Murine Cytomegalovirus Immune Evasion Genes are Functional But Fail to Completely Interfere with Antigen Presentation in Macrophages

by

Diane M. LoPiccolo

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Certificate of Approval

This is to certify that the Master of Science Thesis of

Diane LoPiccolo

has been approved

[redacted]

Ann B. Hill

[redacted]

Ashlee V. Moses

[redacted]

David J. Hinrichs

Table of Contents

Acknowledgements	ii
Abstract	iii
Introduction	1
Materials and Methods	56
Results	64
Figures and Figure Legends	75
Discussion and Summary	83
References	94

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Introduction

CMV Classification

The herpesvirus family is divided into three subgroups: alpha, beta, and gamma, the second of which, CMVs are members. Various distinguishing viral characteristics and genome sequence analyses guide these classifications. Herpesviruses are characterized by their large DNA genomes, and ability to establish a persistent and latent infection in their host. CMVs, in particular, display a tropism for salivary glands, exhibit slow growth kinetics in culture, and are highly species specific (Mocarski, 1999). Indeed a number of different animal species are known to harbor a CMV, including many Old and New World primates, rodents, and even elephants (Alcendor and Hayward, 1999). It is likely that each CMV has evolved with their host over millions of years allowing the virus to develop highly specialized mechanisms to coexist with the host. One important such mechanism is immune evasion.

History of CMVs

Though the history of CMVs likely parallels that of mammalian evolution, our understanding of them begins with early human medical observations. In 1904 H. Ribbert first described HCMV in a retrospective case from 1881. He observed large protozoan-like cells in the kidneys of a stillborn infant. Later that year Jesionek and Kiolemenoglou described protozoan-like cells with nuclear and cytoplasmic inclusions (pocket-like structures in the nucleus and cytoplasm of the cell), in the lungs, kidneys, and liver of an eight-month-old fetus. In 1921 Goodpasture and Talbot reported the same

observation in a 6-week-old baby, not believing that they were protozoan, they applied the term "cytomeglia" meaning large cell, to the condition. In that same year Lipscheutz discovered cytomegalic inclusions he claimed were similar to those found in varicella zoster virus (VZV) and herpes simplex 2 (HSV 2). This suggested a viral basis for cytomeglia. In 1953 Minder first observed by electron microscopy, (presumably viral) particles in cytomegalic cells. These particles were found both in inclusions and in the cytoplasm.

Isolation and growth of CMVs in the laboratory setting was the first step in experimental studies of CMV. Between 1954 and 1956, HCMV was isolated on three separate occasions. HCMV was probably first isolated and propagated by Margaret Smith in 1954. While her publication was held up by review, Weller serendipitously isolated the Davis strain of HCMV while trying to grow Toxoplasma from 3-month-old infant tissues in 1955. Finally, in 1956 while trying to isolate adenoviruses from adenoidal tissue Rowe propagated the AD169 strain of HCMV (Ho, 1991).

While trying to isolate HCMV, Margaret Smith also isolated and propagated a strain of MCMV. The virus was derived from salivary glands of mice and grown in mouse embryonic tissue. Appropriately, the virus was initially reported as murine salivary gland virus (Ho, 1991; Smith, 1954). Later it became known as the Smith strain of MCMV and is widely used in laboratory studies, including ours.

CMV Structure and Genome

CMVs are enveloped viruses with an icosohedral capsid. Glycoproteins dot the outside of the envelope and tegument proteins make up the area between the envelope

and the capsid, which contains the viral genome. CMVs contain the largest genomes of known herpesviruses. In the form of double stranded linear DNA, their sizes reach 229 kilobase pairs (Kb) for human CMV and 230 Kb for murine CMV. Both HCMV (AD169 strain) and MCMV (Smith strain) have been sequenced, revealing the existence of 208 and 170 predicted open reading frames (ORFs) respectively (Chee et al., 1990; Rawlinson et al., 1996). MCMV encodes six major families of genes. Four of these are homologous to HCMV gene families but two exhibit only internal homology (Alcendor and Hayward, 1999). These two families, the m2 and m145 families, contain the known immune evasion genes. *m4* and *m6* are members of the m2 family and *m152* is in the m145 family.

Gene Expression

Following absorption and entry into the cell, CMV DNA moves to the nucleus. Here, transcription and translation of CMV genome depends on cellular machinery but is regulated by the virus. These processes proceed temporally through three distinct phases: immediate early (IE), early (E), and late (L) (Gibson, 1999).

IE genes are the first to be transcribed in an infected cell and do not require previous viral protein synthesis. IE transcripts are operationally defined as those expressed in the presence of a protein synthesis inhibitor, often cyclohexamide (CH). Selective amplification of IE proteins can be achieved by adding CH for a few hours, removing it, and adding an inhibitor of transcription, Actinomycin D (Spear and Roizman, 1980). This way IE genes are transcribed, and following removal of CH, translated, but no E transcripts are made. IE genes are generally responsible for

regulating E and L viral genes and cellular genes (Sinclair and Sissons, 1996). One MCMV IE gene is IE1/pp89, a viral transactivator, and importantly the first known antigen for CD8 T cells, in MCMV-infected BALB/c mice.

Following the rapid IE phase is the E phase. Transcription of E genes requires IE protein synthesis but does not require viral DNA synthesis (Gibson, 1999). E gene products are often involved in viral DNA replication. Notably, MCMV's immune evasion genes are all E genes (Sinclair and Sissons, 1996). Amplification of E gene expression and exclusion of L gene expression is experimentally accomplished by treating cells with a viral DNA synthesis inhibitor, phosphono acetic acid (PAA). Our lab routinely uses PAA to study MCMV's known immune evasion genes, all of which are expressed in the E phase.

The L phase follows the E phase of MCMV gene expression. Transcription of L genes requires synthesis of not only IE and E proteins, but also viral DNA. These are the last transcripts expressed in the replication cycle, and virion assembly and egress follows their translation. Accordingly, many late gene products are structural components of viral assembly (Mocarski, 1996). It is of interest to note that this phase can lag behind the previous two phases depending on cell type. It takes longer to detect L phase MCMV RNA in macrophages, in addition these cells have been shown to replicate CMVs more slowly than fibroblasts (LoPiccolo unpublished data,) (Fish et al., 1995; Hanson et al., 1999).

HCMV Prevalence

HCMV infects anywhere from 50 to 85% of all adults in the United States regardless of socioeconomic standing, or geographic location (CDC). 80-100% of the adult population in urban regions or developing nations is infected worldwide (Mocarski, 1994). Overall the prevalence of HCMV among humans is high compared to many other infectious pathogens. Unlike many other pathogens however, disease due to HCMV infection is rare.

HCMV Infection

Most individuals experiencing a primary HCMV infection will exhibit no symptoms, however, some may display a mild mononucleosis. Three main groups of individuals are at risk for developing cases of severe HCMV infection; neonates, recipients of transplanted organs, and AIDS patients. That none of these groups are operating on a robust immune system is their common thread.

Neonatal infection, called cytomegalic inclusion disease (CID), is rare and results from infection (often primary) of the expectant mother. Symptoms displayed by the neonatally infected infant include hepatosplenomegaly, microcephaly, chorioretinitis, thrombocytopenia, and jaundice. A small percentage of CID infants go on to experience problems due to HCMV during childhood, including mental retardation and deafness (Parslow et al., 2001).

A major cause of disease in transplant patients is HCMV. This problem is particularly severe in bone marrow transplant patients. The virus can originate from the transplanted organ or can reactivate from a latent state in the patient. The type of organ

transplanted seems to partially determine the manifestation of disease, which can include pneumonia, leukopenia, hepatitis, and graft rejection.

HCMV is one of the most important opportunistic infections of patients with AIDS. Because nearly all AIDS patients are HCMV positive, HIV-induced immune suppression results in the development of CMV disease in most AIDS patients. Retinitis is the most common manifestation of disease while infection of the lungs, CNS, and gastrointestinal tract are common as well (Soderberg-Naucler et al., 1999).

In addition to the three major types of severe infection, HCMV is implicated in conditions of the heart including atherosclerosis. The role of the virus in these disease states is controversial and studies at this time are inconclusive. However, it is thought that the virus may be involved in the migration of smooth muscle cells in the aorta, a hallmark of atherosclerosis (Nelson, 2000).

Overall, HCMV disease takes on a variety of forms and affects a variety of organs and tissues. The precise condition of the host immune system in each of the aforementioned cases is different. Neonatal immune systems are underdeveloped overall, specific functions of cells in the immune system are targeted by drugs in transplant patients, and specific immune cells, namely CD4 T cells, are depleted during HIV infection. These differences likely contribute to the form of disease manifested by HCMV.

Likewise different subsets of NK and T cells have been shown to control MCMV in different organs as discussed later. Because CMV causes disease in so many tissues, it is reasonable to assume that it has access to many different areas of the body. The virus

has been shown to infect macrophages, a ubiquitous cell type. Likely the virus uses this cell type to gain access to different organs and tissues.

MCMV as a Model for HCMV

MCMV infection of the mouse is a good model for HCMV infection of the human. The natural host for MCMV is the mouse, and the relationship of MCMV to the mouse is similar to that of HCMV to the human. In both cases the normal course of infection is asymptomatic and the virus eventually establishes a latent state in its host.

Symptomatic HCMV infections of immune compromised individuals can be mimicked with MCMV by infecting neonatal (day old) mice, using mice genetically deficient for components of the immune system, or by inducing immunodeficiency by antibody depletion or irradiation (Koszinowski et al., 1991; Reddehase et al., 1994; Reddehase et al., 1987). Such studies have helped identify elements of the mouse immune system important for fighting MCMV. This knowledge has also aided our understanding of the way human immune systems defend against HCMV.

Recently, it has become possible to address specific aspects of immune evasion by CMVs in the MCMV/mouse model. New viral mutagenesis technology has allowed for faster, more accurate production of MCMVs lacking immune evasion genes (Messerle et al., 1997). Studies using these viruses have already contributed to the limited knowledge of MCMV immune evasion. Further insight to MCMV immune evasion in the mouse will contribute to the understanding of HCMV immune evasion. Overall these studies may assist in the development of therapies and vaccines for this widespread, sometimes very severe disease.

The course of MCMV infection

Unlike a primary HCMV infection, it is possible to track movement of MCMV through the mouse following a primary infection. During the course of infection MCMV targets several organs including the liver, spleen, lungs and salivary glands. The order in which MCMV infects these organs and the degree of infection can vary depending on infectious dose, route of inoculation, and mouse strain. In general higher infectious dose results in more severe infection as does intraperitoneal or intravenous as opposed to intratracheal administration of virus. C57BL/6 and BALB/c are the two strains of mice most commonly used in MCMV studies and it is important to note that the course of primary infection sometimes differs between them. BALB/c are more susceptible than C57BL/6 mice. MCMV infects the spleen and liver of both strains within the first three days of infection (Bukowski et al., 1984; Scalzo et al., 1990; Selgrade et al., 1984; Yuhasz et al., 1994). Liver titers are high for both strains; however, C57BL/6 mice have lower splenic titers than BALB/c mice (Scalzo et al., 1990). The lungs of C57BL/6 mice show a mild infection three to five days post infection while the lungs of BALB/c mice delay but show significant infection 11 to 14 days post infection (Bukowski et al., 1984; Fleck et al., 1998; Lucin et al., 1992; Yuhasz et al., 1994). MCMV titers in the salivary glands of both strains of mice begin to increase at eight days post infection and stay high for five to eight weeks, making the salivary gland the last site to be cleared of infectious virus (Bukowski et al., 1984; Fleck et al., 1998; Yuhasz et al., 1994). Following the resolution of primary infection, no infectious virus is detectable. However, by this time MCMV has established latency and the presence of MCMV genome in various tissues

can be demonstrated (Jonjic et al., 1994; Yuhasz et al., 1994). It is likely that MCMV takes up latent residence in both an organ-associated cell such as an endothelial cell and a ubiquitous cell, the macrophage (see latency section for references).

Host immune control is essential for clearance of infectious virus during primary infection. Early in acute infection, one to three days post-infection, Natural Killer (NK) cells are responsible for controlling MCMV (Bukowski et al., 1984). Later in acute infection T cells begin to play a prominent role; their activation begins at five days post-infection and stays high through nine days (Biron et al., 1996). Antibodies decrease virus titer during acute infection but, like NK and T cells, are unable to prevent MCMV from establishing latency following the acute phase (Jonjic et al., 1994; Reddehase et al., 1994).

In addition to clearing infectious virus during primary infection, the host immune system tightly controls MCMV's reactivation from latency. In fact depletion of NK and T cells from a B cell-deficient mouse latently infected with MCMV results in a resurgence of viral replication in the salivary gland in less than 3 days. Probably the most important immune element in controlling reactivation from latency is the CD8 CTL (Polic et al., 1998).

Given that CTL are important for maintaining the virus in a latent state and the macrophage is one site of MCMV latency, it seems natural to ask how well the immune evasion genes work in the macrophage. First in order to gain perspective on the role of CTL and macrophages in MCMV infection I will review various elements of immune control of viruses and their significance in MCMV infection.

Macrophages as Effector Cells in Viral Infections

Macrophages have been shown to have many effects on viral infections. These cells are part of the innate immune response and can directly decrease viral titers through phagocytosis of viral particles or virus-infected cells. The macrophage can also assist in resolving infection by initiating various cytokine signals to recruit other effector cells, and by acting as a professional antigen presenting cell (APCs) to activate T cells (Gendelman and Morahan, 1992).

Macrophages as Effector Cells in MCMV Infections

Macrophages have a curious role in controlling MCMV infections. As in other viral infections they directly control MCMV titers and elicit cytokine responses. They may also serve a filter-like role preventing infection of other susceptible cell types.

Various studies have examined the effects of macrophages on MCMV infection. These cells have been found to infiltrate the site of infection in an activated state (Heise and Virgin, 1995). This puts the macrophage in the right place to elicit direct anti-viral effects. One such direct effect involves NOS2, an enzyme that catalyzes anti-viral nitric oxide production. This enzyme has been shown to be important for a macrophage's ability to reduce MCMV replication (Noda et al., 2001). In addition, macrophages are likely to play a role in the initiation of various cytokine cascades in response to MCMV infection. Macrophages have been shown to contribute, at least indirectly, to IFNγ and TNFα production (Hanson et al., 1999) however the precise roles of macrophages in initiating cytokine cascades are at this time are unclear.

Hanson et al. (Hanson et al., 1999) propose that macrophages act as filters to protect other susceptible cell types from MCMV infection. They looked at the effects of macrophage depletion on growth of MCMV *in vivo*, during the first three days of infection, using C57BL/6 mice. They used wild type virus and a mutant virus defective in its ability to grow in macrophages, but not fibroblasts, *in vitro*. Compared to wild type virus, this mutant virus also grew less well in mice *in vivo*, interestingly it failed to initiate IFNγ or TNFα production in the spleen. This growth might be explained by the fact that the macrophage is a target for MCMV *in vivo* (Stoddart et al., 1994) and if this virus grows less well in macrophages then it might grow less well in the mouse overall. Therefore one might expect that depleting macrophages would not make a difference in replication of the mutant virus. Surprisingly, however, splenic titers of this virus actually increased to near wild type levels following macrophage depletion. Still, little IFNγ or TNFα was detected in the spleen. On the basis of the above experiments the authors suggest that, in the spleen at least, macrophages serve as a filter, limiting the overall replication of virus and protecting other more susceptible cell types. To add an extra layer of complexity, titers of the mutant virus and wild type virus were virtually unchanged in the liver as compared to the spleen.

As will be discussed below, it has been shown recently that NK cells control viral replication in the first three days of infection and that they use different mechanisms in the liver and the spleen. Given the differences observed in these two organs it is possible that macrophages play a role in initiating different NK effector functions and it would be interesting to investigate these different NK responses in the system described above. Another worthwhile experiment would be to investigate the ability of the mutant virus to

establish latency. It is possible that if this virus cannot replicate well in macrophages, cell types important for latency, that it is less able to establish latency. Lastly, it would also be interesting to assess whether T cell responses are tempered as a result of inefficient macrophage infection or, in the case of the macrophage depletion studies, macrophage loss.

Antibodies in Viral Infections

Antibodies can act directly on viral particles, directly on infected cells, or by helping with antigen presentation to T and B cells. The main contribution of antibodies in a viral infection is to block viral entry or remove infectious virus from circulation. Once antibodies coat viral particles or infected cells, the complement system can clear the particles, or phagocytic cells such as macrophages can engulf them following interaction of cell surface Fc receptors with Fc portions of virus-bound antibodies. In addition, antibody-coated cells are targets for antibody dependent cell-mediated cytotoxicity (ADCC) whereby NK cells recognize Fc receptors of antibodies coating cells and lyse these cells via perforin. Lastly, uptake of antibody-coated particles through the Fc receptors of professional antigen presenting cells can allow for increased antigen presentation to CD4 or CD8 T cells (Guidotti and Chisari, 2001). In a viral infection the first antibody class to appear is the low affinity IgM isotype, later the higher affinity IgG isotype appears and becomes the dominant antibody. In addition to these two isotype classes, IgA inhibits viral invasion at mucosal surfaces (Nash, 1998).

Antibodies and MCMV

Initial studies of antibodies and MCMV examined the anti-viral properties of polyclonal sera from MCMV-infected mice, which include antibodies with a range of specificities and isotypes. Such sera were able to protect mice from lethal challenge and reduce titers in the spleen and liver (Farrell and Shellam, 1991). These phenomena were observed in both genetically susceptible and non-susceptible strains suggesting that susceptibility is not related to antibody production. Interestingly complement did not seem to be required for protection as MCMV titers were similar among wild type mice or mice lacking an element of the complement system.

Jonjic et al. (Jonjic et al., 1989; Jonjic et al., 1994) showed that antibodies were more important in controlling MCMV dissemination during reactivation rather than during primary infection. Initial studies showed that depleting CD4 T cells in a primary infection caused an increase in salivary gland titers as well as a decrease in MCMV-specific antibody titers; which is logical since CD4 T cells augment antibody production. This suggested that either antibodies or CD4 T cells were important in controlling initial infection in the salivary glands. However, infecting mice genetically deficient in antibodies revealed that antibodies played no role in protecting against viral infection in the salivary glands or in preventing the virus from establishing latency (here, CD4 T cells were more important). Instead, they find that antibodies are important in controlling reactivation from latency, as increased salivary gland, lung, spleen and liver titers were observed under reactivation conditions in the absence of antibodies.

Interestingly MCMV encodes an Fc receptor specific for mouse IgG. It was thought that this Fc receptor, known as *m138*, might inhibit the ability of antibodies to

bind infected cells or viral particles by binding them at the Fc fragment instead. As expected an MCMV mutant lacking *m138* exhibits normal viral replication *in vitro* but attenuated replication *in vivo*. Surprisingly, however, infection of mice lacking antibodies resulted in attenuated replication as well. This indicates that *m138* affords MCMV protection from an immune mechanism other than antibody control (Crnkovic-Mertens et al., 1998). Further studies are needed to elucidate the function of this molecule.

Interferons in Viral Infections

One very important element of the immune response to viral infections is IFN. There are two types of interferon: type I which includes IFNα, β, τ and ω, and type II which is IFNγ. IFNs τ and ω are thought to be involved in immune regulation during pregnancy while IFNα, IFNβ and IFNγ play anti-microbial, including anti-viral, roles in immunity. The production of type I IFNs α and β (IFNα/β) occurs in nearly all cell types and can be initiated by viruses, bacteria, protozoa, cytokines and double-stranded RNA (dsRNA). On the other hand activated T cells and NK cells produce the type II IFNγ upon their activation (Samuel, 1991; Sen and Lengyel, 1992).

IFNs α and β bind the same cell surface receptor and IFNγ binds a separate receptor. Most often IFNα/β are involved in directly decreasing virus replication whereas IFNγ coordinates other cells involved in an immune response. Binding of either receptor results in signaling through slightly different pathways involving Jak and STAT molecules. The overall result is selective upregulation of IFN inducible genes. (Kotenko and Pestka, 2000)

Type I and type II IFNs both inhibit viral replication and assist in fighting virus infection by upregulating IFNαβ, iNOS, and IL-12 in macrophages and MHC II in macrophages and dendritic cells. Upregulating MHC II allows for better recognition by CD4 T cells. In a variety of cell types these molecules can upregulate MHC I, transporter associated with antigen processing (TAP), proteasome subunits including LMP2, and β2m all of which are molecules involved in antigen presentation and contribute to CD8 CTL recognition. Interestingly the pathways can also contradict each other. IFNαβ has been shown to counteract induction of MHCII by IFNγ (Inaba et al., 1986) and to negatively regulate IFNγ and IL-12, a cytokine able to induce IFNγ production by NK cells (Cousens et al., 1997).

As previously mentioned type I IFNs directly decrease viral replication. For this they employ special proteins to assist in antiviral responses. Among these proteins are dsRNA dependent Protein Kinase (PKR), 2'5'A synthetase, and Mx proteins. These proteins inhibit viral replication directly. In the presence of IFNα/β, PKR and 2'5'A synthetase are activated by partially double stranded RNA. PKR in turn inhibits mRNA transcription and 2'5'A synthetase activates an enzyme called RNase L to degrade single stranded RNA. Mx proteins are thought to inhibit viral replication by binding or competing for binding with viral polymerase, or preventing nucleocytoplasmic export of RNA. These effects are selective for certain viruses. Although type II IFN can also inhibit viral replication, it is particularly effective in coordinating an immune response. IFNγ affects B cell differentiation and isotype switching. It is also a hallmark of a Th1 immune response and in fact inhibits Th2 responses as discussed later (Samuel, 1991).

Interferons and MCMV

IFNγ plays a significant role in MCMV control. In addition, it becomes particularly important in some of the experiments presented in this thesis. For these reasons I will review the effects of IFNs on MCMV in detail here.

Not surprisingly, IFNs help the mouse keep MCMV in check. These cytokines can work directly to suppress MCMV replication, gene transcription or translation, or to recruit other cells to fight the infection. Many studies address the role of IFNs at these various points of infection as well as in the infected mouse. At the same time MCMV has developed mechanisms to subvert the effects of IFNs. While few studies address these complex mechanisms, they hint at the importance to MCMV of escaping such effects.

Interferons Hinder MCMV's Ability to Replicate

Several studies have illustrated IFN's ability to interfere with MCMV replication in both mouse embryo fibroblasts (MEFs) and bone marrow macrophages (BMMϕ). At least 100U/mL IFNγ in MEFs and BMM was required to observe a decrease in MCMV replication as measured by plaque assay (Lucin et al., 1992; Presti et al., 2001). Overall, IFNγ-pretreated macrophages are drastically less permissive for replication than IFNγ-pretreated MEFs. The importance of this point will become apparent in the results section. IFNγ units are defined based on the ability of the cytokine to inhibit 50% of EMCV growth in mouse L929 cells. IFNα units are defined in a similar bioassay using VSV (Meager, 1987).

The Role of IFNs in MCMV Gene Transcription and Translation

Early steps in MCMV infection such as entry, transport, and release of DNA to the nucleus are unaffected by IFNα or γ. However, viral gene transcription is a candidate IFN target. A decrease in IE transcripts was noticed in the presence of 1000U/mL IFNα or γ in transformed cells, 3T3 fibroblasts, which suggested that it is at this point that IFN interferes with MCMV replication (Gribaudo et al., 1993). In a separate study Lucin et al. (Lucin et al., 1994) make a slightly different conclusion examining protein translation in primary MEFs. They assessed IE gene expression during E stages, using a more modest 50U/mL concentration of IFNγ. They found that IE protein translation was actually enhanced and translation of E MCMV proteins was unaffected. Translation of late protein, gB was markedly decreased by IFNγ. They propose that IFNγ interferes with viral replication at this late step in viral gene expression. In addition to IFNγ they showed a role for TNFα. When the two cytokines were administered together they observed a more significant decrease in replication and gB protein translation suggesting a cytokine synergy. It is unclear whether these groups arrive at different conclusions because they are using different cell types (transformed versus primary), different concentrations of IFN, or are assessing different levels of viral replication (transcription versus translation).

MCMV transcription in macrophages is affected by IFNγ. In contrast to fibroblasts, bone marrow-derived macrophages pretreated with a modest 100U/mL IFNγ (Presti et al., 2001) decreased IE as well as E and L gene transcription. In addition this group presented evidence to suggest that macrophages use a yet undefined IFNγ pathway to control viral replication.

Interferonγ Aids in Cytotoxic T Lymphocyte Recognition

IFNγ's role in augmenting the CTL response to MCMV is clearly demonstrated by Hengel et al. (Hengel et al., 1994). This group contrasted a CTL clone's (this clone recognizes IE antigen in the context of L^d) ability to recognize MCMV-infected, IFNγ-pretreated MEFs with untreated MEFs. They found that the CTL clone failed to recognize untreated MEFs at early times post infection while MEFs treated with only 2U/mL IFNγ were easily recognized. In addition, they showed a dramatic upregulation of MHC class I. They suggested that IFNγ helps control MCMV infection by upregulating MHC class I in infected cells and eliciting the help of CD8 T cells. The importance of this experiment with reference to MCMV's immune evasion genes will become apparent later.

The Effects of Interferons on MCMV *in vivo*

Many studies have addressed the role of IFNγ *in vivo* by depleting mice of IFNγ and subsequently infecting them, or infecting mice genetically deficient of IFNγ or IFNγ receptor. Lucin et al. (Lucin et al., 1992) found that depleting CBA or BALB/c mice of IFNγ resulted in increased MCMV titers in salivary glands two weeks after infection. Because they had previously shown that CD4 T cells were responsible for clearance of MCMV from the salivary glands, (Jonjic et al., 1989) they proposed that this clearance was mediated by CD4 T cell-derived IFNγ. Heise et al. (Heise and Virgin, 1995) showed that IFNα/β or IFNγ depletion in T and B cell-lacking SCID mice resulted in increased viral titers in the spleen and liver 6 to 7 days post infection compared to non-IFNγ

depleted mice. They proposed that the IFNγ depleted from SCID mice was derived from natural killer cells, which may elicit antiviral effects in part by activating macrophages and decreasing their permissivity for MCMV replication.

As previously mentioned, BALB/c mice are known to be more susceptible to MCMV than C57BL/6 mice. Pomeroy et al. (Pomeroy et al., 1998) show that in the more susceptible BALB/c mice, overall IFNγ production began at later times post-infection, and that mortality rates were higher than in the C57BL/6 strain. They also showed that mice lacking the IFNγ gene were more susceptible to infection than wild type mice. Attempts to reconstitute IFNγ in these mice showed protection at modest IFNγ levels.

Presti et al. (Presti et al., 1998) found that MCMV-infected mice lacking the gene for IFNγ receptor were less able to control viral replication than their 129 strain counterparts. In these mice, lethal dose was much lower, and viral titers were higher in the spleen, peritoneal exudate, and salivary glands, up to 5 to 6 months after initial infection. Notably, they presented evidence for IFNγ's role in controlling chronic disease of the great vessels as mice lacking IFNγ receptor exhibited long-term aortic inflammation where wild type mice did not. Overall by reducing mortality rates, decreasing virus titers, and contributing to control over aortic vessel damage, IFNγ plays a key role in controlling MCMV infection *in vivo*.

MCMV Subverts the Effects of IFNs

MCMV antagonizes the effects of IFNs as well. Heise et al. (Heise et al., 1998; Heise et al., 1998) showed in two different papers that MCMV inhibits IFNγ-induced MHC II upregulation on macrophages. MCMV induced IFNαβ -dependent and

independent pathways resulted in inhibition of MHC II upregulation. Heise et al. showed that early in infection, soluble IFNαβ from infected cells inhibited IFNγ-induced MHC II upregulation on uninfected cells. In addition, they showed that direct MCMV infection inhibited IFNγ-induced MHC II upregulation using an IFNαβ-independent mechanism. Importantly, they showed that MHC II interference via this IFNαβ-independent mechanism resulted in a decrease in macrophage antigen presentation to CD4 T cells.

IL-10 and MCMV Infection

It is appropriate to note here yet another MCMV mechanism to downregulate MHC II. Redpath et al. (Redpath et al., 1999) present a completely IFN-independent mechanism by which MCMV decreases MHC II expression on macrophages through host IL-10 production 24 hours after infection. MHC II interference by all three of the above mechanisms likely contributes to the virus's ability to spread and persist by inhibiting early priming of CD4 T cells.

Natural Killer Cells

Natural Killer (NK) cells are important in controlling viral infections; this has been shown to be especially true for herpesvirus infections in both humans and mice. NK cells are non-B, non-T, bone marrow derived cells whose innate immune function does not require gene rearrangement thereby allowing them to make a quicker immune response. Early during viral infection these cells are activated by virus-induced cytokines, IFNα/β and IL-12. IFNα/β elicits an NK cytotoxic response and IL-12 induces NK IFNγ production. The NK cytotoxic response can be achieved by perforin

and granzymes during which perforin forms a pore in the cell membrane and granzymes pass through this pore and activate apoptosis mediating enzymes. Some viruses such as MCMV are particularly potent at inducing NK cells to produce IFNγ. In addition to IFNγ NK cells have been shown to produce TNFα and GM-CSF. Later in infection, as T cells become activated, they help turn off NK cell responses in part by secreting TGFβ (Biron, 1997).

NK Surface Markers

NK cells are commonly identified with the following cell surface markers: NK1.1, asialo GM_1, and DX5. Most MCMV studies have used NK1.1 and asialo GM1 to identify and deplete NK cells. Recent literature suggests that NK1.1 is a more stringent marker for NK cells than asialo GM_1, as asialo GM_1 can also be found on T cells and macrophages (Andrews et al., 2001; Ehl et al., 1996). Three new groups of activating and inhibitory NK receptors have been identified in the mouse, the NKR-P1 molecules including NKR-P1A, B and C, the Ly49 molecules including Ly49A-I, and the CD94/NKG2A/C/E and NKG2D/DAP10 molecules. An NKR-P1 gene encodes the prototype NK marker, NK1.1. All of these molecules are members of the C-type lectin family located in the NK complex on chromosome 6 (Biron and Brossay, 2001; Lanier, 1997).

NK Receptor Function

The NKG2 molecules, NKRP1B, some Ly49 molecules, and NKG2A contain immunoreceptor tyrosine based inhibition motifs (ITIMs) that deliver an inhibitory signal

to the cell. Other NKRP1 molecules, some Ly49 molecules such as Ly49D and Ly49H, and NKG2C/E/D are thought to deliver activating signals (Lanier, 1998; McMahon and Raulet, 2001). A balance between inhibitory and activating signals has been shown to exist. The balance could be tipped either way based on the predominating signal on the target cell. The ligand(s) for NKRP1 molecules are unknown, for Ly49 are self or non-self MHC I molecules, for NKG2A/C/E is the non-classical MHC I molecule Qa-1, and for NKG2D are Rae1 family members and H60. It is thought that NK cells use these ligands to detect virally infected cells, for example cells infected with viruses that downregulate MHC I, including MCMV, become susceptible to attack when inhibitory NK receptors no longer bind their MHC I ligand. It is also possible that virus infection results in an alteration of peptides loaded on MHC I, which could alter NK receptor recognition of MHC I ligand, likely via conformation as opposed to peptide specificity (Janeway et al., 2001).

NK response to MCMV

The first indication that NK cells were important in MCMV came from studies in which mice depleted of NK cells and subsequently infected with wild type MCMV showed increased susceptibility to infection. Bukowski et al. (Bukowski et al., 1984) showed a decreased LD_{50} for anti-asialo GM_1-treated (NK depleted) C57BL/6 mice as compared to wild type mice. In addition MCMV titers were increased in the lungs, liver, and especially the spleens of these mice. Interestingly, salivary gland titers were unchanged in the absence of NK cells and if NK cell depletion was carried out six days after infection, overall viral titers were unchanged. In concordance with their typical

time of activation, NK cells appear to be important in controlling viral replication in certain organs early in infection.

An MCMV Protein Interferes with the NK Cell Response

MCMV encodes a mouse MHC I homolog, which is thought to allow MCMV to evade NK cell attack on virally infected cells. Farrell et al. (Farrell et al., 1997) showed a decrease in viral replication in mice infected with MCMV lacking *m144* (KΔ144) as compared to mice infected with wild type MCMV. Removal of NK cells by anti-asialo GM treatment allowed for a restoration of KΔ144 replication to wild type levels thereby linking the antiviral effects of NK cells to evasion by *m144*. As previously mentioned asialo GM_1 can be found on T cells as well, which means that partial T cell depletion could account for increased replication of KΔ144. It would be interesting to repeat these experiments with recently defined NK markers.

Cmv1^r, MCMV resistance gene in the NK complex

Interestingly, a gene linked to MCMV resistance, Cmv1^r, has been mapped to the NK complex (Scalzo et al., 1995). Expression of NK complex genes can differ from one mouse strain to another. Most notably BALB/c mice do not transcribe NKR-P1 genes and fail to express the MCMV-resistant form of Cmv1^r, expressing Cmv1^s instead. Failure to express Cmv1^r results in increased MCMV titers in the spleen and lower lethal doses of MCMV as compared to C57BL/6 strains. (Scalzo et al., 1990) reviewed in (Lanier, 1998; Ugolini and Vivier, 2000).

NK Cell Effector Functions in MCMV Infection

NK cells use different mechanisms to control MCMV in different organs. This was exemplified in experiments with mice that lack perforin or are depleted of IFNγ (Tay and Welsh, 1997). Perforin-deficient mice are less able to control MCMV in the spleen compared to wild type mice, 3 days post-infection when NK activity is at it's peak. In contrast, mice depleted of IFNγ or administered an inhibitor of nitric oxide synthase, an IFNγ-inducible gene, were less able to control MCMV in the liver. This indicated that NK cells use cytotoxic mechanisms to control splenic infection and IFNγ to control hepatic infection. Possibly IFNγ is activating macrophages in the liver and these macrophages are clearing virus in a NOS-dependent manner. This may be reconciled with the observation that the increase in liver titers of MCMV-infected NOS2-/- mice was much higher than increases in any other organ titers (Noda et al., 2001).

Ly49 Subsets in MCMV Infection

The identification of the Ly49 family of NK markers opened the door for investigations of the roles of Ly49 NK cells in MCMV infection. Initially, investigators (Tay et al., 1999) transferred various combinations of Ly49 NK subsets from naive adults to recipient mice then infected them with MCMV. These transfers all protected suckling mice from lethal MCMV infection indicating that the tested combinations of Ly49 subsets could elicit protective effects in mice that normally lack such cells. More recently investigators (Daniels et al., 2001) depleted various Ly49 subsets, and then infected mice with MCMV. These groups observed that viral titers in MCMV-challenged mice did not generally change suggesting that other subsets can compensate

for depletion. Interestingly, one exception was the Ly49H NK subset which if depleted from the mouse results in increased titers in the liver and especially the spleen.

NK markers on T Cells

Interestingly, expression of some NK receptors is not restricted to NK cells. Emerging in the literature are reports of various populations of T cells with NK markers. Ly49, NKG2A/C/E/D have all been found on subpopulations of T cells (McMahon and Raulet, 2001). It is thought that NK receptors on these cell types may play a role in fine-tuning the T cell response. Interestingly the majority of lymphchoriomeningitis virus (LCMV)-specific CTLs express CD94-NKG2A during infection. Our lab has made the observation that 60-70% of CD8 T cells that respond to MCMV are NKG2A positive (Gold et al manuscript in preparation). It will be interesting to further define roles for NK markers on these T cells.

T cells

T cells are bone marrow-derived lymphocytes that recognize and respond to infected cells. CD8 cytotoxic T cells (CTL) lyse and or secrete cytokines in response to infected cells where CD4 T-helper (Th) cells respond by secreting a set of cytokines determined by the type of infection, and providing help to macrophages or B cells. Among other surface molecules, T cells display a T cell receptor (TCR), and it is through this TCR that the T cell is able to recognize an infected cell. In a viral infection the infected cell displays viral peptides bound to major histocompatibility complex class I

(MHC I), to CD8 CTL and MHC II to CD4 Th cells. Only T cells with TCRs specific for a particular MHC-viral peptide complex will recognize and respond to infected cells.

Naive T cell Activation

Early in the life of a mouse or human, a repertoire of T cells is produced in the thymus. Here, T cells with TCRs specific for self-peptides are deleted, as are cells that lack TCRs that recognize self-MHC. The resulting population consists of T cells that recognize non-self peptides in the context of self-MHC. As long as these T cells have never seen their specific non-self, or antigenic, peptide they are few in number and are called naïve T cells. These cells circulate through the blood and lymphatic systems until they encounter specific antigenic peptide and MHC.

Naïve T cells are activated and can differentiate into armed effector cells during infection. These changes are mediated by potent professional antigen presenting cells (APCs), which are often dendritic cells (DCs), and may be macrophages or B cells. DCs have been shown collect antigen at the site of infection, either through phagocytosis of infected cells or direct infection, and subsequently return to the lymphoid organs to present antigen to T cells. DCs are able to load peptide onto MHC I without synthesizing peptide endogenously. It is thought that DCs use a specialized cross-presentation pathway in which phagocytic vesicles provide the source of peptides for MHC I instead of cytosolic proteins. The exact mechanisms of this process remain to be elucidated. At any rate, circulating naïve T cells move through the lymphoid organs and sample DC-presented antigens. T cells that don't recognize antigen continue to circulate, but T cells specific for antigen stop, proliferate, and become armed effector T cells.

As previously mentioned CD8 T cells recognize antigen in the context of MHC I and differentiate into effector cytotoxic T cells (CTL) and CD4 T cells recognize antigen in the context of MHC II and differentiate into effector Th1 or Th2 cells. In addition to MHC, professional APCs express costimulatory molecules such as the B7 molecules, which provide the essential second signal required to activate naïve T cells. T cells receive this signal through a specific receptor such as CD28, the costimulatory molecule that recognizes B7. CD8 T cells seem to require a stronger costimulatory signal than CD4 T cells and in some cases CD8 activation requires CD4 help. In this case CD4 cells recognize antigen in the context of MHC II, receive a costimulatory signal, and activate the APC. The APC responds by increasing expression of B7 and upregulating other costimulatory molecules. This extra help may contribute to the maintenance of CD8 T cell effectors.

Following recognition of antigen and costimulation, naïve T cells begin to differentiate and proliferate. These actions are driven by IL-2, which is produced by the proliferating cells, and by further T cell interaction with the APC through CD40 ligand/CD40 and 4-1BB/4-1BB ligand pairs. Four to five days after infection this large population of antigen-specific T cells disseminates to the peripheral tissues to mediate control of infected areas. Armed effector cells are now able to recognize their targets through MHC molecules complexed with specific peptide in the absence of costimulation and they respond by either lysing target cells and/or secreting cytokines.

Memory T Cells

Following clearance or control of the infection, antigen-specific effector T cells begin to die and their numbers are usually greatly reduced. It is thought that some of these cells go on to constitute a population of memory cells. These memory T cells are not as poised to attack as effector cells, however they do not need to go through such extensive activation steps as naïve T cells. They remain with the organism for life and prevent resurgent infection with the specific pathogen.

T cell signaling

Upon recognition of MHC with specific peptide, the TCR must use signaling molecules to communicate recognition to the T cell. The TCR α and β chains associate with the four chains that make up CD3: two εs, a γ, and a δ, and two intracellular TCR ζ chains. These associated molecules all possess signaling motifs called immunoreceptor tyrosine-based activation motifs (ITAMs). In addition, two more molecules are recruited to the TCR complex: CD8 or CD4, and CD45 which both assist in signaling to the T cell. Signaling eventually results in transcription, translation and expression of various effector molecules specific for the type of T cell.

T cell Subsets:

CD8 CTL

CD8 CTL are predominantly responsible for recognizing virus-infected cells because of their ability to detect antigen derived from the cytoplasm and displayed on MHC I. Upon recognition of MHC I plus peptide, CTL can carry out cytolytic functions

and secrete cytokines. CTL cytolytic functions are mediated by perforin and granzymes, which work together to destroy infected cells. As previously mentioned perforin forms a pore in the cell membrane and granzymes pass through this pore and activate apoptosis mediating enzymes. CTL also express Fas ligand which, when bound to Fas on target cells, induces apoptosis in the target cell. It is thought that Fas ligand is primarily responsible for removing other lymphocytes bearing Fas. CTL also secrete cytokines including IFNγ, TNFα, and TNFβ. As previously mentioned, IFNγ activates macrophages, upregulates MHC I and MHC II, exhibits direct antiviral properties, activates NK cells, and inhibits Th2 responses. TNFα and TNFβ (LTα) can each synergize with IFNγ to activate macrophages. In addition, both TNFα and TNFβ can induce apoptosis in cells bearing TNF receptors and activate endothelial cells to recruit more immune help.

CD4 Th1 cells

Th0 cells differentiate into either Th1 or Th2 cells. The differentiation of CD4 T cells into Th1 cells predominantly results in a CD8 CTL-mediated immune response. This is generally the best kind of response to control viral infections. Importantly, Th1 cells are efficient at activating macrophages. Activated Th1s recognize specific MHC II and peptide on the macrophage surface. Following this recognition they secrete IFNγ, TNFα, GM-CSF, and bind macrophage CD40 through CD40 ligand. These molecules all contribute to macrophage activation and GM-CSF promotes macrophage differentiation.

An activated macrophage effectively breaks down phagocytic material and becomes a very good APC. The macrophage is able to fuse lysosomes and phagosomes

more efficiently when activated, as well as produce antimicrobial oxygen radicals and nitric oxide. An increase in MHC II, B7, and CD40 on the surface of macrophages allows for better recognition by, and possibly activation of, T cells. Activated macrophages also augment Th1 skewing of the immune response by secreting IL-12, which induces Th1 differentiation. Th1 cells also express Fas ligand to bind Fas on chronically infected macrophages unable to control a bacterial infection thereby allowing release of microbe to fresh macrophages.

Th1 T cells also activate B cells. B cells take up antigen through their B cell receptor and display it on their MHC II molecules. Th1 cells specific for this same antigen activate the B cells to produce IgG2a and IgG2b classes of antibody. Antibodies of these isotypes are hallmarks of a Th1, cell-mediated response.

CD4 Th2 cells

In contrast to Th1 cells, Th2 cells establish a predominantly humoral, or antibody-mediated, response. The antibodies involved in these responses are classes other than those associated with Th1 responses. The Th2 response is effective in controlling some bacterial and parasite infections and is the response invoked in allergic reactions. Th2 cells secrete several B cell-activating cytokines including IL-4 and IL-6. IL-4 induces B cell production of the antibody isotype associated with allergy, IgE. IL-6 induces mucosal IgA antibody production. In addition Th2 cells express CD40 ligand, which binds CD40 on B cells thereby activating them (Janeway et al., 2001).

T Cell Subsets and MCMV *in vivo*:

Adoptive Transfers of T cells Mediate Protection

A series of studies in the late 1970s and early 1980s illustrated the protective potential of T cells in MCMV infection. Initial studies by Starr and Allison (Starr and Allison, 1977) showed that passive transfer of immune lymphocytes could protect mice from MCMV-induced death. Howard et al. (Howard et al., 1978) showed that T lymphocytes derived from MCMV-infected mice proliferated in response to MCMV and were therefore likely MCMV-specific. Quinnan (Quinnan et al., 1978) described MHC I-restricted cytotoxic T lymphocyte responses to MCMV and Ho (Ho, 1980) showed that *in vitro*-generated T cells from immune mice could reduce splenic virus titers in recipient mice. Reddehase et al. (Reddehase et al., 1987; Reddehase et al., 1985) further identified that CD8 positive, CD4 negative, T cells were responsible for protection, and demonstrated that such cells from MCMV-infected donor mice reduced MCMV replication in the lungs, liver, and spleens of irradiated recipient mice in a dose-dependent fashion. In addition this group (Reddehase et al., 1988) showed that this protective capacity extends to CD8 memory cells derived from latently infected donors and restimulated *in vitro*.

In vivo Depletions of CD4 and CD8 Subsets Further Define Roles for T Cells

Jonjic et al. (Jonjic et al., 1989) showed evidence of an organ specific role for CD4s in MCMV infection. Donor mice depleted of CD4 *in vivo* and infected with MCMV supplied, to irradiated recipients, T cells that decreased lung and spleen titers but did not decrease salivary gland titers at all. This is in contrast to cells supplied by non-depleted mice, suggesting that non-CD4 (CD8) T cells are able to control MCMV

replication in the lung and spleen, and CD4 T cells are important in controlling MCMV replication in the salivary glands.

The complexity underlying development of CD4 and CD8 responses becomes apparent in further depletion and transfer studies by Jonjic et al. (Jonjic et al., 1990). As expected, T cells taken from donor mice 10 weeks post infection, and transferred to infected, irradiated mice, decreased splenic titers as compared to mice that received no transfer. Usually the spleen was used as an indicator organ in these studies. If donor T cells are depleted of the CD8 subset *in vitro* and then transferred, mice are not protected. However *in vitro* depletion of the CD4 subset prior to transfer, protected mice, reconfirming the above observations that the CD8 subset is protective. Surprisingly, if the donor animal itself was depleted of CD8 T cells and infected, those T cells were still protective following transfer, as were T cells from donors depleted of CD4 T cells. In addition, mice depleted of CD8 T cells prior to infection took only slightly longer to clear virus from lungs and salivary glands than non-depleted mice. The authors propose that the CD4 T cells in CD8-depleted donors are able to compensate for the lack of CD8s in initial infection.

As previously mentioned, but worth noting again here in the context of T cell subsets, Lucin et al. (Lucin et al., 1992) determined that IFNγ plays a major role in controlling MCMV replication in the salivary glands. They antibody-depleted animals of IFNγ and observed an increase in titers in the salivary glands to nearly the same level as if they had depleted CD4 cells. They propose that the major CD4 subset controlling salivary gland replication is the Th1 subset and IFNγ is an important element of the subset's antiviral activity.

Lymphocyte Subsets Control Reactivation from Latency

Polic et al. (Polic et al., 1998) showed that maintenance of MCMV latency is governed by immune control. They depleted immune subsets, NK1.1, CD8, and CD4, from antibody-deficient C57BL/6 mice. The antibody deficient mice were used in order to increase the sensitivity of their viral titer assay as these mice lack interfering neutralizing antibodies. They found that subsets were able to compensate for each other because the depletion of any one subset rarely led to disease. Depletion of combinations of two subsets revealed a hierarchy of subset importance in controlling reactivation, as well as an indication of the pattern of reactivation in MCMV-affected organs. The CD8 subset was most important in controlling reactivation, followed by the NK1.1 positive subset, and lastly the CD4 subset. Removal of all three subsets induced rapid reactivation as infectious virus was found in the salivary glands in less than three days after depletion. The lungs and spleen displayed infectious virus five and seven days respectively, after removal of three subsets. Interestingly, organs affected by reactivating MCMV seem to follow an order reverse that of primary infection: salivary glands, lungs, spleen and liver. Overall, CD8 T cells seem to play the most prominent role in tight immune control of MCMV reactivation.

MHC Class II:

Normal Antigen Presentation

MHC II molecules present antigen to CD4 T cells. This antigen, in the form of peptide bound to MHC II heavy and light chains, is derived from the endocytic vesicles

of professional antigen presenting cells. Extracellular foreign antigens are taken up by vesicles, and subjected to a drop in pH and degradation by proteases. These vesicles now full of antigenic peptides, then fuse with vesicles of MHC II molecules associated with an invariant chain (Ii) and a peptide fragment known as CLIP. The compartment formed by the fusion of these two vesicles is known as MIIC. Here, Ii is degraded and MHC II exchanges CLIP for antigenic peptide with the assistance of a molecule called HLA-DM. MHC II loaded with antigenic peptide is then exported to the cell surface for CD4 T cell recognition (Janeway et al., 2001).

CMV Interference with Normal MHC II Presentation

Many viruses have evolved mechanisms to interfere with MHC II processing including Adenovirus, HSV, HIV, HCMV, and MCMV (as reviewed in (Alcami and Koszinowski, 2000)). Most studies examining the relationship of CMVs and MHC II have used macrophages. Both HCMV and MCMV have been shown to downregulate MHC II in this cell type. HCMV was first shown to decrease surface MHC II over time on macrophages infected in culture (Fish et al., 1996). Interestingly an HCMV protein known to interfere with MHC I processing, US2, was found to actively translocate MHC II to the cytosol for degradation by the proteasome (Tomazin et al., 1999). As previously mentioned, MCMV interferes with IFNγ-induced MHC II upregulation in two ways, an IFNαβ-independent, and an IFNαβ-dependent mechanism (Presti et al., 1998; Presti et al., 2001). In addition, MCMV interferes with MHC II upregulation in an IFNγ-independent fashion by upregulating host IL-10 production (Redpath et al., 1999).

MHC Class I

Normal Antigen Presentation

CD8 T cells express T cell receptors that recognize MHC class I (MHC I) molecules complexed with peptide. Such peptides are derived from proteins synthesized in the cytosol. These proteins are degraded by a 28-subunit complex called the proteasome. At least three of these subunits can be displaced by three interferon inducible subunits, LMP2, LMP7, and MECL-1, during times of viral infection. These subunits allow the proteasome to create more peptides with preferred MHC I binding motifs. Proteasome-degraded peptides are translocated from the cytosol to the endoplasmic reticulum (ER) via the ATP-dependent TAP transporter, composed of TAP1 and TAP2.

Newly synthesized MHC class I is assembled in the ER following a series of associations with chaperone molecules. Calnexin associates with MHC I heavy chain until β_2 microglobulin (β_2m) binds. The MHC I heavy chain-β_2m bimolecular complex then associates with calreticulin, ERp57, and tapasin chaperones until encountering peptide delivered via TAP. A trimolecular complex consisting of MHC I heavy chain, β2m, and peptide, is then released from the chaperones and TAP, and exported to the cell surface. At the cell surface, the trimolecular complex is subject to encounter with CD8 T cell TCR. (Janeway et al., 2001).

Viral Interference with Normal MHC I Presentation

Interference with MHC I presentation is a logical way to evade the host immune system since CD8 T cells are so important in virus control (Alcami and Koszinowski,

2000). Viruses have evolved an impressive number of mechanisms for this interference. They target several steps in MHC I antigen presentation including antigen processing, TAP transport, and MHC I trafficking.

EBV's EBNA1 protein and HCMV's pp65 protein both interfere with antigen processing. EBNA1 encodes a motif that prevents proteasomal degradation and pp65 is thought to phosphorylate a main HCMV antigenic peptide thereby making it less antigenic. HSV's ICP47 and HCMV's US6 both interfere with peptide transport via TAP. ICP47 prevents peptide binding to TAP in the cytosol and US6 prevents peptide transport through TAP.

Many viruses encode proteins that directly interfere with MHC I trafficking: among them are Adenovirus, HHV-8, MHV-68, HIV, HCMV, and MCMV (as reviewed in (Alcami and Koszinowski, 2000)). Adenovirus's E3/19K was the first known viral protein to interfere with MHC I processing. E3/19K binds MHC I molecules and causes them to be rerouted to the ER by means of a dilysine motif in the E3/19K cytosolic tail. Recent work shows that HHV-8 proteins K3 and K5 selectively downregulate surface MHC I molecules via rapid endocytosis (Coscoy and Ganem, 2000; Ishido et al., 2000). These proteins differentially target specific MHC I molecules. This may be related to the observation that different MHC I molecules play different roles in NK cell control. This thought is further supported by recent evidence that K5 downregulates ICAM1 and B7-2, costimulatory molecules capable of activating NK cells (Ishido et al., 2000). MHV-68 protein K3 is a homolog of K3 and K5 and also reduces cell surface MHC I and prevents CTL recognition (Stevenson et al., 2000). Two HIV proteins are known to interfere with MHC I antigen presentation: Nef, and Vpu. Nef accelerates endocytosis thereby down-

modulating surface expression, and decreasing the half-life of MHC I. Vpu destabilizes MHC I and likely targets it to the cytosol for destruction. Interestingly, both Nef and Vpu have multiple effects on other cell proteins including CD4 (as reviewed in (Ploegh, 1998; Yewdell and Bennink, 1999)).

HCMV encodes four proteins shown to directly interfere with MHC I trafficking: US3, US2, US11, and US6. US3 is synthesized during IE times of infection and retains peptide loaded MHC I in the ER. Transcription of US2 follows that of US3. Both US2 and US11 dislocate MHC I to the cytosol where they are degraded by proteasomes. US6 is synthesized at early after infection and inhibits ATP binding to TAP, thereby preventing MHC I presentation (as reviewed in (Hengel et al., 1998)). MCMV encodes three proteins that directly interfere with MHC I processing and will be discussed in detail below.

The Relationship between MHC I and MCMV:

Mouse MHC I Molecules and Antigenic Peptides

There are three MHC I loci in mice, designated H-2 K, D, and L. Each of these loci exist as multiple alleles, designated by a second superscripted letter. BALB/c mice have the $H2^d$ haplotype, and have K^d, D^d, and L^d MHC I molecules. C57BL/6 mice have the $H2^b$ haplotype, and have K^b, and D^b MHC I molecules (Kavanagh and Hill, 2001). Each MHC I molecule has a preferred peptide binding motif. The identities of enough peptides have been discovered that it is sometimes possible to predict the identities of new MHC I-binding peptides given a limited amino acid sequence. This is an important tool in peptide identification (Rammensee, 1997).

MCMV peptides presented by MHC I

There are five known peptides presented to CD8 CTL in MCMV infection. Four of these are presented by BALB/c MHC I molecules, L^d, D^d, and K^d (Reddehase, 2000). One is presented by C57BL/6 MHC I molecule, D^b (Gold et al., manuscript in preparation)

Much of the early work in identifying MHC I associated peptides was pioneered by the Koszinowski group. They first describe a population of CTL in BALB/c mice that detect antigens formed during the immediate early (IE) stage of viral replication (Reddehase and Koszinowski, 1984) (Reddehase et al., 1984). They were able to clone out a CTL that recognizes an IE protein and showed that this population has functional relevance in infection as it is protective in the lung (Reddehase et al., 1987). Later they show that the peptide recognized by this line is presented by MHC I molecule L^d, and is derived from IE protein pp89 (Del Val et al., 1988). By using vaccinia virus expressing various deletions of pp89 they are able to determine an 18 amino acid sequence that elicits a response from the aforementioned IE-specific CTL clone. By constructing various parts of this protein and testing for CTL recognition they determine the L^d-presented amino acid sequence is YPHFMPTNL (Reddehase et al., 1989). This peptide is relevant to *in vivo* infection as protection from lethal infection can be achieved by vaccinating with vaccinia virus expressing pp89 (Jonjic et al., 1988).

The second MCMV-derived peptide is derived from E protein m4/gp34 and is presented by MHC I molecule D^d (Holtappels et al., 2000). The sequence of this peptide was determined to be YGPSLYRRF. Its identity was deduced by first repeatedly

restimulating memory CTL with HPLC fractions from infected MEFs. This resulted in the growth of a few different populations of CTL, one of which was found to recognize a D^d-restricted peptide. Consequently the entire MCMV genome was scanned for peptides matching a known D^d peptide motif, and the resulting matches were synthesized and tested for CTL recognition. This process of selecting and screening HPLC fractions yielded the aforementioned CTL population. The *in vivo* relevance of this *m4*-specific CTL population was demonstrated in adoptive transfer studies whereby these CTL, as well as IE-specific CTL, reduced virus titers in liver, spleen, lungs, and adrenal glands. Curiously, although not surprisingly, *m4*-specific CTL did not recognize infected MEFs at early stages of viral replication during production of immune evasion gene products. Likely, other cell types, such as DCs, in the mouse are able to present or cross present antigen in order to select for *m4*-specific CTL.

The identity of a third MCMV peptide was revealed following the observation that, in BALB/c mice, protective immunity could be obtained by vaccinating with plasmid encoding M84 (Morello et al., 2000). Given this data, investigators predicted peptide sequences from M84 based on known K^d and L^d motifs (Holtappels et al., 2001). They screened a panel of synthesized peptides deduced that the nonameric peptide sequence is AYAGLFTPL restricted by K^d. The frequency of CD8 CTL recognizing this peptide in MCMV infection is relatively low (0.1%). E protein M84 is a putative homolog of one of HCMV's structural genes, UL83 (Cranmer et al., 1996).

The most recent MCMV peptide identified in the BALB/c mouse is derived from M83, an MCMV protein with some homology to HCMV UL83. This is a structural protein expressed late in infection and unlike M84, plasmids encoding M83 did not

induce protective immunity. However, since the HCMV virion protein UL83 is a source of immunodominant peptides this group (Holtappels et al., 2001) decided to investigate the potential that M83 also encoded a peptide recognized by CD8 CTL. Using methods similar to those outlined for M84 peptide identification they determined that the antigenic peptide derived from M83 is YPSKEPFNF and complexes with L^d. Interestingly, when they examined splenocytes from an infected mouse they found that the frequency of CD8 CTL responding to this peptide was undetectable by cytokine assay, however CTL lines recognizing this peptide are protective in transfer assays. This indicates that CTL responses to this peptide are not likely immunodominant in natural infection.

The first MCMV peptide in the C57BL/6 mouse was discovered in our lab (Gold et al. manuscript in preparation). This peptide is derived from E protein *M45*, a ribonucleotide reductase homolog. D^b is the MHC I molecule by which this peptide, (K)HGIRNASFI, is restricted. This peptide sequence was identified using a novel strategy. Initially, a panel of MCMV-specific CTL clones were isolated and propagated based on their ability to detect MEFs stalled by PAA treatment, in the early phase of MCMV infection. Restriction elements were determined for these CTL clones based on their ability to recognize cells lacking either D^b or K^b. Next, fragments of genomic MCMV were expressed in eukaryotic expression vectors and K41 fibroblasts were transfected with these different vectors. CTL clones then were screened for their ability to recognize various K41 cells transfected with pools of DNA from the library. One D^b-restricted CTL clone was found to recognize a pool of DNA corresponding to *M45*. *M45* DNA was scanned for known D^b motifs and four matches were synthesized and screened to determine the sequence recognized by CTL. Analysis of CD8 CTL in K^b-/- mice

during an acute MCMV infection (8 days post-infection) indicates the importance of this peptide in a wild type infection *in vivo*. At this time a large portion (13%) of total CD8 positive CTL in an acutely infected mouse recognize this peptide.

It is likely that the identities of other MCMV-derived, MHC I-presented peptides will soon be exposed. Several experiments have shown that populations of C57BL/6, BALB/c, spleen, and lung derived CTL, recognize IE, E, and L proteins in MEFs (Del Val et al., 1989; Holtappels et al., 1998; Reddehase and Koszinowski, 1984). In addition, our lab has isolated several CTL clones specific for E timepoint, K^b and D^b-presented MCMV peptides.

MCMV Interference with Antigen Presentation

MCMV encodes genes of three proteins shown to interfere with normal MHC I antigen presentation to CD8 CTL (reviewed in (Hengel et al., 1998; Hengel et al., 1999)). All three are glycoproteins of various weights and are known by their gene and protein name: m152/gp40, m4/gp34, and m6/gp48. *m152* is a member of the m145 family, which includes genes *m145* through *m158* and is located on the right hand side of the MCMV genome. *m4* and *m6* are members of the m2 family, which includes *m2* through *m17* on the left side of the genome. These genes have homology only with other family members and likely have evolved to be highly specialized to modulate the mouse immune system.

These immune evasion proteins are expressed during early stages of viral gene transcription at which point they suppress CTL antigen recognition, an observation first described by Del Val et al. (Del Val et al., 1989). This group used a series of drug

treatments to selectively express IE, E, or L proteins. They observed a complete inability of a pp89-specific CTL clone to detect antigen during E protein expression in mouse embryo fibroblasts despite ample pp89 synthesis. They also showed that pp89-specific polyclonal CTL from both BALB/c and C57BL/6 mice were unable to detect antigen during early protein expression in MEFs. This underscored the importance of these genes in interference of polymorphic MHC I antigen presentation and set the stage to search for proteins responsible for interference with CTL recognition, and their mechanisms.

m152/gp40

The first MCMV immune evasion gene described was *m152*, a 40 kD transmembrane glycoprotein (Ziegler et al., 1997). Of the three known MCMV immune evasion genes *m152* is the first to be expressed, within 2 hours post infection in fibroblasts. This expression peaks at 4 hours post infection and later declines. Interference with normal MHC I processing in *m152*-transfected cells was demonstrated by a decrease of K^k surface expression, and a functional inhibition of pp89-specific CTL recognition of L^d plus pp89 peptide. In addition *m152* was shown to biochemically retain K^d and L^d in an EndoH sensitive compartment. Immunofluorescent colocalization studies revealed that in *m152* transfected cells, K^d appeared in the same compartment as p58, an ER Glogi Intermediate Compartment (ERGIC) protein.

m152/gp40's molecular mechanism is not obvious as it was not possible to demonstrate coprecipitation or complete colocalization with MHC I molecules. Further molecular studies revealed that m152/gp40 itself is rerouted to endosomal compartments where it is degraded (Ziegler et al., 2000). In addition, even after halting m152/gp40

synthesis, MHC I retention is still observed. This group proposes that m152/gp40 imposes a biochemical modification of MHC I thereby tagging it to remain in the ERGIC, however the exact mechanism remains a mystery.

Interestingly a comparison of mouse and human MHC I alleles revealed that m152/gp40 could distinguish between the two. m152/gp40 retained H-2d, H-2k, H-2b, and H-2q MHC I alleles but not human MHC I. This illustrates the high degree of host specificity and hints at the evolutionary history between MCMV and the mouse (Ziegler et al., 1997).

In vivo studies revealed that *m152* plays a role in protecting the virus against CD8 T cell control (Krmpotic et al., 1999). The investigators used wild type Smith strain of MCMV, MCMV deleted for *m152* (ΔMC 96.24) and a revertant form of this deletant (rMC96.27), which matches Smith. The three viruses were shown to have comparable levels of growth *in vitro*, however differences between them were apparent *in vivo*. Newborn mice infected with ΔMC96.24 had lower mortality rates and lower levels of virus in the lungs and spleen compared to wild type viruses, in addition decreased lung virus titers were observed in both BALB/c and C57BL/6 mice. This indicates that *m152* gives the virus a growth advantage *in vivo*. This advantage is likely due to escape from CD8 T cell control because removal of CD8 T cells (via immunodepletion or genetic manipulation) results in restoration of ΔMC96.24 titers to near wild type levels.

Further studies from our lab have investigated the long term CD8 CTL response in mice infected with ΔMC96.24 or ΔMS94.5, a virus lacking 15.8 Kb of viral genome including *m152* (Gold et al. manuscript in preparation). These viruses, like wild type, were never cleared from infected animals and were able to establish latency, as viral

DNA was detected in tissues following resolution of the acute phases of the infections. Interestingly mice infected for 6 months with Δ94.5 had twice as many MCMV-specific, CD8 positive T cells as Smith-infected mice. However, mice infected with ΔMC96.24 do not display this increase in numbers of CD8 T cells. This data indicates that *m152* is not required for MCMV to establish latency and suggests that *m152* does not play a role in dampening the CD8 T cell response over time. Interestingly it seems that there is a gene (or genes) within the Δ94.5 deletion that modulate(s) the numbers of MCMV-specific CD8 positive T cells. The characteristics of such a gene (or genes) would be worth investigating in the future.

m4/gp34

m4/gp34 was first described as a protein associated with MHC I in infected fibroblasts. In MCMV infection *m4* is expressed in a second wave of early proteins following expression of *m152* but, unlike *m152*, its expression is maintained throughout infection (Hengel et al., 1998). Initial studies (Kleijnen et al., 1997) showed that great quantities of m4/gp34 are synthesized and mostly remain in the ER. Some m4/gp34 leaves the ER, but only in association with MHC I when they both move to the cell surface. One hypothesis of this group is that m4/gp34 counteracts the effects *m152* in order to prevent NK cell lysis as a result of low MHC I surface expression. This hypothesis now seems unlikely in light of data reported by our lab (Kavanagh et al., 2001). An analysis of the percent of retained versus exported MHC I (K^b) reveals no difference among wild type-infected or Δ*m4*-infected fibroblasts. This indicates that m4/gp34 does not actively move MHC I to the cell surface and is therefore not likely

counteracting the effects of *m152*. It is possible, however, that m4/gp34 interferes with CTL recognition of MHC I at the cell surface.

Further work in our lab (Kavanagh et al., 2001) demonstrated a function for m4/gp34 in interference with CTL recognition. In addition, this manuscript showed evidence for cooperation of m4/gp34 and m152/gp40 in preventing CTL recognition. Among a panel of CTL clones derived from MCMV-infected mice, some CTL recognized MCMV-derived peptides associated with K^b, others D^b. Both types of CTL clone saw fibroblasts infected with Δ*m152* but not Smith-infected, or uninfected cells indicating a primary role for *m152*. However only K^b-restricted CTL clones recognized Δ*m4*-infected fibroblasts. Biochemical experiments showed that significant amounts of exported K^b were associated with m4/gp34. In addition m152/gp40 has a greater retentive effect on D^b than K^b. Together these data suggested that m152/gp40 is necessary to prevent recognition by D^b and K^b-restricted CTL. However, m152/gp40 is not sufficient to completely prevent antigen recognition by K^b-restricted CTL in the absence of m4/gp34 suggesting cooperation between m152/gp40 and m4/gp34. The precise mechanism of m4/gp34 interference with antigen presentation remains unknown.

More detailed biochemical experiments hinted at the complexity of the m4/gp34 relationship (Kavanagh et al., 2001). Immunoprecipitations of m4/gp34 using different strength detergents revealed two different kinds of m4/gp34-K^b complexes. In MCMV infection much of the exported K^b, that is K^b not retained by *m152*, was associated with m4/gp34 in biochemically tight complexes, stable in strong detergent. K^b retained by *m152*, however, formed a biochemically weak complex with m4/gp34, stable only in weak detergent. The direct implications of these complexes are at this time unknown, but

their existence suggests that m4/gp34 is able to interfere with normal K^b function at two different points.

m6/gp48

Reusch et al. (Reusch et al., 1999) were the first to identify the immune evasion properties of m6/gp48. They indicated that m6/gp48 was synthesized initially within the first 3 hours post infection and, like *m4*, was produced at high levels throughout infection. MHC I (L^q) was shown to have a decreased stability in *m6*-transfected cells, and MHC I surface expression was decreased in such transfectants. In addition, co-immunoprecipitations showed direct association of MHC I and m6/gp48. Immunofluorescence studies with m6/gp48 mutants showed that the LL motif is responsible for directing MHC I and m6/gp48 complexes to lysosomal compartments where they are presumably degraded. This group also reports a reduction in the ability of an L^d-restricted CTL clone to detect cells infected with vaccinia-expressing pp89 and vaccinia expressing *m6*. Further work investigating the effects of *m6 in vivo* has yet to be performed.

MCMV Latency

Today's understanding of MCMV latency is based on a large number of, often conflicting, reports regarding the location of MCMV genome and the status of the virus during latency. A variety of assays have been employed to address the organs and cell types in which MCMV establishes latency based on presence of genome, and whether or not RNA transcripts, viral proteins, and infectious virus are produced during latency. It is

important to bear in mind that the definition of latency, the sensitivity of detection assays, and experimental systems in general vary from one group to another.

The Sites of MCMV Latency- Organs

The fact that organs and/or blood are important sites of CMV latency, is evidenced by the fact that transplant patients are at risk for HCMV infection as a result of transplant from an HCMV positive donor (Soderberg-Naucler et al., 1999). Organ transplants in mice too can transmit MCMV (Hamilton and Seaworth, 1985; Rubin et al., 1984). Early investigators used DNA hybridization and later investigations, PCR assays. Such studies indicated that most organs harbor MCMV DNA in latently infected animals, among them, kidney, heart, spleen, salivary glands, liver, and lungs (Koffron et al., 1998; Kurz et al., 1997; Pollock and Virgin, 1995; Reddehase et al., 1994; Yuhasz et al., 1994).

Interestingly no groups report recovery of MCMV DNA from blood following establishment of latency (Koffron et al., 1998; Kurz et al., 1997; Kurz et al., 1999; Mitchell et al., 1997). Studies of MCMV DNA in the bone marrow give conflicting results. Some groups show evidence of MCMV DNA in the bone marrow (Koffron et al., 1998; Pollock and Virgin, 1995; Yuhasz et al., 1994), however, other groups fail to detect such DNA (Balthesen et al., 1993; Kurz et al., 1999). This is a notable discrepancy, as one idea about CMV latency proposes that pluripotent stem cells in the bone marrow acquire virus and pass it on to certain progenitors of blood cell lineages (Sinclair and Sissons, 1996). Conflicting reports may result from variations in assay sensitivity, cell purity, or the amount of time post-infection that the virus is considered latent.

The State of MCMV Latency

One major question about MCMV latency is whether the virus has actually established a state of molecular latency or a chronic infection. According to Ahmed et al. (Ahmed et al., 1996) latency is defined as a state of infection, usually following an acute infection, during which viruses persist in noninfectious form with intermittent periods of reactivation and shedding. In a chronic infection, the virus is continuously present in tissues.

Early studies attempted to discern the state of latency by assessing the presence of viral mRNA transcripts in various organs of latently infected mice. Three groups found IE1 transcripts in the spleen (Henry and Hamilton, 1993) and lung (Kurz et al., 1999; Yuhasz et al., 1994) and one group founds early-late transcripts and evidence of pp50 viral protein in the spleen and the salivary gland (Yu et al., 1995). None of the animals in these studies were immunosuppressed. These groups suggest a variety of scenarios including abortive infection, chronic infection, low-level persistence, and transient reactivation. The last three scenarios all imply that infectious virus was present in the mouse. Therefore the only way to show that any of the above cases is true is to demonstrate presence of infectious virus.

The standard procedure to measure infectious virus is with a plaque assay on, or coculture with MCMV-infectable cells, usually fibroblasts. Though explants of latently infected organs could be induced to reactivate MCMV (Mercer et al., 1988), most studies were unable to demonstrate evidence of preformed infectious virus in various latently infected organs or cell populations (Balthesen et al., 1993; Yuhasz et al., 1994). Whether the inability to detect virus in latently infected animals was due to lack of virus or low

sensitivities of plaque assays became the focus of two different groups. Pollock and Virgin (Pollock and Virgin, 1995) addressed this problem by establishing an increased sensitivity plaque assay, and a system by which they inject SCID mice (MCMV LD50=2 PFU) with sample organ homogenates. Using these systems they did not detect infectious virus in spleen or kidney, but did demonstrate viral DNA and ability to reactivate virus from these organs. They concluded that MCMV establishes latency without chronic persistence in the spleen and kidney. Kurz et al. (Kurz et al., 1997) reached the same conclusion in the case of the lungs. They too established a plaque assay with increased sensitivity, in this case, due to centrifugal enhancement and RT-PCR. However, doubt still exists in the MCMV community as to the sensitivity of these virus detection assays. Perhaps advances in technology will aid in solving these problems.

Reddehase et al. (Reddehase et al., 1994) emphasized the importance of the lung in latent infection. A comparison of reactivation of MCMV in the lungs, spleen, and salivary glands indicated a correlation between load of latent DNA and risk of recurrence (Reddehase et al., 1994). In this model system, the lungs followed by the salivary glands had the highest amount of latent DNA and the greatest incidence of recurrence. Two papers by Kurz (Kurz et al., 1999; Kurz and Reddehase, 1999) thoroughly explored the state of MCMV latency and reactivation in the lungs. Following bone marrow transplant they infect mice and allow the virus to establish latency. To examine MCMV in the lungs they removed and divided them into 18 sections. They found that during latency viral DNA was evenly distributed but IE1 transcripts were only found in certain sections and no IE3 or gB transcripts were detected at all. The authors suggest that IE1-expressing sections represent foci of aborted or nonproductive reactivation and go on to

present evidence that such foci have the potential to develop active virus during γ irradiation induced-reactivation. Interestingly it appeared that reactivation stayed focused and did not spread to other lung sections over time. Perhaps this indicates that MCMV itself and/or elements of the immune system left intact following γ irradiation are able to partially control reactivation events. It would be interesting to see if this control extends to other organs where reactivation and transmission are likely to occur such as the salivary glands.

The Sites of MCMV Latency- Cells

Many cell types have been implicated as sites of MCMV latency. Early work suggested that B lymphocytes fulfilled the role of latent cell type (Olding et al., 1975) (Olding et al., 1976). Later work showed that neither B nor T lymphocytes carried reactivatable MCMV and pointed instead to splenic stromal cells as latent cell types (Mercer et al., 1988). In addition to demonstrating that splenic stromal cells harbored reactivatable virus, Pomeroy et al. (Pomeroy et al., 1991) show that MCMV DNA is detectable by PCR in such cells. MCMV DNA is detectable in renal tubule cell preparations, which could include endothelial, epithelial or circulating cells (Klotman et al., 1990). Perhaps the most informative study of this type is by Koffron et al. (Koffron et al., 1998). This group uses immunofluorescence and PCR-in situ hybridization to assess the presence of MCMV genome in a wide variety of cell types. They find that MCMV DNA can be detected in endothelial cells (PECAM positive) of the kidney, liver, spleen, and heart. Interestingly, however, MCMV DNA in the lungs was detected not in endothelial cells but in alveolar macrophages (Mac3+).

The ability to detect MCMV in such diverse organs and tissues suggests that a ubiquitous cell type may harbor MCMV and deliver it to other cell types in the body. The favored cell type for this job is the macrophage, a tissue cell whose blood precursor is the monocyte, which is derived from a bone marrow progenitor. The macrophage was first implicated as a site of latency in 1979 (Brautigam et al., 1979). More recent work by Mitchell et al. (Mitchell et al., 1996) showed, using a combination of differential centrifugation, FACS analysis, and PCR, that monocytes (Mac3+) were latent cell types in the blood. Pollock et al. (Pollock et al., 1997) showed, quite convincingly, that among peritoneal exudate cells, the only population containing MCMV genome were F4/80+ macrophages. These cell types could be induced to reactivate and produce MCMV in culture. In addition, this group showed that in their experimental system, bone marrow cells are positive for MCMV DNA. These studies further the argument that, a population of MCMV genome-carrying cells is maintained in the bone marrow and that such cells replenish blood monocytes and tissue macrophages. In conclusion it is likely that there are two cell types in which MCMV can establish latency: organ endothelial cells, and macrophages.

Hypothesis and Rationale:

MCMV encodes a multitude of immune evasion genes that are successful in preventing CTL from responding to infected fibroblasts. Most studies have examined the effects of the immune evasion genes in fibroblasts, however such cells are not a relevant cell type in MCMV infection. The macrophage, one main site of MCMV latency, is a highly relevant cell type in MCMV infection. The question central to this thesis is: do

the immune evasion genes prevent CTL recognition in this important cell type? The hypothesis is that the immune evasion genes will function in macrophages; likely it is to the advantage of the virus to remain hidden from CTL in this site of latency.

Additional Data and Further Rationale:

During the initial stages of this project, a paper was published titled "Macrophages Escape Inhibition of Major Histocompatibility Complex Class I-Dependent Antigen Presentation by Cytomegalovirus" (Hengel et al., 2000). While the title opposes our hypothesis the data contained within the paper do not. The first striking piece of data is that their L^d-restricted, IE antigen-specific, CTL clone recognizes infected macrophages throughout the MCMV replication cycle but MEFs, as previously shown, only prior to E gene expression. They demonstrate expression of m152/gp40 and m6/gp48 proteins ruling out the possibility that escape of inhibition is because no inhibitory proteins are present. They ascertain that macrophages and MEFs are both producing IE peptide, YPHFMPTNL.

The system used by Hengel to analyze the effects of the immune evasion genes on macrophages differs from ours in three ways. First, Hengel et al. do not IFNγ pretreat their fibroblasts prior to CTL assays as we do. Second, their CTL clone recognizes an IE protein and ours recognize E proteins. Third their group looks at the effects of MCMV immune modulation on mice of an $H2^d$ haplotype (BALB/c) and we are looking at effects on mice of an $H2^b$ haplotype (C57BL/6). The significance of these differences is as follows.

As previously mentioned the CTL clone in Hengel's paper recognizes an immediate early protein pp89. This CTL clone recognizes non-IFNγ pretreated, wild type-infected fibroblasts only prior to early gene expression. However, IFNγ pretreatment of these fibroblasts allows the CTL clone to recognize these cells throughout infection (Hengel et al., 1994). Hengel shows that this clone recognizes non-IFNγ pretreated, wild type infected macrophages throughout infection and claims that the immune evasion genes are not functional in macrophages. We considered the possibility that an IFNγ pretreated fibroblast is like a non-IFNγ pretreated macrophage because the macrophage is a professional antigen presenting cell and may be better able to elicit a CTL response without the help of IFNγ. If this is so, the difference that Hengel sees in CTL response to non-IFNγ pretreated fibroblasts and non-IFNγ pretreated macrophages is due to the antigen presenting capacity of the macrophage and not necessarily the effects of MCMV's immune evasion gene products on the macrophage. Our clones do not recognize IFNγ-pretreated or untreated wild type-infected fibroblasts, but do recognize IFNγ pretreated fibroblasts infected with MCMV viruses lacking *m152* and sometimes *m4*, or *m6*, better than wild type MCMV. We feel our system is better set up, and perhaps more sensitive in it's ability to detect an effect of MCMV on macrophages by comparing CTL clone recognition of IFNγ pretreated fibroblasts to untreated macrophages. If the immune evasion genes truly do not work in macrophages, our CTL would see no difference in wild type infected macrophages and macrophages infected with MCMV lacking *m4*, *m6*, or *m152*.

Since pp89 is an IE gene it is likely that there are more pp89 antigen-L^d complexes formed and at the cell surface, by the time the E immune evasion proteins are

expressed. Therefore it is possible that pp89-L^d complexes are detected because they are already sufficiently expressed in macrophages. The gene products recognized by our CTL are expressed near the same time or later than the immune evasion genes and are therefore may be more affected by them. Due to this fact that our system may provide a more sensitive means of detecting whether or not the immune evasion genes can affect macrophage antigen presentation.

Lastly, MCMV's immune evasion genes do not treat all mouse MHC I molecules equally. Our lab has shown recently that K^b and D^b differ in their susceptibility to the effects of *m152* (Kavanagh et al., 2001). Hengel et al. address MCMV immune evasion in macrophages by analyzing MCMV's effects on L^d only. We have the ability to address MCMV's effects on both K^b and D^b as we have both K^b and D^b restricted clones. It was possible that we would observe differences in MCMV's ability to inhibit antigen recognition in macrophages based on MHC I allele.

In addition to the data outlined in the above paragraph, Hengel et al. present a pulse chase experiment in which they suggest that L^d escapes retention by MCMV. However this figure indicates that L^d is slow in moving to the cell surface and the difference between infected and uninfected cells is not apparent. I will demonstrate that both K^b and D^b are retained by m152/gp40 and move to the cell surface in its absence.

The paper by Hengel et al. contains one experiment that is further testament to the importance of bone marrow-derived cells, including macrophages, as it demonstrates that this population is sufficient to process MCMV antigen in organs of infected mice. They show that while L^d-/- mice are unable to process YPHFMPTNL, γ irradiation and a transfusion of L^d+/+ bone marrow allows them to efficiently process the peptide.

For the above reasons we felt it was important to continue this track of research. In the end we conclude that the immune evasion genes do function in macrophages, however, some CTL are able to detect these infected cells at a low level. This data is compatible with that published by Hengel et al. because in both cases, CTL detect wild type-infected cells.

Materials and Methods

Experimental Animals

C57BL/6 mice were purchased from Simonsen Laboratories and maintained according to institutional protocols.

Cell Culture

Mouse Embryo Fibroblasts (MEFs) were grown from trypsin-digested day 12-14 mouse embryos, and used between passage 3 and 4. Balb3T3s (CCL-163) and IC21 SV40-transformed peritoneal macrophages (TIB-186) were obtained from ATCC. MEFs were maintained in DMEM supplemented with 10% fetal calf serum (newborn calf serum for Balb3T3s) and penicillin, streptomycin, glutamine (PSG). IC21s were maintained in RPMI supplemented with 10% fetal calf serum, PSG, 10mM HEPES buffer, 1mM Na Pyruvate (Gibco BRL), and 2.4mg/mL glucose. BMMϕ were derived following the protocol of Bouwer et al. (Bouwer et al., 1997). Femurs were removed from 6 week or older C57BL/6 mice and marrow was flushed from the bone using a DMEM/10%FBS and needle and syringe and then strained through a 70um cell strainer. Cells were washed two times in DMEM/10%FBS and plated out at 1 x 10^7 total cells per 150mm petri plate (Lab-Tek) in DMEM/10%FBS plus 30% GMCSF source, L929 supernatant. (L929 supernatant is derived from L929 cells grown 10 days post-confluency in DMEM/10%FBS) Six days later BMMϕ were harvested by rinsing with room temperature PBS to remove non-adherent cells and then incubating in 4°C PBS for 5

minutes to collect adherent cells. These adherent cells are macrophages as shown in figure 4A.

Generation of mutant MCMVs

Generation and characterization of recombinants ΔMS94.5 (with a deletion of ORFs *m150* to *165*), ΔMC96.24 (with a deletion of ORF *m152*) and rMC96.27 (revertant for ΔMC96.24) MW97.01 wild type MCMV BAC, and recombinant MCMVs Δm4-MW99.03, Δm152-MW99.05 were described previously (Kavanagh et al., 2001; Krmpotic et al., 1999; Thale et al., 1995).

Recombinant MCMV Δm6 was generated by Markus Wagner by transfection of the MCMV BAC plasmids pΔm6 into primary MEFs by calcium phosphate precipitation technique as described previously (Messerle et al., 1997). The MCMV BAC plasmid pΔm6, which encodes an 82% deletion of the *m6* ORF (nt 6392 to 6235), was constructed by insertion of the prokaryotic kanamycin resistance marker (kan^r). The plasmid pCP15 served as template for the kanamycin resistance marker. A linear DNA fragment containing flanking homologies to the *m6* gene fragment and the kan^r was generated by PCR amplification. This fragment was inserted into the wt MCMV BAC plasmid pSM3fr (Wagner et al., 1999) by homologous recombination in *E. coli* to generate the MCMV BAC plasmid pΔm6. Correct mutagenesis was confirmed by restriction enzyme and southern blot analysis (data not shown).

Viruses

Wildtype MCMV, Smith, was obtained from ATCC. Smith and mutant virus stocks were generated by infecting subconfluent MEFs with low-passage seed stock at an

MOI of 0.01. Once the monolayer became 100% infected stocks were harvested by scraping and sonication of cells. Titer of plaque forming units (PFU) was determined by serial dilution and agarose overlay on Balb3T3s.

T Cell Clones

C57BL/6 mice were infected intraperitoneally with 5 x 10^4 PFU MCMV-Smith, ΔMS94.5 (Δm150-m165), or ΔMC96.24 (Δm152). Between 8 and 40 weeks later, spleens were harvested. 10% of splenocytes were infected with MCMV (of the same strain with which mice were infected) and returned to culture with the remaining splenocytes. To derive CTL clones, the cultures were cloned by limiting dilution on day 3 in the presence of irradiated mixed allogeneic feeder splenocytes and 2μg/ml concanavalin A (con A; Sigma). Clones were maintained in cloning medium [RPMI medium with 10% FCS, 5 X 10^{-5} M 2-mercaptoethanol (Sigma), 1% conditioned medium from IL-2-secreting cell line X63.653 (Karasuyama and Melchers, 1988), and 10% conditioned medium from conA-stimulated rat splenocytes], and restimulated with conA and irradiated mixed allogeneic feeders each 10 days. Clones have been maintained in culture for more than 12 months. Clones were screened for anti-viral function based on their ability to specifically kill IFNγ-boosted ΔMS94.5-infected MEFs compared to uninfected MEFs. Clones 3, 11 and 5 are from ΔMS94.5-infected mice (clone 5 is from a different mouse than clones 3 and 11); and clone 55 is from a ΔMC96.24-infected mouse.

Antibodies

Serum 8010 (anti-p8) was generated by immunizing rabbits with synthetic peptide corresponding to exon 8 of K^b. Serum 8139 (anti-m4/gp34) was generated as follows. Serum R123 against the cytoplasmic tail of m4/gp34 (Kleijnen et al., 1997) was used to precipitate m4/gp34 from MCMV (Smith)-infected MEFs. After washing, the immune complex was suspended in complete Freund's adjuvant (Sigma) and used to immunize rabbits subcutaneously. Rabbits were boosted first with immune complex suspended in incomplete Freund's adjuvant (IFA, Sigma), and then by infection with recombinant vaccinia virus expressing m4/gp34 (generated by recombination between modified psc11 plasmid expressing the *m4* gene and WR strain vaccinia virus), and finally with recombinant m4/gp34 protein purified from baculovirus, (the kind gift of Pamela Bjorkman) in IFA. Monoclonal antibody 28.14.8S (ATCC HB-27) was purified from hybridoma supernatant.

RT-PCR Analysis

RNA was isolated from infected cells using Trizol (GibcoBRL), treated with Dnase (GibcoBRL), and cDNA was synthesized using an oligo-dT primer in a SuperScript II Preamplification System (GibcoBRL). cDNA samples were subjected to PCR using the following primers. m4 nucleotide sense: 5'-TAAGGTCCCGTCATCGGATC-3', antisense: 5'-GGAGATTGACGACGACCCAT-3', m6 sense: 5'-TGGCGCTCGTAACTGTGCTA-3', m6 antisense: 5'-AGGGAGCGGATTCATCATGA-3', m152 sense: 5'-TGACCGTAGCGTACCATCCC-3', m152 antisense: 5'-ACGATTGAAGGTGGAAGCGT-3', m144 sense: 5'-

GCCGTATCATCCTCGGTTTC-3', m144 antisense: 5'-AAGGTGATCGGCGTCAAGAC-3', m123 sense: 5'-GTCTCCCAACATGTCCTCCAG-3', m123 antisense 5'-ACCCATCAGACAAGGTGCCA-3', M75 sense 5'-TCCCCCCATGAGATCTGTTC-3', M75 antisense 5'-CTAACGGTCCGTACGCAACTG-3', murine beta actin sense 5'-GCTCCATCTTGGCCTCACTG-3', murine beta actin antisense 5'-CTAGAAGCACTTGCGGTGCA-3', murine beta actin 491frag sense 5'-AAGAGAAGCTGTGCTATGTTGCTC-3', murine beta actin 491frag antisense 5'-CTAGAAGCACTTGCGGTGCA-3'. The cycle parameters were 50 cycles of 95°C for 15 seconds, and 62°C for 1 minute. PCR reactions were carried out in 25uL and contained 50mM KCl, 10mM Tris-HCl (pH 9.0), 1.5mM $MgCl_2$, 0.03u Taq polymerase, 10mM dNTPs, 300nM each primer, and 100ng total cDNA. Products were separated by electrophoresis on a 3% agarose gel.

FACS Analysis

Cells were washed in FACS buffer at 4°C (PBS, 1%FCS, 0.1% NaAzide, 5% normal mouse serum) and incubated for 15 minutes in FACS buffer to block non-specific staining. Cells were washed and incubated with either ratIgG (Sigma) or anti mouse F4/80 (MCAP497 ratIgG2b Serotec) in FACS buffer for 15 minutes. Cells were washed and incubated with FITC-conjugated goat anti ratIgG (Jackson ImmunoResearch) and washed again. Cells were analyzed by flow cytometry using a BD FACS Scan flow cytometer in conjunction with Cell Quest (BD). All further analyses were performed using FlowJo software (Treestar).

Immunofluorescence Analysis

Cells were plated out in 10%FBS DMEM in 6 well dishes with glass coverslips at, $6x10^5$ bone marrow macrophages per well or $9x10^4$, 50U/mL IFNγ-treated, MEFs per well. 24 hours later cells were infected with BAC wild type MCMV at a MOI of 70. Two hours later virus was removed and replaced with 0.3mg/mL PAA-treated medium to prevent late gene expression. At 20 hours post infection coverslips were washed two times with PBS and fixed with fresh-made 2% paraformaldehyde for 8 minutes. Cells were permeablized with 0.2% Triton X 100 (Sigma) in PBS for 2 minutes and washed in PBS. Fc receptors were blocked using 5% normal mouse serum 5% normal goat serum in PBS for 45 minutes at 37°C. Blocking agent was aspirated off and primary antibody added; precleared pre-immune rabbit serum or 8139 (anti-MCMVm4) serum at 1 to 100 in 3% normal goat serum in PBS. Antibodies were precleared by rocking a 1:10 serum to 3% normal goat serum 1x PBS solution over a 150mm plate of fixed, permeablized, and blocked mouse embryo fibroblasts for 24 hours at 4°C. Following this preclear, the same 1:10 solution was then rocked over a 100mm plate of fixed, permeablized, and blocked bone marrow macrophages for 24 hours at 4°C. Following incubation of primary antibody on glass coverslip, cells were washed three times in PBS over five minutes. FITC-conjugated goat-anti rabbit IgG (KPL, Gaithersburg, MD) 12.5mg/mL final concentration in 3% normal goat serum in PBS was added and cells were incubated at 37°C for 45 minutes in dark. Cells were rinsed 3 times with PBS over five minutes and then treated with 2ug/mL Hoechst 2495 (Sigma) in PBS for five minutes at room temperature in the dark. Cells were then rinsed one time with PBS and several times in

dH_2O. Coverslips were dried completely, in dark and mounted on glass slides (Fisherbrand) using Prolong Antifade reagent (Molecular Probes, Eugene, OR). Images were visualized using a 40x water objective on a Bio-Rad 1024UV laser-scanning confocal microscope equipped with an Axiovert-100 (Zeiss). These images or a Nikon Optitek microscope were used for infected cell counting.

Immunoprecipitations

C57BL/6 MEFs or IC21 macrophages were pretreated with recombinant mouse IFNγ at 50 U/mL and BMMϕ were plated in the absence of IFNγ for 24 hours before infection. Virus medium was removed and replaced with cysteine/methionine-free DMEM supplemented with antibiotics, 5%FBS, 0.3 mg/ml PAA plus 0.33mCi ^{35}S cysteine/methionine (NEN). Cells were infected and labeled overnight. Lysis and precipitation steps were carried out at 4°C. Cells were washed with PBS and lysed in NP-40 lysis buffer (0.5% NP-40, 50mM Tris-HCl pH 7.6, 5mM $MgCl_2$). Lysis buffer was supplemented with Complete EDTA-free Protease-Inhibitor Cocktail (Boehringer-Mannheim) just prior to use. Lysates were precleared by incubation with at least 20 μl of normal rabbit serum (NRS) and sometimes normal mouse serum (NMS) and 500 μl of 10% suspension of fixed *Staphylococcus aureus* for 2 hours, and centrifuged for 5 min at 15,000g. Precleared lysates were then subjected to specific immunoprecipitation as indicated in the figures. Unless otherwise indicated, each aliquot of lysate received ~10 μg of antibody plus 150 μl of 5% protein A agarose suspension (Sigma). Immunoprecipitates were washed four times in NET buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA and 0.05% NP40) containing 0.1% SDS. Samples were digested

with Endo H_f (NEB) according to manufacturer's protocol, resuspended in reducing sample buffer and separated by SDS-PAGE on a 12.5% gel.

Cytolytic T cell Assays

MEF or IC21 target cells were plated into 96-well plates at 5,000 cells/well and treated with recombinant mouse IFNγ (50 U/ml, Sigma) and BMMϕ were plated at 20,000 cells/well for 24 hours, infected with MCMV at MOIs indicated in figure legends, and labeled with ^{51}Cr (NEN) overnight, in the presence of 0.3mg/ml phosphonoacetic acid (PAA, Sigma) to prevent expression of viral late genes. CTL clones described here did not kill MEF targets without IFNγ pretreatment (data not shown). T cells were added at the indicated effector-to-target ratios for six hours, after which supernatants were harvested and assayed for γ-irradiation with a Topcount scintillation counter (Packard). Background Cr-release was determined by incubating targets with medium alone, and total Cr release was achieved by lysing targets with medium containing 2% Triton X-100. Percent specific lysis was calculated as (experimental cpm-background cpm)/(total cpm-background cpm). Each data point represents the mean of triplicate wells and error bars represent the standard error of the mean.

Results

MCMV immune evasion genes are transcribed in both fibroblasts and IC21 SV40-transformed macrophages

MCMV infects both fibroblasts and macrophages (Hanson et al., 1999). Our initial hypothesis was that MCMV might differentially regulate immune evasion genes at the level of transcription in these two cell types. Because MCMV gene expression depends on host cell transcription factors and these factors differ among cell types, such a mechanism made sense. We set up an RT-PCR system to test our hypothesis. Following reverse transcription, our procedure was designed to detect the amount of cDNA product through PCR amplification.

We were able to demonstrate that these primers are specific for the immune evasion genes, *m4*, *m6*, and *m152* (Fig. 1). Also included in the assays were MHC I homolog gene *m144*, IE gene *m123*, L gene *M75*, and two primer sets to detect positive control cellular beta actin. One primer set yields a 100 bp product and the other gives a 491 bp product. Successful amplification of the latter demonstrates that 100 bp bands are not a result of contamination due to mixing of primers during PCR reaction set-up, or spill over during gel loading. PCR reactions detected expression of tested genes in mouse embryo fibroblasts (MEFs) infected with MCMV deletant viruses ΔMS94.5, Δm4-MW99.03, Δm6-MWΔm6 and reconfirmed the phenotypes of these deletant viruses (Fig. 1A). ΔMS94.5 lacks 15 genes including *m152*, Δm4 lacks *m4*, and Δm6 lacks *m6*.

To assess expression of immune evasion genes in MEFs compared to IC21 SV40-transformed peritoneal macrophages, these cells were infected with wild type MCMV

(Smith). Both cell types express the immune evasion genes, *m144*, *m123*, *M75*, and cellular β *actin*. As a control we show that uninfected cells express only β *actin* (Figs. 1B and 1C). RT-PCR samples without RT failed to amplify gene product and uninfected IC21 macrophages were negative for immune evasion genes (data not shown). MEFs expressed all tested MCMV genes following an overnight infection while IC21 macrophages required at least a two day infection to express late gene *M75* (data not shown). This delay in gene expression is not surprising however since MCMV growth in macrophages is delayed (Hanson et al., 1999).

While we were testing primers for this quantitative RT PCR assay, Hengel et al. published a paper demonstrating that macrophages escape from MCMV immune evasion. As previously mentioned, this group showed that immune evasion proteins were well expressed in macrophages. They also reported that MHC I molecules were not retained by m152/gp40. We had already demonstrated that macrophages expressed the immune evasion genes at an RNA level. However, we felt that in light of Hengel's recent data, rather than pursuing differences in RNA levels it would be more interesting to examine immune evasion in macrophages at the level of protein and function using $H2^b$ cells and our C57BL/6 CTL clones. We proceeded to assess m4/gp34 protein expression and MHC I retention in IC21 macrophages.

m4/gp34 is expressed and associates with MHC I, and D^b is retained in infected IC21 macrophages

In order to test, first, whether or not *m4* was expressed in macrophages and second, if MHC I was retained, we infected both MEFs and IC21 macrophages with

MCMVΔm6 or left them uninfected. Infecting in the absence of m6/gp48 removes the confounding effect of this protein since it redirects MHC I to the lysosome where they are both degraded. Therefore we used MCMVΔm6 to better detect MHC I retention.

To score for MHC I retention one generally compares EndoH sensitive to EndoH resistant bands. EndoH cleaves N-linked sugars on proteins that have not moved past the medial Golgi since enzymes here modify such sugars, rendering them indigestible or resistant. Likewise proteins in pre-medial Golgi compartments are referred to as EndoH sensitive and indicate retained proteins; and proteins that have advanced past the medial Golgi are denoted EndoH resistant and indicate exported proteins. In this way we can use EndoH digestion to determine retention of MHC I due to MCMV infection.

Figure 2 illustrates a typical immunoprecipitation in which infected or uninfected MEFs and IC21s were methionine S^{35}-labeled overnight, in the presence of DNA synthesis inhibitor PAA, and then lysed in 0.5% NP40 buffer. m4/gp34, K^b, and D^b were immunoprecipitated from the lysates and samples were either treated with EndoH or left untreated and run on a 12.5% SDS PAGE gel.

Immunoprecipitation of m4/gp34 in both MEFs and IC21s revealed a mostly EndoH sensitive band. When undigested, this band runs at approximately 34kD, and is denoted "m4" (Fig 2). The observation that most precipitated m4/gp34 is EndoH sensitive (m4S) indicates that most of this protein is contained in a pre-medial Golgi compartment. Also m4/gp34 was associated with MHC I in both MEFs and IC21s as demonstrated by the presence of MHC I heavy chain and β_2m, in m4/gp34 precipitations.

A reciprocal association was illustrated by the presence of m4/gp34 following immunoprecipitations of D^b and K^b (Fig. 2). m4/gp34 co-precipitating with MHC I was

composed of higher and lower molecular weight populations. The higher molecular weight, EndoH resistant band (m4R), precipitated with exported K^b and the lower molecular weight EndoH sensitive band precipitated with retained K^b as has been shown in the past (Kleijnen et al., 1997).

A comparison of EndoH-treated and untreated D^b immunoprecipitates from uninfected IC21 macrophages revealed that this molecule was mostly EndoH resistant and therefore mostly exported past the medial Golgi (Fig 2). However, in infected IC21s D^b was clearly retained (Endo H sensitive), presumably by m152/gp40. While K^b retention was visible in infected MEFs, immunoprecipitations of K^b from IC21 macrophages resulted in very faint bands and made it difficult to interpret whether or not K^b was retained by MCMV in these cells. Thus we conclude that m4/gp34 is expressed in MCMV Δm6-infected IC21 macrophages and D^b is retained in such cells, presumably by m152/gp40.

m152/gp40 prevents recognition of IC21 macrophages by CTL Clone 3

Having shown that D^b is biochemically retained in infected IC21 macrophages, likely due to m152/gp40, and knowing that MCMV-specific CTL clones recognize Δm152-infected MEFs better than wild type infected MEFs (Ziegler et al., 1997) (Kavanagh et al., 2001; Krmpotic et al., 1999) we predicted that m152/gp40 would function to inhibit CTL recognition of IC21 macrophages. We tested the ability of m152/gp40 to inhibit antigen presentation to an MCMV-specific CTL clone in a Cr^{51}-release assay.

Figure 3 illustrates the results of a typical Cr^{51}-release assay in which infected MEF or IC21 macrophage targets were loaded with Cr^{51} overnight and tested for CTL recognition. In this case, MEF and IC21 targets were left uninfected, or infected with wild type MCMV (MW97.01) or Δm152MCMV (MW99.05) and tested for CTL recognition by CTL Clone 3, a CTL clone that recognizes a peptide derived from E protein M45 (Gold manuscript in preparation) in the context of D^b. As previously demonstrated in our lab, Clone 3 recognized Δm152-infected MEFs much better than wild type-infected or uninfected MEFs (Kavanagh et al., 2001). Interestingly this same pattern was observed in IC21s, indicating that m152/gp40 is also able to prevent CTL recognition in this cell type.

BMMϕ and MEFs express MCMV early proteins at similar levels

Immunoprecipitations and CTL assays suggested that $H2^b$ IC21 macrophages were susceptible to the effects of m152/gp40. This is in contrast to results reported by Hengel using both transformed J744 $H2^d$ BALB/c macrophages and primary bone marrow-derived $H2^d$ BALB/c macrophages. We wanted to make sure that the effect of m152/gp40 in $H2^b$ C57BL/6 IC21s was not due to some artifact of SV40 transformation of this particular line. To rule out this possibility we decided to address the effect of the immune evasion genes in $H2^b$ C57BL/6 primary macrophages.

It was important to establish that we could isolate pure populations of macrophages before we began our experiments. We tested bone marrow-derived macrophages (BMMϕ) for F4/80 expression by FACS analysis. This marker is specifically found on cells that are phenotypically macrophages (Austyn and Gordon,

1981). In addition, Pollock et al. (Pollock et al., 1997) used this marker in their determination that macrophages in the peritoneal cavity carry latent MCMV demonstrating that macrophages bearing this marker are relevant in MCMV infection. Figure 4A demonstrates that macrophages but not MEFs were F4/80 positive.

We next wanted to compare the ability of MCMV to infect and express E protein in MEFs and BMMϕ. We wished to achieve similar percentages of E protein expression in each cell type in order to make comparisons between the two cell types in future assays. Determining the percentage of cells expressing E proteins is a relevant control since both immune evasion proteins (Hengel et al., 1999) and antigens recognized by our CTL clones (Gold, unpublished data) are expressed at early time points.

To assess E protein expression, both cell types were infected with wild type MCMV (MW97.01) at various MOIs. Here the term MOI indicates the number of plaque forming units per MEF or BMMϕ. Plaque forming units were determined by plaque assay on 3T3 fibroblasts. Following infection, these cells were stained for *m4/gp34*, a representative MCMV E protein.

Using an immunofluorescent microscope, a count of at least 200 cells per condition was attained. The number of cells expressing m4/gp34 was compared to the number of cells stained with a DNA-specific stain. These numbers revealed that both cell types expressed MCMV E proteins at similar levels at a given MOI, which will allow for relevant comparisons between macrophages and fibroblasts in future experiments (Figs 4B and 4C). Importantly, both cell types were between 50-75% infected at MOIs ranging from 50 to 100. Subsequent experiments were done with cells infected at MOIs of 45 to 100 in order to ensure that the majority of cells were infected. The m4/gp34 antibody

was specific for infected cells since, it did not stain uninfected cells. This is shown in a field of cells infected at an MOI of 1, an MOI at which the majority of cells are uninfected. In conclusion, a majority of MEFs and BMMϕ are infected by MCMV at MOIs between 50 and 100.

IFNγ-pretreatment of BMMϕ decreases the percent of cells infected with MCMV

IFNγ-pretreatment of MEFs upregulates MHC I, which allows this cell type to be recognized by CTL and enables detection of MHC I in immunoprecipitations. In fact IFNγ-pretreatment of MEFs is required for recognition by all CTL clones used in our lab (Gold, unpublished data). Since GMCSF-treated, bone marrow-derived macrophages are professional antigen presenting cells, and it is possible that they may not need IFNγ pretreatment for CTL recognition or detection of MHC I during immunoprecipitations. In fact there is evidence to suggest that macrophages are refractory to MCMV infection if they are IFNγ-pretreated (Presti et al., 2001). We show in experiments discussed below that BMMϕ not pretreated with IFNγ are detected by CTL. In addition, antibody immunoprecipitations readily detect MHC I in non-pretreated BMMϕ indicating that these assays can be performed without IFNγ.

Interestingly, when we assessed m4/gp34 expression in BMMϕ infected at an MOI of 50 with wild type MCMV (MW97.01) we found that approximately 58% of cells expressed m4/gp34 (fig 4D). This percentage dropped to 16% following IFNγ pretreatment. This could mean that fewer cells were infected, or that fewer cells had progressed to early stages of MCMV protein expression. In either case we feel that IFNγ

pretreating BMMϕ may interfere with future assays because a low percentage of cells are expressing MCMV E proteins.

m152/gp40 retains K^b and D^b in bone marrow macrophages

To determine if MHC I retention in BMMϕ was specifically due to m152/gp40, three viruses were used: wild type MCMV (Smith), Δm152 (ΔMC96.24), and revertant Δm152 (rMC96.27). The phenotype of the revertant Δm152 should match that of wild type MCMV. An effect seen with Δm152 and not with wild type or revertant Δm152 can be directly attributed to m152/gp40.

MEFs and BMMϕ were left uninfected or infected, and metabolically labeled overnight. Next, K^b and D^b were precipitated from all lysates and EndoH treated in order to determine their degree of retention. In figures 5A and 5B we show for the first time that MHC I molecules are retained specifically by m152/gp40 in BMMϕ.

The degree of MHC I retention can be visualized by comparing the amount of EndoH sensitive (retained) MHC I to the total amount of MHC I. Figure 5 shows that m152/gp40 retains MHC I in both cell types. Overall MHC I retention is less striking in BMMϕ than in MEFs. Also retention of D^b is much more apparent than K^b in both cell types infected with wild type or revertant Δm152 viruses. The fact that the MHC I retention pattern for cells infected with revertant Δm152 is similar to that for wild type-infected cells reaffirms the integrity of the Δm152 virus construction and directly attributes retention to m152/gp40. Thus we conclude that m152/gp40 is biochemically functional and retains both K^b and D^b in MEFs and BMMϕ.

m4/gp34 is expressed in bone marrow macrophages and associates with MHC I

In figure 5B we demonstrate findings similar to those in figure 2; K^b and D^b associate with m4/gp34, this time in BMMϕ. These populations represent m4/gp34 associated with retained and exported MHC I. Though these and previously mentioned immunofluorescence experiments illustrate that m4/gp34 is expressed we wanted to further investigate its expression and retention using an m4/gp34-specific antibody.

Figure 5C confirms that the m4/gp34 is expressed in BMMϕ is mostly EndoH sensitive, and again is associated with MHC I. Immunoprecipitations of m4/gp34 were performed on the same lysates as above. We found that MEFs, as expected, express m4/gp34 when infected with any of these viruses, as do BMMϕ. The presence of β2m in lanes with m4/gp34 reiterates its association with MHC I in both cell types infected with any of the shown viruses.

m152/gp40 prevents CTL recognition of BMMϕ

Given that Δm152-infected IC21 macrophages were recognized by CTL Clone 3, and m152/gp40 biochemically retains MHC I in BMMϕ, we predicted that m152/gp40 would also affect antigen presentation in this cell type. To determine whether or not m152/gp40 affected CTL recognition of BMMϕ we performed a ^{51}Cr release assay using either MEFs or BMMϕ as targets and CTL Clones 3 (D^b-restricted) and 5 (K^b-restricted) as effectors. Targets were infected with the same set of viruses used in the above biochemical analysis; wild type MCMV (Smith), Δm152 (MC96.24), or Δm152 revertant (rMC96.27). Figure 6 shows that both CTL clones, Clone 5 (Fig 6A) and Clone 3 (Fig 6B), recognized both MEFs and BMMϕ infected with Δm152 better than those infected

with wild type MCMV or revertant Δm152. This demonstrates that m152/gp40 partially blocks antigen presentation via D^b and K^b in both cell types.

Overall both clones saw Δm152-infected BMMϕ better than Δm152-infected MEFs, suggesting that BMMϕ overall are better antigen presenting cells. Interestingly K^b-restricted Clone 5 was able to recognize wild type and revertant Δm152 -infected macrophages at low levels while D^b-restricted Clone 3 was not. This finding agrees with other data from our lab showing that m152/gp40 is more effective in preventing antigen presentation by D^b than by K^b (Kavanagh et al., 2001). Thus m152/gp40 plays a specific role in preventing antigen presentation in macrophages. However, this gene does not completely prevent antigen presentation since a K^b-restricted clone is able to detect, at low levels, macrophages infected with wild type MCMV.

m4/gp34 and m6/gp48 prevent macrophage antigen presentation to some CTL clones

Two other immune evasion genes, *m4* and *m6*, inhibit antigen presentation in fibroblasts (Kavanagh et al., 2001; Kleijnen et al., 1997; Reusch et al., 1999). In addition we have shown that m4/gp34 was expressed and associated with MHC I in BMMϕ. We investigated the effect of these genes on macrophages' ability to present antigen to CTL clones. BMMϕ or MEF targets were infected with wild type MCMV (MW97.01), Δm4 (MW99.03), Δm6 (MWΔm6), or Δm152 (MW99.05). A ^{51}Cr release assay was performed on these targets with Clones 5, 11, and 55. Figure 7 shows K^b-restricted Clones 5 and 11 were sensitive to the effects of not only m152/gp40, but also m4/gp34, and m6/gp48 as well. D^b-restricted Clone 55 was sensitive to the effects of m152/gp40

and marginally sensitive to the effects of m6/gp48. These results are consistent with previously published data from our lab showing that m152/gp40 interferes more with D^b than K^b and m4/gp34 is needed to completely interfere with K^b-restricted antigen presentation (Kavanagh et al., 2001). Thus m4/gp34 and m6/gp48 prevent antigen presentation to two K^b-restricted clones and m6/gp48 may prevent antigen presentation to a D^b-restricted clone.

Figure 6 indicated that K^b-restricted Clone 5 detected two different wild type MCMVs (Smith and rMC96.27). We show in figure 7 that two K^b-restricted Clones (5 and 11) are also able to detect macrophages infected with a third wild type MCMV (MW97.01). While detection levels were low in all of these cases we are confident that at least some CTL clones are able to see wild type infected macrophages, a phenotype never before observed with fibroblasts. Thus m4/gp34, m6/gp48, and m152/gp40 prevent antigen presentation to K^b-restricted CTL, but are unable to completely block detection of wild type infected macrophages. Also, m152/gp40 and possibly m6/gp48 prevent antigen presentation to the tested D^b-restricted clone.

Figures and Figure Legends

Figure 1

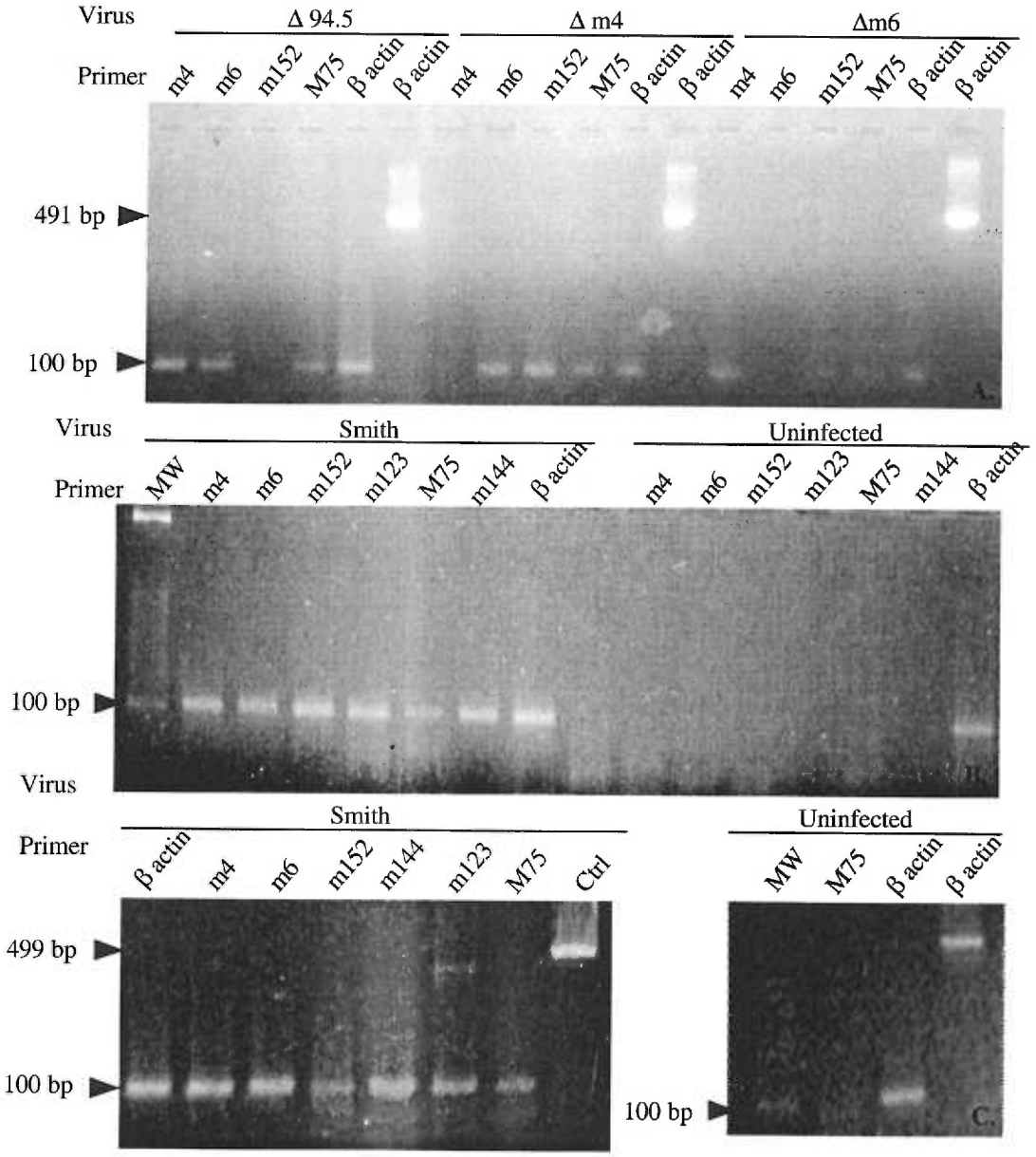


Figure 1. Immune evasion genes are expressed in MEFs and IC21 macrophages. Following infection of MEFs or IC21s, RNA was isolated and subjected to RT-PCR. **A.** Primers are specific for individual genes in MEFs infected overnight at an MOI of 3 with MCMV deletant viruses Δ94.5 (ΔMS94.5), Δm4 (MW99.03), and Δm6 (MWΔm6). This figure also reconfirms the phenotypes of these deletant viruses: Δ94.5 lacks 15 genes including *m152*, Δm4 lacks *m4*, and Δm6 lacks *m6*. **B.** MEFs infected overnight with Smith (wild type MCMV) at a multiplicity of infection (MOI) of 1 express *m4*, *m6*, *m152*, IE gene *m123*, *M75*, MHC I homolog *m144*, and cellular *β actin*. Uninfected MEFs express only cellular β actin. **C.** IC21 macrophages infected with Smith at an MOI of 0.1 for 3 days express *m4*, *m6*, *m152*, *m144*, *m123*, and *M75*. Also, the control cDNA included in the Gibco BRL RT kit amplifies with included primers to give a 499 bp band. Uninfected IC21 macrophages express only *β actin*.

Figure 2

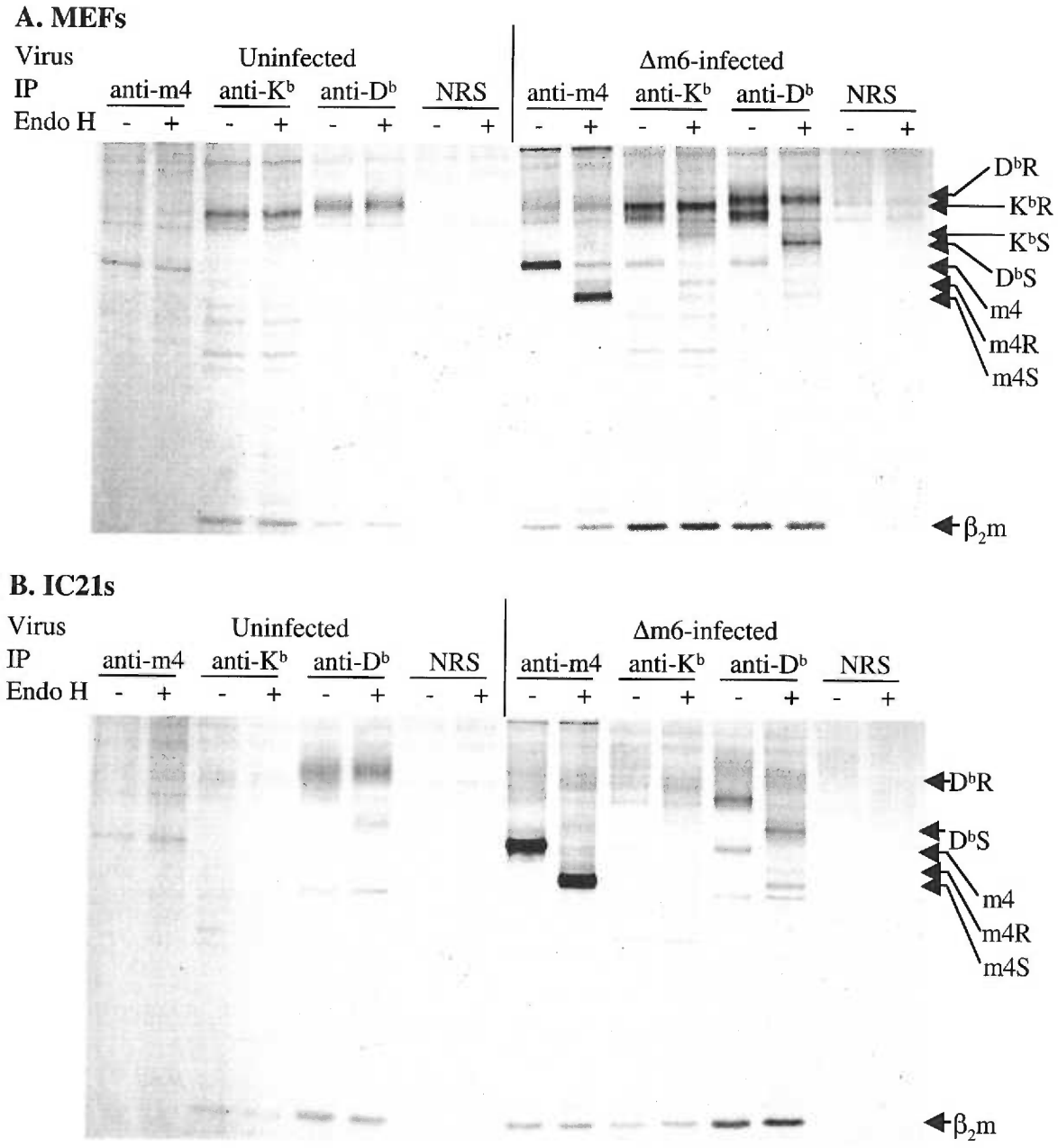


Figure 2. Immune evasion proteins are expressed in MEFs and IC21 macrophages. MEFs (**A.**) and IC21 (**B.**) macrophages were pretreated with 50U/mL IFNγ in order to increase MHC I expression. Cells were either infected with Δm6 (MWΔm6) at an MOI of 5 or left uninfected and S^{35} labeled overnight in the presence of PAA to prevent late gene expression. Cell lysates were subjected to immunoprecipitations using either 8139 anti-m4, 8010 anti-K^b, 2814.8S anti-D^b, or normal rabbit serum (NRS) as a negative control. Immunoprecipitates were either treated with EndoH or left untreated and run on a 12.5% SDS PAGE gel. "R" indicates EndoH resistance and "S" indicates sensitivity. "m4" indicates undigested m4/gp34.

Figure 3

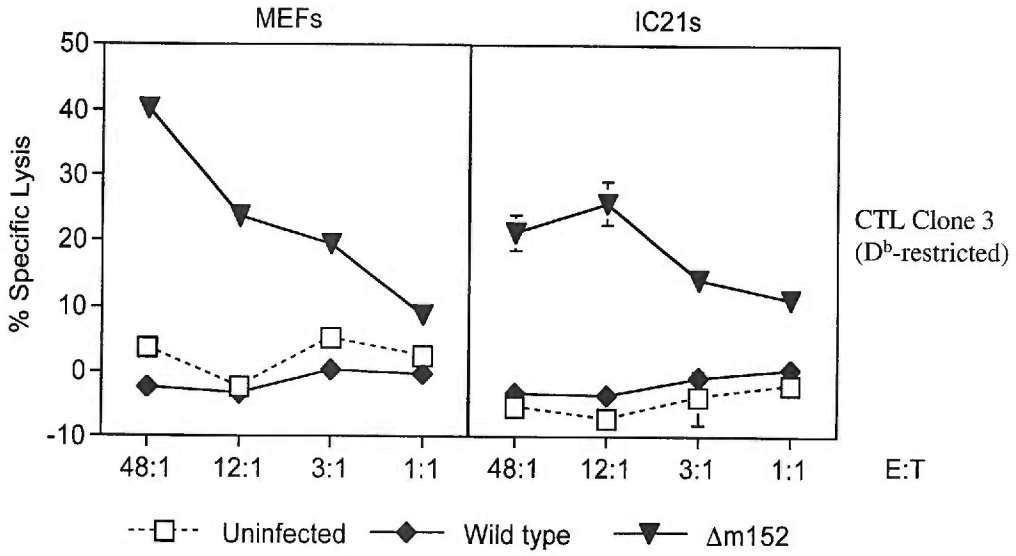


Figure 3. m152/gp40 prevents CTL recognition in IC21 macrophages. IFNγ-pretreated MEFs and IC21 macrophage targets were Cr^{51}-loaded, and infected, at an MOI of 70, overnight, in the presence of PAA, with no virus, wildtype MCMV (MW97.01), or Δm152 (MW99.05). D^b-restricted CTL Clone 3 was tested for its ability to recognize and lyse targets. Error bars represent the standard error of the mean for triplicate wells.

Figure 4

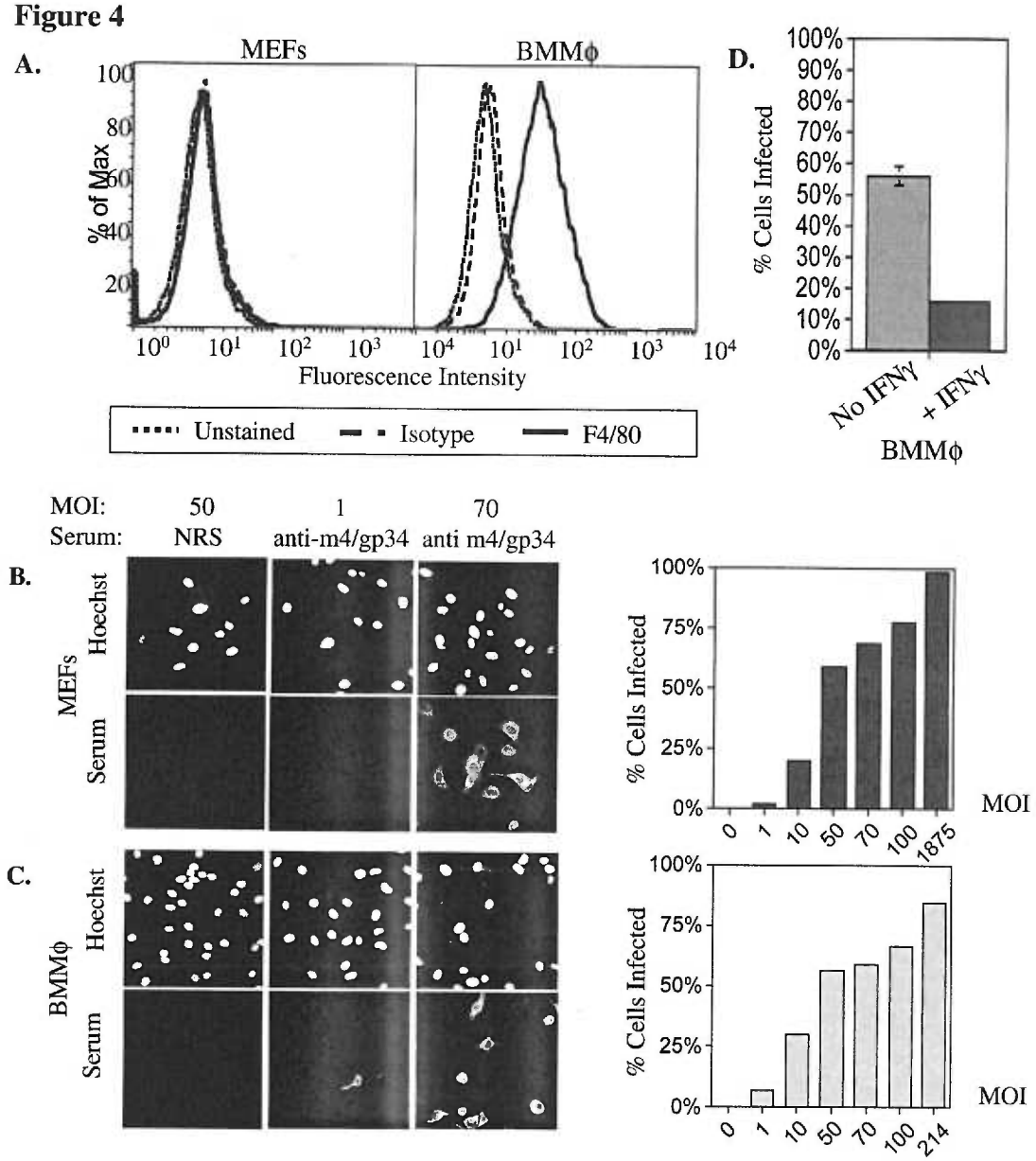
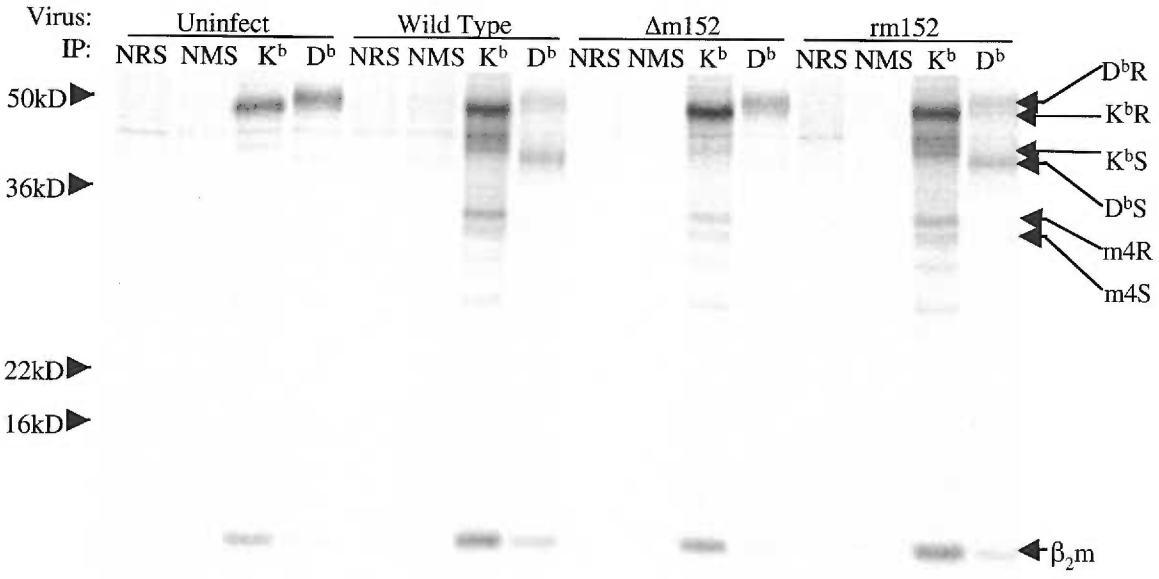


Figure 4. BMMϕ can be infected with MCMV and express E proteins. IFNγ-pretreatment reduces the percentage of BMMϕ expressing MCMV E proteins. **A.** BMMϕ are a pure population, all expressing F4/80. Cells were cultured from bone marrow in the presence of GMCSF for 6 days. BMMϕ and MEFs were stained with isotype control antibody or anti-F4/80 followed by conjugated secondary antibody; these and unstained cells were analyzed by FACS. **B.** IFNγ-pretreated MEFs were plated onto glass coverslips, and infected overnight with wild type MCMV (MW97.01) in the presence of PAA at indicated MOIs. Cells were stained for m4/gp34 expression and treated with Hoescht DNA stain. Normal Rabbit Serum was included as a negative control. Counting m4/gp34-expressing cells compared with Hoechst stained cells in the same field allowed for a quantitation of the percent of cells infected. **C.** BMMϕ were analyzed as for B. **D.** BMMϕ were IFNγ-pretreated or left untreated and infected overnight as above at an MOI of 50. Percent of infected cells was quantitated as above.

Figure 5

A. MEFs



B. BMMϕ

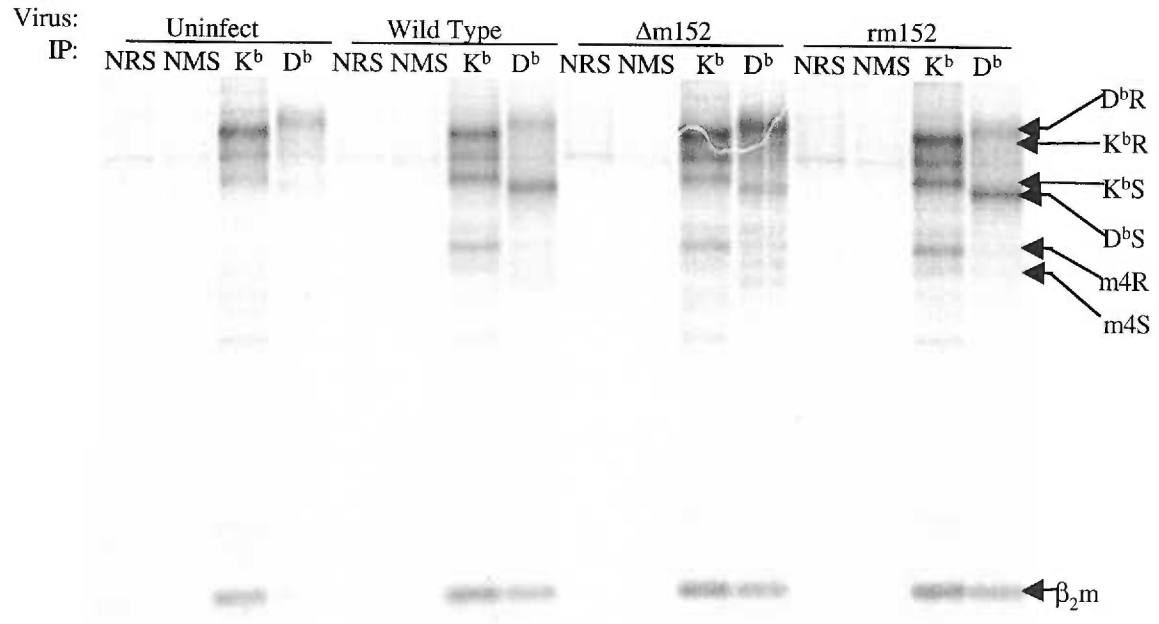


Figure 5
C.

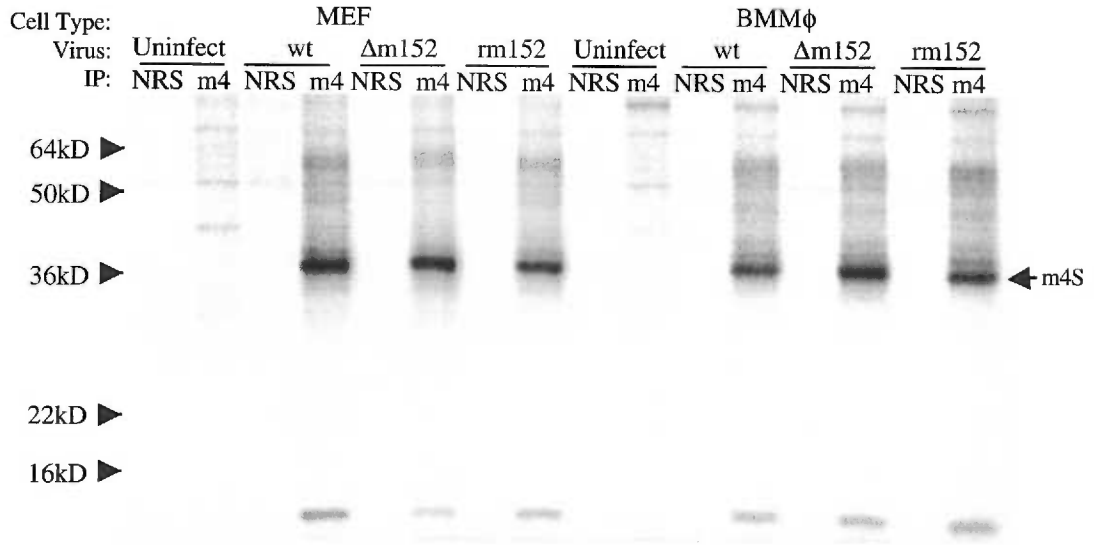


Figure 5. m152/gp40 is responsible for retention of MHC I in MEFs and BMMϕ and m4/gp34 is expressed in BMMϕ and associates with MHC I. MEFs were pretreated with IFNγ for 48 hours. MEFs and BMM were infected with wild type MCMV (Smith), Δm152 (MC96.24), revertant Δm152 (rMC96.27)-denoted rm152, or left uninfected. Cells were S^{35} labeled overnight in the presence of PAA. Cell lysates were subjected to immunoprecipitations, EndoH-treated, and run on a 12.5% SDS PAGE gel. A molecular weight marker depicts approximate weights on the left side of the gels. **A.** MEF lysates were immunoprecipitated with 8010 (rabbit anti-K^b), NRS control for 8010, 28.14.8S (anti-D^b), or normal mouse serum (NMS) control for 28.14.8S. **B.** BMMϕ were analyzed as for A. **C.** MEF and BMMϕ lysates were immunoprecipitated with 8139 (anti-m4) or normal rabbit serum (NRS) as a control for 8139 rabbit serum. m4/gp34 is expressed in all infected cells, is approximately 34kD, and associates with MHC I as evidenced by the presence of β_2m.

Figure 6

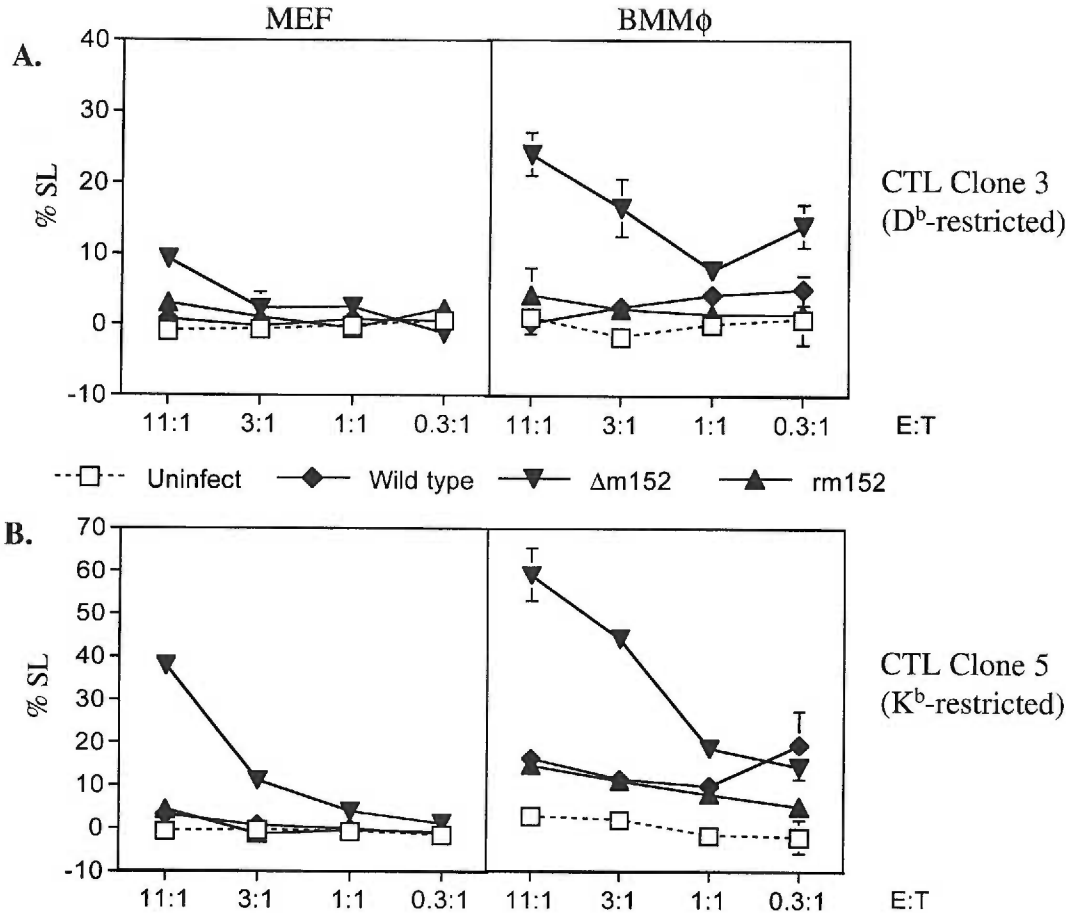


Figure 6. m152/gp40 prevents CTL antigen recognition in BMM.
BMM and IFNγ-pretreated MEF targets were Cr^{51}-loaded, and infected, at an MOI of 45, overnight, in the presence of PAA, with no virus, Smith (wildtype MCMV), Δm152 (MC96.24), or revertant Δm152 (rMC96.27). D^b-restricted CTL Clone 3 and K^b-restricted Clone 5 were tested for their ability to recognize and lyse targets.

Figure 7

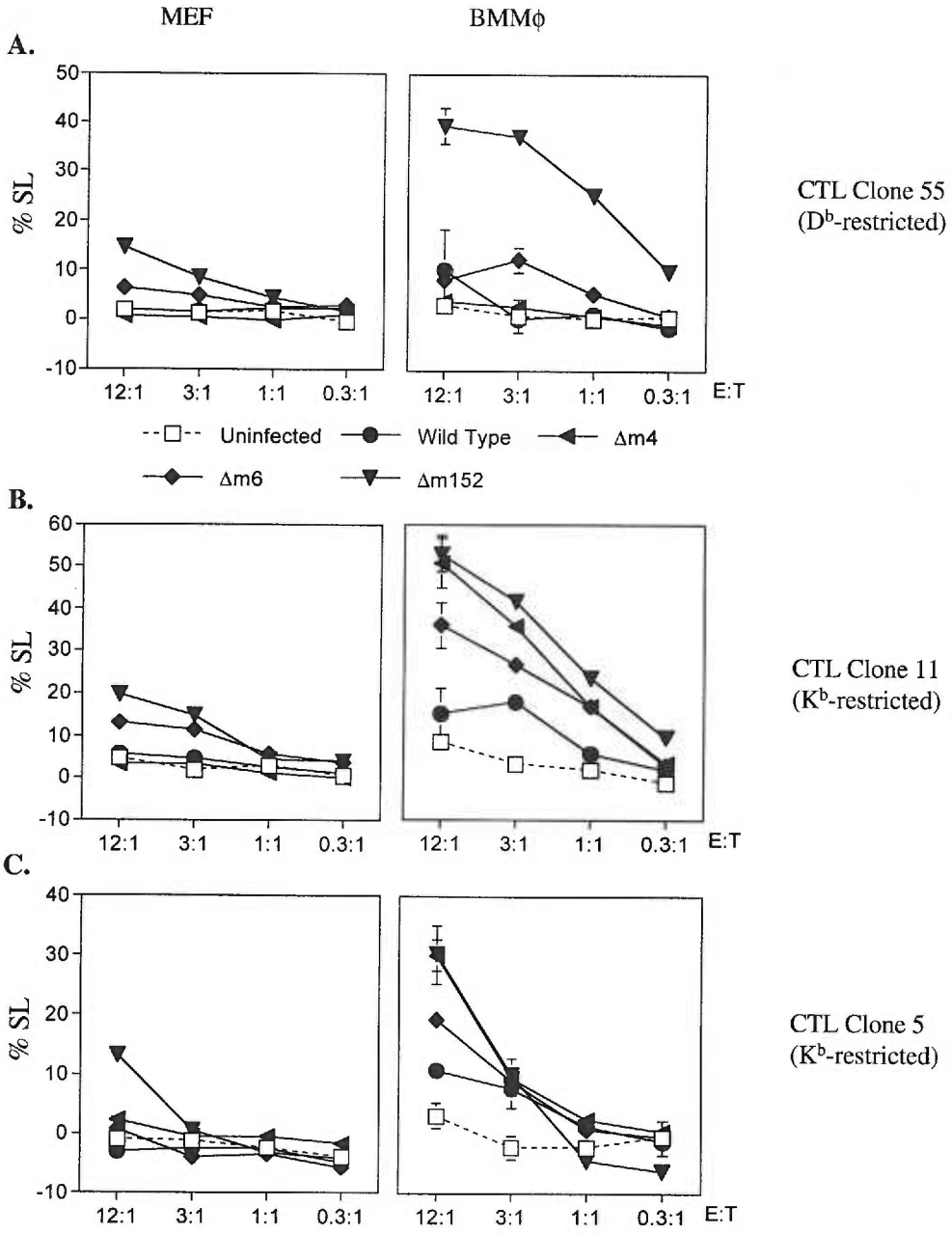


Figure 7. m4/gp34 and m6/gp48 prevent CTL antigen recognition in BMMϕ.
BMM and IFNγ-pretreated MEF targets were Cr^{51}-loaded, and infected, at an MOI of 100, overnight, in the presence of PAA, with no virus, wild type MCMV (MW97.01), Δm4 (MW99.03), Δm6 (MWΔm6), Δm152 (MW99.05). **A.** D^b-restricted CTL Clone 55 and K^b-restricted Clones 11 (**B**) and 5 (**C**) were tested for their ability to recognize and lyse targets.

Discussion and Summary

Five main points can be derived from the data in this thesis. First, the immune evasion genes are transcribed at an RNA level in macrophage cell types. Second, one immune evasion protein, m4/gp34 is expressed and biochemically associates with MHC I in macrophages. Also, this protein interferes with antigen presentation to two K^b-restricted CTL clones. Third, m152/gp40 specifically retains MHC I in macrophages and interferes with antigen presentation to all CTL clones tested. Fourth, m6/gp48 interferes with antigen presentation to K^b, and to a lesser degree, D^b-restricted CTL clones. Lastly, some CTL detect wild type MCMV-infected macrophages although Δm152, and sometimes Δm4 and Δm6 were detected more efficiently.

Prior to the initiation of this project, it was established that *m4*, *m6*, and *m152* worked to prevent antigen presentation in fibroblasts (Kavanagh et al., 2001; Reusch et al., 1999; Ziegler et al., 1997). It was also reported that these genes were not functional in macrophages (Hengel et al., 2000). This study contributes to the understanding of these genes in macrophages in the following ways. First, it clarifies the observation reported by Hengel et al. by demonstrating that immune evasion genes do function in macrophages but do not completely abrogate CTL recognition. Second it corroborates observations reported by our lab that MCMV immune evasion genes do not affect all MHC I molecules equally.

The data outlined in this thesis correlate well with data reported by Hengel et al. because, in both cases wild type-infected macrophages were seen by MCMV-specific CTL clones. It is quite remarkable that some of our CTL clones detect wild type infected

macrophages given that they have never detected wild type infected fibroblasts. CTL detection of wild type-infected macrophages is at a low level, however, when compared with the CTLs' ability to detect Δm152-infected macrophages. In some cases even Δm4 or Δm6-infected macrophages are detected as well as Δm152 by CTL clones. The fact that our CTL clones detect macrophages infected with viruses lacking immune evasion genes indicates that these genes function in macrophages, which initially seems to conflict with Hengel's findings. I will attempt to unite our seemingly disparate findings below.

Following the publication of Hengel's paper we outlined some reasons for continuing with this project even though the published data seemed to address the questions we set out to answer. Most of these reasons are based on differences in our two systems. While it is difficult to determine the significance of these differences I can speculate on their relationship to my findings. One reason for continuing with this project was that we felt our CTL clones might be more sensitive in their ability to detect professional antigen presenting cells based on their ability to see fibroblasts only if they are IFNγ-pretreated. Another reason for continuing was that our CTL clones recognize antigens expressed at the same time as immune evasion genes where Hengel's CTL clone recognizes an IE antigen. A third reason for continuing the project was that we study mice of a different haplotype; we know that MCMV immune evasion functions do not affect all MHC I molecules equally and considered that our studies with K^b and D^b may differ from Hengel's studies with L^d, in that respect.

We had considered the possibility that the pp89-specific CTL clone detected macrophages simply because they were better antigen presenting cells. This idea was

based on the previous report that the pp89-specific CTL clone detected wild type-infected IFNγ-pretreated fibroblasts during immune evasion gene expression (Hengel et al., 1994). We reasoned that IFNγ-pretreated fibroblasts are better antigen presenting cells than non-pretreated fibroblasts, and macrophages are good antigen presenting cells, therefore, macrophages might escape interference with antigen presentation because they are good antigen presenting cells. Our CTL clones are unable to see non-IFNγ-pretreated fibroblasts infected with any virus. They see only IFNγ-pretreated fibroblasts infected with viruses lacking one or more of the immune evasion genes: sometimes Δm4, and Δm6 and always Δm152. We feel our system is therefore more sensitive in detecting the effects of the immune evasion genes. We are able to rule out the possibility that escape from immune evasion gene inhibition is simply due to increased antigen presentation because even IFNγ-pretreated, wild type infected fibroblasts are not seen by our CTL clones. Given this, our prediction was: if the immune evasion genes do work in macrophages then our CTL clones would be sensitive enough to see a difference in macrophages infected with wild type MCMV or Δm152. However, we do not know how sensitive the pp89-specific clone is in this respect. It is worth noting that Hengel's experiments were performed without the benefit of the mutant viruses Δm4, Δm6, and Δm152.

The pp89-specific clone is sensitive to the effect of *m152* when fibroblasts are infected with vaccinia viruses expressing m152/gp40 and pp89 (Ziegler et al., 1997). While this experiment demonstrates that m152/gp40 inhibits antigen presentation, the timing and levels of expression of each protein are not equivalent to that found in MCMV infection of a cell. Another experiment by Del Val et al. (Del Val et al., 1989)

demonstrates that in the context of MCMV infection of a cell, E-expressed immune evasion genes prevent the pp89-specific clone from recognizing a fibroblast. This experiment demonstrates that with normal timing and expression levels, E-expressed immune evasion genes interfere with the pp89 clone's ability to recognize fibroblasts, but this experiment does not demonstrate that this clone is specifically sensitive to the effects of *m152*.

It is possible that, as we see in our system, m152/gp40 is partially blocking antigen presentation by L^d, in macrophages. If this is the case, the pp89-specific clone would better detect Δm152 than wild type-infected macrophages. Alternatively, m152/gp40 may not play a role in blocking L^d antigen presentation to the pp89-specific clone, in which case Δm152 would be detected no better than wild type MCMV. If this second possibility is the case, there are at least two plausible explanations. First because pp89 is an IE-expressed protein and the immune evasion genes are E, there may be enough time to export so many pp89 peptide-L^d complexes to the cell surface prior to m152/gp40 expression and interference with antigen presentation that the pp89-specific clone detects macrophages regardless of m152/gp40 expression. Second, m152/gp40 may not affect L^d as it affects K^b and D^b in the context of an MCMV infection of macrophages. I will elaborate on these two possible explanations and relate them to my findings below.

It's possible that pp89 is detected in macrophages because it is expressed at IE stages of gene expression. By the time the immune evasion genes are expressed, there may already be enough L^d-pp89 antigen complexes formed and at the cell surface for the pp89-specific clone to detect them. We felt that our system may be more sensitive in this

respect as our antigens are expressed at E times, like immune evasion genes, and have not had as much time as pp89-MHC I complexes to build up and travel to the cell surface. While we did not directly assess antigen buildup in our system we saw that some of our CTL clones did detect wild type-infected macrophages indicating that, at least for these clones, there was sufficient antigen production and that the immune evasion genes did not completely prevent antigen presentation in these cells.

MCMV immune evasion functions do not affect all MHC I molecules equally, which has been established at least in the case of *m152* (Kavanagh et al., 2001). Further testament to the differences in immune evasion genes' differential treatment of MHC I molecules can be seen in experiments reported in this thesis. I see that D^b-restricted CTL clones were never able to detect wild type-infected macrophages and K^b-restricted clones were. I cannot make a rule for detection of wild type-infected macrophages based on restriction element, as I only tested two D^b-restricted CTL clone (Clones 3 and 55) which both recognize the same antigen. However, it is certainly possible that D^b is more affected overall by the immune evasion genes in macrophages, given that m152/gp40 is more effective in preventing D^b-restricted antigen presentation in fibroblasts as discussed below. We thought that perhaps by using a system of completely different haplotype we would see different results than Hengel's. While our results were agreeable with Hengel's in that we both observed CTL recognition of wild type infected macrophages we did not prove or disprove that *m152* or any MCMV immune evasion gene differently affects MHC I molecules of different haplotypes, specifically K^b and D^b versus L^d. It would be interesting to determine the effect of m152/gp40 on antigen presentation in the context of L^d. Recently, an L^d-restricted CTL clone that recognizes MCMV E protein

M83, was isolated (Holtappels et al., 2001). It would be possible to use this clone to study the effect of m152/gp40 without the confounding fact that the antigen is expressed earlier than the immune evasion genes as is the case with the pp89-specific clone.

As previously mentioned, this study upholds data recently published from our lab indicating that MCMV's immune evasion genes differently affect MHC I molecules. Kavanagh et al. (Kavanagh et al., 2001) report that m152/gp40 interferes more with D^b-restricted CTL recognition than K^b-restricted recognition of MCMV-infected fibroblasts. In essence m152/gp40 is able to completely prevent antigen presentation by D^b but does not completely prevent presentation by K^b. m4/gp34 is able to make up for this incomplete prevention of antigen presentation by K^b. In this thesis I find that the same phenomenon holds true for macrophages and that interference with antigen presentation by m4/gp34 is even more pronounced. Both K^b-restricted CTL clones recognize Δm4-infected macrophages as well or nearly as well as Δm152-infected macrophages. This was surprising since these clones always see Δm152 much better than Δm4-infected fibroblasts. These findings suggest that m4/gp34 is more important for MCMV immune evasion in macrophages than fibroblasts. Which implies that m4/gp34 has an important role *in vivo* given that the macrophage is an important cell type *in vivo*. Our lab has plans to further investigate possibilities along these lines including the possibility that m4/gp34 may skew CTL immunodominance hierarchies and affect the establishment of latency.

While data demonstrating a MHC I allele preference for m6/gp48 is limited, our lab has some preliminary evidence that this is the case (Gold, unpublished data). Fibroblasts infected with Δm6 are seen moderately well (better than wild type but not as well as Δm152) by K^b but not D^b-restricted CTL clones. Fibroblasts infected with a

double deletant, Δm6Δm152 are better seen than Δm152 by both K^b and D^b-restricted CTL clones. Thus, like m4/gp34, m6/gp40 appears to interfere with antigen presentation to K^b-restricted clones regardless of m152's presence, and to D^b-restricted clones only in the absence of m152. In the case of macrophages, K^b-restricted CTL clones saw Δm6 much better than wild type infected-macrophages. This difference was only moderate for D^b-restricted CTL clones. While m6/gp48's ability to interfere more with K^b than D^b-restricted antigen presentation is less pronounced than m4/gp34's, it is certainly possible that m6/gp48 has a greater effect on K^b than D^b antigen presentation in macrophages as well. Further analyses with D^b-restricted CTL will allow for more confident interpretations of both m4/gp34 and m6/gp48's affects on D^b.

Naturally this study raises several other questions regarding the function of MCMV's immune evasion genes.

First, do m4/gp34 and m6/gp40 always play more important roles in K^b than D^b-restricted antigen presentation by macrophages? Also are wild type-infected macrophages always better detected by K^b than D^b-restricted CTL clones? One problem with this study is the limited amount of CTL clones. We need more data regarding D^b-restricted CTL recognition before we can make substantial claims for the effects of m4/gp34 and m6/gp48 on K^b or the ability of K^b and not D^b-restricted CTL to see wild type-infected macrophages. One way to assess these questions is to isolate and test more D^b-restricted CTL clones, a project on-going in our lab. Another way would be to repeat the assay outlined in figure 6 of this thesis using splenocytes from an MCMV-infected, K^b knockout (only has D^b) or D^b knockout (only has K^b) mouse. At times Cr^{51}-release

assays with polyclonal CTL give high background readings so an alternative assay would be an intracellular cytokine staining assay or IFNγ ELISA using the same cells.

Second, do the immune evasion genes assist MCMV in establishing latency, or reactivating from latency and do their effects in macrophages influence any of these points in infection? Many general questions about CMV latency in macrophages remain unanswered. It would be helpful understand the relationship of MCMV and the macrophage in latency. First, during the establishment of latency does MCMV

A. Replicate in the macrophage, then become latent?

B. Immediately establish latency without replication in the macrophage?

Second, during reactivation from latency does MCMV

A. Periodically reactivate in certain areas like the lungs?

B. Reactivate only in the absence of CTL, and maintain a state of true latency without replication as long as CTL are present?

C. Periodically reactivate in areas sheltered from CTL control, like the salivary glands?

If MCMV replicates in the macrophage before establishing latency and periodically reactivates in certain areas like the lungs, then one would predict that the immune evasion genes work well in macrophages. They would allow MCMV to establish latency and periodically reactivate in the macrophage by keeping it hidden from CTL.

If MCMV immediately establishes latency in macrophages and reactivates only in the absence of CTL then immune evasion genes may not need to work well in macrophages. Conceivably they are not needed to establish latency, as viral genes are not

expressed during this step, and they are likely not needed during reactivation as reactivation only occurs when CTL are not present.

If reactivation only occurs in salivary glands, an area sheltered from CD8 CTL control, then immune evasion genes may or may not work in macrophages as salivary glands are under control of CD4 T cells. The genes may not work well if CTL surveillance is low in salivary glands or if macrophages have nothing to do with reactivation in this site. On the other hand they may work if macrophages replicate MCMV and pass it off to salivary gland acinar epithelial cells and CTL surveillance is adequate in salivary glands.

Third, do the immune evasion genes function equally well in macrophages derived from different organs? Koffron et al. (Koffron et al., 1998) demonstrated that latent MCMV could be found in alveolar macrophages of the lung, but latent MCMV in other organs was found to reside in mostly endothelial cells. This may indicate that MCMV prefers alveolar macrophages to splenic macrophages or Kupffer cells in the liver. It would be interesting to test the effects of the immune evasion genes specifically in these cells. Are they more effective in alveolar macrophages and less so in other macrophages? If there is a difference in macrophage type, we will need to assess how bone marrow-derived macrophages fit into this model.

Fourth, along the same lines as the question posed above, do the immune evasion genes function in other cell types, for example: endothelial cells, salivary gland epithelial cells, or dendritic cells? Because the studies by Koffron and others (see introduction for references) indicate the endothelial cell as a site of latency, it would be interesting to determine if the immune evasion genes were particularly effective in such cells.

Investigations of the immune evasion genes in salivary gland epithelial cells would be interesting because the salivary gland is clearly resistant to CD8 CTL control as shown in studies by Lucin et al. (Lucin et al., 1992). MCMV titers in this organ appear to be controlled by CD4 T cells and IFNγ. I would predict that these cells are resistant to CD8 CTL control because the immune evasion genes are responsible for hiding MCMV in salivary gland cells. Immune evasion in dendritic cells has perhaps the most interesting ramifications. Because dendritic cells are responsible for priming a CTL response, interference with antigen presentation in these cells could result in a change of the entire population of responding CTL. Dendritic cells can phagocytose dead or dying cells and cross present antigen in the context of MHC I to prime naïve T cells. If the dendritic cell is priming naïve T cells while infected with MCMV, and the immune evasion genes are expressed, it is likely that a different population of naïve CTL would be primed than if immune evasion genes were not expressed. Preliminary data (Gold et al. manuscript in preparation) indicates that, in the spleen, the ratio of CTL responding to the Clone 3/55 antigen to CTL responding to MCMV-infected targets, is not significantly different in wild type-infected versus Δm152-infected mice. If such ratios are accurate representations of CTL populations, it is possible that m152 is not playing a role in skewing the population of CTL. This could be explained by a failure of immune evasion genes to work in dendritic cells or the possibility that the majority of dendritic cells priming naïve CTL are not infected and are simply cross presenting antigen. However, there may be other immune evasion genes at work in dendritic cells since differences in the aforementioned ratios are exhibited in Δ94.5-infected mice as compared to wild type infected mice.

Lastly, are there different populations of MCMV-specific CTL in different organs and does this depend on immune evasion in these different organs? Performing tetramer-staining assays on cells from various organs of mice infected with different mutant viruses could test this possibility. Our lab has recently developed a MHC I-peptide tetramer for Clone 3/55. With this tetramer it would be possible to determine the ratio of CTL responding to the clone 3/55 antigen to total CD8 T cells in different organs including the spleen, liver, and lungs of infected mice. These data could be compared to similar data using mice infected with one of the MCMV deletant viruses. As mentioned above, this has been done in our lab for spleenocytes. Skewed populations in other organs of mice infected with a deletant virus might indicate that one or more of the immune evasion genes are responsible for immune evasion in a particular organ.

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