multiple conditions we can only speculate on the reasons for the differences. The conclusions we have come to regarding MCMV immune evasion are based on the cell types we analyzed. I studied 16 different MCMV epitopes and multiple different cell types to determine the immune evasion genes impacts on CD8 T cell effector function. My conclusions are strengthened by the magnitude and comprehensiveness of the studies, which has helped to give us a better understanding of immune evasion gene functions.

Real-time PCR experiments were completed to examine gene expression at different times post infection comparing 2 different cell types. IC-21 and MCVE's, a mouse endothelial cell line (Sapatino et al., 1993) were chosen because each cell type was known to be important for MCMV infection. There were some differences in gene expression profiles between these two cell types. However, the immune evasion genes seem to be expressed equally in the two cell types analyzed. Some of the genes encoding MCMV epitopes had dramatically different transcript levels in the different cell types. For example, there was a large amount of M45 mRNA in MCVE cells while the M45 mRNA was almost undetectable in the IC-21s (data not shown). We have not yet determined if this difference in transcript levels translates into a difference in protein levels, but if this is the case we would expect that blocking all the M45 loaded MHC class from reaching the surface is probably easier in IC-21 than MCVE cells.

A more thorough analysis of MCMV gene expression in multiple different cell types still needs to be completed. The immune evasion genes are not as efficient at inhibiting CD8 T cell killing of infected dendritic cells (DCs), as they are in other cell types. Certain epitope specific CD8 T cell lines, M38 m141 and IE3, can kill wild-type infected DCs. Determining the expression profiles of both the genes encoding these epitopes, and the immune evasion genes, within DCs in comparison to other cell types will help us to determine if the amount of gene expression impacts the ability of the immune evasion genes to block antigen presentation.

m04's unique, and controversial impact of CD8 T cell killing

For those few epitopes, where *m04* had an impact on inhibiting CD8 T cell killing, M33 M36 and M78, it would interesting to compare the CD8 T cell response between the laboratory strain of MCMV and the wild-MCMV isolates described above. We should see an impact of CD8 T cell killing of cells infected with the wild viruses, if *m04* is no longer functional. Our lab has already shown that cells infected with several different *m04* mutant viruses, generated by transposon mutagenesis, are differentially killed by MCMV-specific CTL clones (Ann Hill personal communication). It would be interesting if some of the wild isolates could protect against CTL killing while others did not. Since the sequence variations within the wild isolates are mapped, the differences in protection could be exploited toward understanding which sequences of *m04* are important for protection. In addition, *m04* is required to associate with m168 to traffic to the cell surface with MHC (XiuJu Lu manuscript in preparation). Some of these wild isolates may retain or lack this association, and we could exploit this difference to determine if m168 association was required for *m04* to inhibit CTL effector functions and even if *m04*'s association with MHC on the cell surface is required to inhibit killing.

We still do not know if the immune evasion genes are "seeing" the epitope bound to the MHC, and selectively inhibiting the expression of MCMV-specific epitopes bound MHC. This would be most interesting for explaining the immune evasion strategies of *m04*. In Chapter 4, we have shown that *m06* and *m152* have a much more important role in the downregulation of total MHC class 1 levels. However, *m04* appears not to play a in this type of immune evasion, instead it seems to target specific epitopes, including M33, M36, and M78. Why and how *m04* selectively targets epitopes expressed by these three genes is still unclear.

Both M33 and M78 are G-protein coupled receptors and have homologies to cellular chemokine receptors (Melnychuk et al., 2005; Oliveira and Shenk, 2001). M33 has been shown to be important for smooth muscle cell migration and growth in the salivary glands (Davis-Poynter et al., 1997; Melnychuk et al., 2005). M78 is important for growth in macrophages and is required for IE mRNA induced accumulation and is found within the viron (Oliveira and Shenk, 2001). M36 is a virally encoded anti-apoptotic gene that is required for growth in macrophages (McCormick et al., 2003; Menard et al., 2003). The importance of these individual genes for infection of certain cell types may have some relevance in *m04's* selective impact. Because the studies done in Chapter 2 were preformed in IC-21s, a transformed macrophage line, it is possible we identified an impact of *m04* on immune evasion that we would not have seen if we had used a different cell type. *m04* may inhibit the recognition and CTL killing of other epitopes in other cell types. The strength of *m04*'s effect in macrophages has been confirmed in primary bone marrow macrophages, where the effects of *m04* inhibition of antigen presentation are more dramatic than in other cell types (LoPiccolo et al., 2003; Pinto et al., 2006). M33, M36 and M78 may be expressed at higher levels in these cells and only in these cell types can *m04* play a role in preventing their presentation. The overexpression of other MCMV genes encoding

epitopes in macrophages could be used to determine if *m04* is targeting highly expressed proteins or specific proteins important for viral replication in macrophages. The experiments can be repeated in other cell types to determine if this is a cell type specific phenomenon. However because the genes targeted by *m04*, M33 M36 and M78, are all involved in interfering with cellular processes (chemokine receptor binding and inhibition of apoptosis), over expression of those genes may interfere with the interpretation of the results, and therefore proper controls are absolutely required. Also there are other genes that encode MCMV epitopes that have also been shown to be required for growth in macrophages that do not appear to be affected by *m04*, including m139, and m141. Determination of their expression level in comparison to the genes regulated by *m04* may provide more insight into *m04*'s function. If the expression levels between these genes are similar it could provide more evidence that *m04* is specifically targeting certain genes that can escape the global effects of *m06* and *m152* on inhibiting CD8 T cell killing. Further investigation of how *m04* inhibits presentation of these epitopes may provide more answers about *m04* mechanism's of inhibition of antigen presentation.

The observation that *m04* antagonizes the effect of *m152* has been a very appealing explanation for *m04*'s function. By rescuing the MHC class I retained by *m152*, *m04* presence could explain how priming takes place, and *m04*'s presence may help with restoring class I on the surface of infected cells so as not to induce NK cell responses. When we looked at each of these possibilities, neither one turned out to be accurate. We saw no killing of wild-type infected cells and in a few cases the absence of *m04* alone allowed for recognition of the infected cells suggested that *m04* was not involved in priming. The idea that *m04* rescues class I to protect against NK cell killing was discounted, at least for C57BL/6 mice, when we showed that wild–

type and Δ*m04* infected mice had equal titers on day 4 post-infection (A.K. Pinto unpublished observation).

As demonstrated in Chapter 2 the restoration of killing in the presence of *m04* was not observed, except for one epitope, M38. This result is in contrast with a similar study performed by Holtappels et al, where *m04* appeared to antagonize *m152* in the absence of *m06* in BALB/c mice (Holtappels et al., 2006). We have confirmed their results using T cell lines specific for the BALB/c epitope, pp89 but we failed to see an effect of the restoration of MHC class I in the C57BL/6 system, under our condition. It is also interesting to note that we did not see as pronounced effect of *m152* at inhibiting CTL killing as they observed. Holtappels et al suggest that there is competition for the ERGIC-retained MHC molecules. Normally *m06* takes the retained class I and degrades it in the lysosome, but in the absence of *m06*, *m04* is able to bring the class I to the cell surface. The easiest argument to discount this possibility, is to mention that an infection without *m06* is not physiologically relevant (since all wild isolates of MCMV contain *m06*). Another argument against this possibility is that the pulse chase experiment done with wild-type MCMV and Δ*m152* show no difference in the amount of *m04* associated class I (XiuJu Lu unpublished observation). This argues against the idea that *m04* is acquiring its MHC class I from the ERGIC pools provided by *m152*. *m04* appears to be associating with MHC class I molecules independently of *m152*. However, we do not know *m04's* function in inhibiting antigen presentation it is important to observe its effects under all conditions. It is possible that the time the assays were done and different infection conditions, including the cell types and centrifugal enhancement they used, could lead to the difference in results. We have not repeated all the results, using similar conditions, to determine if the increase in antigen presentation they have observed has an impact on CTL killing.

As discussed above it would be interesting if *m04* can selectively target epitope specific class I molecules to allow their expression on the cell surface. During wild-type MCMV infection *m04* may target self-peptide loaded class I to be presented on the cell surface, selectively avoiding MCMV-epitope specific class I molecules. To address this, future studies could be done where the peptide bound to *m04* associated-MHC molecules could be stripped off and analyzed by mass spectrometry. A comparison of peptides from non *m04* associated MHC class I with *m04* associated MHC class I peptide would show if *m04* has some specificity for the peptide bound to MHC class I. Studies performed in the presence and absence of *m06* would determine if the presence of *m06* affects *m04* association with MHC class I molecules, and if the peptides loaded onto the *m04*-MHC molecules are different. Future experiments with plate-bound peptide loaded MHC class I molecules alone or in association with *m04* could be used to stimulate an antigen specific CD8 T cell response. This experiment would help to determine if *m04* associated MHC class I molecules can stimulate a CD8 T cell response. Currently, the studies we have done to assess the functional outcomes of immune evasion gene expression suggest that the antagonism plays no role in restoring killing in wild-type infected cells for any epitope tested. However the contradictory results may help motivate us to dissect further the role of *m04* in immune evasion.

m06 and m152 downregulate MHC class I and this affects CD8 T cell killing

As predicted from their impact on MHC downregulation, both *m06* and *m152* had a dramatic effect on CTL inhibition of killing. We were surprise to find that *m06* and *m152* had strong effects on both H-2K^b and H-2D^b associated-epitopes. Also the effect of CTL inhibition by *m152* was more pronounced then the inhibition by *m06*. CD8 T cells detected during the

immune response to MCMV are generated against a widely diverse group of genes. It is possible that the immune evasion genes have redundant functions, to ensure that CD8 T cells do not see the virus. The quantity of MHC class I expression varies in different cell types so the overlapping functions of *m06* and *m152* may be important to reduce total class I expression during infections when the level of MHC class I is high in cells. In our system, unlike that of Holtappels et al, we either pre-treated the cells with IFN-γ or used a macrophage cell line with high levels of MHC on the cell surface, which infection with TKO MCMV did not increase. It is conceivable that only one immune evasion gene would be needed to prevent presentation in situations where the MHC class I levels were lower, as was the case in some of the experiments preformed by Holtappels et al, and this might explain why the observed a much more dramatic affect of m152 alone at inhibiting CTL killing. However, during infection when MHC class I levels are higher more than one immune evasion gene is required to completely inhibiting antigen presentation.

m152 is equally, if not more effective than *m06,* at inhibiting CD8 T cell killing of virally infected cells. This result was a bit surprising to us, given our original prediction that the immune evasion gene's impact on MHC class I expression should directly correlate with their impact on CD8 T cell inhibition, at least for *m06* and *m152*. As we struggled for an explanation for this observation, we began to look at the possibility that the immune evasion genes could affect other cell surface molecules and the downregulation of these other proteins could explain the differential impact of the immune evasion on CD8 T cell killing as compared to MHC downregulation. One of the first and most likely candidate proteins were looked at were the family of NKG2D ligands.

m152's downregulation of the NKG2D ligand Rae-1

As discussed in Chapter 3, *m152* can also downregulate the NKG2D ligand Rae-1, and it was presumed that this downregulation had an impact on CTL inhibition. While we did not see much of an impact of NKG2D in this setting, it does not mean NKG2D does not have an effect in other circumstances. While setting up the experiments discussed in Chapter 3 we examined over 20 different cell types to see which cells had the highest NKG2D ligand surface expression, as determined by NKG2D-tetramer staining. Therefore when we infected these cells with wild-type MCMV we saw the greatest downregulation of ligand expression. Most of the cells we tested had 1 to 2 logs lower mean fluorescence intensity (MFI) compared to the IC-21s and the K42 cells we chose to use in our experiments. The reason for our choice was that we felt the cells with the greatest downregulation in ligand expression would have the greatest impact on costimulation. However, it may be worth revisiting the role of NKG2D ligands in costimulation. By using macrophages for most of our studies we may have provided the T cell with adequate co-stimulation, therefore negating any need for NKG2D ligands. If we used cells that expressed lower levels of these other costimulatory molecules we may have seen a more dramatic effect of NKG2D ligand downregulation. Investigations to determine if this hypothesis is correct are currently ongoing in the laboratory.

The mechanism used by *m152* to downregulate the Rae-1 NKG2D ligands is still unknown. Although NKG2D ligands do have some homology to MHC class I, it is not yet clear if *m152* inhibits the Rae-1 surface expression by blocking its progress past the ERGIC, which is the same mechanism that *m152* uses to block MHC class I. If *m152* is able to block Rae-1 surface expression by a different mechanism, we may be able to identify additional cell surface proteins whose expression is also inhibited. Similar to the identification of MHC class I

retention by *m152*, the determination of Rae-1's fate can be made by a combination of immunopercipitation experiments and immunofluorescence (Ziegler et al., 1997). The immunopercipitation studies would be done to look at the glycosylation status of the protein in the presence and absence of *m152*, and the immunofluorescence studies with Rae-1 specific antibodies could be done to determine the localization of Rae-1 in the presence of *m152*. These studies could also give us a good idea about how much ligand is downregulated in comparison to the amount expressed during infection. We may get a better idea of the importance of NKG2D ligand downregulation on inhibiting CD8 T cell costimulation if we could show how much of the ligand is downregulated.

Possible roles for other costimulatory molecules

We have by no means exhausted our examination of cell surface molecules affected by MCMV immune evasion genes. Because the immune evasion genes target MHC class I molecules it would make sense that the immune evasion genes would also impact some class I like molecules. One potential target of the immune evasion genes is the nonclassical class I molecule Qa-1^b. Qa-1^b binds the inhibitory NK cell receptor CD94/NKG2A. CD94/NKG2A is found both on NK cells and activated CD8 T cells, and is known to be expressed on antigen-specific CD8 T cells present in persistent and chronic infections, including MCMV (Byers et al., 2006; Gold et al., 2004; Liao et al., 2006; Moser et al., 2002; Prabhakaran et al., 2005; Suvas et al., 2006). Qa-1^b binding to the NKG2A causes an inhibitory signal to be delivered to the T cell. This inhibitory signal has been shown to affect cytolytic activity while disregarding cytokine secretion (Prabhakaran et al., 2005; Suvas et al., 2006).

m04 could bind Qa-1^b in the ER preventing its retention, or lysosomal degradation, and ensure that it is expressed on the cell surface. Simple Qa-1 blocking experiments were done to show that NKG2A-Qa-1 interactions were not inhibiting killing of IC-21s in our system (A.K. Pinto unpublished observation). However, as with NKG2D, certain cell types may express different amounts of Qa-1 and therefore its cell surface expression level may be more critical for impacting CTL killing. On the cell types that we have examined, Qa-1 expression is extremely low, and we were unable to detect enough on the cell surface to determine if MCMV impacted the expression. To test the hypothesis that *m04* rescues Qa-1 expression immunoprecipitation experiments could be completed to determine if *m04* can bind to Qa-1 and if the associated complex can be exported to the cell surface. If *m04* does allow the export of Qa-1 to the cell surface, then studies with a panel of mutant viruses can be done, in the presence of blocking antibodies, to determine if the NKG2A-Qa-1 interaction plays a role in the inhibition of CTL killing.

ICAM-1 is another very likely target for immune evasion genes. ICAM-1 is essential for much of the observed CTL activation, so it seemed reasonable that MCMV would try and alter its expression. ICAM-1 association with LFA-1, on the T cells, helps stabilize the T cell-APC interaction and is involved in synapse formation. One of the potential problems with the targeting ICAM-1 downregulation is that cytokine production would also be affected. Not surprisingly, we did not see a downregulation of ICAM-1 following infection with either wild-type or TKO. However, we did observe an upregulation of ICAM-1 during virus infection. This upregulation was independent of the immune evasion genes, because it occurred in both wild-type and TKO infected cells. The viral infection increased ICAM-1 expression above what was observed by IFN-γ treatment of uninfected cells.

There is a correlation between ICAM-1 expression and increased atherosclerosis in HCMV patients (Westphal et al., 2006). Increased in ICAM-1 expression altered cell migration and trafficking to the site of infection, this altered migration was linked to an increase in disease severity. However, to my knowledge, an impact of increased ICAM-1 expression on CD8 T cell effector functions has not been formally examined.

What may be occurring in our system is that increases in ICAM-1 levels maybe increasing the duration of CD8 T cell APC interactions, this would prevent the T cell from killing the target then rapidly moving on to other APCs. Holding onto the T cell while being refractory to the lytic granule release maybe another mechanism employed by MCMV to reduce the impact of the T cell response. This theory would require testing by conjugate assays and live video microscopy, to determine if the T cell APC interaction was longer for infected cells compared to uninfected cells. Future experiments to block or reduce ICAM-1 expression would then have to be done, to demonstrate that the increased interaction time was due to increases in ICAM-1 expression levels.

MCMV encodes other genes to downregulate costimulatory molecules as part of its immune evasion strategy. In our studies we did not examine the impact of these genes on CD8 T cell recognition and killing, however it is important to be aware of their function in immune evasion. Studies have shown that m147.5 downregulates CD86 (Loewendorf et al., 2004), and m138 downregulates CD80 (Mintern et al., 2006). The reduction or absence of these costimulatory molecules maybe contributing to the inhibition of CTL killing we are observing. There are several experiments that should be completed to address the contribution of m147.5 and m138 to the inhibition in killing we have observed. Viruses mutated for these genes could be used to determine if our T cell lines are able kill cells infected with the mutant viruses.

Similar to the experiments proposed above, it will be important to see how strong the downregulation of CD80 and CD86 is in different cell types. If there are variations in the effects in different cell types, the result may help explain some of our observations in DCs compared to other cells. If we could show that the costimulatory molecules on DCs were still functional and aided in this DC phenotype, we could justify our assumptions regarding DCs status as professional APCs.

Emerging is a picture of where MCMV genes downregulate multiple costimulatory molecules, blocking CD8 T cell activation by multiple different pathways. MCMV immune evasion strategies are often cell type specific. The complexity and the redundancy of the system may be necessary for immune evasion to work within the whole host. MCMV has multiple stages of infection; acute, latent or persistent, and reactivation within the host. It is almost certain that the virus has devised separate immune evasion strategies to deal with each of these stages. The immune response during acute infection involves both innate and naïve adaptive cell types, while the response generated against reactivating virus is primarily the recall response of antigen experienced effector cells. I have focused my studies on the evasion of the effector T cell response, believing that the virus survives the acute response and has devoted most of its immune evasion energies toward developing strategies to maintain persistence.

Timing of MCMV gene expression

The immune evasion genes are expressed at early times post infection, so how well do they impact epitopes encoded during the IE phase of gene expression? A long term clone generated against the BALB/c IE antigen pp89 has been shown to recognize and kill MCMV infected MEFs when early gene expression is blocked (Reddehase et al., 1986), but in situations

where all the immune evasion genes are expressed pp89 specific-CD8 T cell to fail recognize wild-type infected cells (Holtappels et al., 2006). When we looked at this question we saw that the immune evasion genes were able to efficiently inhibit presentation of IE gene products. For our studies we used an epitope encoded by the IE3 gene. The reason for this was that we did our studies in C57BL/6 mice, where pp89 is not an epitope. We attribute the inhibition of IE3 killing to the fact that there is not a lot of IE3 protein before transcripts of the immune evasion genes *m06* and *m152* can be detected.

m06 and *m152* transcripts can be detected by 2 hours post-infection, although experiments have proven that *m06* and *m152* are E genes because their expression can be inhibited by cyclohexamide. To support this argument, the experiments in Chapter 4, Figure 2 show that if cells are infected with wild-type or TKO for 3 hours prior to performing the ^{51}Cr assay antigen specific CD8 T cells can kill TKO infected cells but not wild-type infected cells. This indicates that the viral antigens are being synthesized but the immune evasion genes can inhibit the presentation. However when we exogenously add cognate peptide the wild-type infected cells are now killed. In this case we have been able to overwhelm the immune evasion genes at early time post infection. If we wait longer after infection we no longer can see recognition of wild-type infected cells even with the addition of exogenous cognate peptide. This result supports our hypothesis that the immune evasion genes can inhibit IE gene expression because there is not a lot of IE3 peptide present before the immune evasion genes are expressed. This result also supports some of my previous statements that the effects of the immune evasion genes vary at different times post infection, and this could lead to alternate interpretations of immune evasion gene function.

To prove that the immune evasion genes are able to inhibit small amounts of IE gene expression, future experiments can be done by transfecting a plasmid encoding IE3 and increase the amount of peptide expressed before the immune evasion genes are expressed. I would predict that by increasing the amount of IE3 peptide in the cell at early times post MCMV infection we could detect killing of the infected cell, however at later times after infection the immune evasion genes would be able to inhibit even the IE3 peptide expression.

Priming in the presence of immune evasion genes

One of the most difficult questions we have to address is why I see such a strong impact of immune evasion genes on CD8 T cell killing, yet we see no effect on the priming of a strong CTL response. Long term analysis of CTL responses in wild-type MCMV infected mice compared to TKO infected mice revealed only a few differences in epitope specific responses, which may be explained by the large variation the responses detected within the mice (Munks manuscript in print). However in BALB/c mice there was a higher amount of wild-type virus in the salivary glands compared to TKO, and the increase in viral load was due to the control of TKO by CD8 T cells. TKO viral titers were increased to the level of wild-type following the depletion of CD8 T cells (Lu et al., 2006b). In C57BL/6 mice viral transcripts for both wild-type and TKO could be detected after 6 weeks of infection, although the amounts of virus could not be determined (Gold et al., 2004). So how is the inhibition of killing we see in vitro related to, or important for, the in vivo infection. Put another way, why does MCMV encode immune evasion genes? I think we are a long way from answering this question and it is my hope that the work contained within this dissertation moves us closer toward answering this very vexing question.

A hypothesis strongly favored recently is that priming of CD8 T cells occurs through cross-presentation, which would negate the effects of immune evasion genes. During acute infection MCMV infected cells would be phagocytozed by DCs and the antigens could then be cross-presented. There is a large body of literature supporting the role of cross presentation in priming CD8 T cell responses to infection {Reviewed in (Rock and Shen, 2005)}, and many people believe that most, if not all, priming occurs by cross-presentation. In addition to cross-presentation, experiments that I have done show that the immune evasion genes do not inhibit killing of wild-type infected DCs by some epitope specific CD8 T cells (Munks manuscript in print). DCs, by virtue of the professional antigen presentation status, seem to be more resistant to the effects of the immune evasion genes. So either direct or cross-presentation of epitopes by DCs maybe responsible for priming the CD8 T cell response.

Our lab has tried to test the role of cross-presentation for MCMV priming without much success as of yet. It is very difficult to establish a model where cross-presentation is not occurring but direct presentation can still occur. One possibility that might be explored is depleting the total DC population from the mouse at different times during infection. While this will not directly address the importance of cross-priming it will help to determine the role of DC in priming. As I have shown, antigen specific T cells can kill DCs infected with wild-type MCMV. If we deplete DCs with antibodies, or use the CD11c-DTR transgenic mice (Jung et al., 2002) to remove the DCs after diphtheria toxin treatment at different times during MCMV infection, we could determine if the DCs are the primary mediators of priming the CD8 T cell response. Controls must be done to account for disruptions in the NK cell response as well as potential differences in viral titers in the absence of DCs. These experiments would be useful in determining a role for DCs during MCMV infection. If we did see differences in either the

amount of priming or the hierarchy of the response we could begin to conclude that the immune evasion genes are not functioning in DCs, and from there we could begin to look specifically at DCs ability to directly prime versus cross–priming a CD8 T cell response.

When do the immune evasion genes function during infection?

MCMV has to achieve 2 main goals in order to survive. 1) Infect the host and establish a persistent infection and 2) spread to a new host. The immune evasion genes could be involved in protecting the cells important for viral spread from being killed by antigen specific CD8 T cell responses and not as involved in the acute phase or the establishment of a persistent infection. We have focused so much of our attention on the establishment and maintenance of a persistent infection that we have fail to recognize that without the ability to spread to a new host MCMV would have gone extinct a very long time ago.

The immune evasion genes probably do help in the establishment of a latent virus pool but that may not be their primary function. Their presence may help seed a larger latent pool by decreasing the ability of the CD8 T cell response to detect the infected cells. Studies still need to be done to determine if in the presence of an intact immune system there is a difference in the amount of virus between wild-type and TKO infected mice. The difficulty in finding latent viral genomes has hampered much of our understanding of the establishment of latency. Data that we have now proves that TKO MCMV genomes can be detected 6 weeks after infection, and after any virus can be detected by plaque assay (Gold et al., 2004), suggesting that the immune evasion genes are not needed for viral persistence.

As was shown by Lu et al, the immune evasion genes are important for getting virus to the salivary glands (Lu et al., 2006b). The immune evasion genes may help the infected cells get

to the salivary glands without detection by the CD8 T cell response. If the primary purpose for encoding immune evasion genes is to allow the virus to spread then in the absence of the immune evasion genes there should be no viral spread. This very simple concept is very difficult to prove. Studies looking at viral spread are very challenging with inbred mouse populations and depletion of immune responses to increase viral titers defeats the purpose of the immune evasion genes. Transmission does occur in wild mouse populations, as shown by multiple MCMV isolates being detected in wild mice populations. To determine the possible role of the immune evasion genes in transmission outbred mice could be infected with TKO, wild-type, and the wild MCMV isolates. The differences in virus titers would be measured to determine the possible role of the immune evasion genes in viral spread.

Currently we are left with several incontrovertible facts, which we must fit the function of the immune evasion genes into: 1) In vitro, cells infected with a virus containing all three immune evasion genes are not killed by antigen specific CD8 T cells (Pinto et al., 2006). 2) Viral transcripts of both wild-type and TKO can be detected 6 weeks post-infection (Gold et al., 2004). 3) Wild-type MCMV is found at higher titers within the salivary glands than TKO (Lu et al., 2006b). 4) The immune response to wild-type and TKO is not significantly different at different times post infection (Munks et al., 2006). Fitting all of these observations into one unifying theory is still a long way off, and my work and the work of many others has only helped to narrow down the possibilities for how the immune evasion genes are functioning.

Immune evasion genes impact on IFN-γ

In this discussion I have focused on the role of the immune evasion genes in the inhibition of lytic granule release, and I have not touched on the cytokine response generated

against MCMV. The immune evasion genes do not appear to affect the IFN-γ response to MCMV (C.M. Doom unpublished observation and A.K. Pinto chapter 4). As discussed in the introduction it is much easier to induce CD8 T cells to kill than it is to get them to secrete IFN-γ. Why then do we see cytokine production but no killing?

This discrepancy has helped to establish our new perspective on immune evasion and viral infection. Very few studies have shown a dissociation of these two CD8 T cell effector functions, and those that have, have found CD8 T cells that can kill but not produce cytokines. In our case the T cells can make a detectible cytokine response but do not kill infected cells. That does not mean that the T cells are not being stimulated to kill infected cells. The granzyme B ELISPOTs shown in Chapter 4, Figure 7 demonstrates that the CD8 T cells are releasing granzyme B in response to cognate peptide on the surface of wild-type infected cells, however the number of cells and the amount of granzyme released is lower in comparison to TKO infected cells. An equal number of T cells are making IFN-γ however the amount of IFN-γ, as determined by MFI shown in Chapter 4, Figure 5 suggests that the T cells are making less IFN-γ in response to uninfected and wild-type infected cells pulsed with peptide. Unfortunately the ELISPOT data for IFN-γ does not confirm the ICCS results shown in Chapter 4, Figure 7. Background problems associate with the IFN-γ ELISPOT assays have caused me to question these results, and the assay needs to be repeated to clarify the results. If CD8 T cells are making less IFN-γ in response to less cognate peptide, we can demonstrate that the effector functions, lytic granule release and cytokine production, of the responding CD8 T cells are not disconnected.

The IFN-γ released in response to MCMV infection is a potent anti-viral. However, MCMV also encodes a gene, M27, which interferes with the STAT signaling pathway and

blocks many of the effects of IFN-γ (Zimmermann et al., 2005). Similar to the inhibition of death observed following the release of granzymes, MCMV-infected cells seem to be refractory to many of the anti-viral affects of IFN-γ. In the absence of M27 MCMV replication is greatly reduced in the presence of IFN-γ. The immune evasion genes maybe functioning to reduce the amount of IFN-γ being secreted in response to infected cells and M27 is then able to block the anti-viral action of the IFN-γ that is secreted. Because more IFN-γ maybe secreted in response to TKO it would be interesting to determine if the STAT pathway is equally affected in TKO and wild-type infected cells.

MCMV infected cells highly are resistant to granzyme B

MCMV has multiple ways in which it interferes with the apoptosis pathway. The importance for keeping the cell alive during infection has obvious advantages for the virus, for survival and replication. Until recently we had not considered the inhibition of apoptosis as a possible immune evasion strategy for MCMV. Cells infected with MCMV have historically been resistant to CTL mediated lysis. We have always believed that this resistance to lysis was due entirely to a block in CTL lytic granule release due to the reduced class I levels on the surface of infected cells. However the reduction in class I surface levels mediated by the immune evasion genes is not complete and there is still a substantial amount of class I on the cell surface. CD8 T cells can release lytic granules in response to very low amounts of cognate antigen (Purbhoo et al., 2004).

The complete block in death of MCMV infected cells did not correlate with the low level of MHC class I still present on the surface of infected cells. After the addition of exogenously added cognate peptide failed to restore killing of wild-type infected cells, we felt that MCMV

must be altering the CD8 T cell effector functions in some way as to inhibit lytic granule release, because we did see cytokine release in response to wild-type infection. However after analyzing the granule release by granzyme B ELISPOT we were able to show that CD8 T cells were releasing granzyme in response to cognate pMHC. How then were the wild-type infected cells not dying while uninfected cells, that received equal if not less granzyme, were sensitive to death.

We believe that wild-type MCMV infected cells are resistant to death, because MCMV blocks the apoptosis induced by the granzymes released by CD8 T cells in response to wild-type infection. To prove this we demonstrated that wild-type and TKO infected cells are equally resistant to death from granzyme B and that uninfected cells are much more sensitive to granzyme B mediated death. This experiment proves that the immune evasion genes are not involved in blocking apoptosis. The reason that TKO infected cells are killed by lytic granule release is that the amount of granzyme they receive is much greater than that of wild-type infected cells.

In future experiment it would be interesting to determine how much if any granzyme B is being released in response to endogenous pMHC in wild-type infected cells compared to TKO infected cells. While this will not help us to determine exactly how much epitope specific MHC molecules are present on the surface of an infected cell, we have seen a titration in granzyme release that corresponds to the SIINFEKL peptide titration. We may be able to estimate how much of each peptide-epitope is present on the surface of the wild-type infected cell. There is a concern however that the ELISPOT may not be sensitive enough to detect very low amounts of lytic granule release that will accompany low levels of pMHC. Other more sensitive assays,

such as calcium flux analysis, may need to be done as a surrogate for granzyme release to measure T cell activation.

CD8 T killing of TKO compared to uninfected cells

We have also noted in than is needed to kill uninfected cells. While this is related to the observation that it is easier to kill uninfected cells in comparison to infected cells, this result also has great significance when we Chapter 4 that the duration of signaling events required to kill TKO infected cells is much longer think about what makes an effective T cell response. By disrupting the CD8 T cell signaling events during a ^{51}Cr assay we noticed that there was at least a 2 hour difference between the death of uninfected and TKO. Up until now we have measured cytolytic activity after a 4-6 hour incubation with T cells and APCs. By doing experiments in this way we would have said that T cells could kill TKO infected cells just as well as uninfected cells pulsed with peptide. Through the experiments with PP2 we have just begun to show that our previous assessment of an effective T cell response may be inaccurate and that although it requires a very small stimulus to induce the killing of an uninfected cells, the threshold stimulus required to kill an infected cell is much higher.

There are several possible problems with the experiments shown in Chapter 4 Figure 10. First PP2 is a src kinase inhibitor, and may be altering other cellular and viral processes other then just T cell stimulation. To show that PP2 does not affect granzyme B mediated cell death, uninfected, wild-type, and TKO infected cells were treated with PP2 during the transfection with granzyme B and no difference was seen.

The disruption of cell signaling with PP2 does not tell us if the CD8 T cells require sustained contact with infected cells in order to kill them or if the TKO infected cells are

receiving multiple hits by CD8 T cells which then add up to the eventual death the infected cell. To address this future experiments could be done to look at conjugate formation. Conjugate assays done by flow cytometry at different times after the incubation of the T cells with the infected APCs would determine if the T cells are interacting with infected APCs in equal numbers to uninfected APCs. In conjunction with these experiments, calcium levels could be measured in the T cells to determine if the response to the different infection conditions is equivalent. Live cell imaging studies of T cells interacting with infected an uninfected APCs should be completed. These experiments would allow us to determine if continuous contact is required for the killing of infected cells or if cell death is a result of multiple hits by the same or different T cells.

Importance of total MHC class I levels

The experiments performed in Chapter 4 also highlight the importance of total MHC class I levels for the induction of a strong CTL response. These results provide us with some evidence that the immune evasion genes may be important in the resistance of MCMV to immune responses. In vivo experiments still need to be done, but it is possible that *m06* and *m152* function by reducing the class I on the surface of infected cells by just enough to reduce the effector CD8 T cell response, protecting the infected cell from death. We have demonstrated that reduced total MHC class I levels on infected cells protects the cells from CTL-mediated death, and if we increase the total MHC levels the infected cells can now be killed. In the experiments shown in Chapter 4 Figure 12 it appears that if class I levels are increased above an MFI of 250, the infected cells are susceptible to CTL responses.

Future experiments need to be done to determine why total MHC class I levels are important for mediating killing by CD8 T cells. Experiments are currently ongoing to examine the structure of the synapse with infected and uninfected cells, in collaboration with David Parker's laboratory. Hopefully with these studies we will be able to determine if infection results in altered immune synapses. The reduction in class I may lead to a weaker synapse with a looser interaction between the T cell and the APC. There is also a possibility that we will see a difference in the synapse structure between TKO infected cells and uninfected cells suggesting that MCMV encodes other genes which alter the synapse formation to aid in the resistance of infected cells to lytic granule release. By visualizing the synapse we will hopefully gain some idea as to how the immune evasion genes are reducing T cell effector functions. One possibility is that MHC on the APC helps to recruit the CD8 co-receptor on the T cell into the synapse. Increased CD8 co-receptor levels would lead to an enhancement in the T cell signal that would result in a stronger T cell response. There is evidence in the literature for this model, for both the CD4 and CD8 T cell responses, where total amounts of class I or II have been shown to be important in stimulating a T cell response (Krogsgaard et al., 2005; Yachi et al., 2006). However only in the MHC class I system was the CD8 co-receptor interaction with noncognate pMHC shown to be important for stimulating a CD8 T cell response (Yachi et al., 2006). In our system, experiments still need to be completed to determine the role of noncognate pMHC in stimulating a CD8 T cell response but based on these studies and our demonstration that the CD8 T cell response is weaker when the total MHC levels are reduced, it seems very likely that inhibition of CD8 recruitment to the synapse is a likely cause of our block in killing. With the demonstration that the immune evasion genes, *m06* and *m152,* impact CD8 co-receptor

recruitment to the synapse we will hopefully be one step closer answering the question of why MCMV encodes immune evasion genes.

Conclusion

MCMV is a very large persistent virus that encodes many different genes, only some of which are required for viral replication. Many of the genes encoded by MCMV are present specifically to interact with the host environment. There is a danger in thinking that the relationship between MCMV and it host is tenuous and that even subtle disruptions of the relationship will have enormous consequences for both the host and the virus. When we introduce disruptions, such as removing all of the immune evasion genes, we would expect the virus to fold under the perceived dominance of the immune response. However, the virus remains and the immune response is seemingly unaltered. Studies of MCMV's relationship with its mouse host have shown us that there are many immune evasion strategies employed by MCMV to combat the diverse immune responses generated. It is very hard to disrupt the balance between MCMV and the mouse without completely disrupting the immune response. This balance between the host and CMV has developed over millions of years, so it may be a bit naïve of us to think that we understand all that is going on between the virus and the host after only a relatively short period of study.

Through the course of my thesis work I have characterized the functional effectiveness of the immune evasion genes, determined the importance of NKG2D costimulation on the CD8 T cell response to MCMV and demonstrated that CD8 T cells are responding to MCMV infected cells, but the infected cells have become resistant to the response. I have added to our knowledge of how the virus and the host immune response interact and have suggested a novel

way in which MCMV evades the host immune response. MCMV does not completely block CD8 T cell effector functions, rather it dampens them, so that other genes encoded by the virus can counteract the effector responses. With this knowledge future experiments can be designed to incorporate as well as challenge this new paradigm in order to extent not only our understanding of immune responses to MCMV but hopefully to many other viruses that persist by actively evading/containing the immune system.

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