IMMUNE CONTROL AND VIRAL COUNTERMEASURES IN MURINE CYTOMEGALOVIRUS INFECTION

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)
ADAR	dsRNA-specific adenosine deaminase
AIDS	acquired immunodeficiency syndrome
AP-1	activator protein-1
APC	antigen presenting cell
ATP	adenosine triphosphate
β2-m	β2-microglobulin
BAC	bacterial artificial chromosome
BAP31	B cell receptor-associated protein
BFA	brefeldin A
BM(T)	bone marrow (transplant)
CCL5	chemokine (C-C motif) ligand 5
CD	cluster of differentiation
CD40L	CD40 ligand
CDR	complementarity determining region
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
CXCL-1	chemokine (C-X-C motif) ligand-1
DAG	diacylglycerol
DC	dendritic cell
DNA	deoxyribonucleic acid
DRiP	defective ribosomal product
ds	double-stranded
E	early (gene)
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERAAP	endoplasmic reticulum amino-peptidase
ERAD	endoplasmic reticulum-associated protein degradation
ERGIC	endoplasmic reticulum-golgi intermediate compartment
FACS	fluorescence activated cell sorter
FasL	Fas ligand
γ-HV68	γ-herpesvirus 68
gB	glycoprotein B
GBP-1	guanylate binding protein-1
GEF	guanine exchange factor
gH	glycoprotein H
GM-CSF	granulocyte macrophage colony-stimulating factor
Grz	granzyme
GVHD	graft versus host disease
HAART	highly active antiretroviral treatment
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSP	heat shock protein
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
ICCS	intracellular cytokine stain
IE	immediate-early (gene)
IFN(-α/β/γ)	interferon(-α/β/γ)
Ig(G)	immunoglobulin (G)
IHC	immunohistochemistry
i.n.	intranasal
i.p.	intraperitoneal
IP3	inositol triphosphate
ISG	interferon-stimulated genes
ISH	*in situ* hybridization
ITAM	immunoreceptor tyrosine-based activation motifs
kB	kilobase
KSHV	Kaposi's sarcoma associated herpesvirus
L	late (gene)
LAT	linker of activation in T cells
Lck	leukocyte-specific protein kinase

LCMV	lymphocytic choriomeningitis virus
LD_{50}	lethal $dose_{50}$
LIR	leukocyte Ig-like receptor
LFA	lymphocyte function associated-antigen
MAP	mitogen-activated protein
MCMV	murine cytomegalovirus
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MIP	macrophage inflammatory protein
MNV	murine norovirus
MOI	multiplicity of infection
MPV	mouse parvovirus
mRNA	ribonucleic acid
MTOC	microtubule organizing center
NFAT	nuclear factor of activated T cells
NK cell	natural killer cell
NOS	nitric oxide synthase
NV	norovirus
ORF	open reading frame
PAA	phosphonoacetic acid
PACS1	phosphofurin acidic cluster sorting protein 1
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PFU	plaque forming unit
p.i.	post-infection
PKR	dsRNA-activated protein kinase R
PLC-γ	phospholipase C-γ
p.o.	*per orad* (by mouth)
qPCR	quantitative polymerase chain reaction
RAE-1	retinoic acid early-inducible gene-1
RAG	recombination-activating gene
RBC	red blood cells
RFLP	restriction fragment length polymorphism
RT-PCR	real time PCR
s.c.	subcutaneous
SEM	standard error of the mean
SG	salivary glands
SIV	simian immunodeficiency virus
SPF	specific pathogen-free
SMAC	supramolecular activating complex
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TC	tissue culture
TCR	T cell receptor
TNF(-α)	tumor necrosis factor(-α)
VCAM-1	vascular cell adhesion molecule-1
vICA	viral inhibitor of caspase activation
vMIA	viral mitochondrial inhibitor of apoptosis
VZV	varicella zoster virus
WNV	West Nile virus
WT	wild type

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ABSTRACT

Murine cytomegalovirus (MCMV) is a well-studied model of β-herpesvirus infection. It generates a robust CD8 T cell response and asymptomatic infection in immunocompetent mice. The CD8 T cell response is established even though, like all herpesviruses studied, MCMV encodes genes that interfere with antigen presentation via major histocompatibility complex (MHC) class I. *In vitro*, these genes robustly prevent CD8 T cells from killing infected cells. Therefore, it was expected that a mutant virus lacking the MHC class I immune evasion genes would have impaired viral fitness *in vivo*. The mutant virus has previously been shown to be impaired in a bone marrow transplant model of MCMV infection. Our laboratory is interested, however, in the immunobiology of MCMV infection of immunocompetent mice.

For my thesis work, I set out to identify the impact of these immune evasion genes *in vivo* by studying the MCMV-specific CD8 T cell response and viral loads following infection with both wild type and mutant virus. I first embarked on a study to further characterize the CD8 T cell response to wild type MCMV infection of mice on a variety of genetic backgrounds. It was not entirely clear what factors contributed to the magnitude and immunodominance hierarchy of the CD8 T cell response, and my findings suggest that genes outside of the MHC complex play a significant role. I then asked how the MHC class I immune evasion genes impact acute and chronic infection of mice with different genetic backgrounds. This work found no new evidence for a role for these genes *in vivo*, but corroborated the finding that the genes benefit viral growth in the salivary glands of MCMV-susceptible mice.

To further probe why the MHC class I immune evasion genes are conserved in MCMV given the relatively subtle phenotype in the salivary glands, I established a model of more natural

infection conditions. I expected that the evolutionary benefit of the genes might be more apparent under infection conditions that were more similar to those under which the virus evolved. Mice infected under more natural conditions (low dose and different routes of infection) appear to control the mutant virus better than wild type virus, although immune control of both viruses was variable and no firm conclusions could be drawn. These studies have supported the conclusion that the MHC class I immune evasion genes are conserved in order to benefit viral transmission in immunocompetent mice. My work also called attention to the variability in our model and this has led us to reexamine the benefits of our model and what questions it is best suited to answer.

Chapter 1: INTRODUCTION

1.1 Overview

A striking feature of many descriptions of the immune system is the use of warfare vocabulary. On one side—that of the pathogen—many infectious agents have developed "counterattack" mechanisms that disable the weapons of the immune system and protect them from harm. Yet others have evolved strategies for avoiding detection to begin with, because if the immune system cannot detect intruders—or even not see them well—it cannot launch an effective offensive. The herpesviruses are a virus family renowned for their immune evasion tactics. Every herpesvirus studied in detail possesses gene(s) that encode proteins that target the major histocompatibility complex (MHC) class I pathway of antigen presentation. Human cytomegalovirus (HCMV), for example, employs at least three mechanisms to inhibit MHC class I-dependent antigen presentation. This dissertation examines the role of MHC class I immune evasion in the mouse model, murine cytomegalovirus (MCMV). The following work can be divided into two main topics. First, what is the global impact of MHC class I immune evasion in MCMV infection? And second, how well suited is our model to the questions we are asking?

Following the war metaphor, MHC class I serves as a sentinel for the immune system and alerts the host to infection. MCMV completely disables the ability of cluster of differentiation (CD)8 T cells from killing infected targets *in vitro*. *In vivo*, however, the impact of the MHC class I immune evasion genes has been less striking. Thus, while the presence of these genes of these has long been appreciated, their true evolutionary role is not as clear as was originally supposed. For my dissertation project, I endeavored to identify a role for the MHC class I immune evasion proteins by determining the phenotype of a mutant virus *in vivo*, working under the supposition that MCMV would not retain these genes if they did not provide an advantage to the virus *in vivo*.

Using both different strains of mice and various doses and routes of infection, I compared the CD8 T cell response and viral control following both acute and chronic infection with wild

type MCMV and a mutant that lacks the MHC class I immune evasion genes, Δm04+m06+m152-MCMV. None of these scenarios offered a clear phenotype for the MHC class I immune evasion genes, except that they provided a one-log growth advantage in the salivary glands of BALB/c mice, verifying a finding reported by a former member of our laboratory (Lu et al., 2006). Some of the experiments described in this dissertation highlight the experimental variability we see in our model. This variability led me to address more directly the strengths and weaknesses of the model itself and to question our assumptions when working with inbred mice in specific pathogen-free (SPF) colonies and virus strains that have been extensively passaged in the laboratory. The experiments will be presented in detail in the following chapters, but they will first be introduced by a comprehensive description of the cytomegaloviruses, our particular animal model, MHC class I antigen presentation, the ensuing CD8 T cell response, and finally, the immune response to—and immune evasion in—MCMV infection.

1.2 Cytomegaloviruses

The herpesvirus family, after the Greek *herpein*, "to creep," includes more than 100 large, ancient DNA viruses that infect vertebrates. There are eight known herpesviruses that infect humans, human herpesviruses (HHV) 1-8, most of which also have common names, such as herpes simplex virus-1 (HHV-1) or cytomegalovirus (HHV-5). The herpesviruses have evolved with their hosts for 180-220 million years (McGeoch et al., 1995). All herpesviruses genomes consist of 100-200 genes and are packaged as double-stranded (ds), linear DNA within an enveloped, icosahedral capsid. They are further divided into three subfamilies based on both biological characteristics and genome sequence. The α-herpesviruses have a broad host range and a short reproductive cycle *in vitro*, they are neurotropic, and they are cytotoxic to the cells they infect. The β-herpesviruses have a restricted host range, a long reproductive cycle *in vitro*, and cause enlargement of infected cells. *In vivo*, they cause minimal disease in immunocompetent

hosts. Finally, the γ-herpesviruses also have a narrow host range and a relatively long reproductive cycle *in vitro*. They can be cytotoxic to fibroblasts and epithelial cells, and they are the only subfamily known to encode oncogenes.

All three subfamilies cause life-long infection, most of which go through cycles of lytic and latent infection, interrupted by periods of reactivation. For the α-herpesviruses, such as herpes simplex virus (HSV)-1 and -2 and varicella zoster (VZV), viral genomes are maintained in cells such as neurons in the absence of viral protein expression, and the sites of latency are well understood. In contrast, the γ-herpesviruses, such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) express a subset of proteins required for maintaining the latent cycle of infection. For the β-herpesvirus, such as cytomegalovirus, whether true latency is established is controversial; the cell type harboring latent virus is unclear and reactivation may be a constant process whereby persistent, infectious virus is present at very low levels.

The herpesviruses have characteristics in common with a variety of other virus families. The pox- and adenoviruses also include large dsDNA viruses. The poxviruses have similar, impressive coding capacities and encode a wide range of genes involved in immune evasion, including MHC class I immune evasion. Unlike the herpesviruses, however, they replicate in the cytoplasm, grow rapidly with dramatic cytopathic effect *in vitro*, and cause acute infections. Adenoviruses are smaller than herpesviruses, but still encode 30-40 genes, some of which function in immune evasion. In fact, the first MHC class I immune evasion mechanism was described in adenovirus type II (Andersson et al., 1985; Burgert and Kvist, 1985). Papillomaviruses are small, sometimes oncogenic dsDNA viruses, although they are similar to the herpesviruses in that they are ancient and very species-specific. They also cause chronic infection and can interfere with MHC class I-dependent antigen presentation. Finally, while very different from the herpesviruses in many ways, the retrovirus, human immunodeficiency virus (HIV),

shares some similarities in that it leads to lifelong infection and relies on numerous immune evasion mechanisms.

HCMV

Our laboratory is interested in the β-herpesvirus, cytomegalovirus. The cytomegaloviruses have a narrow host range whereby a given virus will only infect one species. The human CMV genome is large—around 230 kilobases (kB), which constitute approximately 165 open reading frames (ORFs), depending on the particular strain (Reddehase, 2002; Strauss and Strauss, 2002). Viral gene expression is temporally regulated and characterized in three stages: immediate early (IE), early (E), and late (L). IE genes are the "transactivators." They are the first genes transcribed by the cellular RNA polymerase and are expressed between one and six hours post-infection (p.i.); the IE proteins then transactivate the E gene promoters. The E genes are non-structural proteins that are expressed between four and 18 hours p.i., and they tend to suppress IE transcription (Soderberg-Naucler, 2000; Manley et al., 2004). Finally, the L genes are structural proteins that are transcribed after 12 hours of infection, upon viral DNA synthesis.

Infection of cells by HCMV leads to two pathognomonic cytologic signs: cytomegaly, or enlargement of the cells, and "owl's eye" intranuclear inclusion bodies, named for the appearance of the cells by hematoxylin and eosin (H&E) staining. HCMV cell tropism *in vitro* depends on the strain of virus used; the laboratory-adapted strains, such as AD169 and Towne, are restricted to human fibroblasts. Clinical isolates, however, also have epithelial, endothelial, smooth muscle, and leukocyte tropism (Plachter et al., 1996; Gerna et al., 2004; Sinzger et al., 2008). *In vivo*, HCMV infects fibroblasts, hepatocytes, smooth muscle cells, neuronal cells, hematopoietic cells, and the epithelial cells of most organs (Soderberg-Naucler, 2000; Manley et al., 2004). Epithelial cells are the predominant cell type infected, including endothelial cells, pneumocytes, enterocytes, and glandular epithelial cells of the salivary glands (Soderberg-Naucler, 2000; Reddehase, 2002). As mentioned, whether HCMV establishes true viral latency is somewhat

controversial, however, a number of studies, have identified cell types from which HCMV DNA can be detected in the absence of replicating virus. Such reservoirs include myeloid progenitor cells, monocytes, macrophages, dendritic cells (DCs), and some types of epithelial cells, including endothelium and salivary glands ductal cells (Soderberg-Naucler et al., 1997; Gerna et al., 2004).

Transmission and superinfection

Like other members of the herpesvirus family, CMV is globally distributed, with infection rates approaching 100% in some developing regions of the world. In less developed countries, most of the population is infected by age three, whereas in a U.S. study, CMV seroprevalence was 36% in six to 11 year olds, but 91% in those greater than 80-years-old (Staras et al., 2006). Transmission can be either horizontal or vertical via intimate contact with an individual excreting virus. HCMV is shed in myriad bodily fluids, including saliva, urine, blood, tears, semen, and breast milk (Tebourbi et al., 2001). The salivary glands are particularly important organs in HCMV infection because they are the organs of dissemination. Oral or nasal routes of infection are thought to be the most common in horizontal transmission via contact with hands contaminated with infectious fluids (Hutto et al., 1986). Notably, in a meta-analysis of the rate of HCMV shedding in children in U.S. daycare centers, HCMV viruria was detected in between 10-72% of urine samples (Adler, 1988). The virus can also be transmitted by bone marrow or solid organ transplant and blood transfusions (Soderberg-Naucler, 2000). Vertical transmission of HCMV occurs via breastmilk or, much more commonly, across the placenta (Reynolds et al., 1973; Hamprecht et al., 2001; Fisher et al., 2000).

Individuals—particularly the immunocompromised—are often infected with more than one virus strain, most likely due to serial exposure and reinfection (Chou, 1989b; Baldanti et al., 1998). Boppana *et al.* investigated pregnant women and found evidence of superinfection by analyzing strain-specific neutralizing antibodies and the sequence of HCMV glycoprotein H (gH)

(Boppana et al., 2001). A majority (69%) of the subjects had antibodies to gH epitopes from two different HCMV laboratory strains (AD169 and Towne). Another study found that 21.3% of blood donors—either healthy volunteers or potential renal transplant donors or recipients—had antibodies to gH epitopes from AD169 and Towne (Ishibashi et al., 2008). Interestingly, the study noted that most of the samples (61%) with antibodies toward both virus strains were from subjects over 50-years-old.

There is also evidence of superinfection in children. Bale *et al.* reported that 19% of children in area daycare centers were infected with more than one HCMV strain as assessed by restriction fragment length polymorphism (RFLP) analysis of serial samples (Bale et al., 1996). A study of AIDS patients also showed by RFLP analysis that 46% of patients were infected with more than one HCMV strain, however no immunocompetent control patients were coinfected (Baldanti et al., 1998). Importantly, this study also showed that in 67% of the coinfected patients, there is sequential evidence for mixed viral populations, suggesting superinfection—rather than primary coinfection—occurred. Finally, in organ transplant between HCMV seropositive donors and seropositive recipients, superinfection is more frequent than reactivation (Chou, 1989a). Acute kidney transplant rejection and CMV disease was more frequent in cases where seropositive donors and seropositive recipients had HCMV strain mismatches, which suggests indirectly that superinfection, rather than reactivation, leads to worse outcomes (Ishibashi et al., 2007).

Morbidity and mortality

Once HCMV has been transmitted to an uninfected individual, the course of infection varies depending on the immune status of the individual. HCMV is very immunogenic, and it induces a robust innate and adaptive immune response. HCMV-specific responses can encompass up to 10% of the total, peripheral CD8 T cell response in seropositive individuals, with up to 4% of CD8 T cells responding to a single HCMV epitope (Gillespie et al., 2000; Sylwester et al.,

2005). Because of multiple layers of immune control, infection is relatively benign in immunocompetent hosts. Primary infection may be associated with mononucleosis-like symptoms (*e.g.* fever, fatigue, sore throat, adenopathy, splenomegaly), but it is commonly asymptomatic. Individuals typically remain asymptomatic unless they become immunosuppressed, resulting in decreased control and unchecked growth of HCMV. Under these circumstances, HCMV can cause life-threatening, multi-organ disease.

HCMV is a particularly fearsome complication in three patient populations: HIV/AIDS patients, transplant recipients, and pregnant women. Before highly active antiretroviral treatment (HAART), HCMV was the most frequent opportunistic infection seen in AIDS patients (Reddehase and Lemmermann, 2006). The result of HCMV infection of AIDS patients most often involves retinitis, hepatitis, and colitis (Reddehase and Lemmermann, 2006). In fact, before HAART, 21-44% of AIDS patients developed retinitis (Jacobson, 2008). HCMV is also thought to be the most important pathogen in transplant patients and has been dubbed "the troll of transplantation" (Trulock, 2008). The source of virus is often an endogenous HCMV infection of the recipient that resurges in the face of chemical immunosuppression, however, the virus can also be transmitted from the transplanted organ to the recipient. Solid organ transplant recipients commonly experience pneumonitis, hepatitis, anemia, and leukopenia, as well as high rates of graft rejection and graft versus host disease (GVHD) as a result of HCMV infection. Before effective prophylaxis, such as ganciclovir, existed (much like before HAART in AIDS patients) CMV seropositive, allogeneic hematopoietic stem cell (HSC) transfer recipients had a 70-80% risk of HCMV reactivation, and one-third of those developed CMV disease (Anaissie, 2008). Finally, primary or superinfection of pregnant women can lead to devastating outcomes for the fetus, even in the absence of overt disease in the mothers and in the presence of pre-existing immunity (Boppana et al., 2001; Reddehase and Lemmermann, 2006). Congenital HCMV infection is the leading infectious cause of birth defects and the most common viral cause of brain damage in the U.S. Congenital infection impacts between one to three percent of babies born in

the U.S. each year, and five to 15% are symptomatic at birth (Reddehase and Lemmermann, 2006; Demmler, 2008). Complications of congenital infection include spontaneous abortion, premature delivery, intrauterine growth restriction, anemia, and hepatosplenomegaly (Reddehase and Lemmermann, 2006). Over 60% of those symptomatic at birth will have neurological sequelae, including, most commonly, hearing loss, but also microcephaly, mental retardation, seizures, and an abnormal neurological exam (Reddehase and Lemmermann, 2006; Demmler, 2008). Damage, particularly of the central nervous system, can develop after delivery and/or progress for the first few years of life (Reddehase and Lemmermann, 2006). The chance of congenital transmission is greatest for women who experience primary infection during their pregnancy. The transmission rate is 40% for mothers with primary infection—and those infants are more likely to be symptomatic at birth—while it is less than one percent for women experiencing recurrent infection (Demmler, 2008).

Vaccines

It is the severity of disease caused by HCMV in these clinical scenarios that has placed HCMV as a "highest priority" for vaccine development by the Institute of Medicine (Stratton et al., 2001). The 1999 report estimated the cost of treating HCMV-related symptoms in the United States to be more than four billion dollars per year. There are multiple challenges to developing an HCMV vaccine, not least of which is deciding on the goal of vaccination. Due to the prevalence and severity of congenital disease, much of the focus has been on developing a traditional vaccine to prevent primary infection and induce sterilizing or protective immunity (Reddehase and Lemmermann, 2006). However, some argue that it is more prudent to focus on particular target populations with the goal of boosting preexisting immunity or preventing reactivation or superinfection. A second major challenge is identifying and agreeing upon the components of a successful vaccine. There is general agreement that such a vaccine would include viral proteins (and immunodominant antigens) that induce both neutralizing antibody and

a T cell response. Glycoproteins B (gB) and H (gH) have been targeted in HCMV vaccine development. Both elicit a neutralizing antibody response; gB also elicits a strong CD8 T cell response, although it is not clear whether it is protective (Hopkins et al., 1996; Liu et al., 1991; Britt et al., 1990; Borysiewicz et al., 1988). The HCMV proteins pUL83 (pp65), which is both immunodominant and highly conserved, and pUL123 (IE1) are also vaccine candidates for inducing a CD8 T cell response (Wang et al., 2004; Sylwester et al., 2005; Khan et al., 2002; Berencsi et al., 2001). Multiple types of vaccines are in some stage of development, including: live attenuated, live recombinant, and recombinant gB subunit vaccines, as well as viral vector-based, peptide-, and DNA-vaccines (Zhong et al., 2008).

The consequences of HCMV infection are one of the many reasons why we study MCMV. It is a disease that can be devastating in a number of clinical scenarios and still boasts a startling list of unknowns regarding its immunobiology, its ability to evade the immune system, and the best way in which to generate a vaccine. MCMV has many similarities to HCMV, which make it a very useful animal model. And, though we use MCMV as a model with the hope of making discoveries that are relevant to HCMV, we also use MCMV for its own merits to better understand basic immunology. While findings in MCMV infection may not translate directly to HCMV infection, discoveries regarding the mechanisms of MHC class I antigen presentation or the characteristics of a memory CD8 T cell response have far-reaching implications that also affect human health. The details of using MCMV as a model—as well as the benefits and limitations—will be described in the following section.

1.3 Models of MCMV infection

Many human diseases have animal models that are widely used for research into mechanisms of disease, pathogenesis, and vaccine strategies. A majority of these experiments are impossible to do in humans for technical, and more importantly, ethical reasons. While many discoveries have been made using HCMV *in vitro*, there is a point at which it becomes necessary

to manipulate both host and viral genetics in an exquisitely controlled fashion. Much of the work described in this dissertation compares infection of mice with wild type MCMV and with a mutant virus that lacks the MHC class I immune evasion genes—experiments that would be impossible to perform in humans. Therefore, as mentioned above, we use the mouse model as a proxy to understand MHC class I immune evasion in HCMV infection, but also as a system to understand the general principles of MHC class I immune evasion.

The basic virology of the human and mouse CMVs is similar regarding characteristics such as virion structure, tissue tropism, and gene expression patterns. The Smith strain of MCMV, like HCMV, is ~230 kB in size, and the two viruses are colinear over the central 180 kB of the genome (Cha et al., 1996; Rawlinson et al., 1996). HCMV and MCMV are similar at a genetic and nucleotide level. MCMV-Smith codes for ~170 ORFs, 78 of which have significant amino acid homology with HCMV, ranging from 15-65% identity (Rawlinson et al., 1996). Most importantly for our interests, HCMV and MCMV behave very similarly in their respective hosts. MCMV goes through the same stages of infection as HCMV, resulting in asymptomatic, chronic infection in wild type mice. In immunocompromised mice, however, infection can be severe and results in the same clinical complications seen in HCMV infection of immunodeficient patients (*e.g.* pneumonitis).

In part, because of the similarities in virus characteristics and behavior in the host, the mouse model has been widely employed for studying HCMV immunobiology over the past 40 years. MCMV is a natural mouse pathogen and the only herpesvirus to naturally infect the *mus musculus* species, which makes it particularly valuable. Many experiments on the immunobiology of CMV are arguably done most efficiently and most easily in the mouse, based in part on the availability of reagents, the short gestational period of mice, the ease and relatively low cost of ordering commercially available mice, and the extensive supply of mutant mouse strains. Thus, in terms of using an animal model at all, the mouse is a prolific one, particularly for studying CMV.

Other animal models have been pursued and are in use, such as the rat, guinea pig, and non-human primate. Each of these models has their own advantages, and some are more appropriate for studying certain aspects of HCMV infection than the mouse model. Mouse physiology, for example, prevents the study of transplacental transmission of MCMV, but guinea pig CMV can be transmitted across the placenta. It could be argued that rhesus CMV is the best model to study HCMV based on a much closer genetic similarity between humans and primates than humans and mice. The realities of the rhesus model, including the expense, facilities requirements, and reagent availability, certainly contribute to it being less commonly used. With those caveats, the mouse is the best animal model with which to ask our overall questions of CMV immunobiology.

Transmission

MCMV was first recovered and propagated *in vitro* in 1954 by Margaret Smith (Smith, 1954). She isolated the virus from laboratory mice that had been naturally infected by colony contamination. That strain, Smith, is the most commonly used strain of MCMV in research laboratories. In the wild, between 60 and 90% of mice are infected with MCMV—similar to the prevalence of HCMV in humans and ~34% of mice are infected with multiple isolates (Singleton et al., 1993; Moro et al., 1999; Gorman et al., 2006; Singleton et al., 2000). The natural routes of infection of mice have not been definitively established. It is clear, however, that mice do not transmit MCMV across the placenta, but horizontal and vertical transmission is common, likely via saliva and breastmilk (Booth et al., 1993; Singleton et al., 1993; Moro et al., 1999; Farroway et al., 2005). Thus, it has been speculated that the oral (p.o.), intranasal (i.n.), and subcutaneous (s.c.) routes (due to biting behavior) are the most likely natural routes.

Laboratory mice were able to naturally transmit MCMV, as first evidenced by Smith's discovery that some animals in her colony were contaminated. Natural transmission studies in Swiss Webster mice showed that, while less than one percent of the colony was contaminated

with MCMV, MCMV could readily be transmitted between cagemates (Mannini and Medearis, 1961). When mice, intravenously infected with MCMV-Smith 49 days prior, were caged with uninfected animals, virus was recovered from throat swabs of all of the control animals by 16 days p.i.; none of the mice housed in an adjacent cage became infected. Transmission rates were similar when mice infected by p.o. and i.n. routes were caged with uninfected animals. More recently, Farroway *et al.* reported that wild mice can transmit the wild isolate, MCMV-G4, to other wild mice or to BALB/c cagemates when paired as potential breeders for at least 21 days (Farroway et al., 2005). BALB/c mice also appear to transmit another wild isolate, MCMV-N1, to BALB/c cagemates (Farroway et al., 2005). Contemporary MCMV-Smith, however, does not naturally transmit to cagemates in at least some strains of immunocompetent, laboratory mice infected (Ann Hill, personal communication). Because of this, and in order to control the dose of inoculation, the most common experimental models of MCMV infection involve infecting BALB/c mice with high dose ($\geq 10^4$ plaque forming units (PFU)) tissue culture (TC)-passaged MCMV-Smith by either an intraperitoneal (i.p.) or s.c. inoculation. Our laboratory and others have more recently advanced the C57BL/6 (B6) mouse model, in large part because of the wealth of tools available for this particular strain.

Pathogenesis

The salivary glands are the central organs for replication and transmission of MCMV; specifically, the virus infects the acinar, glandular epithelial cells of this organ (Henson and Strano, 1972). MCMV infects many cell types, but epithelial cells, endothelial cells and macrophages are especially important sites of virus production. Infection is characterized by three stages: 1) acute infection, whereby virus titers in the visceral organs peak at three to five days p.i., and the CD8 T cell response peaks at seven days p.i. 2) persistent infection, whereby virus is generally controlled in the visceral organs, but titers peak in the salivary glands at ~ 21 days p.i. 3) and latent infection, whereby viral genes are expressed and may be detectable by quantitative

polymerase chain reaction (qPCR), but infectious virus is only sporadically produced and detectable by plaque assay or by *in vitro* reactivation assays (Pollock and Virgin, 1995; Kurz et al., 1997; Koffron et al., 1998).

Upon i.p. infection of BALB/c or B6 mice, the virus acutely replicates in multiple sites, including the spleen, liver, adrenal glands, lung, and salivary glands. Initial studies on infection of immunocompetent, adult BALB/c mice reported the kinetics and magnitude of viral load following i.p. infection with high dose, salivary glands-derived, MCMV-Smith. Virus peaks in the spleen and liver at around four days p.i., drops to the limit of detection by nine days, and then recurs at a lower titer a few weeks later (Mercer and Spector, 1986). Virus in the salivary glands develops with slower kinetics, peaking at day nine p.i, and then gradually declining until it is no longer detectable at day 32 p.i.; the peak viral load is two to three logs higher in the salivary glands than in the spleen or liver.

The pathogenesis is somewhat different with TC-passaged MCMV-Smith infection of adult BALB/c mice. The titers in the salivary glands and lungs are the highest in acute infection (Reddehase et al., 1994). By two weeks p.i., there is no detectable virus by plaque assay in the lung, liver, or spleen; the only site in which virus is found is the salivary glands, where it peaks around three weeks p.i. and becomes undetectable by two months (Reddehase et al., 1985; Reddehase et al., 1994). Control of acute infection and persistence in the salivary glands is different in mice infected as neonates, which is discussed in more detail below. Overall, the differences seen between salivary glands and TC-derived virus are attributed to differences in purity and the inflammatory response induced by salivary glands-derived viruses.

Latent MCMV DNA is found in many organs, including the salivary glands, adrenal glands, brain, spleen, heart, kidney, and lungs (Collins et al., 1993; Reddehase et al., 1994; Pollock and Virgin, 1995; Bevan et al., 1996; Kurz et al., 1997; Koffron et al., 1998). The lungs have been reported to be a particularly important latent virus reservoir; viral loads in the lungs are high in acute infection and reactivating viral loads are higher here than other organs, correlating

reactivation with the acute viral titer (Reddehase et al., 1994). The specific cell types involved in latency had been theorized for some time; it was assumed that the cells important in HCMV latency would also be important for MCMV. This has held true, in general, as endothelial cells, as well as bone marrow (BM) and alveolar cells of the monocyte lineage, are reported sites of MCMV latency (Koffron et al., 1995; Pollock et al., 1997; Koffron et al., 1998).

Our laboratory has focused on the B6 model of MCMV infection, using high dose, i.p. infection with TC-derived MCMV strain MW97.01. This strain was generated by bacterial artificial chromosome (BAC) technology using the Smith strain, although the HindIII E' fragment is derived from MCMV-K181. MW97.01 will be referred to throughout this dissertation as MCMV-BAC (Wagner et al., 1999). MCMV-BAC can be detected by plaque assay in the spleen, lung, liver, kidney, and heart of B6 mice at four days p.i., although not consistently in all mice (see Chapter 5 and unpublished observation). Virus in the salivary glands is found consistently up to two weeks p.i. (Jonjic et al., 1994). By qPCR analysis, viral genomes peak in the lung, kidney, and liver around four days p.i. and are detectable through 15 days p.i. (Gold et al., 2004). During chronic infection of B6 mice, virus usually cannot be detected by plaque assay in any organ. However, MCMV-Smith and -K181 establish persistent salivary glands infection in B6 mice (Bukowski et al., 1984; M. Degli-Esposti, unpublished observation).

While there are various strengths and weaknesses of our model, there is a significant body of literature utilizing the model, which gives us insights into both. My dissertation research was undertaken with some knowledge of the limitations of the model, but it has also turned into a more formal exploration of some of them. I chose to study, almost entirely, the *in vivo* response to MCMV infection. As with most *in vivo* systems, this automatically adds a dimension of complexity, if not experimental variability. However, in our model, unmasking the *in vivo* impact of the MHC class I immune evasion genes—if one exists—still seems to me one of the most compelling areas of investigation. The paradox of our *in vitro* and *in vivo* findings regarding MHC class I immune evasion by MCMV will be discussed further at the end of this introduction.

The following section first describes MHC class I antigen presentation pathway in enough detail to then discuss how it is targeted by viral immune evasion genes.

1.4 Role of MHC class I in immunobiology

As discussed above, the herpesviruses have impressive coding capacity and have exquisitely evolved with their hosts. These two features likely contribute to the myriad immune evasion mechanisms the herpesviruses have developed. Individual herpesviruses employ immune evasion strategies that are common to the virus family (but may differ in exact mechanism), and they also employ strategies that are unique to individual viruses. All herpesviruses examined, however, encode genes that impact the MHC class I pathway of antigen presentation. A central question of my thesis is: what is the real biological role of these genes? In order to address this question, it is necessary to summarize the current knowledge about the role of MHC class I antigen presentation in the immune response.

MHC class I molecules comprise three α heavy chains and one invariant light chain, β2-microglobulin (β2-m). The molecule is part of the major histocompatibility complex, a linked group of genes on chromosome 17 that encode the α-chain of MHC class I, the α- and β-chains of MHC class II, and a number of other genes that tend to have immunological function. The class I genes include the L, D, and K α-chains, as well as the locus for tapasin. The β2-m chain of MHC class I is not coded for in the complex and is located on another chromosome. β2-m is also polymorphic; at least seven alleles have been described for the mouse (Parnes and Seidman, 1982; Hermel et al., 1993). The class II genes include the A and E α- and β-chains, as well as the O and M α- and β-chains and genes that code for the transporter associated with antigen processing (TAP)1, TAP2, and Lmp. M and O (equivalent to human DM and DO) are non-classical MHC class II molecules. The Lmp genes encode for proteasome subunits. The MHC class III genes encode for components of the complement system, cytokines (tumor necrosis

factor (TNF)-α and TNF-β), as well as an enzyme important in steroid biosynthesis. Finally, the MHC class Ib genes are a group of more than 50 genes that exhibit much less polymorphism than the rest of the MHC complex, not all of which have known immune function.

MHC class I pathway of antigen presentation

Naïve CD8 T cells are activated, in part, by recognizing antigenic peptide in the context of MHC class I molecules. MHC class I molecules are expressed on all nucleated cells in the body, so that any cell type is able to present antigen when necessary. Three cell types, however, are considered to be professional antigen presenting cells (APCs): B cells, macrophages, and DCs. DCs seem to be the most important APCs for viral infection because they efficiently acquire and present antigen, and they can be induced by viruses to express the co-stimulatory molecules necessary for naïve CD8 T cell activation. The manner by which a cell processes a virus or intracellular bacterium into peptide fragments for display on MHC class I, regardless of cell type, is exquisitely orchestrated. Endogenous polypeptides are ubiquitinated and marked for degradation by the immunoproteasome. The exact source of these polypeptides is somewhat disputed. It has been suggested that newly translated polypeptides—those either with errors in translation, those that have been misfolded, or defective ribosomal products (DRiPs)—are the primary source of proteins for the proteasome (Schubert et al., 2000; Yewdell and Nicchitta, 2006). Others have reported, however, that newly synthesized polypeptides are protected from the proteasome during and after translation, suggesting that preexisting proteins are the primary source of proteins for the proteasome (Vabulas and Hartl, 2005).

What the proteasome subsequently does is not disputed, however. Larger polyproteins are degraded into peptides between around eight to 40 amino acids long (Glithero et al., 2006). The peptides are then transported from the cytosol into the endoplasmic reticulum (ER) via TAP. Longer peptides must be trimmed before binding to MHC class I, as the optimal peptide length for MHC class I is eight to 10 amino acids. This trimming is done in the ER by the endoplasmic

reticulum amino-peptidase (ERAAP) molecule (Rock et al., 2004; Chang et al., 2005). ERAAP is able to recognize the carboxy terminus of a peptide and then trims the amino terminus to its final length. Nascent MHC class I molecules await peptide loading in the ER. It is not until MHC class I is loaded with peptide that it is an optimally stabilized molecule—peptide binding stabilizes protein folding and optimizes glycosylation and transport to the cell surface (Carven et al., 2004; Glithero et al., 2006; Vyas et al., 2008).

The three α chains assemble with each other in the ER and are stabilized by the membrane-bound chaperone, calnexin. Then, β2-m assembles with the α chains, calnexin dissociates, and the MHC class I loading complex begins to form. The role of this complex is twofold. First, as suggested by its name, it assists in coupling peptides with MHC class I. Second, it provides quality control, through peptide exchange and editing, before export of the MHC class I complex to the cell surface (Jensen, 2007). The complex includes calreticulin, tapasin, TAP, and ERp57, and binding is cooperative. Calreticulin is a chaperone molecule required for proper assembly and loading, and it is thought to stabilize the MHC class I heterodimer (Hughes et al., 1997; Lindquist et al., 1998; Morrice and Powis, 1998). Tapasin binds to both MHC class I and TAP, physically linking the two and stabilizing TAP. ERp57 is a thiol reductase associated with tapasin via a disulfide bond; ERp57 is thought to maintain disulfide bond structure in MHC class I heavy chains, which stabilizes the peptide binding groove and allows the heavy chains to bind β2-m and peptide (Sijts and Pamer, 1997; Solheim, 1999). After dissociating from the peptide-loading complex, peptide-MHC class I complexes aggregate at ER exit sites and bind to the transport receptor, B cell receptor-associated protein (BAP)31 (Antoniou and Powis, 2008). The complex then travels via regulated vesicular transport through the Golgi compartment to the cell surface. This process is clearly a complicated orchestration of specific steps and multiple molecular players. The existence of so many steps and important proteins provides multiple

targets for viral immune evasion genes, which may have contributed to the development of an alternative way for which antigens can be presented via MHC class I.

Cross-presentation

Cross-presentation, or cross-priming, is a second pathway for peptide antigens to be presented by MHC class I to CD8 T cells, and it has particular relevance for MCMV infection that will be discussed below. Cross-presentation is a way in which *exogenous* proteins can be presented, generally by DCs, via MHC class I. DCs take up infected cells or cellular debris, they process the exogenous proteins into peptides by the mechanism described above, and present the peptides on their MHC class I molecules. $CD8^+$ DCs are the subset of murine DCs specialized for cross-presentation, although there are reports that macrophages, B cells, neutrophils, and endothelial cells can also cross-present (Basta and Alatery, 2007). Many different forms of antigen have been implicated in cross-presentation, including soluble protein, immune complexes, defective ribosomal products, heat shock protein (HSP)-bound peptides, and apoptotic cells (Yewdell et al., 1996; Regnault et al., 1999; Khan et al., 2001; Basta and Alatery, 2007). It is currently believed that the primary source of cross-presented antigens are cell-associated proteins that require further processing (*i.e.* not peptides or HSP-bound peptides, for example) (Basta and Alatery, 2007; Jensen, 2007).

There are multiple mechanisms by which DCs can acquire exogenous proteins for cross-presentation, including via receptor-mediated endocytosis, gap junctions and, more commonly, phagocytosis and macropinocytosis (Basta and Alatery, 2007; Savina and Amigorena, 2007). Once in the DC, there are at least two pathways defined for the trafficking of acquired antigen to display on MHC class I. One is TAP-independent and the other, considered to be more relevant *in vivo*, is TAP-dependent (Lin et al., 2008). In the TAP-independent, or "vacuolar," pathway, antigen is processed into peptide within the phagosome (Rock and Shen, 2005; Lin et al., 2008). Cathepsin S, a cysteine protease, plays an essential role in peptide generation, but the process by

which the peptides encounter MHC class I molecules is still not clear. In the TAP-dependent pathway, a number of models exist to explain how endocytosed, exogenous antigens are ultimately presented on MHC class I, all of which implicate the translocon Sec61 at some point in the process. The leading model is one whereby antigen is exported from the endosome to the cytosol and then is processed and presented just as described for the classical MHC class I pathway (Lin et al., 2008). The "endosome-ER" model suggests that antigen is delivered from the endosome directly to the ER and then accesses the cytosol in an endoplasmic reticulum-associated protein degradation (ERAD)-like process (Lin et al., 2008). Finally, there is the "ER-phagosome fusion" model, which describes an ER-phagosome compartment equipped with all of the components of the MHC class I peptide loading complex (Cresswell et al., 2005; Lin et al., 2008). The existence of an ER-phagosome compartment has recently been challenged, but the evidence in support of it shows that antigen in the ER-phagosome is retrotranslocated to the cytosol. Once in the cytosol, antigen is processed into peptides by the immunoproteasome, and the peptides are then transported via TAP back into the ER-phagosome for loading onto MHC class I (Houde et al., 2003).

Cross-presentation is a mechanism by which DCs that are not themselves infected present antigen to CD8 T cells. Importantly, because the DCs are not infected, they are not subject to any immune evasion mechanisms employed by the infecting pathogen. One might conclude that cross-presentation is the immune system's response to the development of such immune evasion mechanisms by pathogens. Cross-presentation had actually been suggested to be the primary method of antigen presentation in MCMV infection, although despite my and others' efforts, this has not yet been demonstrated experimentally (Gold et al., 2004). In order to further discuss antigen presentation and the resulting CD8 T cell response in MCMV infection, I will first describe the process of CD8 T cell antigen recognition, activation, and effector function in the following section.

1.5 Recognition of presented antigen by CD8 T cells

Peptide-MHC class I complexes present antigen to CD8 T (and natural killer (NK)) cells. CD8 T cells recognize this complex through the T cell receptor (TCR), which is ubiquitously expressed on the T cell surface. Under the right circumstances, this interaction forms an "immune synapse" that activates cell signaling pathways, which result in the activation of CD8 T cell effector mechanisms. In this section, I will describe the structure of the TCR and the immune synapse, outline the signaling pathways, and discuss the two types of effector mechanisms used by CD8 T cells. While NK cells recognize MHC class I in an important (and sophisticated) manner, this interaction will be discussed only briefly, as my thesis focuses almost exclusively on CD8 T cells. NK cells express both inhibitory and activating NK receptors. When an activating NK receptor is triggered, the NK cell is stimulated and can be directly cytotoxic and or secrete interferon (IFN)-γ, as CD8 T cells do. Inhibitory receptors recognize MHC class I. If MHC class I is expressed on a cell, that implies that the cell is "normal"—*i.e.* that its MHC class I is not pathologically downregulated by viral infection or malignancy. An interaction with MHC class I triggers inhibitory NK receptors, which can override an activating signal and prevent the NK cell from killing normal cells.

One function of CD8 T cells is to travel through peripheral lymphoid organs and sample the peptide-MHC class I molecules on APCs. Each CD8 T cell expresses a TCR specific for a particular peptide epitope; an individual cell can express up to ~30,000 clonal TCR on its cell surface (Janeway, 2005). The TCR itself is structurally reminiscent of immunoglobulin molecules and also undergoes gene segment rearrangement to insure a diverse repertoire. It is a heterodimer of either an α and a β chain (TCR α/β) or a γ and a δ chain (TCR γ/δ). TCR α/β is considered to be the predominant, classical TCR; TCR γ/δ is believed to recognize unprocessed antigens either in the context of non-classical MHC class I or without MHC class I at all and will not be further addressed (Janeway, 2005). Each chain (α, β) has a constant and a variable region. The variable

regions contain three complementarity-determining regions (CDRs); CDR1 and 3 interact specifically with the peptide-MHC class I surface, while CDR2 interacts with conserved regions of the MHC class I molecule.

TCR signaling and the immune synapse

The TCR is associated with three other types of molecules that are essential in transmitting a signal upon binding of a cognate peptide-MHC class I complex. They include the CD3 signaling complex, the CD8 co-receptor, and various co-stimulatory molecules, such as lymphocyte function-associated antigen (LFA)-1, CD28, and CD40. These molecules contribute to the immune synapse—the physical interface between an APC and a CD8 T cell—which will be described in greater detail below. The CD3 complex is constitutively associated with the TCR and initiates signaling after antigen recognition. The complex is made up of at least six chains; CD3 ε/δ and CD3 γ/ε are cell-membrane bound to either side of the TCR, and CD3 ζ/ζ is adjacent to the tails of the TCR in the cytosol. The CD8 "co-receptor" is what defines a CD8 T cell; it is adjacent to the CD3 complex and its binding to a conserved region of MHC class I is required for T cell activation. The co-receptor is typically a heterodimer comprised of an α and a β chain (CD8 α/β), but can also be an α/α homodimer.

A signaling cascade begins when peptide-MHC class I is bound to both its cognate TCR *and* CD8-α/β, as described in more detail in Figure 1.1. The direct outcome of this signaling is increased transcription and translation, chromatin remodeling, actin polymerization, and cytoskeleton rearrangement. However, for this signaling to proceed as depicted and to result in activation of the effector functions of a naïve CD8 T cell, there must also be co-stimulatory signals. The only cells that can deliver the proper co-stimulation are B cells, macrophages, and DCs. The most critical co-stimulatory signals for CD8 T cells are mediated through LFA-1 and intercellular adhesion molecule (ICAM)-1, CD28 and B7.1 or B7.2, and CD40 and CD40 ligand

(CD40L). ICAM-1 binds LFA-1 on the CD8 T cell; this interaction is important for cellular adhesion and in the formation of the immunological synapse, which will be described below. The CD40-CD40L interaction maintains signaling, driving T cell proliferation and differentiation, and it also increases B7 expression on APCs, which allows for enhanced co-stimulation via CD28.

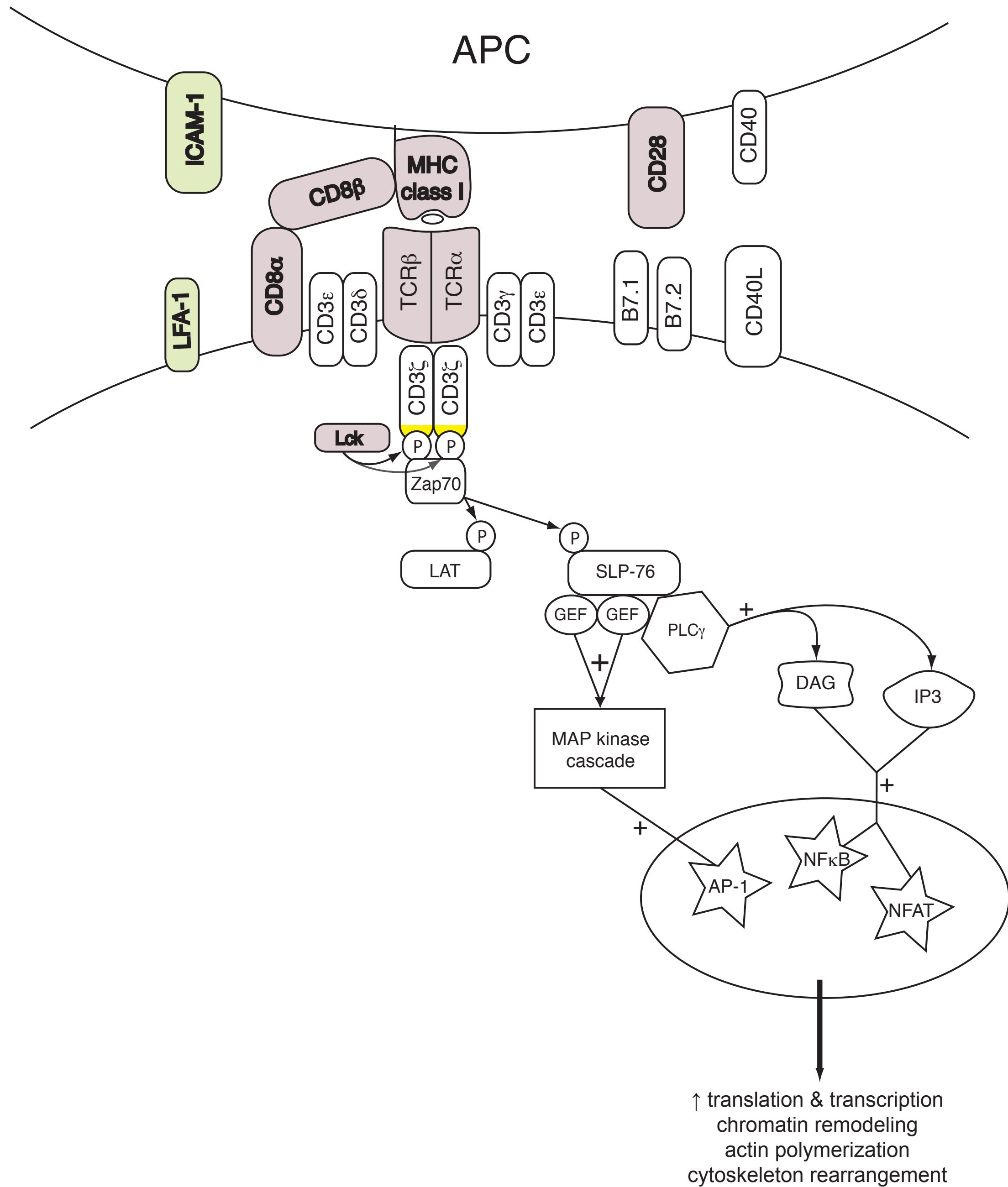


Figure 1.1. The immune synapse, TCR activation, and the subsequent signaling cascade. When peptide-MHC class I is bound to both its cognate TCR and to CD8-α/β, the signaling cascade begins with recruitment of the protein kinase, Lck, to the immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 complex. Lck phosphorylates the ITAMs, which allows the tyrosine kinase Zap70 to bind and be activated. Zap70 phosphorylates the adaptor proteins, linker of activation in T cells (LAT) and SLP-76. SLP-76 subsequently binds to and activates guanine exchange factors (GEFs) and phospholipase C-γ (PLC-γ). The GEFs go on to activate a mitogen-activated protein (MAP) kinase cascade, ultimately activating the transcription factor, AP-1. PLC-γ, on the other hand, activates diacylglycerol (DAG) and inositol triphosphate (IP3), which ultimately activate the transcription factors NFκB and nuclear factor of activated T cells (NFAT). Molecules shaded in pink are located within the central supramolecular activating complex (SMAC). Molecules shaded in green are found in the peripheral SMAC. ICAM-1: intracellular adhesion molecule 1. LFA-1: lymphocyte-function associated antigen 1. Lck: leukocyte-specific protein kinase. AP-1: activator protein-1.

Without the appropriate co-stimulation, on a molecular level there can be modified phosphorylation of the CD3 complex and recruitment of an inactive form of the protein tyrosine kinase Zap70 to the complex (Janeway, 2005). In a more global sense, naïve T cells receiving a signal through their TCRs in the absence of co-stimulation become anergic or "exhausted." It is important to point out that co-stimulatory signals are required by *naïve* T cells for effector function but are not required by already primed, effector or memory T cells. In the case of primed T cells, they only require cognate peptide-MHC class I recognition and CD8-α/β binding and adhesion via LFA-1 and ICAM-1 to initiate signaling, formation of an immune synapse, and ultimately, an effector response.

The signaling events described above all occur in a defined space, the immune synapse. The synapse sets up a contained, central location from which effector molecules are secreted to act on a target cell. The cytoskeletal changes that occur during T cell activation play an essential role in the development of the immune synapse. Reorganization of the microtubule organizing center (MTOC) and the Golgi to the side of the T cell in contact with the APC makes it possible for directed secretion of effector molecules. The synapse itself is a structure made up of the supramolecular activating complex (SMAC), which is further broken down into rings defined by their position relative to the TCR and MHC class I. Signaling molecules make up the central (c)SMAC, while the peripheral (p)SMAC contains molecules involved in adhesion. While the CD4 T cell synapse has been studied more than the CD8 T cell synapse, it has been shown that, in particular, the TCR, peptide-MHC class I, CD28, and leukocyte-specific protein kinase (Lck) are found in the cSMAC and LFA-1 and ICAM-1 are found in the pSMAC of the CD8 T cell synapse (Monks et al., 1998; Stinchcombe et al., 2001; Potter et al., 2001; Huppa and Davis, 2003). Because of the immune synapse, an activated CD8 T cell is in direct contact with its target infected or cross-presenting cell. Thus, as the T cell executes one of its effector functions, discussed below, it does so in a directional fashion, decreasing the risk of killing uninfected, neighboring cells.

CD8 T cell effector functions: cytotoxic granules

Once activated, CD8 T cells have two effector functions: the first is to directly kill cells via cytotoxic granule release or Fas ligation, and the second is to secrete antiviral cytokines that can impact viral fitness without killing the infected cell. The cytotoxic granules contain a number of preformed molecules, the most characterized of which are granzymes (Grz) A and B and perforin. Because they are preformed and stored in vesicles, these cytotoxic molecules are ready for immediate release. Lytic granules can be released within five to 10 minutes after antigen recognition (Poenie et al., 1987; Purbhoo et al., 2004). They are also synthesized upon CD8 T cell activation to replenish exhausted stores, which allows the cells to kill other cells in the area within a short period of time (Valitutti et al., 1995; Isaaz et al., 1995).

Granzymes are serine proteases found in lytic granules; Grz A and B are the most abundant and most well-studied. Granzymes are somewhat redundant, and the specific contribution of granzymes beyond Grz A and B is still being established. Once in the target cell, Grz B activates the caspase cascade via caspases-3, -7, and -8, which results in apoptosis. Grz A also induces apoptosis, but in a caspase-independent manner. Grz A targets the nucleus, damages nuclear structural integrity, and causes single-stranded DNA nicks. The other main lytic granule molecule is perforin, a protein with structural and functional homology to complement. At high concentration, perforin multimerizes and inserts itself into the target cell membrane. It was thought to disrupt the integrity of the membrane, effectively forming a “chunnel” from the extracellular space to the cytosol. Perforin is required for the function of granzymes and is the only molecule known that can deliver granzymes to a target cell. It was thought that granzymes entered the cell through the perforin chunnel, however, this assumption is not supported by direct data. It is not clear what, if anything, passes through the perforin pore, and size restrictions may preclude the passage of granzymes. Grz B can enter a cell in the absence of perforin, either by endo- or macropinocytosis (Froelich et al., 1996). Apoptosis is not induced, however, unless

perforin is present. Thus, a few models have since been suggested to describe the function of perforin: to act as a pore for granzymes to enter, to release granzymes from endosomes when the endosomal membrane is used in cell membrane repair mechanisms, or some combination of both (Pipkin and Lieberman, 2007).

In controlling viral infection, perforin is often required for CD8 T cell-mediated cytotoxicity. Perforin$^{-/-}$ mice are immunocompromised and are more susceptible to various pathogens. A few groups have reported that perforin$^{-/-}$ mice are sensitive to MCMV infection. They have increased viral loads in the spleen, liver, and lung—but not the salivary glands—at between three and six days p.i. and have increased mortality compared to wild type mice (Tay and Welsh, 1997; Loh et al., 2005; van Dommelen et al., 2006). Grz A/B$^{-/-}$ mice have also been shown to be sensitive to MCMV infection with similar increases in viral loads seen in the visceral organs, but MCMV infection is not fatal in these mice (van Dommelen et al., 2006). An additional group has reported, however, that, at a later time point (15 days p.i.), perforin$^{-/-}$, Grz A/B$^{-/-}$, and double knock-out mice have increased viral loads in the salivary glands compared to wild type mice but have no detectable virus in the lung or spleen and are not susceptible to lethal infection (Riera et al., 2000). These differences could be attributed to the different times at which the mice were analyzed, as well as different methods of preparing virus.

While many viruses are sensitive to perforin and granzymes, there is another mechanism of CD8 T cell-mediated cytolysis. This second mechanism is the Fas-Fas ligand (FasL) pathway. When FasL, expressed on CD8 T cells, ligates Fas on target cells, the caspase cascade is activated and the cell dies by apoptosis. The Fas system is relied upon relatively less for viral pathogens than the perforin/granzyme mechanism, however it plays an important role in dampening the inflammatory response (Kagi and Hengartner, 1996). FasL has been shown to be completely dispensable in controlling MCMV in the salivary glands, spleen, and lung at 15 and 30 days p.i. (Riera et al., 2001). A second group has shown that Fas$^{-/-}$ mice clear MCMV in the salivary glands, liver, kidney, lung, and peritoneal exudate with the same kinetics as wild type mice (Fleck

et al., 1998a; Fleck et al., 1998b). The lack of a functional Fas system leads to chronic inflammation in these tissues, however, because of the lack of Fas-mediated regulation of the ongoing immune response.

CD8 T cell effector functions: cytokines

One may imagine that in some infections, it would benefit the host not to kill infected cells. In HSV-1 infection of the trigeminal ganglia, for example, it would be preferable to dispense of the virus without killing neurons. The second mechanism for CD8 T cell effector function, cytokine secretion, makes that possible. Cytokines are secreted proteins that can have autocrine, paracrine, or endocrine effects, which range from establishing an inflammatory environment to maturing antigen-presenting cells to preventing pathogen attachment to inducing transcription factors (Harty et al., 2000). It is by inducing transcription factors that cytokines can directly affect viral fitness by decreasing viral gene expression or restricting replication.

Since the effects of cytokines can be systemic, this effector mechanism is not as directed as CD8 T cell cytolysis; however, to function, cytokines must bind to specific receptors expressed on the surface of target cells, so they do not exert their functions without restriction. Many cell types produce cytokines, and cytokine cascades that are started during the innate immune response often impact the cytokine responses of adaptive immune cells. Unlike perforin and granzyme, cytokines are synthesized *de novo* upon CD8 T cell activation. IFN-γ and tumor necrosis factor (TNF)-α secretion can be detected between 30 minutes and one hour after antigen presentation, and are maximally detected within six to 18 hours of activation (Lalvani et al., 1997; Slifka et al., 1999; Harty et al., 2000; Raue et al., 2004). Cytokine secretion terminates when the CD8 T cell breaks contact with the target cell, but T cells can produce cytokine again as soon as they associate with another target cell (Slifka et al., 1999). Resumed cytokine production occurs at maximal levels within one hour of reassociation.

CD8 T cells can produce IFN-γ, TNF-α and -β, and the chemokines, chemokine (C-C motif) ligand 5 (CCL5, or RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β. Chemokines are a subset of cytokines, although they specifically function in cell homing and recruitment; in particular, they activate and recruit macrophages and neutrophils to the site of inflammation. Of the cytokines released by CD8 T cells, the most is known about IFN-γ and TNF-α. TNF-α is made in soluble and membrane-associated forms. TNF-α upregulates the co-stimulatory molecule and member of the immune synapse, ICAM-1, as well as vascular cell adhesion molecule (VCAM)-1. The upregulation of VCAM-1 attracts circulating T cells to the site of infection. TNF-α also activates macrophages, stimulates DC maturation and migration to lymph nodes, and induces nitric oxide synthesis (Janeway, 2005).

IFN-γ is a ubiquitous cytokine that possesses a substantial list of functions. For the purposes of this dissertation, only a subset of its functions will be mentioned. IFN-γ is a type II interferon; IFN-α and -β are type I interferons and the two types share functional, but not genetic or structural characteristics. The type I IFNs are clustered genetically and type II IFN is located on a separate chromosome. Their receptors are also different and non-redundant; type I IFNs bind to the receptor subunits IFNAR-1 and -2, while type II IFN binds to IFNGR-1 and -2. Most cell types produce IFN-α and -β, whereas specific immune cells, such as NK cells, CD4 T_H1 cells, and CD8 T cells produce IFN-γ (Katze et al., 2002). Both types have two main roles: specific antiviral activity and immunomodulatory activity (Teixeira et al., 2005). The antiviral function of IFN-γ is actually argued to be secondary to its immunomodulatory function. An important immunomodulatory property includes stimulating more effective antigen processing and presentation by upregulating expression of a variety of molecules involved in antigen presentation, including MHC class I, MHC class II, TAP, ERAAP, and proteasome subunits (Janeway, 2005). IFN-γ is the primary cytokine activator of macrophages and it is an important product and activator of NK cells. As an antiviral, like the type I interferons, IFN-γ upregulates

expression of IFN-inducible genes, which go on to produce an "antiviral state" via the dsRNA-activated protein kinase R (PKR), dsRNA-specific adenosine deaminase (ADAR), guanylate binding protein (GBP)-1, and inducible nitric oxide synthase (NOS)/NOS2.

IFN-γ has some notable interactions with MCMV. IFN-γ decreases IE1/pp89 mRNA in infected NIH 3T3 fibroblasts and macrophages (but affects L gene mRNA in primary fibroblasts) (Gribaudo et al., 1993; Presti et al., 2001). MCMV, however, has developed mechanisms to counteract some of the effects of IFN-γ. In addition to IFN-γ, MCMV infection induces type I interferons. The type I interferons impair IFN-γ production, making IFN-γ less effective at activating macrophages (Heise et al., 1998b). More directly, MCMV encodes a protein that makes the virus less sensitive to both types of interferons (Khan et al., 2004; Zimmermann et al., 2005). The protein, M27, binds and downregulates signal transducer and activator of transcription (STAT)-2, a signaling molecule that is directly activated by IFN-γ. One of the ramifications of this is decreased expression of the IFN-γ-inducible proteasome subunits, thus preventing the formation of the immunoproteasome.

Relative importance of cytotoxicity and cytokine release by CD8 T cells

While it may be a general assumption that both of the effector functions discussed above are employed during an immune response, it is important to consider how and when they may be uncoupled. A master transcriptional regulator, *Eomesodermin*, was recently identified and its expression leads to both effector functions in CD8 T cells, suggesting a certain degree of coupling (Pearce et al., 2003). Different requirements for the two functions, however, have been identified in experiments studying the threshold of T cell effector function. Valitutti *et al.* reported a decade ago using a human cytotoxic T lymphocyte (CTL) line that T cells are cytotoxic *in vitro* at cognate peptide concentrations as low as 10^{-12} to 10^{-15} M (Valitutti et al., 1996). IFN-γ production (and a proliferative response to IL-2) requires greater than 10^{-9} M peptide. This suggests that

cytotoxicity is a more sensitive effector mechanism than cytokine production. In a different context, cytotoxicity was shown to be more sensitive to the length of antigenic stimulation of CD8 T cells. While cytokine production remains fairly constant regardless of the length of stimulation, cytotoxicity is enhanced with longer antigen exposure (>24 hours) (Usharauli and Kamala, 2008).

There are a series of reports in chronic viral infections that suggest that CD8 T cells can secrete IFN-γ in the absence of cytotoxicity. Lancaster *et al.* showed that, while the ability to produce IFN-γ is the same, CD8 T cells from patients with resolved, acute hepatitis C (HCV) infection are much more cytotoxic (and to a broader range of peptides) than T cells from patients with chronic HCV (Lancaster et al., 2002). In a hepatitis B (HBV) model, the difference in effector function was epitope-specific. Chen *et al.* demonstrated in a mouse model that CD8 T cells specific for one HBV epitope ($HBcAg_{87-95}$) can execute both effector functions, while T cells specific for a second epitope ($HBcAg_{131-139}$) produce IFN-γ in the absence of cytotoxicity (Chen et al., 2005). Interestingly, mice that receive transferred $HBcAg_{87-95}$-specific CD8 T cells show signs of liver impairment—presumably due to direct cytotoxicity—when compared to mice that receive $HBcAg_{131-139}$-specific CD8 T cells. CD8 T cells from $HIV^{+}HCMV^{+}$ patients produce equivalent amounts of IFN-γ to both HIV and HCMV antigens (Appay et al., 2000). However, HIV-specific CD8 T cells express lower amounts of perforin and are impaired in their ability to lyse specific targets when compared to HCMV-specific CD8 T cells from the same patient. Finally, in HSV-1 infection, virus-specific CTL can execute both effector functions *in vitro* when stimulated with either infected splenocytes or corneal fibroblasts, but if stimulated concomitantly, the CTL lose their cytotoxic function (and increase IFN-γ production) (Knickelbein et al., 2007). The loss of cytotoxic function is not due to abnormal lytic granule release, but decreased production and storage of Grz B. Infected fibroblasts alone cause CTL to polarize and release lytic granules towards the infected cells, resulting in apoptosis; infected neurons alone, however,

cause CTL to directionally release lytic granules, but this does not result in apoptosis and latent virus is still controlled (Knickelbein et al., 2008). In a novel function for Grz B, it was recently reported that Grz B inactivates the virus without killing the cell by degrading the HSV-1 E protein, ICP4.

The previous two sections have described in detail the way in which CD8 T cells recognize antigen and the consequences of activation. These processes are important to make clear because in the following sections I will describe the specific ways in which viruses have attempted to avoid these consequences. First, I will contrast the role that CD8 T cells play in various virus infections, particularly in MCMV infection. Then, I will introduce the myriad immune evasion strategies of cytomegaloviruses before, more specifically, discussing MHC class I immune evasion mechanisms and the impact they have on MCMV infection.

1.6 Role of CD8 T cells in virus infection

CD8 T cells have been shown to be important in the control of many different viral infections, including lymphocytic choriomeningitis (LCMV), influenza, γ-herpesvirus 68 (γ-HV68), and HIV (Lukacher et al., 1984; Jamieson et al., 1987; Ehtisham et al., 1993; Koup et al., 1994; Ciurea et al., 2001; Thomas et al., 2006). The specific effector function that is required for control of these different pathogens can vary, however. Furthermore, while it is outside of the scope of this dissertation, instead of always fighting infection, CD8 T cells can also contribute to disease by causing massive cell death in an organ (*e.g.* hepatitis) or by augmenting immunopathology.

LCMV generates an infamously strong CD8 T cell response in mice. CD8 T cells eliminate acute infection with the Armstrong strain by eight to 10 days p.i.; β2-m$^{-/-}$ mice do not control the virus and become persistently infected (Matloubian et al., 1994). The chronic strain of LCMV (clone 13) also generates a robust CD8 T cell response. Wild type mice eventually clear

clone 13 from most tissues, although the CD8 T cell cytotoxic response becomes ineffective–what is now known to be "exhaustion" from chronic antigen stimulation (Matloubian et al., 1994; Klenerman and Hill, 2005). CD4 T cells are not required for CD8 T cells to mount a response to acute, LCMV-Armstrong infection, but CD4 T cell depletion of mice infected with clone 13 go on to have high viral titers in various tissues (Matloubian et al., 1994).

Influenza A infection stimulates a robust CD8 T cell response that is detected in the infected airway by five to seven days p.i. and is maintained until at least 570 days p.i. (Thomas et al., 2006). Adoptive transfer of both CTL and T_H1 clones protects mice from lethal infection with influenza A (Lukacher et al., 1984; Graham et al., 1994). CD8 T cell depletion of influenza-infected mice, however, leads to slightly delayed clearance, but in these and in $\beta2\text{-m}^{-/-}$ mice, the virus is cleared by day 10 p.i. (Eichelberger et al., 1991). In CD4-depleted mice, viral clearance is also slightly delayed. A CD8 T cell response develops in the absence of CD4 T cells, although the magnitude of the recall response is significantly decreased (Thomas et al., 2006). Either CD8 or CD4 T cells are required from protection from influenza, however; when both are depleted, mice succumb to infection (Eichelberger et al., 1991).

CD8 T cells are required to clear acute infection with γ-HV68 in a perforin-independent manner (Harty et al., 2000); without CTL, there is unchecked replication in the lungs that can lead to death (Ehtisham et al., 1993). $\text{IFN-}\gamma^{-/-}$ mice clear acute infection and develop latent infection (Harty et al., 2000). In CD4-depleted mice, acute viral clearance is slightly delayed but there is no defect in CD8 T cell generation or maintenance. Long-term, however, CD4 depletion eventually leads to a chronic, lethal wasting disease (Stevenson et al., 1998). This is not due to CD8 T cell exhaustion; if CD8 T cells are depleted from $CD4^{-/-}$ mice with reactivating virus, the virus titers increase even further and the animals become more ill.

HIV induces a strong CD8 T cell response, although the exact role of CTL protection has been difficult to decipher. In patients with recent onset HIV infection, those who controlled

viremia within the first month also have a significant HIV-specific CD8 T cell response, predominantly to one epitope (Borrow et al., 1994). Chronically infected individuals typically have a high frequency of circulating, activated HIV-specific CD8 T cells that can inhibit HIV replication *in vivo*, although their ability to produce IFN-γ decreases with disease progression (Benito et al., 2004). In human longitudinal studies, there is a correlation between low or undetectable CD8 T cell activity and the reappearance of infectious virus in the blood. Studies in simian immunodeficiency virus (SIV) have tested this correlation by CD8-depleting rhesus monkeys (Schmitz et al., 1999). The kinetics and magnitude of peak viral RNA is the same with or without CD8 T cells, but in monkeys with lasting CD8 T cell-depletion, there is no control of virus replication. In monkeys with short-term depletion, however, as the CD8 T cells reappear, there is a corresponding decrease in viremia. Furthermore, in animals chronically infected with SIV, CD8 T cell depletion leads to a rapid increase in viral RNA (Schmitz et al., 1999).

Role of CD8 T cells in MCMV infection

The role of T cells in MCMV infection of immunocompetent BALB/c mice has also been studied by depleting lymphocyte subsets, either alone or in combination. Particularly interesting findings to come out of these experiments are that in the absence of CD4 T cells, virus in the salivary glands is not controlled by 12 weeks p.i. (Jonjic et al., 1989; Jonjic et al., 1990); in the absence of CD8 T cells, there is only a modest impact on the acute control of virus in the lung (Jonjic et al., 1990). In the absence of CD4 cells, if CD8 T cells are depleted after chronic control has been established, 100% of mice have virus in both their lungs and salivary glands at 10 weeks p.i. (Jonjic et al., 1989).

There is relatively less literature on T cell subset control in B6 mice, in large part due to their robust NK cell response. The NK cell response accounts for a distinct difference between the immune response of B6 and BALB/c mice. Because the experiments in this dissertation involve both BALB/c and B6 mouse strains, it is important to briefly highlight this difference.

BALB/c mice are considered to be MCMV "susceptible," while B6 mice are "resistant," due to a difference in their Ly49H status. B6 mice express Ly49H, an activating NK cell receptor that recognizes the *m157* gene product of MCMV (Arase et al., 2002; Smith et al., 2002). Expression of this viral protein activates Ly49H on NK cells, leading to the lysis of infected cells and, thus, more robust NK cell control in B6 mice (Shellam and Flexman, 1986; Quinnan and Manischewitz, 1987). $Ly49H^-$ strains (129/SvJ, BALB/c, etc.) are much more susceptible to MCMV infection because they lack this activation.

We now know, however, that the overall picture in B6 mice is the same as in BALB/c mice: CD8 T cells are rather dispensable for control of MCMV in immunocompetent mice. When the C57BL substrain, C57BL/10 mice, are depleted of T cell subsets, either alone or in combination, then infected with high dose MCMV-K181, there is no effect on the virus load in the spleen or liver (Lathbury et al., 1996). When B6 mice are depleted of these subsets and infected with high dose MCMV-Smith, CD4 depletion results in impaired control of virus in the salivary glands at three weeks p.i., while CD8 depletion has no effect (Jonjic et al., 1994). Cell subsets were also depleted from chronically infected, antibody-deficient (μMT/μMT) mice, in order to assess the role of T cells with the layer of antibody protection removed. Depleting CD4 T cells, CD8 T cells, or NK cells demonstrated that they contribute to controlling latent virus in a redundant fashion. Depletion of any two of these cell types leads to detectable virus reactivation within days, whereas depletion of any single subset does not (Polic et al., 1998).

These findings lead to several conclusions: first, CD8 T cells are not required to control infection in immunocompetent hosts. In fact, CD8 T cells educated in the absence of CD4 T cells *cannot* control virus in the salivary glands. As a corollary, when CD4s are primed in an environment lacking CD8 T cells, they are able to fully compensate by eliciting an effective antiviral response, including control of virus in the salivary glands (Jonjic et al., 1989; Jonjic et al., 1990; Koszinowski, 1991). Second, CD4 T cell help is not required for priming a CD8 T cell response or for immediate maintenance of CD8 T cell memory, although it is required for the

development of antibody (C. Snyder, unpublished observation; Jonjic et al., 1990). CD4 T cells may also be needed for long-term maintenance of the CD8 T cell response in MCMV infection, but their exact role in this is not yet clear.

T cell control of MCMV in immunosuppressed, adult mice

The accumulated data indicate that there is a high degree of redundancy in immune control of MCMV, no doubt a result of millions of years of co-evolution to achieve a highly buffered equilibrium between the virus and its host. The fact that no single arm of the immune system is uniquely required for virus control does not mean that each of these arms is ineffectual, merely that if it is absent it can be compensated by another arm. To understand the capability of each immune component against MCMV, it has been necessary to study severely immunocompromised hosts and to selectively add back each component. This has been done by developing a bone marrow transplant (BMT) model of MCMV infection, which also serves to model clinical CMV disease in transplant and immunosuppressed patients.

The most commonly used model, developed by the Reddehase group, involves irradiated BALB/c "indicator" mice and the transfer of different subsets of primed lymphocytes either at the time of or six days after infection with high dose MCMV-Smith (Reddehase et al., 1985). Irradiated, BALB/c mice that are infected with MCMV without any hematopoietic cell transfer unfailingly succumb to lethal CMV disease. There is infection of the liver, lung, spleen, kidney, adrenal glands, gastrointestinal tract, and salivary glands, both evidenced by virus found in these organs and by histopathology seen by immunohistochemistry (IHC) and *in situ* hybridization (ISH) (Reddehase et al., 1985; Reddehase et al., 1988; Jonjic et al., 1989; Alterio de Goss et al., 1998). Irradiated mice can be rescued from lethal disease by CD8 T cells from acutely or chronically infected mice (Reddehase et al., 1985; Reddehase et al., 1987; Jonjic et al., 1989). This rescue is dependent on the number and type of cells transferred and results in decreased viral loads in visceral organs. By two weeks after lymphocyte transfer, titers in the spleen, liver, and

lung are below detection and viral titers in the salivary glands decrease by greater than two logs (Reddehase et al., 1985; Jonjic et al., 1989). Transferring CD8 T cells alone is as effective in reducing viral loads in the lung, adrenal glands, and spleen as transferring both CD8 and CD4 subsets (Reddehase et al., 1987; Koszinowski et al., 1987b; Reddehase et al., 1988; Jonjic et al., 1989; Jonjic et al., 1990). Depleting CD8 T cells, however, or CD4 *and* CD8 T cells, results in organ immunopathology, virus replication in all organs tested, and 100% lethality (Reddehase et al., 1987; Jonjic et al., 1989).

Fewer studies have been reported regarding the immune response to MCMV in immunosuppressed B6 mice. Holtappels *et al*. reported, however, that adoptive transfer of CD8 T cells specific for the D^b-restricted M45 MCMV epitope, HGIRNASFI, does not impact the virus load in the spleen or lung, nor does it impact the number of infected liver cells at 12 days p.i. (Holtappels et al., 2004). Transferring the total CD8 T cell population *does* confer protection though, indicating that CD8 T cells at least of some specificities can exert antiviral activity in B6 mice. Thus, in contrast to the unique role for CD4 T cells seen in immunocompetent mice, when mice are severely immunocompromised, CD8 T cells are both necessary and sufficient to control MCMV infection and to decrease histopathology.

Control of MCMV in neonates

The outcomes of MCMV infection has also been studied in neonates. Much of the original work studied the susceptibility of neonates to MCMV infection compared to adults and showed that neonatal mice of a variety of strains have a much lower lethal dose (LD_{50}) than weanling or adult mice of the same strain (Grundy et al., 1981; Shellam and Flexman, 1986). For example, the LD_{50} in newborn BALB/c mice infected i.p. with MCMV-K181 is only two PFU, while it is $5x10^4$ PFU in adult BALB/c mice (Fitzgerald et al., 1990; Fitzgerald and Shellam, 1991). Some groups then became interested in what cell types were involved in the neonatal immune response. When 12-hour-old BALB/c pups are infected i.p. with low dose (100 PFU)

MCMV-Smith, there is a high viral load in the spleen and lung eight days p.i., which correlates with the time at which they begin to succumb to the infection (Krmpotic et al., 1999). When CD4 and CD8 T cells are depleted from four-day-old pups, the viral load in the lung increases by about one log. In B6 neonates, adoptive transfer of adult splenocytes one day after i.p. infection with high dose, salivary glands-derived MCMV-Smith leads to decreased viral load in the spleen and a benefit in survival (Bukowski et al., 1985). There is little benefit from splenocytes transferred from 17-day-old mice, but intermediate benefit from splenocytes transferred from 35 day-old-mice. When both CD4 and CD8 T cells are depleted from the transferred splenocytes, there is still a survival advantage, but when NK cells are depleted, there is no protection and increased viral load in the spleen. When the role of CD8 T cells was evaluated in B6 $CD8^{-/-}$ neonates, titers in the lung are again about one log higher than in wild type B6 neonates (Krmpotic et al., 1999).

Redundancy of CD8 T cells with other components of immunity in MCMV infection

Control of acute, primary MCMV infection is independent of an anti-MCMV antibody response (Lawson et al., 1988; Jonjic et al., 1994). This is, in part, due to the development of a strong neutralizing antibody response *after* infectious virus had already spread to target organs (Lawson et al., 1988; Jonjic et al., 1994). Control of the latent virus pool is also independent of antibody, and antibody does not provide protection from superinfection with different strains of MCMV (Farroway et al., 2005; Gorman et al., 2006). However, prophylactic antibody treatment via transfer of serum or induced by vaccination with inactivated virus or recombinant glycoproteins does offer protection (Tolpin et al., 1980; Farrell and Shellam, 1991; Rapp et al., 1992; Geoffroy et al., 1996; Xu et al., 1996). Antibody *is* thought to play a role either in limiting viral reactivation or limiting replication after reactivation, however (Reddehase et al., 1994). This was determined, in part, by studying chronically infected B cell-deficient (µMT/µMT) mice, immunosuppressed by irradiation and depletion of T and NK cells. Virus starts to reappear in the salivary glands three days after immunosuppression (Polic et al., 1998). Two weeks after

immunosuppression, B cell-deficient mice have two to three log higher viral loads in their spleen, lung, and salivary glands than B6 controls (Jonjic et al., 1994; Polic et al., 1998). This is reversed by treating the mice with anti-MCMV sera.

Natural killer cells

NK cells are important in the first four to five days p.i., at which point the adaptive immune response begins to contribute. BALB/c and 129/SvImJ mice can succumb to high dose, i.p. infection, while B6 mice infected i.p. with up to $5x10^6$ PFU show no signs of morbidity (C. Doom, unpublished observation). Furthermore, restoring NK cell function to BALB/c mice makes them more "resistant" (Scalzo et al., 1995; Lathbury et al., 1996; Krmpotic et al., 2002; Iizuka et al., 2007), while blocking or inhibiting NK cell function in B6 mice makes them more "susceptible" (Bukowski et al., 1984; Shanley, 1990; Welsh et al., 1991; Scalzo et al., 1992). NK cell control also provides an example of the organ specificity of the immune response to MCMV. One of the biggest differences in viral load between resistant and susceptible mice is found in the spleen; titers are two to three logs higher in BALB/c and congenic BALB.B mice than B6 mice for the first six days p.i (Allan and Shellam, 1984; Scalzo et al., 1992; Lenac et al., 2008).

The above data highlights the importance of CD8 T cells in controlling virus infection, which leads to the presumption that persistent viruses, like the herpesviruses, must dismantle CD8 T cell function to avoid eventual clearance. When analyzed in immunocompetent animals, CD8 T cells play a relatively minor role in MCMV infection. That finding could be attributed to the significant redundancy seen in the immune response to MCMV infection or to the fact that MCMV encodes MHC class I immune evasion genes, making the CD8 T cell response less effective. Because the CD8 T cell response to MCMV and its MHC class I immune evasion capabilities are central to my dissertation work, I will conclude the introduction with a discussion of MHC class I immune evasion mechanisms and what is known about this form of immune evasion in MCMV infection.

1.7 Viral immune evasion

When compared with other virus families, herpesviruses seem particularly intent on interfering with the MHC class I pathway, however, other viruses also interfere with this pathway. It may be a less common form of immune evasion in other viruses because some viruses spread so rapidly that CD8 T cells do not exert enough evolutionary pressure to encode such genes or because many acute viruses have very small genomes with restricted coding capacity. Adenoviruses, HIV, poxviruses, and all subfamilies of herpesviruses encode MHC class I immune evasion genes. While there are differences in the exact mechanisms for inhibiting MHC class I antigen presentation, there are clear themes that are shared among very different viruses. There are four general mechanisms for MHC class I immune evasion, including: preventing MHC class I trafficking to the cell surface by retaining it in an organelle, marking mature MHC class I or its components for degradation, accelerating endocytosis of peptide-loaded MHC class I on the cell surface, and blocking TAP and thus preventing peptide loading of MHC class I. Most of these mechanisms result in the ultimate degradation of MHC class I molecules, although steady state levels may not be impacted because of the constant production of new molecules. The four mechanisms will be addressed briefly below and are also included in Table I. There are two additional forms of MHC class I immune evasion, the production of MHC class I homologs and decreased transcription of MHC class I, which will not be discussed in detail.

Retention: MHC class I immune evasion was first described for adenovirus type II. The adenovirus protein AdE3/19K retains MHC class I in the ER (Andersson et al., 1985; Burgert and Kvist, 1985; Yewdell and Hill, 2002). The HCMV US3 gene product also causes retention of MHC class I in the ER, and both AdE3/19K and US3 can also interfere with tapasin. While the HCMV US10 protein does not cause complete retention of MHC class I, it does delay trafficking out of the ER (Antoniou and Powis, 2008).

Redirection/degradation: Two ER-resident, HCMV proteins, US2 and US11, increase the degradation rate of MHC class I heavy chains. They both induce ubiquitination of the heavy chains, which leads to ERAD and retrotranslocation of the heavy chains into the cytosol for degradation (Wiertz et al., 1996a; Wiertz et al., 1996b; Antoniou and Powis, 2008). Their specific mechanisms are unique—they use different members of the ubiquitin system, different domains of their respective proteins are required, and US11 alone induces the unfolded protein response. The K3 gene product of γ-HV68 is a membrane-associated E3 ubiquitin ligase located on the cytosolic face of the ER. It associates with not only MHC class I heavy chains, but also TAP and tapasin, and ubiquitinates these molecules, targeting them for degradation (Stevenson et al., 2000; Boname and Stevenson, 2001; Antoniou and Powis, 2008). Finally, HIV Nef disrupts trafficking of MHC class I to the cell surface by redirecting molecules to the lysosome via adaptor protein-1 (AP-1) (Antoniou and Powis, 2008).

Endocytosis: KSHV proteins, K3 and K5, are members of the same viral protein family as γ-HV68 K3, and both ubiquitinate cell surface-expressed MHC class I (Coscoy and Ganem, 2000; Ishido et al., 2000). This leads to accelerated endocytosis by increasing internalization and directing the MHC class I molecules to lysosomes. The downregulation mediated by K5 is selective and only leads to downregulation of some MHC class I alleles (Antoniou and Powis, 2008). It diverts MHC class I from the cell surface to the *trans*-Golgi network via a clathrin-independent recycling pathway (Schwartz et al., 1996; Collins et al., 1998; Piguet et al., 2000).

TAP inhibition: The HSV protein, ICP47, disrupts MHC class I antigen presentation by preventing peptide delivery to the ER. ICP47 acts as a molecular stopper for the TAP molecule and binds with high affinity to the cytosolic peptide-binding site. The HCMV protein US6 also inhibits TAP, although it acts in the ER lumen, and prevents the binding of ATP to TAP.

MHC class I immune evasion in MCMV infection

MCMV encodes three known genes that interfere with antigen presentation through the MHC class I pathway: *m04*, *m06*, and *m152*. The gene products either prevent the peptide-MHC class I complex from reaching the cell surface or associate with the complex at the surface of infected cells. m06 redirects MHC class I molecules to the lysosome (Reusch et al., 1999), while m152 retains MHC class I molecules in the ER-Golgi intermediate compartment (ERGIC) (Ziegler et al., 1997). Expression of either one or both of these proteins results in downregulation of cell-surface MHC class I molecules (Wagner et al., 2002; Holtappels et al., 2006; Pinto et al., 2006). The mechanism by which m04 acts is not yet known; however, m04 forms complexes with MHC class I molecules, which are exported and stay stably associated for many hours on the cell surface. Unlike *m06* and *m152*, expression of *m04* does not lead to decreased cell surface expression of MHC class I; rather, it may even lead to increased levels (Wagner et al., 2002). In fact, as will be discussed further below, m04 has been reported to act as a positive regulator of MHC class I expression in the face of m152 expression (Holtappels et al., 2006).

Other immune evasion strategies of the CMVs

My thesis is concerned with MHC class I immune evasion, but that is certainly not the only mechanism of immune evasion employed by the cytomegaloviruses. Table II includes a list of additional immune evasion mechanisms. It is not an exhaustive list and not all gene products with duplicate mechanisms were included. The mechanisms are varied, although one may note a clear theme of evading the NK cell response. MCMV and/or HCMV encode a number of cellular homologs (MHC class I, IL-10, and chemokines), Fc receptors, and inhibitors of apoptosis. Since my experiments are *in vivo*, in mice with redundant, fully intact immune systems, all components of the immune system are potentially in use. These additional immune evasion mechanisms are important to consider as they continue to play a role in interpreting my and others' results.

1.8 Literature concerning the impact of MHC class I immune evasion on MCMV infection

Following the discovery of the MHC class I immune evasion genes, due to their evolutionary conservation and due to the importance of CD8 T cells in controlling infection in the BMT model, it became a pressing question to determine the impact of these genes on CD8 T cell function. These experiments were made possible by the development of knockout viruses that lack one or more of the MHC class I immune evasion genes and by the identification of the MCMV epitopes to which CD8 T cells generate an effector response (Krmpotic et al., 1999; Wagner et al., 2002).

Immunodominance and magnitude of the CD8 T cell response in MCMV infection

As MCMV MHC class I-restricted antigens were individually identified, it became straightforward to study the effector function of MCMV-specific CD8 T cells *in vitro*. H2-D^d-restricted epitopes from BALB/c mice were first identified (Reddehase and Koszinowski, 1984; Reddehase et al., 1986; Holtappels et al., 2002). Then, Marielle Gold, a former graduate student in our laboratory, identified the first B6 epitope, an MHC class I D^b-restricted epitope derived from the MCMV M45 gene (Gold et al., 2002). Michael Munks, another former graduate student, then provided us with a powerful tool to extend our experiments beyond a single antigen-specific CTL response. He identified 26 additional MCMV K^b- and D^b- restricted epitopes that elicit a CD8 T cell response strong enough to be detected directly *ex vivo* in acute, MCMV-BAC infection of B6 mice (Munks et al., 2006b).

The MCMV epitopes have been used to quantify CD8 T cell priming and to characterize the immunodominance hierarchy of the CD8 T cell response to MCMV infection. The immunodominance hierarchy is the pattern with which T cells respond to pathogen-specific peptide antigens. It is influenced both by the peptide antigens and the TCR. Specific determinants of immunodominance include the MHC class I haplotype, the peptide affinity for MHC class I, the cell surface stability of the peptide-MHC class I complex, and T cell avidity. How the determinants ultimately come together to establish a hierarchy, however, is not yet well-

understood. The biological importance of this hierarchy is also not entirely clear, although the fact that an epitope is immunodominant establishes that it is significantly antigenic and that the T cells specific for that antigen vigorously proliferate.

Table I. MHC class I immune evasion mechanisms used by different viruses

Gene	MHC class I immune evasion mechanism
MCMV	
m04	complexes with MHC class I in the ER, accompanies it to the cell surface; E gene (Kleijnen et al., 1997; Kavanagh et al., 2001b)
m06	decreases MHC class I cell surface expression, binds MHC class I and redirects it to the lysosome; E gene (Reusch et al., 1999)
m152	decreases MHC class I cell surface expression by retaining it in the ERGIC; also reduces cell surface expression of H-60 and the Rae-1 family of NKG2D ligands; E gene (Ziegler et al., 1997; Ziegler et al., 2000; Kavanagh et al., 2001a; Krmpotic et al., 2002; Lodoen et al., 2003)
HCMV	
US2	decreases MHC class I cell surface expression, induces translocation of α-heavy chains from the ER to the cytosol for degradation by the proteasome; E gene (Fruh et al., 1999; Khan, 2007)
US3	decreases MHC class I cell surface expression, retains MHC class I in the ER, impairs assembly of the loading complex; IE gene (Ahn et al., 1996; Fruh et al., 1999; Khan, 2007)
US6	decreases MHC class I cell surface expression, inhibits TAP by binding with it in the ER lumen and prevents ATP-dependent peptide translocation (Ahn et al., 1997; Fruh et al., 1999; Mocarski, 2002; Khan, 2007)
US10	delays trafficking out of ER (Khan, 2007)
US11	decreases MHC class I cell surface expression, induces translocation from the ER to the cytosol for degradation by the proteasome; E gene (Fruh et al., 1999; Khan, 2007)
HSV	
ICP47	binds cytosolic side of TAP and prevents peptide translocation into the ER; empty MHC class I molecules are then degraded (York et al., 1994; Hill et al., 1995)
adenovirus	
E3/19K	retains MHC class I in the ER and prevents docking with TAP, mediated by tapasin (Andersson et al., 1985; Burgert and Kvist, 1985; Yewdell and Hill, 2002)
KSHV	
K3	accelerates endocytosis of MHC class I by ubiquitylating its tail, followed by degradation in endolysosomes; selective downregulation of HLA allotypes (Coscoy and Ganem, 2000; Ishido et al., 2000; Yewdell and Hill, 2002)
K5	ubiquitylates MHC class I tail and targets it to lysosomes; also targets ICAM-1 and B7-2 (Yewdell and Hill, 2002)
HIV	
Nef	accelerates endocytosis of cell surface MHC class I, uses the phosphofurin acidic cluster sorting protein (PACS)1 sorting protein to remove MHC class I and sequester it in the trans-Golgi (Piguet et al., 2000)
Vpu	degrades newly synthesized MHC class I (Yewdell and Hill, 2002)
γ-HV68	
K3	accelerates endocytosis of MHC class I; binds MHC class I in the ER, induces retrotranslocation to the cytosol for degradation by the proteasome; lytic gene (Stevenson et al., 2000; Boname and Stevenson, 2001)

Table II. Other immune evasion mechanisms used by cytomegaloviruses

Gene	Immune evasion mechanism
MCMV	
unknown	inhibition of MHC class II proteins via induction of IFN-β and IL-10 production (Heise et al., 1998a; Redpath et al., 1999)
M27	downregulates STAT-2 by binding it and inducing ubiquitylation and proteasome degradation (Zimmermann et al., 2005)
m38.5	protects from Bax-, but not Bak-mediated apoptosis; a viral mitochondrial inhibitor of apoptosis (vMIA) (Jurak et al., 2008)
M45	homolog of ribonucleotide reductase, prevents apoptosis in macrophages and endothelial cells; inhibits TNF-α-induced activation of NFκB and caspase-independent cell death (Brune et al., 2001a; Mack et al., 2008)
m131/m129	chemokine homolog similar to CC chemokines; monocyte/macrophage-attracting, pro-inflammatory (MacDonald et al., 1999; Mocarski, 2002)
m138	immunoglobulin G (IgG) Fc receptor (Thale et al., 1994)
m144	MHC class I homolog, interferes with NK cell-mediated clearance (Farrell et al., 1999)
m147.5	decreases surface expression of the co-stimulatory molecule CD86 (B7.2) (Loewendorf et al., 2004)
m152	binds NKG2D ligands (H-60, Rae-1 family), preventing activation of NK cells; does not work in Ly49H$^+$ mice (Krmpotic et al., 2002; Lodoen et al., 2003)
m155	decreases expression of the NKG2D ligand H-60, preventing activation of NK cells (Hasan et al., 2005)
m157	binds to the inhibitory NK cell receptor, Ly49I; escape mutants develop that can no longer bind to the NK activatory receptor, Ly49H (Arase et al., 2002)
HCMV	
unknown	viral IL-10 homolog (Mocarski, 2002)
US2	blocks translocation of MHC class II proteins (Tomazin et al., 1999)
UL16	binds non-classical MHC class I molecules (MICB, ULBP1, ULBP2), preventing them from binding NKG2D (Mocarski, 2002)
UL18	MHC class I homolog, possible inhibitory effect on leukocyte Ig-like receptors (Leong et al., 1998; Mocarski, 2002; Khan, 2007)
US28	receptor for most human CC chemokines and fractaline (CX3C) (Mocarski, 2002)
UL36	viral inhibitor of caspase activation (vICA), inhibits caspase-8 activation (Skaletskaya et al., 2001)
UL37x	vMIA, acts in manner similar to members of the Bcl family (Goldmacher et al., 1999)
UL40	encodes a peptide that binds to host MHC class I molecules, which then bind the inhibitory NKG2A receptor (Mocarski, 2002)
UL69	induces a block in the G1 phase of the cell cycle (Hayashi et al., 2000)
UL112	microRNA that downregulates an NKG2D ligand (Khan, 2007)
UL118/119	IgG Fc receptor (Mocarski, 2002)
UL141	blocks expression of CD155, a ligand for an activating NK cell receptor (Khan, 2007)
UL146/147	viral chemokine ligand (CXCL-1) homolog, induces chemotaxis of neutrophils and degranulation (Mocarski, 2002)
IE1, IE2	prevents TNF-induced apoptosis (Murphy et al., 2000; Mocarski, 2002; Yu and Alwine, 2002)

In viral infections in which the immunodominance hierarchy has been measured, there is typically a reliable order of the CD8 T cell response, whereby one (dominant) or perhaps several (co-dominant) epitopes drive a significant proportion of the response, while subdominant epitopes constitute the rest. The priming of CD8 T cells to the MCMV epitopes falls into an immunodominance hierarchy that is remarkably similar from mouse to mouse. In BALB/c mice, the acute CD8 T cell response to MCMV-Smith is co-dominated by epitopes derived from the IE1/pp89 and m164 proteins, and there are five known additional subdominant epitopes (Reddehase and Koszinowski, 1984; Reddehase et al., 1986; Holtappels et al., 2002; Holtappels et al., 2002). In acute MCMV-BAC infection of B6 mice, the largest population of MCMV-specific CD8 T cells is generated against the M45-derived peptide, followed by epitopes from m139, M57, and m141 (Munks et al., 2006b).

We now also know that the CD8 T cell response to some epitopes increases over time and the cells acquire an effector memory T cell phenotype, a phenomenon dubbed "memory inflation" (Karrer et al., 2003). In BALB/c mice, the co-dominant CD8 T cell response to IE1/pp89 and m164 increases over time (Holtappels et al., 2000; Karrer et al., 2003). In B6 mice, there is a different pattern. The response to the immunodominant epitope in acute infection, M45, actually decreases over time, while the response to the subdominant epitopes, m139 and M38, increases or "inflates" over time (Munks et al., 2006a; Snyder et al., 2008). Finally, the response to IE3, which is essentially undetectable in acute infection inflates to become co-dominant in chronic infection (Munks et al., 2006a).

As will be shown in Chapter 3, the acute CD8 T cell response is larger in B6 than in BALB/c mice. The responses to individual antigens cannot be directly compared, as the two strains have different MHC class I restriction and, thus, a different panel of epitopes. What determines the magnitude of the acute CD8 T cell response to a pathogen is not entirely clear. The size of the antigen load, the size and availability of the APC population, the CD8 T cell precursor frequency, T cell avidity, and the presence of proper co-stimulation and cytokine

signals have all been reported to impact the size of the acute response (De Boer et al., 2001; Kaech and Ahmed, 2001; van Stipdonk et al., 2001; Wong and Pamer, 2001; Badovinac et al., 2002; Foulds et al., 2002; Wherry et al., 2002; Badovinac et al., 2003; Haring et al., 2006; Prlic et al., 2006; Badovinac et al., 2007; Andrews et al., 2008; Masson et al., 2008).

CD8 T cell priming versus recognition in MCMV infection

The immunodominant epitopes in MCMV infection were identified by infecting BALB/c or B6 mice with wild type MCMV. Because wild type MCMV contains the full spectrum of MHC class I immune evasion genes, the immunodominance hierarchy observed after infection represents the CD8 T cell response generated in spite of hindered antigen presentation. The MHC class I immune evasion genes are not equally expressed throughout the viral cycle—they are all E genes. It was expected that an epitope from a gene expressed *before* the MHC class I immune evasion genes should escape any effect of the immune evasion proteins and would be a good candidate for an immunodominant epitope in MCMV infection. This is not the case in B6 mice, as none of the immunodominant epitopes in acute infection come from IE genes. The very fact that MCMV-specific CD8 T cells can be primed and indentified tells us one very important thing: MHC class I immune evasion is not absolute enough to completely prevent a primary CD8 T cell response. It remained to be determined, however, how effective CD8 T cells primed against wild type MCMV would be when compared to T cells primed against a virus lacking one or more of the MHC class I immune evasion genes.

Gold anticipated that since M45 was so immunodominant in B6 mice, it would also be protective. However, she found that M45-specific CD8 T cells can only lyse fibroblasts infected with a virus that lacks *m152* (Δm152-MCMV, strain MW99.05) and cannot lyse fibroblasts infected with MCMV-BAC (Gold et al., 2002). This result was unexpected because it provides evidence of CD8 T cell priming to a peptide that the primed T cells can then not recognize on infected cells. This finding had only been observed with the M45 peptide, so it was unclear

whether the difference in priming and recognition was a universal paradox or a result confined to this particular epitope. Once the additional MCMV epitopes were available, we wanted to quickly determine whether the immune evasion genes had the same effect on CTL responses specific to each of the newly identified epitopes, that is: Do the immune evasion genes affect *in vitro* cytotoxicity, while having little impact on priming a CD8 T cell response *in vivo*?

Once we had a peptide panel to work with, we were ready to address whether the M45 paradox held true for a variety of epitopes and determine what role the MHC class I immune evasion genes held. Amelia Pinto, a former graduate student, pursued this question and found that the phenotype is not subtle. Using infected IC21 targets (a macrophage-like, transformed cell line) and a standard assay for antigen recognition, the ^{51}Cr release assay, she demonstrated that 16 different MCMV peptide-driven CD8 T cell lines can kill cells infected with a virus lacking the three MHC class I immune evasion genes, Δm04+m06+m152-MCMV, but cannot kill cells infected with MCMV-BAC (Pinto et al., 2006). This finding has also been repeated using primary macrophages (Pinto et al., 2006). Therefore, MCMV-specific CTL are able to kill infected cells *in vitro* only when the MHC class I immune evasion genes are absent.

A number of other studies have shown that MCMV-specific CD8 T cells can kill cells infected with a mutant virus lacking one or more of the MHC class I immune evasion genes but not cells infected with wild type MCMV (Krmpotic et al., 1999; Hengel et al., 2000; Kavanagh et al., 2001a; Gold et al., 2002; LoPiccolo et al., 2003; Gold et al., 2004; Holtappels et al., 2004; Pinto et al., 2007). It should be noted, however, that this phenotype is not absolute. The Koszinowski and Reddehase groups have reported that MCMV-Smith-infected mouse embryonic fibroblasts (MEFs), primary macrophages, and a macrophage cell line can be killed by IE1/pp89-specific CD8 T cells from BALB/c mice (del Val et al., 1992; Hengel et al., 2000; Holtappels et al., 2002). Our laboratory also has evidence that some primary DCs infected with MCMV-BAC can be killed by CD8 T cells of some specificities (A. Pinto, personal communication). The full implications of these results remain to be explored. However, when wild type MCMV and MHC

class I immune evasion mutants have been compared side by side, killing of mutant virus-infected cells is almost always much more efficient.

In vivo data comparing wild type MCMV and MHC class I immune evasion mutants

While *in vitro* experiments are important in delineating the functions of individual genes, the ultimate test is what happens *in vivo*. Before discussing CD8 T cell control, however, a second biological function for *m152* must be mentioned. In addition to its MHC class I immune evasion function, m152 also impacts NK cell control in BALB/c mice by downregulating expression of RAE-1 ligands for the activatory receptor, NKG2D (Krmpotic et al., 2002). Therefore, when BALB/c mice are infected with *m152*-expressing MCMV-Smith, they lack NK cell activation both by the Ly49H *and* NKG2D receptors. m152 does not impact NKG2D receptor-mediated activation in B6 mice (Krmpotic et al., 2002), thus in wild type MCMV infection of these mice, activation via Ly49H and NKG2D is intact. NK cell function can be somewhat restored by infecting BALB/c mice with Δm152-MCMV (*i.e.* the activatory receptor ligand is no longer downregulated). Under these conditions, the virus is better controlled by NK cells, as evidenced by lower titers in the lungs in the first few days of infection (Krmpotic et al., 2002). Thus, NK cell control of mutant MCMVs could contribute to any differences seen between wild type MCMV and mutant virus infection, so it was necessary to identify the cell type(s) responsible via antibody-mediated cell subset depletion studies.

<u>Immunocompromised BALB/c mice</u>

As described above, because of the redundancy in immune control of MCMV, the antiviral potential of T cells has been best demonstrated in immunocompromised mice. Accordingly, the impact of the MHC class I immune evasion genes on T cell control of virus is also most clearly seen in immunocompromised animals, which have been manipulated so that the burden of control falls most heavily on CD8 T cells. When irradiated mice infected with either

Δm152-MCMV or a revertant (wild type phenotype) virus receive transferred lymphocytes isolated from the spleens of latently-infected mice, Δm152-MCMV is always better controlled in the lungs at two weeks p.i. (Krmpotic et al., 1999). In the liver, transfer of non-primed lymphocytes completely controls Δm152-MCMV, but not revertant virus; when primed cells are transferred, both viruses are completely controlled. This was the first *in vivo* evidence that the immune evasion gene, *m152*, has an impact on CD8 T cell control.

Immunocompromised C57BL/6 mice

B6 mice have also been used in the model of hematoablation by γ-irradiation. The role of *m152* was tested by comparing virus loads in visceral organs following MCMV-BAC or Δm152-MCMV infection after transfer of D^b-restricted M45-specific CD8 T cells (both peptide-driven lines or memory cells taken directly *ex vivo*) (Holtappels et al., 2004). In the spleen, transfer of low numbers of M45-specific CD8 T cells completely controls Δm152- but not MCMV-BAC at 12 days p.i.; in the lung, there is over a two log decrease in Δm152-MCMV viral load dependent on the number of transferred cells. In the liver, instead of looking at virus titers, an elegant two-color ISH technique was used after co-infection of B6 mice with mutant and revertant virus. Liver sections are stained for virus and the readout for Δm152-MCMV infection is red and for the Δm152-MCMV revertant, black. Thus, infected cells can be individually visualized and the infecting virus identified. M45-specific cells can again kill cells infected with Δm152-MCMV, but have no impact on the cells infected with wild type revertant virus. At the highest number of cells transferred, the number of Δm152-MCMV infected cells in the liver is at the limit of detection. In another set of experiments by Holtappels *et al*. using the same model, a Δm04+m06-MCMV mutant could not be controlled in the lung or the liver by M45-specific CD8 T cells, but a Δm06-MCMV mutant could be controlled with graded numbers of transferred cells (Holtappels et al., 2006). These studies suggest that m152 is the principal negative regulator of antigen presentation, that m04 is a positive regulator—restoring presentation in the face of m152

expression, but that m06 (which is present in all wild isolates) (Smith et al., 2006) can overrule the positive effect of m04.

Immunocompetent C57BL/6 and BALB/c mice

CD8 T cell response

The MHC class I immune evasion genes allow virus to escape otherwise stringent CD8 T cell control in severely immunocompromised mice when adoptively transferred CD8 T cells are, in effect, the only functioning arm of the immune system. There has been rather little evidence, however, for a phenotype of these genes in MCMV-BAC-infection of immunocompetent mice. It was expected that when mice were infected with Δm04+m06+m152-MCMV, there would be a different immunodominance hierarchy and/or a different magnitude of the CD8 T cell response compared to mice infected with MCMV-BAC. The first surprising finding—given the robust MHC class I downregulation in MCMV-BAC-infected cells and the impact on cytotoxicity *in vitro*—was that BALB/c and B6 mice mount strong and virtually identical acute CD8 T cell responses to MCMV-BAC and Δm04+m06+m152-MCMV (see Chapter 3; Gold et al., 2002; Holtappels et al., 2004; Munks et al., 2007).

These results have led to a presumption that the CD8 T cell response to MCMV must be primed *in vivo* by cross-presented antigen, although this remains to be verified. However, the real question of interest in evolutionary terms is how the immune evasion genes impact viral fitness. Two types of readout have been used to identify a potential role of the MHC class I immune evasion genes—the CD8 T cell response and viral load measured by plaque assay or IHC/ISH. As mentioned, the CD8 T cell response in chronic infection undergoes “memory inflation” (Karrer et al., 2003). The size and phenotype of the CD8 T cell response in chronic infection may be the best indicator of virus activity; therefore, we use the antigen-specific CD8 T cell response as a readout for immune control of the virus—a proxy measurement for virus activity. In analyzing

the CD8 T cell phenotype and response in chronic infection of B6 mice, however, it is nearly identical following infection with wild type MCMV, Δm152-MCMV, or Δm04+m06+m152-MCMV (see Chapter 3; Gold et al., 2002; Gold et al., 2004; Munks et al., 2007). The CD8 T cell response following chronic infection with MCMV-BAC or Δm04+m06+m152-MCMV in acute and chronic infection is also the same in BALB/c mice (see Chapter 3; Reddehase et al., 2004). Thus, the ongoing immune response gives no sign that there is any difference in the activity of wild type or mutant MCMV during latent infection.

Might the MHC class I immune evasion genes not affect the CD8 T cell response, but still impact viral control? Finally, the answer, in some circumstances is, “Yes.”

Viral control

Immunocompetent BALB/c mice infected i.p. with high dose TC- or salivary glands-derived Δm04+m06+m152-MCMV control the virus in the salivary glands more effectively than MCMV-BAC between two and four weeks p.i. (Lu et al., 2006). Importantly, the difference in virus control is CD8 T cell-dependent, as anti-CD8 antibody depletion equalizes the titers. This is key *in vivo* evidence that the MHC class I immune evasion genes *do* benefit the virus in the face of a fully intact, functional immune system—at least in the salivary glands, the site of viral dissemination.

In immunocompetent B6 mice, no differences have been detected in the virus genome copy number between infection with MCMV-BAC and Δm04+m06+m152-MCMV. This was determined in the lung, liver, and kidney between days zero and 14 p.i.; the viruses show the same kinetics in these organs and all peak around three to five days p.i. (Gold et al., 2004). In our hands, we rarely find any virus in the salivary glands of B6 mice by plaque assay.

Neonates: BALB/c and C57BL/6

Interestingly, the first report of the function of the MHC class I immune evasion genes described infection in neonates, in both BALB/c and B6 pups. BALB/c pups were infected i.p. with low dose virus at 12 hours of age (Krmpotic et al., 1999). Their survival and organ viral titers were measured, and at day eight p.i., Δm152-MCMV is better controlled in the lungs and spleen than revertant, wild type MCMV. This translates into a survival difference: mice infected with wild type MCMV-Smith have much lower survival rates through day 30 p.i. In slightly older, four-day-old BALB/c pups, Δm152-MCMV is again better controlled than wild type MCMV in the lungs at day 10 p.i. When CD4 and CD8 T cells are depleted, the titers increase and equalize, implicating a role for T cell control. In four-day-old B6 pups infected under the same conditions, lung titers at day 10 p.i. are also lower in Δm152-MCMV infection, and this difference is abrogated with CD4 and CD8 T cell depletion (Krmpotic et al., 1999). Since infection of neonates is likely an important means of virus transmission in the wild, the ability of immune evasion to impact infection in this setting probably has real biological significance.

1.9 Conclusions

MCMV-specific CD8 T cells are better able to control MCMV infection *in vitro* and *in vivo* if at least one of the MHC class I immune evasion genes is missing. However, in a fully immunocompetent animal, infection with high dose MCMV-BAC or a mutant lacking the three MHC class I immune evasion genes results in the same global CD8 T cell response to the virus. The CD8 T cell response magnitude and specificity and the amount of virus in different organs is exactly the same in B6 mice. In BALB/c mice, the response is the same except for the salivary glands titers, whereby Δm04+m06+m152-MCMV is somewhat better controlled.

We thus know two settings in which MHC class I evasion genes have a phenotype *in vivo* in immunocompetent mice: in the periphery of neonates and in the salivary glands of adult mice. Both of these could offer clear selective advantage to the virus. However, the phenotype is rather mild and does not necessarily justify the striking evolutionary conservation of these genes. This

consideration led me to ask: Are there other biologically relevant conditions under which the MHC class I immune evasion genes have a phenotype *in vivo*?

Chapter 2: MATERIALS AND METHODS

Cells

All cells were grown at 37˚C in the presence of 5% CO_2. The following cell types were grown in "DMEM-complete" (Dulbecco's minimal eagle medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units penicillin/mL, and 100 µg/mL streptomycin): K41 (SV-40-transformed H-2^b fibroblasts, a kind gift from Marek Michalak, University of Alberta, Edmonton, Canada), B16-F1 (murine melanoma cells, a kind gift from Glen Dranoff, Harvard Medical School, Cambridge, MA), $K^{b-/-}$ fibroblasts (SV-40-transformed dermal fibroblasts from adult $K^{b-/-}$ mice), BALB/c 3T3 (immortalized murine embryonic fibroblasts, American Type Culture Collection (ATCC)), L929 (murine fibrosarcoma cell line, ATCC), 293A (human embryonic kidney cells), and primary murine embryonic fibroblasts (MEFs). RAW 264.7 (a macrophage-like cell line, a kind gift from Fred Heffron, OHSU, Portland, OR) were grown in DMEM-complete supplemented with 10 mM HEPES. IC21 cells, an SV-40-transformed H-2^b macrophage cell-line (Cavanaugh et al., 1996) were a kind gift from Ann Campbell (East Virginia Medical School, Norfolk, VA). They were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 100 units penicillin/mL, and 100 µg/mL streptomycin. gL-expressing 3T3s were generated by and were a kind gift from Jane Allan (The University of Western Australia, Fremantle, Australia). They were grown in DMEM-complete under 700 µg/mL Geneticin selection.

Primary MEFs were generated by harvesting embryos from C57BL/B6 dams at between 11 and 16 days gestation. Embryos were extensively minced and digested in trypsin for 18 hours at 4˚ C. They were seeded at a concentration of 10^6 cells/mL and grown until approximately 80% confluency, harvested, aliquoted, and frozen at a concentration of $5x10^6$ cells/mL for further use between passages 2 and 6. Primary bone marrow DCs were harvested from the femurs of female

B6 mice in a modification of Lutz *et al*. (Lutz et al., 1999). To increase the percentage of DCs in the bone marrow, mice were injected s.c. with $5x10^5$ B16-F1 tumor cells 10-12 days before harvest, when a tumor approximately 2-3 mm in size was palpable. The B16-F1 cells have been transfected with Flt3L, which has been shown to enrich cells with a DC phenotype *in vivo* (Maraskovsky et al., 1996; Shi et al., 1999). Cold RPMI complete was flushed through the femurs and the marrow plug was resuspended by flushing it through a 30-gauge needle. The cells were grown on 150 mm, non-tissue culture-treated plates in RPMI-complete supplemented with 50 μM β-mercaptoethanol, 25 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF), 5 ng/mL IL-10, and 50 ng/mL IL-4. Eight to 10 days later, adherent cells were harvested by vigorous pipetting and used in experiments. The phenotype of the cells was checked before use in experiments by fluorescence-activated cell sorting (FACs). Primary bone marrow macrophages were isolated as described above for DCs and grown in DMEM-complete supplemented with 30% macrophage colony-stimulating factor (M-CSF) from L929 supernatant. The supernatant was harvested from L929 cells grown for 10 days after reaching confluence in serum-free media. Six to 10 days after bone marrow harvest, adherent cells were harvested by incubating them in cold PBS for 5 minutes at 4˚C and used in experiments. The phenotype of the cells was checked before use in experiments by FACs.

Viruses

Wild-type, BAC-derived MCMV strain MW97.01 (“MCMV-BAC”), Δm139-141-MCMV (Menard et al., 2003), and Δm04+m06+m152-MCMV (Wagner et al., 2002) were generated by infecting subconfluent, primary B6 MEFs, grown in DMEM-complete, at a multiplicity of infection (MOI) of 0.01. When the monolayer showed 100% cytopathic effect (CPE), the plates were scraped and all cells and supernatant were purified by ultracentrifugation at 22,000 rpm through a 15% sucrose cushion. MCMV strain K181 (a kind gift from Ed Mocarski, Stanford University, Palo Alto, CA) and MCMV strain N1 (a kind gift from Alec

Redwood, The University of Western Australia, Fremantle, Australia) were grown on B6 MEFs. Crude viral lysates of these 2 strains were frozen and thawed three times and aliquoted. The final virus titers were calculated by taking the mean of at least three virus titrations.

MNV-1 strain CW3 and MNV-1 strain CR6 (a kind gift from H.W. Virgin, Washington University, St. Louis, MO) were grown on RAW 264.7 cells and infected at an MOI of 0.05. Cell lysates were frozen and thawed three times and clarified by centrifugation at 3000 rpm for 20 minutes. For concentrated stocks, lysates were centrifuged at 27,000 rpm for 3 hours at 4˚C. Virus stocks were titered on RAW 264.7 cells as described below. The final virus titers were calculated by taking the mean of three virus titrations. Mice were infected p.o. with $3x10^7$ PFU in a volume of 10 µL, unless otherwise specified. ΔgL-MCMV was a kind gift from Jane Allan and was propagated on gL-expressing 3T3s or by co-infection with a gutless adenovirus expressing gL (Ad-gL, described below).

Mice

Female C57BL/6, 129/SvJ, BALB/c, BALB.B (C.B10-*H2b*/LilMcdJ), and B6.C*H2d*/bByJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Charles River (Wilmington, MA) and maintained in our animal facilities at Oregon Health and Science University (Portland, OR). BALB/c $H\text{-}2^{bxd}$ mice were the F1 generation of a BALB/c and BALB.B (C.B10-*H2b*/LilMcdJ) cross. B6 $H\text{-}2^{bxd}$ mice were the F1 generation of a C57BL/6 and B6.C*H2d*/bByJ cross. BALB/c pups were infected between 12 hours and 5 days of age and housed with the original dam. $CD40^{-/-}$ mice ($H\text{-}2^{d}$, BALB/c background) were a kind gift from Dave Hinrichs, Veterans Affairs Hospital, Portland, OR). All mice were housed and maintained in our animal facilities at Oregon Health and Science University according to the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee guidelines. Mice were given access to food and water *ad libitum* and housed in a specific-pathogen-free facility that monitors sentinel animals for 13 different pathogens every 3 months. None of the sentinels

for the animals used in these experiments were ever positive for any of the monitored pathogens.

Antibodies

PE-conjugated anti-IFN-γ (XMG1.2) and CyChrome-conjugated anti-CD8-α (53-6.7) were purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Anti-Ly49H was a kind gift from W. Yokoyama, Washington University, St. Louis, MO. Anti-IE1/pp89 (Del Val et al., 1988) was purified on a protein A column (Sigma-Aldrich, St. Louis, MO) and conjugated to FITC (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

CTL lines

Female C57BL/6 mice were infected with 5×10^6 PFU of MCMV-BAC or Δm04+m06+m152-MCMV. Mice were infected for at least 4 weeks before spleens were harvested for CTL lines. Upon harvest, spleens were dissociated into single-cell suspensions with nylon mesh strainers and cultured in RPMI-complete with 50 μM β-mercaptoethanol. "Feeder" spleens were harvested from uninfected mice that had been injected s.c. with 5×10^5 B16-F1 tumor cells 10-14 days before harvest. Feeder splenocytes were dissociated into single-cell suspensions, cultured in RPMI-complete, γ-irradiated (3000 rads), and pulsed with 10^{-8} M peptide. Peptides were synthesized by Jerini (Berlin, Germany), suspended in DMSO at a concentration of 2 mg/mL, and stored at -20˚C. The H-2^d-restricted peptides used include: IE1/pp89$_{168-176}$ (YPHFMPTNL), m164$_{257-265}$ (AGPPRYSRI), M18$_{346-354}$ (SGPSRGRII), M45$_{507-515}$ (VGPALGRGL), M83$_{761-769}$ (YPSKEPFNF), M84$_{297-305}$ (AYAGLFTPL), and m04$_{243-251}$ (YGPSLYRRF). The H-2^b D^b-restricted peptides used include: M45$_{985-993}$ (HGIRNASFI), M86$_{1062-1070}$ (SQNINTVEM), M36$_{213-221}$ (GTVINLTSV), m04$_{1-9}$ (MSLVCRLVL), M33$_{47-55}$ (GGPMNFVVL), M44$_{130-138}$ (ACVHNQDII), and m164$_{267-275}$ (WAVNNQAIV). The H-2^b K^b-restricted peptides used include: m139$_{419-426}$ (TVYGFCLL), m141$_{16-23}$ (VIDAFSRL), M78$_{8-15}$

(VDYSYPEV), $M57_{816-824}$ (SCLEFWQRV), $M38_{38-45}$ (STYTFVRT), $M97_{210-217}$ (IISPFPGL), $M100_{72-79}$ (RIIDFDNM), and $m164_{283-290}$ (GTTDFLWM). Feeder splenocytes were mixed with splenocytes from MCMV-infected mice. On the third day of culture, 10 U/mL of recombinant IL-2 (eBioscience) was added; IL-2-containing media was replenished every 2 days thereafter. Twenty-four to 48 hours prior to use in an experiment, cells were taken out of IL-2 containing media. After 10 to 14 days of culture, cells were used for intracellular cytokine staining or an ELISA measuring IFN-γ.

Plaque assay

MCMV: BALB/c 3T3 cells were seeded at 1.5×10^5 cells/well in 6-well plates (BD Biosciences, San Jose, CA) and grown overnight. Either purified virus preparations or 10% organ homogenates that had been clarified by centrifugation were added in 10-fold dilutions to 1 mL of freshly-replaced media/well. Ninety minutes later, the wells were overlaid with 4 mL of a 3:1 mixture of media and 15% w/v carboxymethylcellulose (CMC; Sigma-Aldrich). When assaying viral titers from organ homogenates, the media was removed from the wells before overlay. On day 6, the plates were rinsed with PBS and stained with 0.1% crystal violet in 1% formalin (BD Biosciences) for 5 minutes. The plates were rinsed again in PBS and allowed to dry. Plaques were then visualized on a light source and counted.

MNV: RAW 264.7 cells were seeded at 2×10^6 cells/well in 6-well plates and grown overnight. Either purified virus preparations or 10% organ homogenates that had been clarified by centrifugation were added in 10-fold dilutions to 500 µL of freshly-replaced media/well. After rocking for 1 hour at room temperature, each well was overlaid with 2 mL of a 1:1 mixture of 2X MEM and 3% Seaplaque (Lonza, Basel, Switzerland). Two days later, each well was overlaid with 2 mL of a 1:1 mixture of 2X MEM, 3% SeaKem (Lonza), including 0.01% neutral red dye. Twelve hours later, plaques were visualized by microscope and counted.

Intracellular cytokine staining (ICCS)

Effector cells were either CTL lines or splenocytes taken directly *ex vivo* from MCMV-infected mice. Effectors cells (2-6x10^6) were stimulated directly with 10^{-6} M peptide or with MCMV-infected cells in the presence of brefeldin A (BFA or "Golgi Plug," BD Biosciences) for 7 hours. MCMV-infected stimulator cells included K41, IC21, or primary MEFs, infected overnight at an MOI of 10, 3, 1, 0.3, and 0.1 with MCMV-BAC or a mutant MCMV virus in the presence of 0.3 mg/mL phosphonoacetic acid (PAA, Sigma-Aldrich) to inhibit viral DNA replication and late protein synthesis. Mutant viruses included Δm139-141-MCMV and Δm04+m06+m152-MCMV. T cells were incubated in round-bottom 96-well plates in a total volume of 200 µL. After stimulation, cells were stained with anti-CD8-α antibody, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with anti-IFN-γ. Cells were acquired by flow cytometry on an LSR-II flow cytometer (BD Biosciences) using Cell Quest acquisition software (BD Biosciences). All further analysis was done using FloJo software (Treestar, San Carlos, CA).

Enzyme-linked immunosorbent assay (ELISA)

To test for MCMV or MNV seropositivity, high-binding Immulon 4HBX microtiter plates (Thermo, Milford, MA) were coated either with MCMV-infected cell lysate (or mock-infected cell lysate) or semi-purified MNV virions. Sera was serially diluted at least 4 times and tested in triplicate. The primary antibody, horseradish peroxidase-conjugated, goat anti-mouse IgG (heavy and light chains, Biorad, Hercules, CA), was diluted 1:1000. The substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Calbiochem, Gibbstown, NJ) was used for the following ELISAs, and chemiluminescence was measured at 405 nm by a Biorad Benchmark Plus spectrophotometer (Biorad). Data was acquired using the Microplate Manager 5.2 software program (Biorad). All sera used in ELISAs were collected by retro-orbital bleeding. Blood

samples were allowed to coagulate overnight at 4˚C and then were spun at 2 rpm for 20 minutes. The sera were aliquoted into multiple tubes for further use. Some sera samples were also sent for commercial testing to University of Missouri Research Animal Diagnostic Laboratory (RADIL; Columbia, MO).

IFN-γ: Effector cells were CTL lines; they were stimulated either directly with 10^{-6} M peptide or with MCMV-infected cells. MCMV-infected stimulator cells included K41, IC21, primary macrophages, primary DCs, or primary MEFs, infected overnight at an MOI of 10, 3, 1, 0.3, and 0.1 with MCMV-BAC or a mutant MCMV virus in the presence of 0.3 mg/mL PAA to inhibit viral DNA replication and late protein synthesis. Mutant viruses included Δm139-141-MCMV and Δm04+m06+m152-MCMV.

MNV virion preparation

MNV virions were purified by the method describe in Wobus *et al*., with some modification (Wobus et al., 2004). Briefly, RAW 264.7 cells were seeded at $4x10^7$ cells/well in T175 flasks (Falcon) and grown overnight. The cells were infected at an MOI of 0.05 with MNV-1 CW3 and incubated for 48 hours. The cells and supernatant were harvested and frozen (-80˚C) and thawed 3 times before being centrifuged at 3000 rpm for 20 minutes. The supernatant was then centrifuged through a 30% sucrose cushion at 27,000 rpm for 3 hours at 4˚C. The supernatant was removed and virus was resuspended in a total of 2 mL of PBS, aliquoted, and stored at -20˚C.

Polymerase chain reaction (PCR)

PCR to verify the genotype of MCMV-BAC (MW97.01) and Δm04+m06+m152-MCMV virus preparations was performed with *pfu* polymerase (Stratagene, La Jolla, CA) and primers for *m04*, *m06*, and *m152*. The PCR cycles included 1 round of denaturation at 94˚C for 2 minutes, 35 rounds of amplification (94˚C for 1 minutes, 58˚C for 1.5 minutes, and 72˚C for 2.5 minutes), and

one round of extension at 72˚C for 10 minutes. Bands representing *m04*, *m06*, and *m152* were visualized by 1.5% gel electrophoresis.

Construction of Ad-gL

PCR to amplify the MCMV *gL* gene were performed on MCMV-BAC (MW97.01) with *pfu* polymerase and the primers: TCA CGG TCT CTT TCG TTG ATA TTG A and CCA CCA TGG CTT TAT TAT TGC TCA TAC TGC TGT CCA C. The PCR cycles included one round of denaturation at 94˚C for 5 minutes, 80˚C for 5 minutes, 30 rounds of amplification (94˚C for 1 minute, 58˚C for 1 minute, and 72˚C for 2 minutes), and 1 round of extension at 72˚C for 10 minutes. The PCR product was visualized by 1.5% gel electrophoresis and the band representing *gL* was cut out and the DNA extracted using a QiaQuick gel extraction kit (Qiagen, Valencia, CA). A *gL*-expressing adenovirus (Ad-gL) was constructed using a ViraPower Adenoviral Expression System (Invitrogen), per manufacturer's instructions. Briefly, the cloned *gL* gene was inserted into a pENTR/SD/D-TOPO vector and transformed into One Shot Mach1 competent *E. coli*. The correct insert was confirmed by sequencing, recombined into a pAD/PL-DEST gutless adenovirus vector under an EF1α promoter (made by A. Pinto), and transformed into DH10B cells. The insert was again sequenced and then transfected into 293A cells using Fugene (Roche) to make a crude viral lysate. The lysate was used to infect 293A cells to make an amplified adenovirus stock.

Detection of virus in tissues

Tissues were harvested in an aseptic manner in a biological safety hood. Unless otherwise specified, the spleen and salivary glands were harvested into 1 mL DMEM complete and stored on ice. Other organs were harvested into Eppendorf tubes and flash frozen in liquid nitrogen. The spleen and salivary glands were homogenized on the day of harvest using tissue grinders (Wheaton, Millville, NJ), aliquoted, stored at -80˚C, and frozen and thawed 3 times before titers

were measured by plaque assay. Snap frozen organs were thawed, suspended in 1 mL of DMEM complete (except for the liver, which was suspended in 2 mL of DMEM complete), homogenized, and frozen and thawed 3 times before titers were measured by plaque assay. On the day of use, aliquots were centrifuged at 300 rpm for 20 minutes to clarify the supernatant.

Real time-PCR

Viral DNA was extracted using a High Pure Viral Nucleic Acid kit (Roche, Basel, Switzerland). Real-time PCR was performed according to the manufacturer's instructions using a Quantitative PCR ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) to detect the MCMV IE1/pp89 gene exon 1. The following IE1/pp89 primers were used: TCG CCC ATC GTT TCG AGA and TCT CGT AGG TCC ACT GAC GGA. PCR product was detected with the probe ACT CGA GTC GGA CGC TGC ATC AGA AT labeled with 6-FAM and black hole quencher-1 (Biosearch Technologies, Novato, CA). A standard curve was generated using an IE1/pp89-expressing plasmid (a kind gift from Jay Nelson, OHSU, Portland, OR). Viral genome copy numbers were determined by reference to the standard curve.

Statistics

Unpaired, two-tailed t-tests were used to analyze the statistical significance of the CD8 T cell response. The non-parametric Mann-Whitney test was used to analyze organ viral titers by plaque assay. All statistical analyses and were performed using GraphPad Prism (La Jolla, CA).

Chapter 3. THE IMPACT OF NON-MHC GENES AND MHC CLASS I IMMUNE EVASION GENES ON THE CD8 T CELL RESPONSE TO MCMV INFECTION

The extensive literature on the immune response to MCMV provides us with an important body of information and a framework to use in planning additional investigations. When the work for this dissertation began, it was known that: mice of different strains have varying susceptibility to MCMV; the adaptive immune system is redundant in its control of MCMV; CD8 T cells are important, but not required, for control of MCMV; and, MCMV encodes three known genes that affect MHC class I. The expression of the MHC class I immune evasion genes might explain *why* CD8 T cells are not more necessary for the control of MCMV infection. We were interested in understanding more about the CD8 T cell response to MCMV and in determining how the MHC class I immune evasion genes impacted this response, both mechanistically and on a physiological level. With the help of MCMV epitope identification, the CD8 T cell response could be analyzed for size and the particular antigen-specific response. The development of mutant viruses lacking one or more of the MHC class I immune evasion genes made it possible to address our questions about the CD8 T cell response and the impact of the immune evasion genes on the immune response of this T cell subset (Wagner et al., 1999; Wagner et al., 2002).

3.1 Examination of the magnitude and antigen-specific CD8 T cell response to MCMV infection among different strains of mice

Introduction

A series of papers from Tony Scalzo's and Geoff Shellam's groups reported on the susceptibility of a variety of strains of mice to MCMV infection (Chalmer et al., 1977; Grundy et

al., 1981; Grundy et al., 1982; Allan and Shellam, 1984; Shellam et al., 1985; Lawson et al., 1988; Scalzo et al., 1990). From these findings came the discovery of Ly49H and the differences in NK cell control of MCMV in different mouse strains, as discussed in Chapter 1. The viral load of MCMV in the spleen four days p.i. is nearly four logs higher in mice lacking Ly49H (*e.g.* BALB/c) than in mice that express Ly49H (B6) (Scalzo et al., 1990). Treating B6 mice with anti-NK1.1 monoclonal antibody before infection with MCMV abolishes viral control in the spleen and their viral loads are similar to BALB/c mice (Scalzo et al., 1992).

Despite its sensitivity to the virus, a number of laboratories have employed the BALB/c mouse model of MCMV infection. The first H-2^d-restricted, MCMV-specific CD8 T cell epitope was described for BALB/c mice in 1987 (Koszinowski et al., 1987a). Various other epitopes have been reported since then, and now there is a well-characterized panel of seven MCMV-derived peptides recognized by CD8 T cells following MCMV infection in BALB/c mice (Holtappels et al., 2002). Our laboratory has primarily focused on MCMV infection of immunocompetent B6 mice and this effort led to the identification of a panel of H-2^b-restricted epitopes recognized by CD8 T cells (Munks et al., 2006b).

I was first interested in examining what factors might contribute to the size and immunodominance hierarchy of the CD8 T cell response to MCMV infection. It was evident that there were significant differences in the ability of various mouse strains to control MCMV replication, but it was not clear how this affected the CD8 T cell response in these strains. There were reports that the CD8 T cell response in BALB/c mice was lower than the response in B6 mice, however, no direct comparisons had been reported. Furthermore, the H-2^b-restricted peptide panel was so new that it had not yet been used to determine the epitope dominance in other mice strains with the H-2^b haplotype. So, I took the opportunity to ask the following questions: Does the relative susceptibility of different mice to MCMV correlate with the size of their MCMV-specific CD8 T cell response? How do differences within or outside of the MHC complex impact the immunodominance hierarchy?

Results

Genes outside of the MHC complex impact the magnitude and immunodominance hierarchy of the acute CD8 T cell response to MCMV-BAC infection

I began my *in vivo* experiments in the laboratory by asking how the epitope dominance and the magnitude of the CD8 T cell response is impacted by genes outside the MHC complex. To do this, I measured the CD8 T cell response to acute MCMV-BAC infection of three different H-2^b mouse strains: C57BL/6 ("B6"), 129/SvJ ("129") and BALB.B10 (Figure 3.1A). BALB.B10 mice have a BALB/c background but are congenic for the H-2^b complex. Of the three strains, only B6 mice express the Ly49H molecule. 129 mice have been shown to be highly susceptible to MCMV infection, BALB/c mice intermediately susceptible, and B6 mice resistant (Chalmer et al., 1977; Morello et al., 1999). In this experiment and throughout this chapter, the response at the peak of "acute" infection was measured at seven days p.i. by harvesting the spleen and performing ICCS for IFN-γ.

Surprisingly, the CD8 T cell responses elicited following acute MCMV infection were different among the three mouse strains. Each strain made a CD8 T cell response to each of the peptides tested, with the exception of M36 (in 129 and BALB.B10 mice). The hierarchy of the epitope response in BALB.B10 mice was similar to the hierarchy in B6 (where M45 is dominant followed generally by m139), but in BALB.B10 mice M45 and m139 were essentially co-dominant. The magnitude of the BALB.B10 CD8 T cell response mirrors that of BALB/c mice and was much lower than in B6 mice; the highest percentage of any epitope-specific response was 1% in BALB.B10 versus 3% in B6 mice. In 129 mice, the hierarchy was unique. The m139 response was dominant (results that are repeated and described below), followed by M45; the rest of the response was shared among epitopes that appeared to be equally subdominant. The

magnitude of the response in 129 mice was intermediate between B6 and BALB.B10, with the highest percentage of any epitope-specific response measuring 2%.

I also examined how relative immunodominance is impacted in B6 and BALB/c mice if their MHC class I haplotype differences were neutralized. H-2^{bxd} mice were generated either by crossing BALB.B10 mice with wild type BALB/c mice ("BALB H-2^{bxd} mice") or by crossing C57BL/6 mice with B6.BALB-H-2^{d} mice ("B6 H-2^{bxd} mice"). All mice have a pure background of their indicated strain but are heterozygous at the H-2 locus and express both the H-2^{b} and H-2^{d} haplotypes. The mice were infected with MCMV-BAC and the CD8 T cell response was measured to a combination of H-2^{b} and H-2^{d}-restricted peptides. BALB/c H-2^{bxd} mice responded to the immunodominant H-2^{d}-restricted peptides, IE1/pp89 and m164 (see Figure 3.1B). Interestingly, B6 H-2^{bxd} mice responded codominantly to H-2^{b}- and H-2^{d}-restricted peptides. This supports the above findings that non-MHC class I genes have a significant impact on the immunodominance hierarchy. Interestingly, the magnitude of the response to *all* of the epitopes (H-2^{b}- or H-2^{d}-restricted) was larger in the B6 H-2^{bxd} mice than the BALB H-2^{bxd} mice. This strongly suggests that non-MHC genes—and something in the B6 genotype—has a powerful impact on the magnitude of the CD8 T cell response.

Comparison of genes outside of the MHC complex on the magnitude and immunodominance hierarchy during chronic infection with MCMV-BAC infection

Because of the changes in the CD8 T cell response over time in B6 mice, I also measured the CD8 T cell response in chronic infection of different mouse strains. As mentioned in Chapter 1, the immunodominance hierarchy changes during chronic infection in B6 mice. Figure 3.2A shows that the chronic response in B6 mice was dominated by m139 and M38; the response to an IE3 epitope is also dominant in the memory response, but it had not been identified at the time of this experiment. The magnitude of the m139- and M38-specific responses in chronic infection were essentially the same as the responses to these epitopes in acute infection (compare to Figure

3.1A). The CD8 T cell response to m139 often varies slightly over time, but it remains a dominant epitope in the memory response (Munks et al., 2006a; Munks et al., 2007; Snyder et al., 2008). The M38 response typically continues to increase over time, although not dramatically so (Munks et al., 2006a; Munks et al., 2007). That the size of the response to M38 did not increase in my analysis of chronically-infected B6 mice is within the normally variability we see in the CD8 T cell response. What happens over time with MCMV infection of 129 mice had not previously been studied. As described above, the acute response in 129 mice was dominated by m139, followed by M45. In chronic infection, similar to B6 mice, the response was dominated by m139 and M38. The magnitude of the m139-specific response increased slightly, while the M38-specific response rose from <1% in the acute response to nearly 2% in the memory response. As expected, the response in BALB/c mice was co-dominated by IE1/pp89 and m164. The magnitude of the response increased and, interestingly, underwent greater inflation than the response in B6 mice.

I also measured the CD8 T cell response from the splenocytes of H-2^{bxd} mice infected with MCMV-BAC for 18 months. Figure 3.2B shows that the CD8 T cell response in long-term infection of BALB/c H-2^{bxd} mice mirrors acute MCMV infection; the dominant responses were to IE1/pp89 and m164, although the magnitude was increased, signifying memory inflation. The largest response to an H-2^{b}-restricted peptide was to m139, also seen in acute infection, though the response to m139 did not inflate over time as it does in wild type B6 mice. Surprisingly, while B6 H-2^{bxd} mice made a response to m139 in acute infection, that response was not maintained. It is clear that in chronic infection, the only peptides significantly driving the memory response in B6 H-2^{bxd} mice were H-2^{d}-restricted IE1/pp89 and m164. The response to IE1/pp89 and m164 in B6 H-2^{bxd} mice also underwent inflation. Interestingly, while the inflation was larger in the BALB/c H-2^{bxd} mice (similar to wild type BALB/c mice), the magnitude of the response to the H-2^{d}-restricted epitopes in both strains of mice was more similar in chronic than in acute infection.

The memory responses in both strains of H-2^{bxd} mice therefore appear to be driven by H-2^{d}-restricted epitopes, and nearly exclusively so in B6 H-2^{bxd} mice.

Blocking Ly49H impacts the magnitude, but not the immunodominance hierarchy, of the CD8 T cell response in C57BL/6 mice

The above results suggest that the hierarchy is not simply determined by the MHC haplotype, but that genes outside the complex influence which epitopes are the most dominant. MHC class I restriction also does not determine the magnitude of the response, as it is varied among strains with the same MHC haplotype. We hypothesized that the magnitude of the CD8 T cell response might be most affected by the ability of NK cells to control infection among different strains of mice. Originally, we expected that the CD8 T cell response would be stronger in mice with poor NK cell control (*e.g*. BALB/c mice), as there is more virus present when the CD8 T cell response is being primed. The finding that the CD8 T cell response is *lower* in BALB/c was surprising, and the reasons for it were still unclear.

In order to directly compare how NK cell control would impact the magnitude and immunodominance hierarchy of the CD8 T cell response within the same mouse strain, I treated B6 mice with an anti-Ly49H blocking antibody. When B6 mice are treated with anti-NK1.1 antibody and infected with MCMV-BAC, the virus loads at three days p.i. in the spleen, lungs, and liver increase by approximately two logs (Bubic et al., 2004). When B6 mice infected with a Δm157-MCMV mutant (that cannot engage Ly49H), the titers in the spleen and lungs are similar or even higher than the titers seen after NK cell depletion. B6 mice were mock-injected or treated with antibody before infection with MCMV-BAC. Figure 3.3 shows that the immunodominance hierarchy was nearly identical in B6 mice acutely infected with MCMV-BAC regardless of anti-Ly49H antibody treatment.

CD40-mediated co-stimulation is not required to prime a CD8 T cell response or to maintain a normal memory CD8 T cell response

Our previous studies show that the magnitude of the CD8 T cell response to MCMV infection is influenced by a number of variables, particularly the Ly49H status. As mentioned above, the magnitude of the acute CD8 T cell response is likely driven by multiple factors, including the efficiency or amount of co-stimulation. CD40 is a member of the TNF receptor superfamily and is expressed on B cells, professional APCs, and on activated CD8 T cells. Its ligand, CD40L (or CD154) is predominantly expressed on CD4 T cells. The CD40 and CD40L interaction is important in APC "licensing" and is an important mechanism by which CD4 T cells provide help to a developing immune response. CD40L stimulates maturation of APCs via CD40 ligation, and the interaction also plays a key role in inducing an antibody response (Fang and Sigal, 2005). It has been shown in some mouse models of infection that CD40-mediated co-stimulation is required for normal priming of a CD8 T cell response (polyoma virus), while in others (*Listeria monocytogenes*, LCMV, γ-HV68) priming is CD40-independent (Whitmire et al., 1996; Brooks et al., 1999; Montfort et al., 2004; Kemball et al., 2006). The role of CD40 in acute MCMV infection has only been reported in the context of B cell activation and was shown to be unnecessary (Karupiah et al., 1998).

In order to determine the requirement for CD40-mediated co-stimulation in MCMV infection, I infected wild type BALB/c and BALB/c CD40$^{-/-}$ mice with MCMV and measured the magnitude and epitope specificity of the CD8 T cell response following acute and chronic infection. Figure 3.4A shows that following acute infection, both strains exhibited a similar pattern of response to the H-2^{d}-restricted epitopes. However, the magnitude of the response was significantly greater in CD40$^{-/-}$ mice. While the background (no peptide stimulation) response was somewhat higher in CD40$^{-/-}$ mice (p=0.037), the IE1/pp89-, m164-, and M45-specific CD8 T cell responses were all significantly higher in CD40$^{-/-}$ mice (p=0.0025, 0.0019, and 0.005, respectively). In chronic infection of BALB/c or CD40$^{-/-}$ mice, again the immunodominance

hierarchy was the same, but the magnitude of the CD8 T cell response had equalized and was also identical between BALB/c and CD40$^{-/-}$ mice (see Figure 3.4B). While this is not an exhaustive study of the role of CD40 in MCMV infection, these data strongly suggest that CD40-mediated APC maturation and/or antibody induction are not necessary to prime a response to MCMV or to maintain a normal memory CD8 T cell response.

3.2 The MCMV MHC class I immune evasion genes have no impact on the priming of CD8 T cells under standard laboratory infection conditions

Introduction

Despite a number of alterations in the magnitude of the CD8 T cell response, we found it interesting that none of the situations tested above unveiled a large impact on the immunodominance hierarchy in MCMV infection. It should be pointed out that the MHC class I immune evasion genes were present in all of the viruses used in the above experiments. A number of possibilities exist for how or when the immune evasion genes might affect the immune response *in vivo*. They could affect the ability of CD8 T cells to prime an immune response to MCMV, affect the ability to establish a chronic, lifelong infection, affect the ability to contain reactivating virus, or some combination of the three. Work in our lab and others has ruled out an absolute role for the MHC class I immune evasion genes at two of these stages of viral infection, simply by virtue of the fact that MCMV-BAC-specific CD8 T cells were identified and characterized from MCMV-infected mice—implying priming (Gold et al., 2002; Munks et al., 2006b)—and that virus is rarely detected at late time points p.i.—signifying the ability of the immune response to keep reactivating MCMV under control (M. Gold, unpublished observation).

While these findings ruled out an absolute role for the MHC class I immune evasion genes, they did not rule out a quantitative or qualitative advantage provided to MCMV by the genes at any of the stages of infection. A number of studies have shown that under a few specific

circumstances, there is a role for the MHC class I immune evasion genes *in vivo*. The first evidence that an MHC class I immune evasion gene provided a growth advantage to MCMV *in vivo* was reported by Krmpotic *et al*. Irradiated BALB/c mice that received transferred lymphocytes isolated from the spleens of latently-infected mice control Δm152-MCMV (ΔMC95.24) better than revertant, wild type MCMV (rMC96.27) in the lungs at two weeks p.i. (Krmpotic et al., 1999). In the same model using B6 mice, transferred M45-specific CD8 T cells could again better control (sometimes completely) Δm152-MCMV than wild type, revertant MCMV in the spleen, lung, and liver (Holtappels et al., 2004).

I hypothesized that the immunodominance hierarchy to the peptide panel would show changes upon infection with a virus that lacked the MHC class I immune evasion genes. We expected to see differences in priming in response to MCMV-BAC and Δm04+m06+m152-MCMV infection. The antigenic peptides come from proteins with different timing of gene expression, different functions, and different biophysical properties. These factors, in addition to any changes in antigen presentation of Δm04+m06+m152-MCMV, could contribute to changes in the immunodominance hierarchy. The MHC class I immune evasion genes were so powerful *in vitro* that it was expected that the responses primed to the panel of epitopes would be affected by their absence.

Results

MHC class I immune evasion genes have no effect on priming a CD8 T cell response in immunocompetent mice

To determine if these immune evasion proteins impacted the CD8 T cell response that develops following MCMV infection, I infected B6 mice i.p. with $5x10^6$ PFU of MCMV-BAC or Δm04+m06+m152-MCMV. On day seven p.i., I measured priming of CD8 T cell responses to the 13 most immunodominant epitopes by ICCS. This had been done before for MCMV-BAC

infection using the full epitope panel, but not as a side-by-side comparison with Δm04+m06+m152-MCMV. The CD8 T cell responses, as measured by size and immunodominance hierarchy, were nearly identical in MCMV-BAC and Δm04+m06+m152-MCMV-infection (Figure 3.5). This data suggested that the MHC class I immune evasion genes do not have an effect on priming the CD8 T cell response to any of the known H-2^{b}-restricted epitopes under our infection conditions. I repeated these experiments in 129 mice and was surprised to find, again, that the magnitude and hierarchy of the CD8 T cell response was not altered in Δm04+m06+m152-MCMV infection (Figure 3.5).

As reported above, the magnitude of the acute CD8 T cell response was significantly higher in CD40$^{-/-}$ mice when compared to wild type BALB/c. To determine whether the immune evasion proteins were better able to function in an environment lacking proper CD40-mediated DC licensing, I infected CD40$^{-/-}$ mice using the same infection conditions described above. Compared to wild type BALB/c mice, Δm04+m06+m152-MCMV-infected CD40$^{-/-}$ mice had a larger CD8 T cell response to each of the peptides tested, although the no-peptide control was also higher in CD40$^{-/-}$ mice than in wild type BALB/c mice (no pep: p=0.0012, IE1/pp89: p=0.0009, m164: p=0.019, M45: p=0.034, and M18: p=0.005) (see Figure 3.6). Therefore, the higher, acute CD8 T cell response seen in CD40$^{-/-}$ mice could not be explained by the presence of the MHC class I immune evasion proteins. In addition, there were no differences in the magnitude of the acute CD8 T cell response in BALB/c mice between MCMV-BAC or Δm04+m06+m152-MCMV-infection, nor were there any differences in the immunodominance hierarchy in either BALB/c or CD40$^{-/-}$ mice in the absence of the MHC class I immune evasion genes.

The MHC class I immune evasion genes do not affect the chronic CD8 T cell response

In the absence of a phenotype for the MHC class I immune evasion genes in priming an immune response, I hypothesized that they are required for the virus to establish and maintain

lifelong persistence. It was possible that while there was no impact on the CD8 T cell response in acute infection, that a virus lacking the MHC class I immune evasion genes would drive a different CD8 T cell response. To address whether the MHC class I immune evasion genes allow virus to persist in the host and what impact they have on the antigen-specific response, I measured the memory CD8 T cell response in 129 and BALB/c mice chronically infected with MCMV-BAC or Δm04+m06+m152-MCMV. Mice were infected i.p. for at least four weeks before T cells were assayed for IFN-γ production by ICCS. 129 mice infected with Δm04+m06+m152-MCMV underwent the characteristic memory inflation of the M38-specific CTL response and had an altered immunodominance hierarchy (see Figure 3.7). In chronic infection of BALB/c $CD40^{-/-}$ mice, the CD8 T cell responses to both IE1/pp89 and m164 inflated, and the overall magnitude equalized in wild type BALB/c and $CD40^{-/-}$ mice (see Figure 3.8). The responses in both BALB/c mouse strains were also identical between MCMV-BAC and Δm04+m06+m152-MCMV infection. Therefore, the MHC class I immune evasion proteins do not prevent the establishment of latency, nor do they appear to benefit latent virus undergoing reactivation.

Discussion

The influences of MHC and non-MHC genes on the immunodominance hierarchy and magnitude of the MCMV-specific CD8 T cell response

The results from these experiments make four important points regarding the CD8 T cell response to MCMV infection. Genes within and outside of the MHC complex impact both the immunodominance hierarchy and magnitude of the CD8 T cell response to MCMV infection. In addition, and rather surprisingly, the MHC class I immune evasion genes do not impact the immunodominance hierarchy or magnitude of the CD8 T cell response.

That the immunodominance hierarchy is different in mice that share their MHC class I haplotype may be surprising at first blush. The mice have the same MHC class I molecules and, therefore, the peptide sequences, peptide-MHC class I affinity, and cell surface stability should be the same. However, different mouse strains have different susceptibilities to MCMV, and some mice harbor much higher viral titers than others (Chalmer et al., 1977; Allan and Shellam, 1984). An altered amount of virus could lead to different immunodominance hierarchies, as there may be a different levels of inflammation and associated cytokines present (*e.g.* IFN-γ and greater induction of the immunoproteasome) and simply more antigen present to influence which antigens are ultimately presented. Genetic differences between mouse strains could also contribute to differences in the efficiency of antigen presentation, T cell avidity for antigen, and the T cell repertoire. The TCR genes are polymorphic and encoded outside the MHC complex, so the TCR repertoire may be a very good candidate to explain the different responses to MCMV. I found it particularly interesting that when mice that do not naturally share MHC class I haplotypes are engineered to do so (the H-2^{bxd} mice), there are still notable differences in the acute CD8 T cell immunodominance hierarchy. This highlights how important the non-MHC genes are in influencing the CD8 T cell response.

I have not looked extensively at the immunodominance hierarchy in different strains of mice in chronic infection, although a number of comparisons were made and reported above. The hierarchies in chronic infection are altered from their hierarchies in acute infection in B6 and 129 mice, but the hierarchy does not change in BALB/c mice. Memory inflation in B6 mice has been well-documented by our laboratory. Memory inflation has not been documented *per se* for 129 mice, but the data in this chapter show that the T cell response in chronic infection of 129 mice is very similar to the response in B6 mice in that the M45-specific response decreases, while the m139- and M38-specific responses increase. We do not yet understand what drives memory inflation, but the fact that the m139- and M38-specific CD8 T cell responses inflate in both B6 and 129 mice either suggests that these antigens are particularly abundant in chronic infection or

that the T cells specific for these epitopes have a competitive advantage over others. Again in chronic infection, the response in the H-2^{bxd} mice was rather intriguing, because while the acute response in B6 H-2^{bxd} mice was co-dominated by both H-2^{b}-restricted and H-2^{d}-restricted epitopes, the response to H-2^{b}-restricted epitopes did not inflate over time.

The second general conclusion from these experiments is that genes outside of the MHC complex also dictate differences in the size of the CD8 T cell response to MCMV. What determines the size of the CD8 T cell response—like immunodominance—is not a simple rubric. BALB/c mice, for example, are MCMV-susceptible and this has been explained by the lack of an effective NK cell response. This led us to predict that BALB/c mice would mount a larger CD8 T cell response to MCMV—in order to compensate for the lack of NK cell control and perhaps to combat the larger viral load. However, BALB/c and BALB.B10 mice mount a CD8 T cell response that is, at most, half the size of a B6 T cell response. 129 mice, which also lack Ly49H and are highly susceptible to MCMV infection, have an intermediately-sized CD8 T cell response. While there are certainly other genetic differences that may account for a smaller, acute CD8 T cell response in 129 mice, the observation that it is more similar in size to a BALB/c CD8 T cell response could also be explained by their substandard NK cell control.

My experiments in CD40$^{-/-}$ mice showed that there was not a requirement for CD40 in the priming and maintenance of the CD8 T cell MCMV-specific response, as least as measured by the IFN-γ-producing effector response. CD40 is not required for priming nor generating or maintaining a memory CD8 T cell response following a bacterial infection (Montfort et al., 2004). In the context of persistent viral infections, CD40L is necessary for normal priming of the CD8 T cell response to polyoma virus, but is not required to generate or maintain the memory response (Kemball et al., 2006). In LCMV infection, CD40L is not required for a normal, acute CD8 T cell response, but ultimately virus replication is uncontrolled (Wherry and Ahmed, 2004). There did not appear to be a similar phenomenon occurring in our system; MCMV viral titers in the spleen and salivary glands were the same in BALB/c and CD40$^{-/-}$ mice in both acute and

chronic infection (data not shown). The most surprising finding from these experiments was that the magnitude of the acute CD8 T cell response was higher in CD40$^{-/-}$ mice. This did not impact the magnitude of the memory response, as, by six weeks p.i., the CD8 T cell response in BALB/c and CD40$^{-/-}$ mice looked nearly identical. The presence of the MHC class I immune evasion proteins did not explain the increased, acute CD8 T cell response in CD40$^{-/-}$ mice, as acute infection with Δm04+m06+m152-MCMV also led to a larger CD8 T cell response when compared to BALB/c mice. CD40$^{-/-}$ mice do have significantly decreased numbers of CD4^{+} CD25^{+} regulatory T cells, so it is possible that the absence of regulatory T cells allowed for a stronger acute response.

MHC class I immune evasion and its impact on the immunodominance hierarchy and magnitude of the MCMV-specific CD8 T cell response

It was possible that, in addition to the host's genetic background, the viral genetics could impact the magnitude and immunodominance hierarchy of the CD8 T cell response. As discussed in Chapter 1, MCMV's three MHC class I immune evasion genes very efficiently target and impair CD8 T cell function *in vitro*. In all of the experiments discussed above, mice were infected with wild type MCMV-BAC, which encodes the MHC class I immune evasion genes. It was possible that the differences in the magnitude and immunodominance hierarchy could be due to differences in the ability of the MHC class I immune evasion genes to function in different strains of mice. I hypothesized that the real differences in the magnitude of the CD8 T cell response and the immunodominance hierarchy would become apparent upon comparing the response to MCMV-BAC and Δm04+m06+m152-MCMV infection.

Despite an expectation for the highly-conserved MHC class I immune evasion genes to have a phenotype *in vivo*, the above experiments do not support this conclusion. I hypothesized that infecting mice with Δm04+m06+m152-MCMV would result in an altered immunodominance hierarchy, a decrease in the size of the CD8 T cell response, or both. Neither of these differences

was observed in acute of B6, 129, or BALB/c mice or chronic infection of 129 or BALB/c mice. One explanation for why the responses were the same is that all priming in MCMV infection occurs by cross-presentation. Cross-presentation is a process that has served to answer questions about mounting an immune response to pathogens that do not infect professional APCs. While MCMV infects professional APCs, its interference with MHC class I expression may mean that MCMV antigens are only efficiently presented by cross-presentation. It is difficult to theorize why cross-presentation would also be occurring exclusively in Δm04+m06+m152-MCMV infection when MHC class I expression is not downregulated. However, the myriad other immune evasion mechanisms of MCMV would be functional in both MCMV-BAC and Δm04+m06+m152-MCMV, and perhaps one or more of them drives Δm04+m06+m152-MCMV (as well as MCMV-BAC) to be predominantly cross-presented. A good candidate would be m138, which downregulates B7.1 (CD80) from the surface of DCs (Mintern et al., 2006).

I tried to test the cross-presentation hypothesis; my intention was to utilize mice reported to be deficient in their ability to cross-present K^b-restricted epitopes ($K^{bY\rightarrow F}$ mice) (Lizee et al., 2003). I hypothesized that if cross-presentation was occurring to a significant degree in MCMV infection, the CD8 T cell response to all K^b-restricted epitopes should be decreased in $K^{bY\rightarrow F}$ mice. This was not seen following MCMV-BAC infection of $K^{bY\rightarrow F}$ (data not shown). Furthermore, the $K^{bY\rightarrow F}$ mice made normal inflating, memory CD8 T cell responses and $K^{bY\rightarrow F}$ bone marrow-derived DCs were able to cross-present soluble ovalbumin *in vitro* (Chris Snyder, personal communication), indicating that this was not a good model for cross-presentation deficiency. A report has come out during the writing of this dissertation, however, that there is another mouse deficient in cross-presentation. This mouse lacks the *Batf3* transcription factor, which prevents the development of the professional cross-presenting cells, CD8-α^+ DCs (Hildner et al., 2008). This mouse model will be useful in addressing the role of cross-presentation in MCMV infection in the near future.

If cross-presentation is *not* the only form of antigen presentation in MCMV infection, then direct presentation must be occurring at some level to explain how a T cell response is primed. This was initially hard to reconcile with the data that showed that the MHC class I immune evasion genes were so effective at preventing CD8 T cell cytotoxicity *in vitro*. In parallel *in vitro* experiments described more fully in Appendix A, however, I show that MCMV-specific CTL lines could make IFN-γ in response to MCMV-BAC-infected cells, although less efficiently than in response to Δm04+m06+m152-MCMV-infected cells. This data provides critical evidence that cells infected with MCMV-BAC—both professional APCs and fibroblasts—can directly present antigen to T cells. Therefore, if MCMV-BAC is directly presented at some level to CD8 T cells *in vivo*, it may be enough to prime the CD8 T cell response we see. While this observation could explain how the CD8 T cell responses are the same in MCMV-BAC and Δm04+m06+m152-MCMV-infection, it still leaves an important question. If CD8 T cells can be primed in the presence of the MHC class I immune evasion proteins, why are they encoded?

As discussed earlier in this chapter, it is possible that the role of the MHC class I immune evasion proteins is to interfere with the later stages of infection, such as the response in chronic infection or in reactivation from latency. If the proteins impact the chronic CD8 T cell response or MCMV reactivation, we would expect either the T cell response to be altered (magnitude and/or immunodominance hierarchy) in Δm04+m06+m152-MCMV-infection, greater evidence of reactivating virus in MCMV-BAC infection, or both. The results from this chapter exclusively addresses the CD8 T cell response. I show that the MCMV-specific CD8 T cell response is virtually identical in chronic infection of 129 and BALB/c mice with MCMV-BAC and Δm04+m06+m152-MCMV. Our laboratory has further established that the MCMV-specific CD8 T cell response is virtually identical in chronic infection of B6 mice.

Another important assessment of viral control is measuring virus titers from the organs of infected mice. When this is done at late times p.i., it can serve as a measurement of spontaneous viral reactivation. Reactivation is difficult to detect in MCMV and is not as well understood as

reactivation of other herpesviruses, such as HSV-1 and -2 and EBV. Infectious virus is rarely detected in MCMV-BAC-infected B6 or BALB/c mice beyond four weeks p.i. Any virus that is detected after four weeks is thought to represent foci of reactivating virus, often restricted to only one organ at any given time. Notably, it is difficult to detect either MCMV-BAC or Δm04+m06+m152-MCMV from the organs of chronically-infected mice (see Chapter 4 and 5; also M. Gold, unpublished observation). This supports my CD8 T cell data, which showed that the MHC class I immune evasion proteins did not impact the CD8 T cell response in chronic infection, suggesting, albeit indirectly, that the proteins do not dramatically impact CD8 T cell control of latent virus. Therefore, based on CD8 T cell responses and virus titer measurements, the MHC class I immune evasion proteins appeared to have no impact on establishing an infection, priming a CD8 T cell immune response, maintaining the T cell response, or contributing to virus reactivation.

There is one scenario in immunocompetent animals where the immune evasion genes have a clear impact on viral fitness *in vivo*, and it was reported as the experiments in this chapter were being completed. At the peak of virus load in the salivary glands of MCMV-susceptible BALB/c mice, MCMV-BAC titers are between one and two logs greater than Δm04+m06+m152-MCMV titers and the effect is CD8-dependent (Lu et al., 2006). The timing of this difference suggests that the MHC class I immune evasion proteins may function to enhance transmission of the virus via saliva. This data, however, and virtually all of the contemporary MCMV literature has been generated following very high inoculating doses of MCMV ($\geq 10^5$ PFU). With a focus on MHC class I immune evasion and its evolutionary purpose, it seemed important to reconsider whether our model was optimized to reveal the role of the MHC class I immune evasion genes. These considerations led me to ask: Do the MHC class I immune evasion genes have a phenotype in infection of mice under more natural infection conditions? The experiments presented in the following chapter were designed to answer that question, with the hope of unmasking the role for these genes.

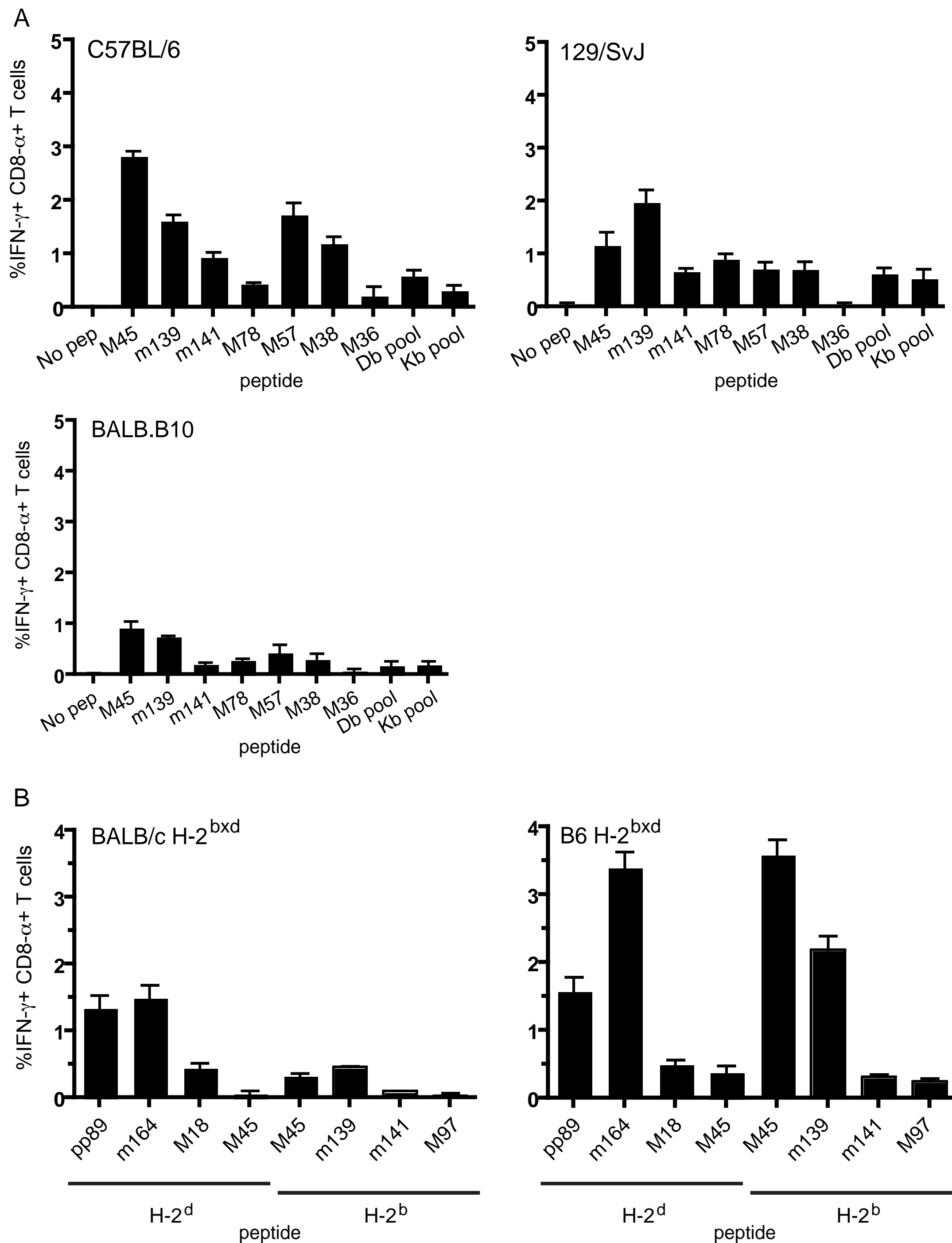


Figure 3.1. The magnitude and immunodominance hierarchy of the acute MCMV-specific CD8 T cell response is different in various mouse strains. A) C57BL/6, 129/SvJ, or BALB.B10 and B) BALB/c or C57BL/6 H-2^{bxd} mice were infected i.p. with $5x10^4$ PFU MCMV -BAC. A week later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells was measured by flow cytometry on an LSR II and analyzed by FloJo software. K^bpool: M97, M100, m164. D^bpool: m04, M33, M44, m164. n=3 for each group. Error bars indicate SEM. B) is a reproduction of an experiment performed by M. Munks and published in Munks *et al.* JI (2006) 176:3760.

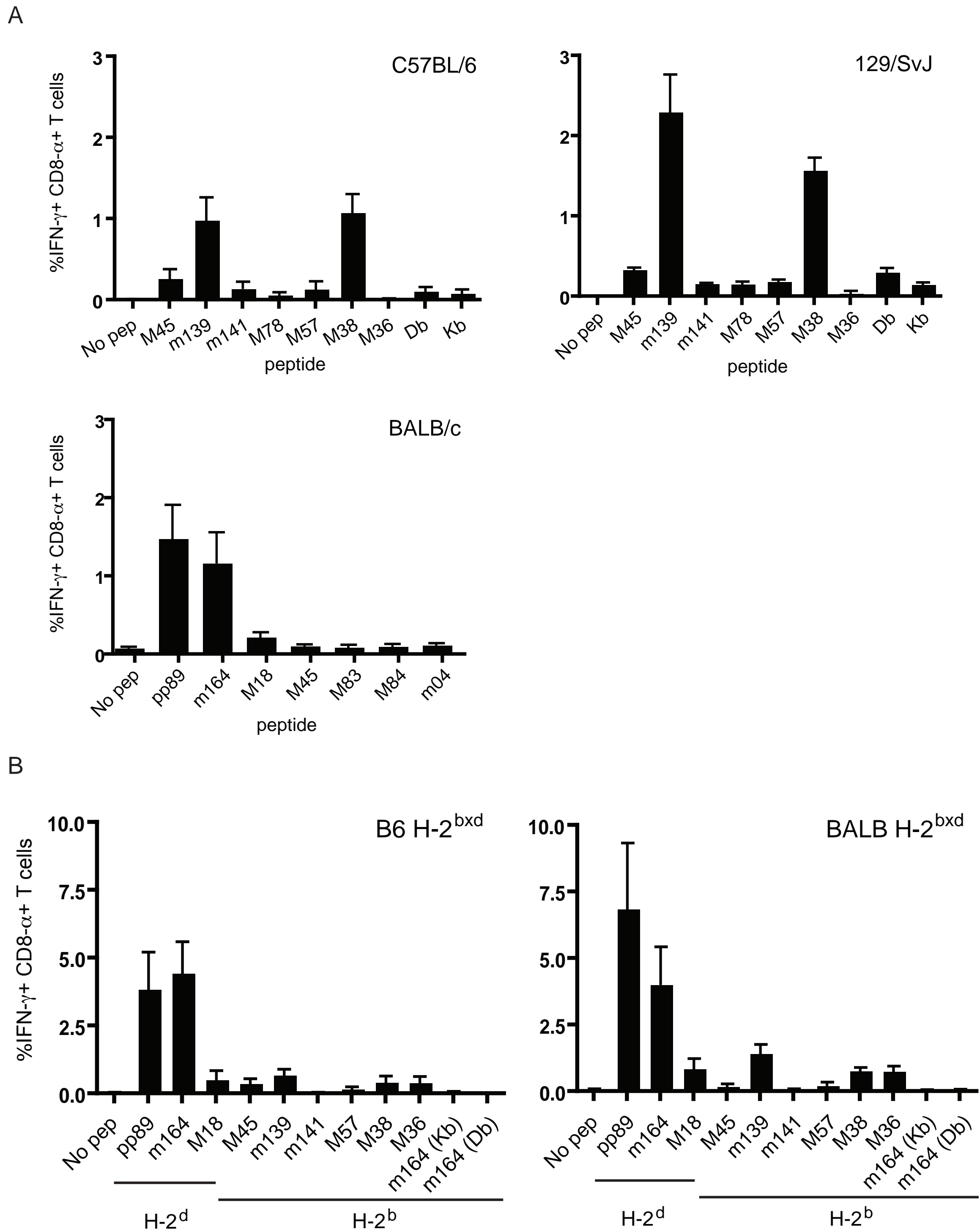


Figure 3.2. The MCMV-specific CD8 T cell immunodominance hierarchy changes in chronic infection in various strains of mice. A) C57BL/6, 129/SvJ, or BALB/c or B) C57BL/6 H-2^{bxd} or BALB/c H-2^{bxd} mice were infected i.p. with MCMV-BAC. C57BL/6 mice were infected with $5x10^6$ PFU; all other strains were infected with $5x10^4$ PFU. A) After at least 4 weeks or B) 16 months later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells was measured by flow cytometry on an LSR II and analyzed by FloJo software. K^bpool: M97, M100, m164. D^bpool: m04, M33, M44, m164. n=3 for each group. Error bars indicate SEM.

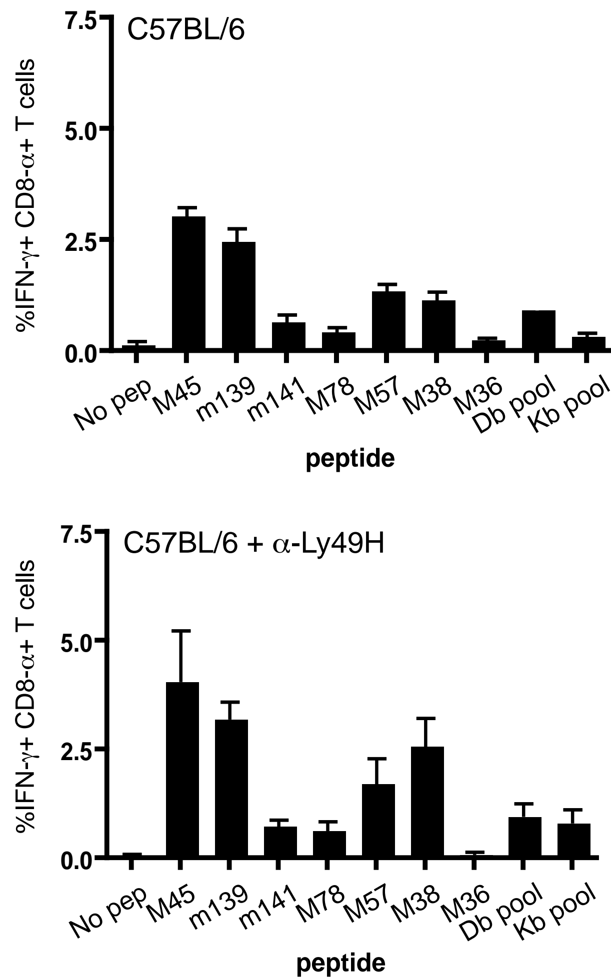


Figure 3.3. Blocking Ly49H does not impact the acute MCMV-specific CD8 T cell response magnitude or immunodominance hierarchy in C57BL/6 mice. C57BL/6 mice were treated with 200 μg anti-Ly49H antibody 2 days before i.p. infection with $5x10^4$ PFU MCMV-BAC. A week later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6}M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells was measured by flow cytometry on an LSR II and analyzed by FloJo software. K^b pool: M97, M100, m164. D^b pool: m04, M33, M44, m164. n=3 for each group. Error bars indicate SEM.

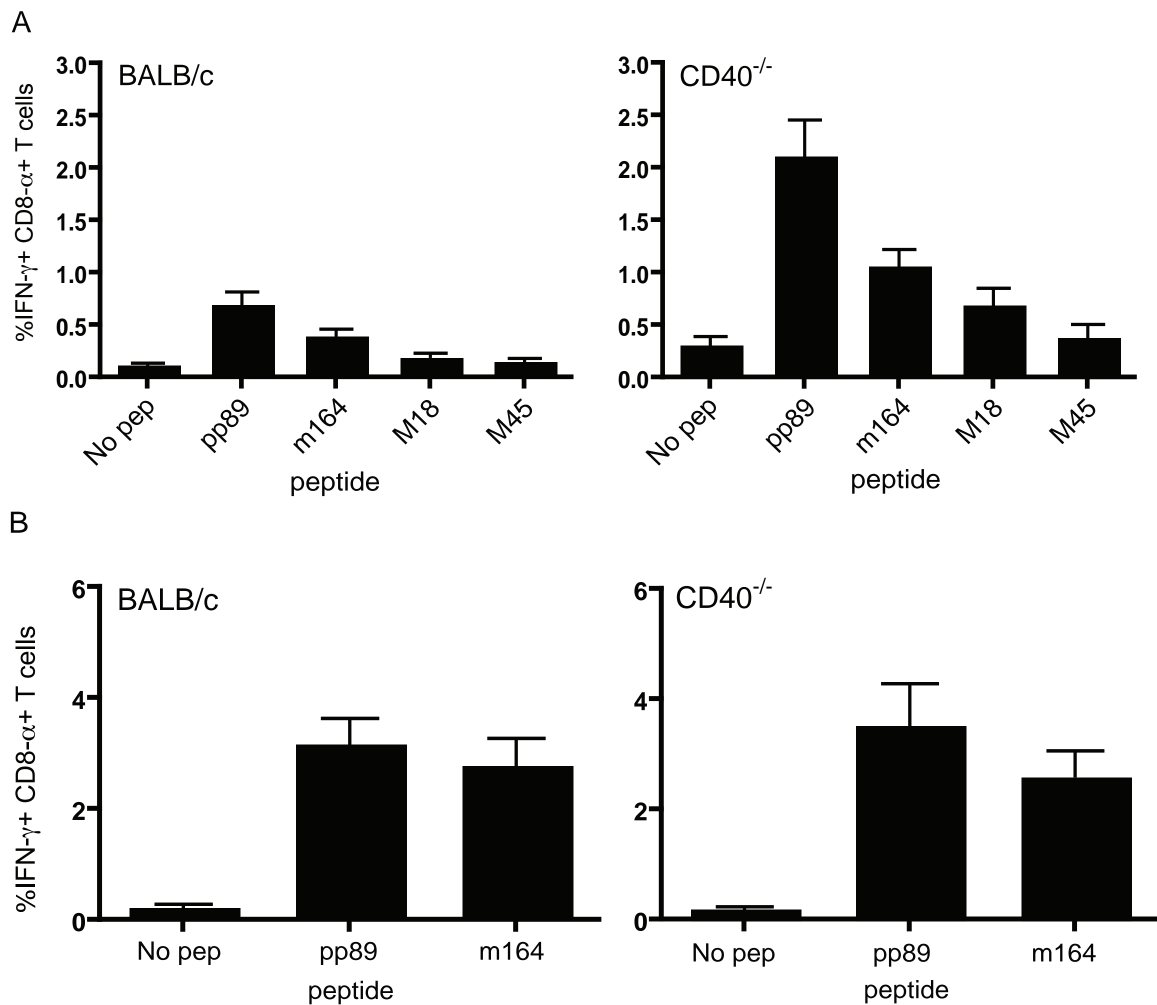


Figure 3.4. The magnitude of the acute MCMV-specific CD8 T cell response in CD40$^{-/-}$ mice is higher than the response in BALB/c mice. BALB/c (left) or CD40$^{-/-}$ mice (right) were infected i.p. with 3x10^{5} PFU MCMV-BAC. A) One week or B) six weeks later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. n=6 for each group. Error bars indicate SEM. Results are representative of at least 2 experiments.

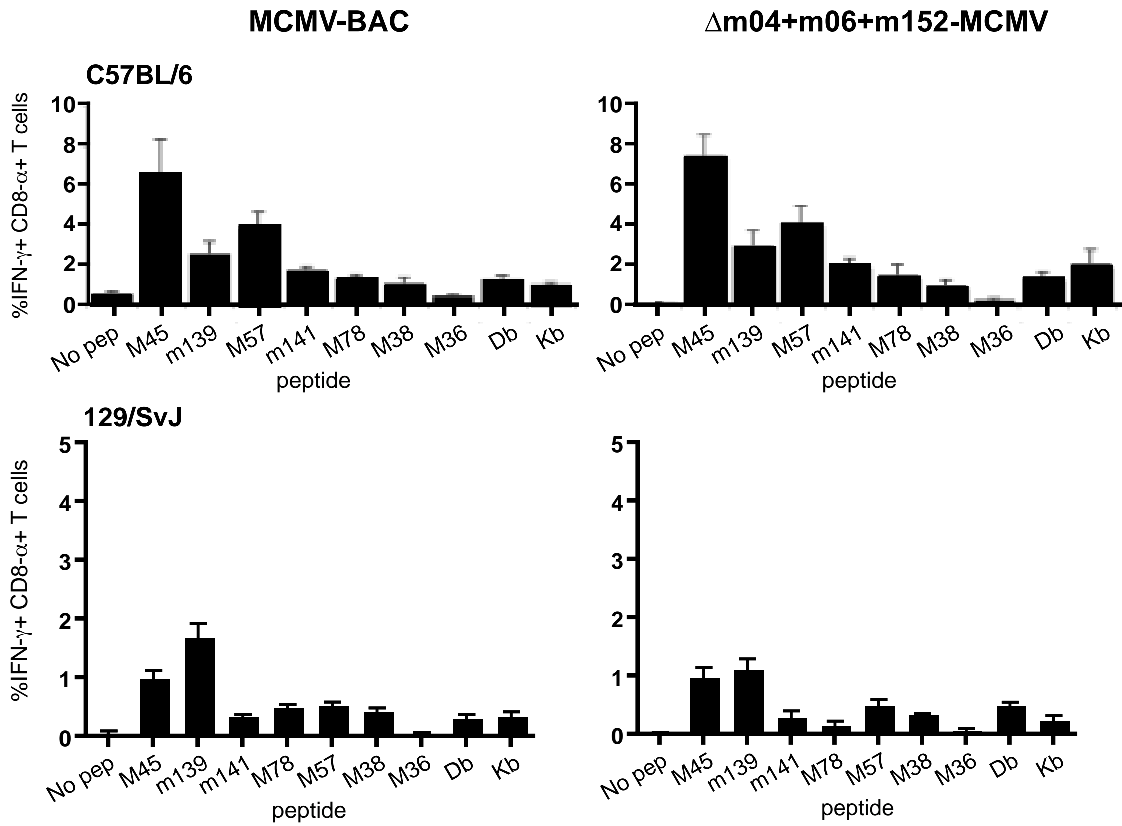


Figure 3.5. The MHC class I immune evasion genes do not alter the acute CD8 T cell response in C57BL/6 or 129/SvJ mice. All mice were infected i.p. with MCMV-BAC (left) or Δm04+m06+m152-MCMV (right). C57BL/6 mice (top) were infected with $5x10^6$ PFU; 129/SvJ mice (bottom) were infected with $5x10^4$ PFU. A week later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6}M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells was measured by flow cytometry on an LSR II and analyzed by FloJo software. K^b pool: M97, M100, m164. D^b pool: m04, M33, M44, m164. n=3 for each group. Error bars indicate SEM.

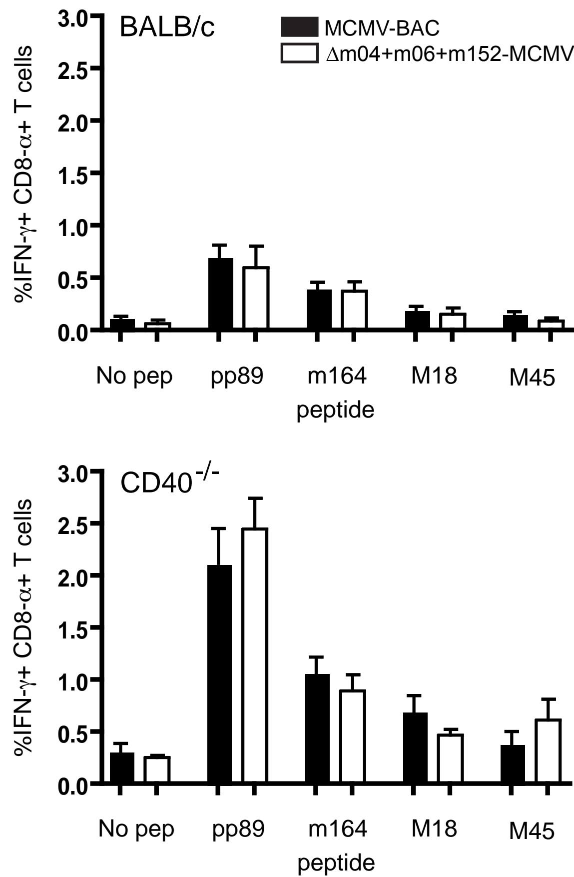


Figure 3.6. The MHC class I immune evasion genes do not impact the acute MCMV-specific CD8 T cell response in BALB/c or CD40$^{-/-}$ mice. BALB/c (top) or CD40$^{-/-}$ mice (bottom) were infected i.p. with 3x10^{5} PFU MCMV-BAC (black bars) or Δm04+m06+m152-MCMV (white bars). A week later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6}M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. n=6 for each group. Error bars indicate SEM. The results are representative of at least 2 experiments. The CD8 T cell response to MCMV-BAC infection depicted here is the same as that shown in Figure 3.4.

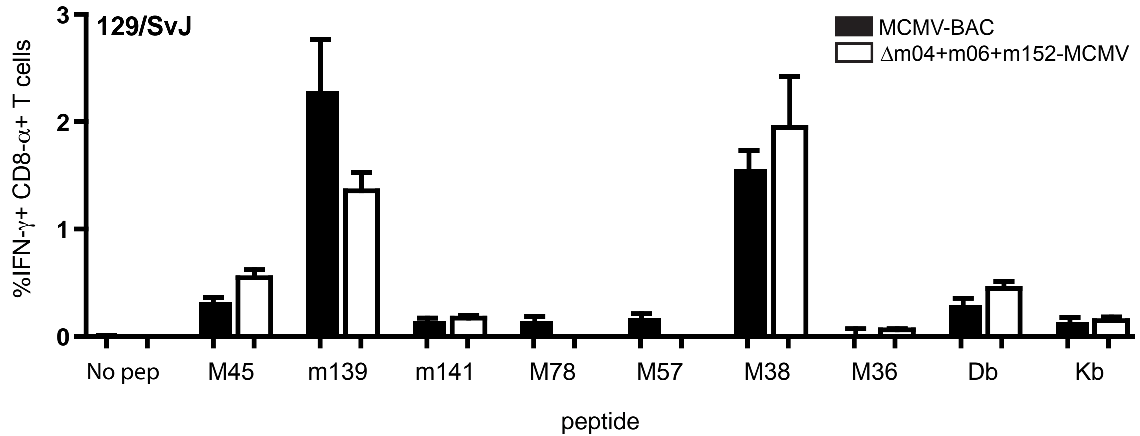


Figure 3.7. The MHC class I immune evasion genes do not impact the chronic CD8 T cell response in 129/SvJ mice. 129/SvJ micewere infected i.p. with $5x10^4$ PFU MCMV-BAC (black bars) or Δm04+m06+m152-MCMV (white bars). After at least 4 weeks, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6}M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. K^b pool: M97, M100, m164. D^b pool: m04, M33, M44, m164. n=3 for each group. Error bars indicate SEM. The results are representative of at least 2 experiments.

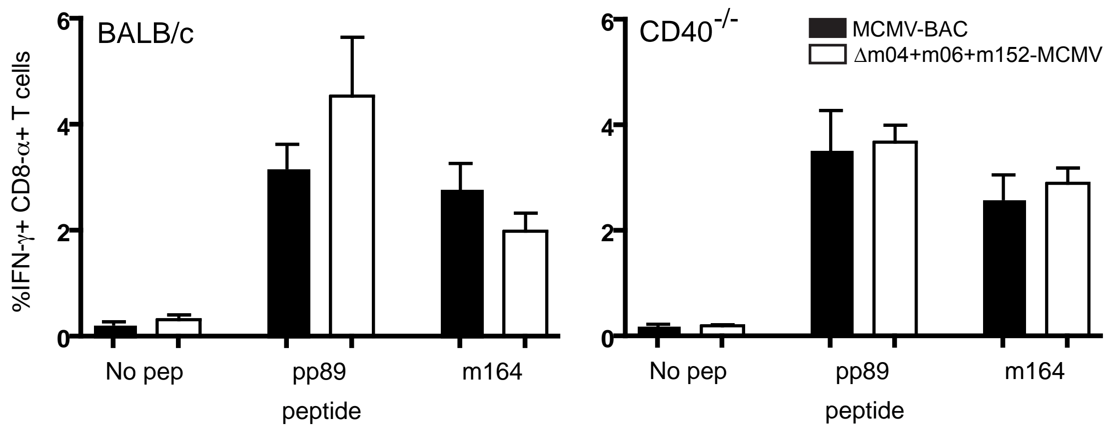


Figure 3.8. The MHC class I immune evasion genes do not impact the chronic MCMV-specific CD8 T cell response in BALB/c or CD40$^{-/-}$ mice. BALB/c (left) or CD40$^{-/-}$ mice (right) were infected i.p. with 3x10^{5} PFU MCMV-BAC (black bars) or Δm04+m06+m152-MCMV (white bars). Six weeks later, spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. n=6 for each group. Error bars indicate SEM. Results are representative of at least 2 experiments. The CD8 T cell response to MCMV-BAC infection depicted here is the same as that shown in Figure 3.4.

Chapter 4: THE IMPACT OF MHC CLASS I EVASION IN MCMV INFECTION UNDER NATURAL INFECTION CONDITIONS

Introduction

There are two settings in which MHC class I evasion genes have a phenotype *in vivo* in immunocompetent mice: in neonates (Krmpotic et al., 1999) and in the salivary glands of adult mice (Lu et al., 2006). Both of these could offer a clear selective advantage to the wild type virus. However, the difference in viral load is only about one log and does not necessarily justify the striking evolutionary conservation of these genes. Animals models are developed to achieve reproducible measurements, and our laboratory initially sought a reliable method for measuring the CD8 T cell response to MCMV infection that was established by using high dose, intraperitoneal infection. Perhaps the function of the MHC class I immune evasion proteins are obscured by the high dose infection we use and the unnatural route by which we infect. If their impact is at all mild, flooding an animal with a massive quantity of antigen may be enough to overwhelm the proteins' function and obscure what could be a more significant role in natural infection. The relatively artificial nature of our model in the context of the evolutionary persistence of MHC class I evasion genes led me to ask whether there are other biologically relevant conditions under which the MHC class I immune evasion genes have a phenotype *in vivo*?

As was discussed in Chapter 1, MCMV is a natural pathogen of mice. Therefore, there are certainly more biologically relevant conditions to test—those of infection in the wild. The experimental conditions most MCMV researchers use do not even attempt to model natural infection, which is likely not such a high inoculating dose and is certainly not delivered by an i.p. route. Modeling natural infection has not been that important because of the questions being asked, but the continued defiance of the MHC class I immune evasion genes to show a clear

phenotype *in vivo* led me to examine our model itself. I hypothesized that if a lower dose infection by a more natural route was used, a more evident selective advantage for the MHC class I immune evasion genes might be unmasked.

High dose infection may simply overwhelm the system, providing so much antigen that CD8 T cells become activated even in the face of MHC class I immune evasion. At low infecting doses, MCMV-BAC may be better able to establish infection due to its immune evasion abilities, which would be clear if more mice became infected with MCMV-BAC- than Δm04+m06+m152-MCMV at limiting doses. Alternatively, MCMV-BAC may be more successful at reactivating from latency under these conditions, which would be evidenced by a higher CD8 T cell response and/or higher titers of virus in the salivary glands at chronic time points. Because I was looking for the role of MHC class I immune evasion genes in low dose infection, I wanted to study the response to MCMV right at the threshold of virus infectivity (*i.e.* when not all mice are infected). In designing experiments of this kind, it was essential to perform a power analysis to determine the appropriate group size based on the expected standard deviation and the expected effect size. It was expected that the variation both within a group and between repeat experiments would be low.

Natural infection model

These experiments were designed to ask: when we are at the threshold of virus infectivity and when virus is delivered by a natural route of infection, do the MHC class I immune evasion genes impact the ability of the virus to establish infection? A secondary question was: do these genes impact virus titers or the size of the immune response in acute or chronic infection? I set out to explore natural infection conditions in two ways. The first was to infect adult BALB/c and B6 mice with low doses of MCMV-BAC or Δm04+m06+m152-MCMV by the most likely natural routes (i.n., s.c.) or by the i.p. route. The i.p. route was included as a positive

control, as it causes a robust infection and we know we can establish infection at low doses (Selgrade et al., 1984).

Both strains of mice were studied in adults because both have historically been used in MCMV research and both models have their advantages. B6 mice were included because little is known about low dose infection of B6 mice and because they would serve as a bridge to the rest of the work in our lab, which has been focused on the B6 model of MCMV infection. The magnitude of the MCMV-specific CD8 T cell response is also larger in B6 than in BALB/c mice and there is a very clear pattern of CD8 T cell memory inflation. In B6 mice, the responses to some peptides are low ($M38_{316\text{-}323}$) or undetectable ($IE3_{416\text{-}423}$) in acute infection but become dominant at later time points p.i. (Munks et al., 2006a; Snyder et al., 2008). Thus, *de novo* development of the IE3 response at ~four weeks p.i. is an excellent indicator of virus activity during chronic infection. BALB/c mice were included because they are more susceptible to MCMV infection and more closely model MCMV infection of wild mice (Smith et al., 2006; Smith et al., 2008). Furthermore, while the CD8 T cell response magnitude is lower in BALB/c mice, the organ viral titers are higher, and persistent salivary glands infections (between two to four weeks p.i.) are much more readily detected.

I approached the question using multiple measurements of infection: 1) viral titers in the salivary glands to most directly measure viral control of MCMV-BAC and Δm04+m06+m152-MCMV, 2) the CD8 T cell response to establish that infection occurred and to use as a proxy measurement of viral activity, 3) an antibody response, also for evidence of established infection. I chose to assay for virus in the tissues by plaque assay instead of the more sensitive quantitative PCR method because I was interested in measuring the amount of infectious virus present.

<u>Virus control in neonates</u>

The second approach was to orally infect BALB/c neonates with MCMV-BAC or Δm04+m06+m152-MCMV. Only the oral route was used with pups, assuming vertical

transmission occurs via the breastmilk and/or saliva as discussed in Chapter 1. Vertical transmission in the wild makes MCMV infection of neonates an important model for biologically relevant infection. It has been suggested that newborn mice of all strains can be considered MCMV susceptible; resistance to infection develops in the first few weeks of life (Fitzgerald et al., 1990). Neonatal mice of a variety of strains have a much lower LD_{50} than weanling or adult mice. BALB/c neonates have decreased survival, higher viral titers in the spleen, salivary glands, and lung, and delayed viral clearance when compared to adults (Reddehase et al., 1994). These findings are the most pronounced in the salivary glands. In the neonatal experiments described below, I measured viral titers in the salivary glands of BALB/c mice, expecting that even with low dose infection, virus would be readily detectable in that organ.

Results

Because such low doses were being titrated, it was important to carefully characterize the virus preparations used in these experiments. The same virus preparations of TC-derived, MCMV-BAC or Δm04+m06+m152-MCMV were used for all of the following experiments and were titered by plaque assay four times to be certain of their titers. In order to verify that the particle-to-PFU ratio was similar for both virus preparations, quantitative PCR was performed and showed that both virus preparations had a similar genome copy number to PFU ratio (see Figure 4.1).

Once the viruses were characterized, a set of dose-titration experiments was performed to determine the lowest dose via each route at which a CD8 T cell response and/or viral load in the salivary glands could be detected in at some, but not all, of the mice. Figures 4.2-4.4 show the CD8 T cell response to the immunodominant m164 epitope from BALB/c mice or the M38 epitope in B6 mice infected by each of the different routes. There was evidence of infection by each of these routes at as low as 10 PFU, however, it was far more uncommon than not, and thus considered to be below the threshold of infection. Unique to the i.n. route of infection, the CD8 T

cell response took longer to reach detectable levels; the response shown in Figure 4.3 is from four weeks p.i. The response measured at one and two weeks p.i. showed little evidence of infection. By either CD8 T cell response or plaque assay, the threshold of infection of BALB/c mice by the i.n. and s.c. routes was between 100 and 1000 PFU, while it was as low as 10 PFU for i.p. infection. Using this dose titration data, I decided to infect larger cohorts of mice with either 100 or 1000 PFU, comparing MCMV-BAC and Δm04+m06+m152-MCMV infection of BALB/c and B6 mice by the i.n., s.c., and i.p. routes.

Adult mice

The results for infection of adult BALB/c mice with 100 or 1000 PFU of MCMV-BAC or Δm04+m06+m152-MCMV by different routes of immunization are summarized in Tables III and IV, and the results for B6 mice are shown in Tables V and VI. Infection with 1000 PFU gave a higher rate of infection across all routes, however, there was not a clear dose titration effect. For virus to be detected in the salivary glands of B6 mice, at least 1000 PFU was required, but even then detection was rare. In BALB/c mice, there were higher viral titers in the salivary glands at 21 days and 13 weeks p.i. after 1000 PFU infection than 100 PFU infection across all routes tested. The CD8 T cell responses were also higher after infection with 1000 PFU, although they cannot be directly compared between experiments. Of note, the CD8 T cell response appeared to be a more sensitive measurement than viral titers as more mice within a given group mounted a detectable CD8 T cell response than had detectable virus in the salivary glands at any of the time points tested. This observation supports our laboratory's assertion that the size and phenotype of the CD8 T cell response, particularly in chronic infection, is the best indicator of virus activity.

In BALB/c mice, in eight out of 13 experiments where any virus was detected, viral titers were higher in MCMV-BAC infection, as hypothesized, but this observation was not absolute and the standard deviation of the data is far too large to be conclusive. In B6 mice, though, there was only one instance of virus detected in the salivary glands, and it was Δm04+m06+m152-MCMV

(1000 PFU, i.p., at 13 weeks p.i.). Unfortunately, these experiments were plagued by an unexpected degree of variation in the measurements taken within an experiment, as well as between cages and experiments. As an example, there were two experiments in which BALB/c mice were infected i.p. with 1000 PFU of MCMV and analyzed at 21 days p.i. (see Table III, route: i.p., time: 21 days). In the first experiment, one of six mice infected with MCMV-BAC had virus in their salivary glands, whereas all six of six mice infected with Δm04+m06+m152-MCMV did. In a repeat of that experiment, done a few months later, six of six mice infected with *either* virus had virus detectable in their salivary glands.

Because this degree of variability was not anticipated, there is simply not enough statistical power to make valid comparisons between the CD8 T cell responses and viral titers in the salivary glands of mice infected with MCMV-BAC or Δm04+m06+m152-MCMV. This led us to think of different ways in which to approach this data. One is to look at these infections as we would look at human data, whereby we know that there are many confounding variables, and we do not know the route, dose, or day of infection. With this in mind, data from these experiments have been analyzed in just this fashion, *i.e.* pooling the results of the different infectious routes and doses (see Tables VII-X). The overall observation from this study is that the immune evasion genes did not impact the ability to establish infection. From the pooled data, I have drawn the following conclusions:

1. The percentage of mice making a CD8 T cell response (see Table IX) or that was seropositive (see Tables III-VI), was very similar for the two virus infections in both BALB/c and B6 mice. Although the percentage of mice that made CD8 T cell responses did not differ between MCMV-BAC and Δm04+m06+m152-MCMV infection, the overall size of the response tended to be higher in MCMV-BAC infection in BALB/c mice (Tables III-IV). This might be due to a higher degree of viral activity in BALB/c mice, but a larger sample size is needed to draw any firm conclusions. In both strains of mice, the percentage that made a CD8 T cell response decreased when only the natural routes (s.c., i.n.) were included, suggesting that they are not as

efficient as the i.p. route. However, the comparison of the percentages for mice infected with MCMV-BAC or Δm04+m06+m152-MCMV remained very similar. From this data I conclude that there is no evidence to suggest that Δm04+m06+m152-MCMV is impaired in its ability to establish an infection.

2. MCMV-BAC appears to be more poorly controlled in the salivary glands in BALB/c mice: virus was detectable in a higher percentage of mice, and the titers of those that were infected were higher in most individual experiments (see Tables III, IV, VII, and X), although the latter trend was not statistically significant. This is consistent with our previous results using high dose i.p. infection (Lu et al., 2006). As shown in Table X, again, a smaller percentage of BALB/c mice had detectable virus in the salivary glands when only the natural routes were included. In this subanalysis, MCMV-BAC was detected in more mice than was Δm04+m06+m152-MCMV; the difference actually grew when only the s.c. and i.n. routes were included. Virus was only once detected in the salivary glands (but in two of six B6 mice) and it was by the i.p. route. This is not surprising given the relative resistance of B6 mice to MCMV and the observation that MCMV is rarely detected in the organs of B6 mice infected i.p. with up to $5x10^6$ PFU.

Neonates

BALB/c pups were infected orally (p.o.) by pipetting 10 µL of virus into the oral cavity. Table XI summarizes the experiments in which neonatal mice were infected orally. Similar to the adult data described above, the ability to infect neonates with either virus was highly variable. Virus was rarely detected in the salivary glands after p.o. infection with 1000 PFU, which was unexpected since I was using susceptible BALB/c mice. This suggests that the dose or efficiency of oral infection may be too low for use as a model. In any case, the percentage of pups infected with MCMV-BAC that had measurable virus in their salivary glands (18.2%) was again greater than that of pups infected with Δm04+m06+m152-MCMV (12.5%). Furthermore, the average titer in MCMV-BAC-infected mice (11950 PFU) was higher than Δm04+m06+m152-MCMV-

infected mice (636 PFU), although it was not statistically significant (p=0.4807). Both of these observations, however, offer support to the conclusion that MCMV-BAC is more poorly controlled in the salivary glands than is Δm04+m06+m152-MCMV.

Discussion

These analyses suggest that the published data concluding that the MHC class I immune evasion genes do not significantly impact the CD8 T cell response or virus titers in central organs are not artifacts due to the experimental conditions, such as high dose, i.p. infection. A very recent report by the Reddehase group describes the impact of MHC class I immune evasion in very low dose infection by the intraplantar route (Bohm et al., 2008). Those experiments reveal an advantage (although also statistically insignificant) for MCMV-BAC over Δm04+m06+m152-MCMV in the draining lymph node based on IE1/pp89 transcript number. They also show a tendency for mice infected with MCMV-BAC to have more IE1/pp89-specific T cells in the draining lymph node and spleen by ELISPOT, although the total number of responding CD8 T cells is very low. The data presented here support the notion that in this dissertation, as least in BALB/c mice, the impact of the MHC class I immune evasion genes is on viral control at the site of dissemination.

MCMV-specific CD8 T cells are better able to control MCMV infection *in vitro* and *in vivo* if at least one of the MHC class I immune evasion genes is missing. However, in a fully immunocompetent animal, infection with high dose MCMV or a mutant lacking the three MHC class I immune evasion genes results in the same CD8 T cell response to the virus, as shown in Chapter 3 (Munks et al., 2007). The CD8 T cell response magnitude and specificity and the amount of virus in different organs is exactly the same in B6 mice. In BALB/c mice, the response is the same except for the titers in the salivary glands, in which Δm04+m06+m152-MCMV is somewhat better controlled. I hypothesized that under more natural infection conditions, the MHC class I immune evasion proteins would exert a more detectable phenotype. Under these

conditions, they did not exert a stronger phenotype, but the suggestion remains that the MHC class I immune evasion genes provide a survival advantage to the virus in the salivary glands.

I originally considered three main possibilities for the role of the MHC class I immune evasion genes *in vivo*, which will be discussed in more detail in Chapter 6: 1) they allow for better establishment of initial infection, 2) they allow the virus to survive in the host after the adaptive immune response has developed, or 3) they allow for more efficient transmission. My studies summarized above do not support the first possibility. The number of mice that had a detectable CD8 T cell response at any of the time points measured was very similar between both MCMV-BAC and Δm04+m06+m152-MCMV infection in both strains of mice (see Table IX). This suggests no difference in the ability of either virus to establish infection or to continue to drive a CD8 T cell response in chronic infection. More BALB/c mice infected with MCMV-BAC had virus detected in their salivary glands at 13 weeks p.i. than mice infected with Δm04+m06+m152-MCMV (see Table VII); this could indicate that MHC class I immune evasion proteins do allow the virus to survive or reactivate more readily once the adaptive immune response has developed. Evading an established T cell response seems a more likely goal for the proteins than aiding in establishment of infection. The CD8 T cell response takes time to develop; it is the innate immune response the virus would want to avoid in order to increase its chance at establishing infection.

The third possible role for the MHC class I immune evasion genes is that they allow for more efficient transmission of the virus. The salivary glands are central to MCMV replication and transmission. CD8 T cells and the MHC class I immune evasion genes have a known impact on the viral load in the salivary glands in high dose, i.p. infection of immunocompetent animals, findings that I repeated in my studies (data not shown; Lu et al., 2006). This suggestion that the genes are conserved to aid in transmission was a consideration when I was designing these experiments. Ideally, I would have studied the natural transmission of MCMV-BAC and Δm04+m06+m152-MCMV. Using natural transmission would, of course, be preferable for

studying the natural immunobiology. However, the BAC-derived viruses have never been reported to transmit from mouse-to-mouse and do not in our hands, therefore, I was restricted to studying intentional infection. Regardless of that constraint, however, my data most strongly supports the argument that the MHC class I immune evasion genes aid in transmission. Perhaps the genes function in the salivary glands to prevent effective CD8 T cell control. Mice infected with MCMV-BAC were more likely to have detectable virus in their salivary glands than mice infected with Δm04+m06+m152-MCMV, particularly when comparing mice infected by the more natural s.c and i.n routes (see Tables VII and X). The evolution of pathogens is often thought of in terms of a competition between the virus and the host immune system. It would be prudent to also consider competitive evolution *between* viruses—perhaps the MHC class I immune evasion genes have been conserved simply because MCMV is constantly competing with itself.

I was unable to make a stronger conclusion about the role of the MHC class I immune evasion genes in transmission because analysis of this study was hampered by the rules of statistical power. It is important to point out, however, that the experiments were deliberately designed to examine the threshold of infection. If the MHC class I immune evasion genes had provided a significant advantage in establishing or maintaining infection under natural conditions as I hypothesized, it would have been clear: mice infected with MCMV-BAC would establish and maintain infection (almost always) and mice infected with Δm04+m06+m152-MCMV would not (almost never). The results of this study did not show such an absolute difference, which can be explained by two things. First, perhaps the MHC class I immune evasion genes do contribute to virus transmission, although even under natural infection conditions, the phenotype is mild. Second, a definite phenotype exists, but the degree of variability seen in these experiments made it difficult to identify.

The variability suggested that there was a factor in these experiments—probably in our mouse colony—that could impact infection outcomes for which we could not control. In the

midst of analyzing these results, we learned that the recently discovered mouse pathogen, murine norovirus (MNV), had contaminated our mouse facility. Therefore, I took the opportunity to test the hypothesis that an adventitious agent was contributing to the variability seen in our *in vivo* model. In the following chapter, I address the possibility that MNV exposure was impacting our model of MCMV infection by infecting mice with MNV and measuring outcomes of MCMV infection in a number of different experimental scenarios.

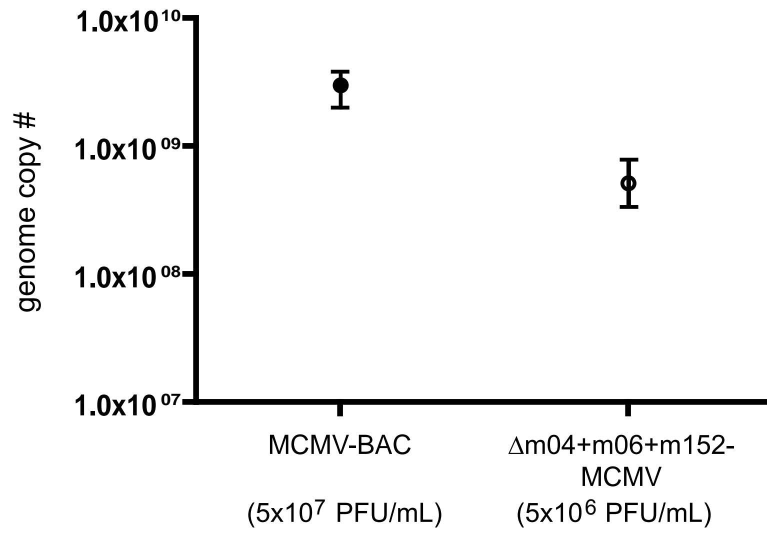
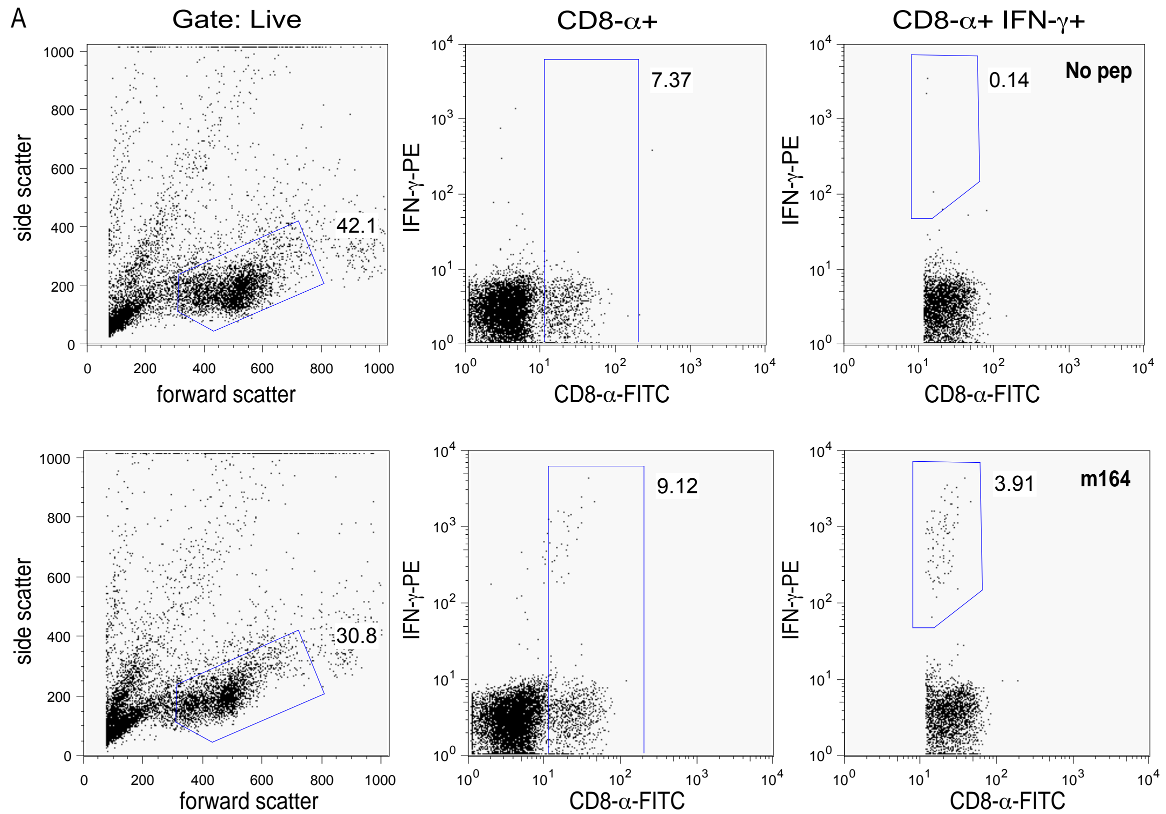


Figure 4.1. Analysis of the viral particle to PFU ratio of wild type and mutant virus preparations by quantitative PCR. Viral DNA was extracted from the indicated virus preparations and analyzed by real-time PCR using a DNA probe specific for the IE1 gene. Values were assessed in reference to a standard curve generated using an IE1-expressing plasmid. Titers of the virus preparations are listed below the x-axis. Error bars indicate SEM.



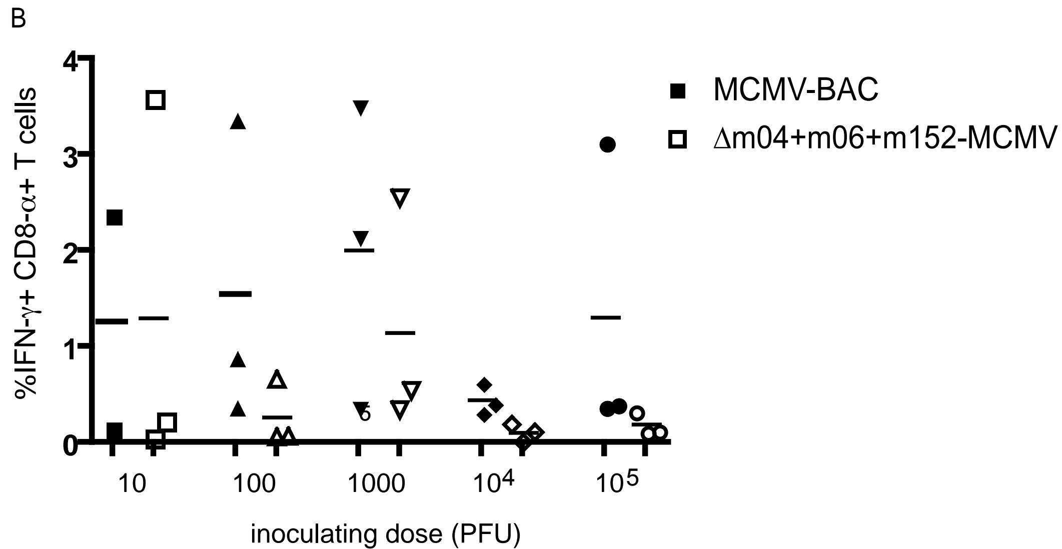


Figure 4.2. Very low doses of MCMV can generate a detectable m164-specific CD8 T cell response by the i.p. route of infection. BALB/c mice were infected with the indicated doses of MCMV-BAC or Δm04+m06+m152-MCMV. One week later, their spleens were harvested and splenocytes were incubated with the m164 peptide (10^{-6} M) for 7 hours in the presence of brefeldin A. Cells were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. A) Representative FACS plots for a mouse infected with 10 PFU of Δm04+m06+m152-BAC. The top plots show splenocytes stimulated with media and brefeldin A. The bottom plots show splenocytes stimulated with m164 peptide. B) The CD8 T cell response for individual mice is shown. Each symbol indicates an individual mouse and the horizontal bar indicates the mean. n=3 for each group. i.p.: intraperitoneal.

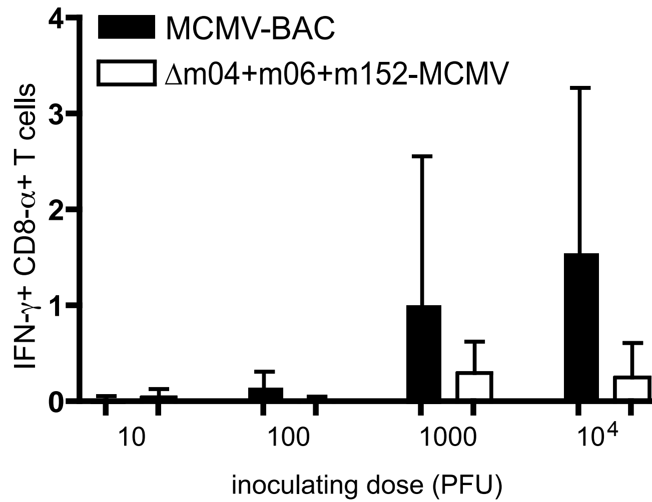
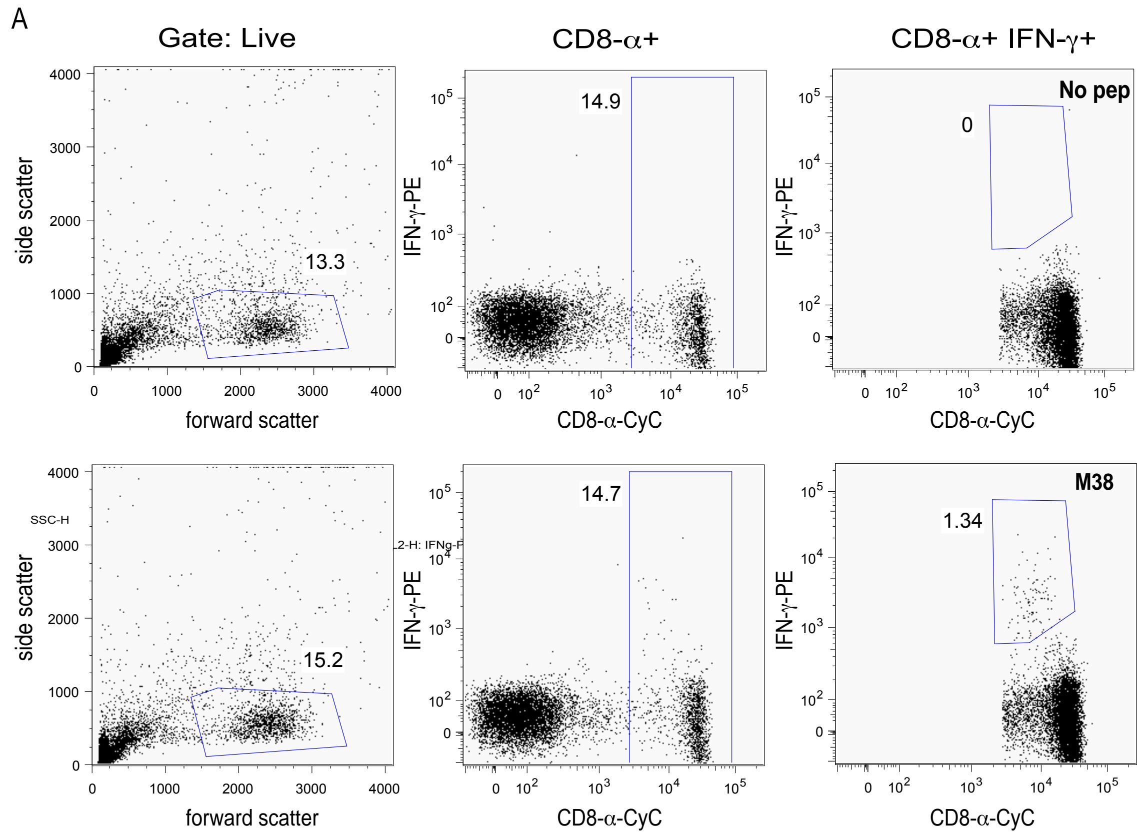


Figure 4.3. Very low doses of MCMV can generate a detectable m164-specific CD8 T cell response by the i.n. route of infection. BALB/c mice were infected with the indicated doses of MCMV-BAC or Δm04+m06+m152-MCMV. Four weeks later, their spleens were harvested and splenocytes were incubated with the m164 peptide (10^{-6} M) for 7 hours in the presence of brefeldin A. Cells were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. The mean CD8 T cell response is shown. Error bars indicate SEM. n=3 for each group. i.n.: intranasal.



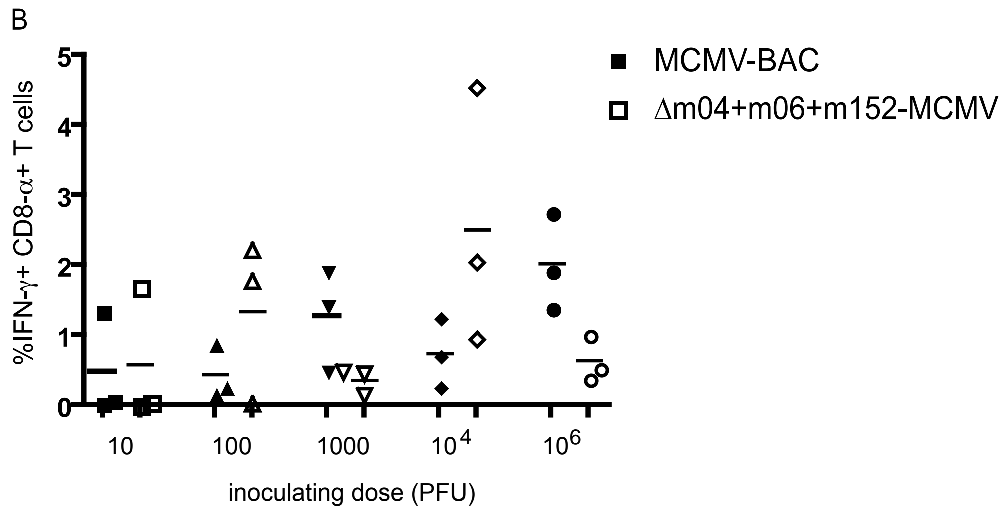


Figure 4.4. Very low doses of MCMV can generate a detectable M38-specific CD8 T cell response by the s.c. route of infection. C57BL/6 mice were infected with the indicated doses of MCMV-BAC or Δm04+m06+m152-MCMV. Two weeks later, their spleens were harvested and splenocytes were incubated with the M38 peptide (10^{-6} M) for 7 hours in the presence of brefeldin A. Cells were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. A) Representative FACS plots for a mouse infected with 10 PFU of MCMV-BAC. The top plots show splenocytes stimulated with media and brefeldin A. The bottom plots show splenocytes stimulated with M38 peptide. B) The CD8 T cell response for individual mice is shown. Each symbol indicates an individual mouse and the horizontal bar indicates the mean. n=3 for each group. s.c.: subcutaneous.

Table III. Comparison of 1000 PFU MCMV-BAC and Δm04+m06+m152-MCMV infection of BALB/c mice by various routes based on the CD8 T cell and antibody response and salivary glands titers

i.p.[a]	7 days[b]		21 days		13 weeks	
	MCMV-BAC	TKO[c]	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with SG virus[d]	1/2	0/5	1: 1/6[f] 2: 6/6	1: 6/6 2: 6/6	3/5	5/6
average titer in SG[e]	100 ± 0	0	1: 300±0 2: 1430±763	1: 1000 ± 880 2: 1150 ± 644	400 ± 265	420 ± 377
CTL response[g]	6/6	1/1	6/6	6/6	6/6	5/6
% IFN-γ^+ $CD8^+$ T cells	0.54 ± 0.3	0.22 ± 0	2.51 ± 0.47	1.45 ± 0.42	3.37 ± 0.97	1.11 ± 0.59
seropositivity[h]	0/6	0/6	1: 0/6 2: 6/6	1: 0/6 2: 4/6	6/6	3/5
s.c.						
# of mice with SG virus	0/6	0/6	1: 2/6 2: 2/6	1: 0/5 2: 0/6	0/4	1/6
average titer in SG	0	0	1: 350 ± 70.7 2: 700 ± 0	1: 0 2: 0	0	20 ± 0
CTL response	0/6	0/6	1: 6/6 2: 6/6	1: 5/5 2: 6/6	4/4	6/6
% IFN-γ^+ $CD8^+$ T cells	N.A.[i]	N.A.	1: 3.5 ± 1.14 2: 6.12 ± 1.68	1: 1.28 ± 0.59 2: 3.49 ± 4.22	1.49 ± 0.51	0.88 ± 0.61
seropositivity	0/6	0/6	N.D.	N.D.	3/5	2/6
i.n.						
# of mice with SG virus	N.D.[j]	N.D	0/6	0/6	3/6	2/6
average titer in SG	N.D	N.D	0	0	4470 ± 1360	1050 ± 495
CTL response	N.D.	N.D.	2/6	2/6	5/6	3/5
% IFN-γ^+ $CD8^+$ T cells	N.D.	N.D.	0.87 ± 0.88	0.68 ± 0.62	1.15 ± 0.77	0.28 ± 0.31
seropositivity	N.D.	N.D.	0/6	0/6	4/6	0/6

[a]Mice were infected i.p., s.c. (at the nape of the neck), or i.n. Virus was diluted to 1000 PFU in a total volume of 200 µL for i.p. and s.c. infection; for i.n. administration, 10 µL were instilled in each nare.

[b]On the indicated day p.i., mice were sacrificed, salivary glands harvested, and 500 µL of blood taken for sera and peripheral blood mononuclear cells (PBMC).

[c]TKO: the "triple knockout virus," Δm04+m06+m152-MCMV

[d]10% salivary glands (SG) homogenates were prepared in DMEM (10% FCS, supplemented with penicillin and streptomycin) and assayed for virus by a standard plaque assay on BALB 3T3 fibroblasts. The number of mice with virus in their salivary glands over the total number of mice per group is listed. The limit of detection is 100 PFU.

[e]The average only includes mice that had virus present in the salivary glands.

[f]Experiments under the exact same conditions performed on different days are shown individually to demonstrate experiment-to-experiment variation.

[g]The cytotoxic T cell (CTL) response to the IEI/pp89 antigen was measured from PBMC after red blood cell (RBC) lysis by intracellular cytokine staining for IFN-γ and analyzed on an LSR II flow cytometer, as described (Gold et al., 2002). The ratio of mice with a positive CTL response to the total number of mice per group is listed. The average % of IFN-γ^{+} $CD8^{+}$ T cells is also listed; this is taken from the mice with positive responses only. CD8 T cell responses were only considered positive if background was ≤ 0.2% and the IE1/pp89 response was ≥ 2X background.

[h]Antibody to MCMV was assayed by ELISA using MCMV- or mock-infected cell lysate as a plate antigen. Serostatus was determined from a 1:100 dilution of straight sera and was considered positive if it was ≥ 1.5X the background response to mock antigen. The ratio of mice with positive sera to the total number of mice per group is listed.

[i]N.A.: not applicable because the CD8 T cell response was not greater than background.

[j]N.D.: not done

Table IV. Comparison of 100 PFU MCMV-BAC and Δm04+m06+m152-MCMV infection of BALB/c mice by various routes based on the CD8 T cell and antibody response and salivary glands titers

i.p.[a]	7 days[b]		21 days		13 weeks	
	MCMV-BAC	TKO[c]	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with SG virus[d]	3/5	0/6	1/6	1/6	4/6	1/6
average titer in SG[e]	100 ± 0	0	100 ± 0	1200 ± 0	225 ± 150	420 ± 377
CTL response[g]	5/5	6/6	N.D.	N.D	6/6	6/6
% IFN-γ^+ $CD8^+$ T cells	0.45 ± 0.27	0.35 ± 0.13	N.D	N.D.	2.11 ± 1.6	2.31 ± 0.93
seropositivity[h]	0/5	0/6	0/6	0/6	1/6	6/6
s.c.						
# of mice with SG virus	0/6	0/6	0/6	1/6	1/6	0/6
average titer in SG	0	0	340 ± 378	100 ± 0	100 ± 0	0
CTL response	2/6	2/6	1. 6/6 2. 6/6	1. 3/6 2. 5/6	6/6	6/6
% IFN-γ^+ $CD8^+$ T cells	0.28 ± 0.12	0.19 ± 0.16	1. 3.89 ± 1.6 2. 2.15 ± 0.81	1. 0.68 ± 0.64 2. 0.82 ± 0.66	1.48 ± 0.38	0.58 ± 0.27
seropositivity	3/5	6/6	N.D.	N.D.	6/6	1/6
i.n.						
# of mice with SG virus	N.D.[i]	N.D	0/6	0/6	0/6	0/6
average titer in SG	N.D	N.D	0	0	0	0
CTL response	N.D.	N.D.	0/6	2/6	1/6	3/5
% IFN-γ^+ $CD8^+$ T cells	N.D.	N.D.	N.A.	N.A.	0.62 ± 0	0.26 ± 0.17
seropositivity	N.D.	N.D.	0/6	0/6	0/6	0/6

[a]Methods are the same as Table III, except that mice were infected with 100 PFU of virus.

Table V. Comparison of 1000 PFU MCMV-BAC and Δm04+m06+m152-MCMV infection of C57BL/6 mice by various routes based on the CD8 T cell and antibody response and salivary glands titers

i.p.	7 days		21 days		13 weeks	
	MCMV-BAC	TKO	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with SG virus	0/4	0/5	0/4	0/1	0/3	2/5
average titer in SG	0	0	0	0	0	200 ± 0
CTL response[a]	5/6	6/6	5/6	1/6	4/5	5/6
% IFN-γ^+ CD8$^+$ T cells	0.54 ± 0.29	1.03 ± 0.80	2.68 ± 1.57	1.02 ± 0	2.43 ± 0.12	4.02 ± 1.88
seropositivity	N.D.	N.D.	2/5	0/6	4/5	4/6
s.c.						
# of mice with SG virus	0/6	0/6	0/6	0/6	N.D.	N.D.
average titer in SG	0	0	0	0	N.D.	N.D.
CTL response	5/5	4/5	4/6	4/6	N.D.	N.D.
% IFN-γ^+ CD8$^+$ T cells	0.69 ± 0.38	0.75 ± 0.47	1.35 ± 0.69	1.05 ± 0.34	N.D.	N.D.
seropositivity	0/5	0/5	3/6	0/6	N.D.	N.D.
i.n.						
# of mice with SG virus	0/6	0/6	N.D	N.D	0/5	0/6
average titer in SG	0	0	N.D.	N.D.	0	0
CTL response	0/6	0/6	N.D.	N.D.	2/6	0/6
% IFN-γ^+ CD8$^+$ T cells	0	0	N.D	N.D.	2.32 ± 0.76	0
seropositivity	0/6	0/6	N.D.	N.D.	0/5	0/6

[a]Methods are the same as Table III, except that the CD8 T cell response to the M38 antigen was measured from PBMC.

Table VI. Comparison of 100 PFU MCMV-BAC and Δm04+m06+m152-MCMV infection of C57BL/6 mice by various routes based on the CD8 T cell and antibody response and salivary glands titers

i.p.	7 days		21 days		13 weeks	
	MCMV-BAC	TKO	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with SG virus	0/6	0/6	0/2	0/6	0/6	0/6
average titer in SG	0	0	0	0	0	0
CTL response[a]	6/6	5/6	4/6	1/6	4/6	2/6
% IFN-γ^+ CD8$^+$ T cells	0.94 ± 0.57	1.00 ± 0.77	0.92 ± 0.87	5.43 ± 0	1.76 ± 0.69	2.4 ± 2.55
seropositivity	N.D.	N.D.	0/6	0/6	4/6	4/6
s.c.						
# of mice with SG virus	0/6	0/6	0/6	0/5	N.D.	N.D.
average titer in SG	0/6	0/6	0	0	N.D.	N.D.
CTL response	2/6	4/6	4/6	1/6	N.D.	N.D.
% IFN-γ^+ CD8$^+$ T cells	0.97 ± 0.62	0.7 ± 0.41	1.97 ± 1.12	0.67 ± 0	N.D.	N.D.
seropositivity	N.D.	N.D.	3/6	1/6	N.D.	N.D.
i.n.						
# of mice with SG virus	N.D.	N.D.	N.D.	N.D.	0/5	0/6
average titer in SG	N.D.	N.D.	N.D.	N.D.	0	0
CTL response	N.D.	N.D.	N.D.	N.D.	0/4	0/6
% IFN-γ^+ CD8$^+$ T cells	N.D.	N.D.	N.D.	N.D.	N.A.	N.A.
seropositivity	N.D.	N.D.	N.D.	N.D.	0/5	0/6

[a]Methods are the same as Table III, except that mice were infected with 100 PFU of virus and the CD8 T cell response to the M38 antigen was measured from PBMC.

Table VII. Comparison of MCMV-BAC and Δm04+m06+m152-MCMV infection of BALB/c mice across all routes and doses based on the CD8 T cell response and titers in the salivary glands

combined routes, doses	7 days		21 days		13 weeks	
	MCMV-BAC	TKO[a]	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with a CTL response	13/23	9/19	44/54	39/53	28/34	29/34
# of mice with SG[b] virus	4/19	0/23	29/54	19/53	20/34	9/36
average SG titer	100	0	668 ± 739	1014 ± 732	1418 ± 2057	511 ± 451

[a]TKO: Δm04+m06+m152-MCMV
[b]SG: salivary glands

Table VIII. Comparison of MCMV-BAC and Δm04+m06+m152-MCMV infection of C57BL/6 mice across all routes and doses based on the CD8 T cell response and titers in the salivary glands

combined routes, doses	7 days		21 days		13 weeks	
	MCMV-BAC	TKO[a]	MCMV-BAC	TKO	MCMV-BAC	TKO
# of mice with a CTL response	11/17	10/17	9/12	5/12	6/11	5/12
# of mice with SG[b] virus	0/20	0/23	0/24	0/22	0/18	2/23
average SG titer	0	0	0	0	0	200 ± 0

[a]TKO: Δm04+m06+m152-MCMV
[b]SG: salivary glands

Table IX. Comparison of BALB/c and C57BL/6 mice that had a detectable CD8 T cell response at any time point following infection with MCMV-BAC or Δm04+m06+m152-MCMV

# mice with a detectable CD8 T cell response	**BALB/c**		**B6**	
	MCMV-BAC	Δm04+m06+m152-MCMV	MCMV-BAC	Δm04+m06+m152-MCMV
all routes	73/99 (73.7%)	67/94 (71.3%)	45/74 (60.8%)	3/77 (42.9%)
"natural routes" only	44/70 (62.9%)	43/69 (62.3%)	17/39 (43.6%)	3/41 (31.7%)

Table X. Comparison of BALB/c and C57BL/6 mice that had MCMV in their salivary glands at any time point following infection with MCMV-BAC or Δm04+m06+m152-MCMV

# of mice with detectable salivary glands virus	**BALB/c**		**B6**	
	MCMV-BAC	Δm04+m06+m152-MCMV	MCMV-BAC	Δm04+m06+m152-MCMV
all routes	27/88 (30.7%)	22/94 (23.4%)	0/34 (0 %)	2/35 (5.7%)
"natural routes" only	12/58 (20.7%)	4/47 (8.5%)	0/23 (0 %)	0/24 (0 %)

Table XI. Comparison of titers in the salivary glands in MCMV-BAC and Δm04+m06+m152-MCMV oral infection of BALB/c pups mice across doses and age at infection

# of mice with virus in the SG[a]	MCMV-BAC	Δm04+m06+m152-MCMV
21d (postnatal)[b]	1) 1/4 2) 0/4	1) 2/5 2) 0/4 3) 0/2
22d	1) 0/4 2) 0/8	1) 0/3
23d	1) 1/4 2) 5/5 3) 0/5 4) 1/9	1) 1/4 2) 3/5 3) 0/5 4) 0/10
24d	1) 0/3 2) 2/4	1) 0/2 2) 0/2
26d	1) 0/5	1) 0/6
total # of mice with virus in the salivary glands[c]	10/55 (18.2%)	6/48 (12.5%)
average titer	11950 ± 778	663 ± 404

[a]Pups were infected p.o. between postnatal day 1 and 5 with either 100 of 1000 PFU of MCCMV-BAC or Δm04+m06+m152-MCMV in 5 µL. SG: salivary glands

[b]21 days p.i., the salivary glands were harvested and 10% homogenates were prepared in DMEM (10% FCS, supplemented with penicillin and streptomycin) and assayed for virus by a standard plaque assay on BALB 3T3 fibroblasts. The ratio of mice with virus in their salivary glands to the total number of mice per group is listed. The limit of detection is 100 PFU.

[c]The ratio is given of the total number of mice with virus in the salivary glands to the total number of mice infected with that virus, combining doses and age at infection.

Chapter 5: THE IMPACT OF MURINE NOROVIRUS INFECTION ON OUR MODEL OF MCMV

Introduction

Animal models of infection and disease have been central to the study of pathogenesis, vaccine development, and drug treatment. While there are drawbacks to these models—particularly that they may not precisely model human disease—they are indispensable for making precise statements about the agent being studied, because they allow the investigator to control confounding variables. Mutant pathogens and animals can be generated and the impact of genetic polymorphisms, the timing and dose of infection, and exposure of animals to other pathogens can all be strictly controlled by using inbred mouse strains and housing them in SPF facilities.

SPF facilities are particularly important for immunological and pathogenesis studies, as unintentional infections can alter immune system functioning and confound experiments using other infectious agents. Use of SPF mice for this sort of study has been virtually universal for decades. SPF mice are typically screened for pathogens such as mouse parvovirus (MPV) and mouse hepatitis virus (MHV) that are known to spread endemically and affect experimental results (Rowe et al., 1959; Riley et al., 1960; Biggart and Ruebner, 1970; Bonnard et al., 1976; McKisic et al., 1993). But how do we define a pathogen? Agents that cause overt disease in healthy animals are automatically classified as pathogens and are excluded from animal facilities. However, a range of other microorganisms can colonize animals, including normal gastrointestinal flora, some of which can cause disease in immunocompromised animals. Some agents, such as *Helicobacter (H.) hepaticus* and *H. bilis* are part of the regular screens performed to monitor SPF facilities, and yet they are not eradicated by many facilities, including our own. Eradication of agents from a facility can be extremely costly, frequently requiring elimination of most of the colony and rederivation of breeder stock. In practice, investigators and facility managers are generally motivated to eradicate new agents by evidence that the agents impact experimental outcomes.

Murine norovirus (MNV) was discovered in SPF mice at Washington University by Karst *et al*. five years ago when untreated recombination-activating gene 2 and signal transducer and activator of transcription 1 knockout (RAG-$2^{-/-}$/STAT-$1^{-/-}$) mice within an SPF colony started dying of a lethal infection of unknown cause (Karst et al., 2003). MNV-1 was subsequently isolated and identified as the first calicivirus to infect rodents. It has since been suggested that MNV infection of laboratory mice is endemic, presumably being spread by the oral-fecal route as in humans (Perdue et al., 2007). A recent study of mice in research facilities within North America found that 22% of serum samples were MNV seropositive, making MNV the most common mouse colony contaminant known (Hsu et al., 2005). Caliciviruses are single-stranded, positive-sense RNA viruses that are non-enveloped and non-segmented. There are ~7400 base pairs that code for three ORFs and eight known proteins. The original strain was named MNV-1; 26 MNV-1 genomes have since been isolated from different institutions around the world and sequenced (Karst et al., 2003; Wobus et al., 2006; Thackray et al., 2007). Of the 15 unique strains identified by Thackray *et al.*, there is a maximum of 13% nucleotide divergence at the whole genome level (Thackray et al., 2007). Minor amino acid changes can quite significantly impact the virulence of strains, however, as mutation in only two amino acids can attenuate a virulent strain (Bailey et al., 2008). However, all known strains form a single genotype, genogroup, and serotype. It is important and convenient that serological cross-reactivity exists, because infection with any of the isolated strains can be identified using a single antibody (Hsu et al., 2006; Lochridge and Hardy, 2007; Thackray et al., 2007).

There has been much concern, particularly in the immunology community, about the potential impact of MNV on infection and disease models in mice and thus, the need for its eradication. As a consequence, I embarked on the current study to investigate how MNV-1 impacts the outcome of experiments carried out in our own laboratory. MNV seems most likely to pose a problem for experiments involving the immunological pathways that are important for its control. It is clear that the IFN response is important in this regard, as MNV-1 infection is lethal

to mice that lack the receptors for both type I and type II interferons (IFN-$\alpha/\beta/\gamma R^{-/-}$). MNV-1 is also lethal to mice that lack STAT-1, a transcription factor activated by IFN signaling that ultimately induces expression of interferon stimulated genes (ISG) (Karst et al., 2003). MNV is not lethal, however, to mice that have *either* the IFN-α/β (type I) or the IFN-γ (type II) system intact (Karst et al., 2003; Muller et al., 2007). Mice that lack other aspects of the innate or adaptive immune systems, such as inducible NOS$^{-/-}$ or PKR$^{-/-}$ and RAG-1$^{-/-}$ or RAG-2$^{-/-}$, respectively, do not succumb to infection (Karst et al., 2003).

MNV infection can be either acute or persistent; persistence is both virus strain- and mouse strain-specific. For example, wild type 129/SvJ mice clear MNV-1 CW after acute infection, but 129/SvJ RAG-2$^{-/-}$ mice are persistently infected with MNV-1 CW and have viral RNA in multiple tissues up to 90 days p.i. (Karst et al., 2003). B6 RAG-1$^{-/-}$ mice are also “chronic secreters” of MNV-1 CW, but wild type B6 mice clear the virus by day seven p.i. (Karst et al., 2003; Thackray et al., 2007). Strains that are acutely cleared in wild type animals include MNV-1 CW1, CW3, and WU11, while strains CR3, CR6, and CR7, and WU11 have been shown to be persistent (Thackray et al., 2007).

MNV infection is not as symptomatic in mice as its human counterpart, which causes gastrointestinal illness. There are a few examples where mice show overt signs of infection, however. Before STAT-1$^{-/-}$ mice succumb to MNV-1 CW3 infection, they display gastrointestinal symptoms, including diarrhea, decreased gastric emptying, and decreased fecal weight contents, in addition to splenitis and pneumonia at 72 hours p.i. (Karst et al., 2003; Mumphrey et al., 2007). While MNV-1 infection has a clear impact on the morbidity and mortality of some immunodeficient, knock-out mice, the nature of infection and immune response in immunocompetent animals is still under investigation. MNV-1 CW3-infected B6 and 129/SvJ mice are asymptomatic, but infected 129/SvJ mice have evidence of splenic red pulp hypertrophy and granulocyte infiltration in the intestine at three days p.i. (Mumphrey et al., 2007). Viral RNA

is detectable in the liver, spleen, and intestine up to three days p.i. in 129/SvJ mice and up to seven days p.i. in B6 mice (Karst et al., 2003; Mumphrey et al., 2007). MNV-1 infection induces a strong serum type I IFN response in 129/SvJ mice, which peaks at 24 hours p.i. (Thackray et al., 2007). This cytokine response suggests that silent MNV infection could have a strong and polarizing effect on the immune system of immunocompetent mice.

MNV is present in most laboratory mouse colonies in North America, but now most major commercial suppliers are free of MNV. The virus is thought to be readily transmitted by fecal dust that is spread by normal handling of mouse cages. Thus, mice purchased for experiments have a significant chance of undergoing acute MNV infection at some stage during an experiment. Another laboratory at our institution experienced a dramatic change in the LD_{50} of their mice for West Nile virus (WNV) at about the same time that their colony became infected with MNV (Janko Nikolich-Zugich, personal communication). Our laboratory routinely measures the CD8 T cell response by cytokine production and viral control by comparing MCMV titers in various organs. There is natural variability in this system, and we have the impression that responses and titers seem to be higher amongst an entire experimental cohort in some experiments than in others (see Chapter 4; Doom and Hill, 2008). The variability is greater than might be expected using inbred mice and standard, TC-derived virus. When MNV was added to the panel of agents for which our sentinel mice are routinely tested, we became aware of the presence of MNV in our mouse colony. I therefore wondered whether MNV contamination of our experimental mice was responsible for some of the variation we see in our model.

The following study was performed in order to assess the effect on MNV on MCMV infection and the ensuing immune response. I tested two strains of mice (BALB/c and B6) and two strains of MNV (one persistent, one acute), varying the timing of both infections to model potential random MNV contamination.

Results

Handling of mice to avoid inadvertent MNV infection

Because these experiments were performed in an animal room in which some sentinel mice had tested positive for MNV, it was important to prevent accidental MNV infection of the study mice. MNV is thought to be transmitted within an animal facility by means of infectious fecal material spreading through the air as dust. The Virgin laboratory has established a strict protocol for handling mice that enables infected and uninfected mice to be housed in the same room without spread of MNV, and I followed these procedures in our facility. Mice were housed in standard cages with filter tops and were only handled in a biosafety cabinet dedicated to these experiments and only by two individuals who did not handle other mice in the colony. All cage equipment was autoclaved as a unit and stored in the autoclave bag, which was opened in the biohazard cabinet to prevent exposure to airborne fecal dust. The water was autoclaved and supplied in individual bottles and all food was irradiated. Surfaces and supplies were disinfected before and after use for at least 10 minutes in a 10% bleach solution. Whenever mice were handled, infected, or harvested, naïve mice were handled first, followed by MCMV-infected mice, followed by MNV- (± MCMV-) infected mice to prevent any contamination.

Mice were tested for MNV seropositivity before use in an experiment and on the day of sacrifice. Seroconversion generally occurs by day 21 p.i, but does not peak until at least day 35 p.i. (Karst et al., 2003; Thackray et al., 2007); therefore, serological testing could not exclude inadvertent infection for many experiments. In total, 272 mice were used in this study. Over half of them (152) were in the facility for greater than 21 days; the average length of time in the facility before sacrifice was 63 days. For all of these experiments, the only mice that were seropositive at sacrifice were mice that had been intentionally infected with MNV. Furthermore, no sentinels from the rack of mice used in these experiments ever tested positive for MNV or any other pathogen tested. Therefore, although I cannot conclusively exclude the possibility of inadvertent MNV infection shortly before sacrifice, the fact that I found no evidence of

unintentional infection indicates that it is likely that the vast majority of the control mice (MNV-non-infected) in this study remained MNV-free.

MNV infection does not impact the timing or magnitude of MCMV viral titers

In order to ask whether MNV contamination impacts our model of MCMV infection, it was important to model a number of scenarios. I considered that mice purchased commercially arrive at our facility free of MNV, but may become infected at any time thereafter. I thought that MNV contamination would be the most likely to impact our experimental results if acute MNV infection occurred around the time of initial infection with MCMV. Hence, the first set of experiments tested whether MNV infection at the time of or immediately before MCMV infection would impact the course of MCMV infection. Mice were infected p.o. with MNV-1 strain CW3, which causes acute, cleared infection in immunocompetent mice (Karst et al., 2003; Mumphrey et al., 2007). Either the same day or one or two days later, I infected these and control mice i.p. with $5x10^5$ PFU MCMV-BAC (Figure 5.1). I tested both B6 and BALB/c mice to determine whether MNV infection had an impact in either strain. I also performed a similar experiment whereby B6 mice were infected p.o. with MNV-1 strain CW3 and two days later these and control mice were infected i.p. with $2x10^5$ PFU MCMV-K181 (Figure 5.2). MCMV titers were measured in multiple organs four days post-MCMV, the peak of acute viral titers. Because the goal of these experiments was to determine the impact of MNV on MCMV infection, only *MCMV* viral titers were measured. I am confident that the plaques assessed as MCMV were in fact MCMV and not MNV, because MNV is very poorly infectious for the BALB 3T3 fibroblast cell line that we use to plaque MCMV. In addition, all plaques on BALB 3T3s from representative experiments were positive for the MCMV protein, IE1/pp89, by immunofluorescence (data not shown).

As expected, MCMV titers were much higher in BALB/c than B6 mice. In B6 mice, the presence of virus by plaque assay at early time points is quite variable at the infecting dose used of $5x10^5$ PFU. Multiple experiments are shown to highlight the variability seen in detecting

MCMV-BAC in the organs at acute time points (compare the spleen titers in Figure 5.1 BALB/c i. and iii.), which I hypothesized could be explained by concomitant MNV infection. Occasionally, comparisons revealed a small but significant difference between titers in individual organs within an experiment. For example, in Figure 5.1 C57BL/6 ii., four out of 12 mice infected with MCMV only had MCMV detectable in the liver (mean titer = 125 PFU), while 10 out of 12 mice infected with MCMV+MNV had MCMV detectable in the liver (mean titer = 220, p=0.001). However, in the same experiment, no other organs showed a significant difference in titers. Furthermore, in a parallel experiment (Figure 5.1 C57BL/6 iii.), the titers did not differ in the liver (0.5476). Overall, there was no consistent impact of concomitant MNV infection on MCMV titers at day four post-infection. In addition, acute titers of the more virulent MCMV-K181 strain were not impacted by MNV-1 CW3 exposure two days prior to MCMV infection in B6 mice (Figure 5.2).

While MNV did not alter the earliest course of MCMV infection, it was possible that MNV could impact later aspects of MCMV infection, particularly, the ability of MCMV to spread to and replicate in the salivary glands. Therefore, I investigated the impact of MNV on the kinetics of acute MCMV infection in B6 mice at three, seven, and 14 days post-MCMV (Figure 5.3) and 21 days post-MCMV (the peak of salivary glands infection) in both BALB/c and B6 mice (Figure 5.4A). Figure 5.4A shows that the MCMV-BAC titers at the peak of the viral load in the salivary glands were nearly identical with or without MNV infection in both strains of mice. MCMV was detected in the spleens of some mice at this late time point, which is a sporadic, yet common, finding that contributes to the variability seen in our model of MCMV infection. The presence of MCMV in the spleen cannot be explained by exposure to MNV, however, as MCMV was also found in the spleens of mice infected with MCMV alone. These results suggest that acute MNV infection does not impact viral control in early MCMV infection, nor does acute MNV infection impact whether MCMV can establish infection or spread to and replicate in the salivary glands. Most importantly, these results provide no evidence that acute MNV infection is

the cause of the variability seen in our model, including the occasional presence of virus in the spleen at 21 days post-MCMV-BAC.

To ask whether infection with a persistent strain of MNV-1 could impact subsequent MCMV infection, I infected B6 mice as described above with MNV-1 strain CR6 (passage 2). CR6 persists in the distal ilea and mesenteric lymph nodes of B6 mice up to 35 days p.i. (Thackray et al., 2007). At 21 days p.i. with MCMV-BAC, despite ongoing MNV-1 shedding in the feces (data not shown), no MCMV was detected in any organ except for the kidney (Figure 5.4B). These data highlight two important points. First, as seen before, the presence of MCMV-BAC in the salivary glands of B6 mice at 21 days p.i. was variable (compare to Figure 5.4A). Second, neither cleared MNV-1, nor ongoing MNV-1 replication, impacted the MCMV viral burden in the salivary glands.

Acute MNV affects the acute, immunodominant CD8 T cell response to MCMV

While MCMV virus titers were not impacted by concurrent MNV-1 infection, it was possible that MNV-1 infection could affect the CD8 T cell response to MCMV infection. CD8 T cell responses can be affected by cytokines, including IFN-α and IL-12, and MNV-1 has been shown to induce a strong IFN-β response in 129/SvJ mice, which peaks at 24 hours p.i. (Mumphrey et al., 2007). To determine whether acute MNV-1 infection impacts either the magnitude of the CD8 T cell response or the immunodominance hierarchy, mice were infected p.o. with MNV-1 strain CW3 and control mice were infected i.p. two days later with MCMV-BAC. A week later, the CD8 T cell response to MCMV was measured in splenocytes by ICCS for IFN-γ (Figure. 5.5). In BALB/c mice, the percentage of IFN-γ^+ CD8-α^+ T cells specific for the two most immunodominant peptides, IE1/pp89 and m164, was modestly but significantly lower in mice infected with MNV (p=0.005 and 0.0003, respectively). The same result was seen in two separate experiments using T cells isolated from the peripheral blood (data not shown). Similarly,

in B6 mice, the magnitude of the response to the most immunodominant peptide, M45, was lower in MNV-infected mice (p=0.0446). The immunodominance hierarchy was not affected by MNV in either mouse strain. Thus, concomitant MNV infection did affect the CD8 T cell response to MCMV infection, although the impact was modest.

MCMV does not reactivate in response to MNV infection

By four weeks p.i., MCMV-BAC is generally undetectable in any organ by plaque assay, although latent infection—and perhaps undetected, persistent, replicative infection—continues (see Chapter 4; M. Gold, unpublished observation). Reactivation of latent MCMV is an important, yet still poorly understood, part of the infectious cycle that can be experimentally induced by a number of stimuli. Notably, inflammatory stimuli, such as bacterial infection, LPS, TNF-α, and IL-1 have been shown to induce MCMV reactivation (Koffron et al., 1999; Cook et al., 2002; Simon et al., 2005; Cook et al., 2006). Our laboratory has occasionally found several mice housed within the same cage with MCMV viral loads in the spleen or other organs at a time p.i. at which the virus is usually undetectable (X. Lu, unpublished observation). These findings have bedeviled experimental reproducibility and made us suspect a non-random, adventitious event.

Because MNV-1 infects some of the cell types in which MCMV has been shown to be latent (Mercer et al., 1988; Pomeroy et al., 1991; Pollock et al., 1997; Koffron et al., 1998; Hanson et al., 1999; Wobus et al., 2004), and because it induces inflammatory cytokines, I thought it possible that MNV may induce reactivation of latent MCMV. In order to test this, mice were infected with MCMV-BAC for at least four weeks, after which half of the mice were also infected with acute MNV-1 CW3 (Figure 5.6A and B). MCMV reactivation models have different peaks of reactivation depending on the stimulus used, therefore, viral titers were measured either one or two weeks post-*MNV* in the salivary glands, lung, and spleen—the organs in which MCMV reactivation is most readily detected (Balthesen et al., 1993). Nevertheless, I

detected no difference in viral titers in the presence or absence of MNV stimulation, suggesting that MNV infection did not induce reactivation of MCMV in either BALB/c or B6 mice.

While the original report of MCMV-BAC indicated that it behaved as wild type Smith strain both *in vitro* and *in vivo* (Wagner et al., 2002), in our hands, infection with MCMV-BAC leads to lower viral load in the organs than with the Smith or K181 strains. I therefore repeated the reactivation experiments with the more virulent K181 strain (Figure 5.6C). K181 caused persistent infection of the salivary glands in B6 mice, as has been reported (M. Degli-Esposti, unpublished observation). However, MNV infection did not cause any alteration of virus titers to indicate reactivation in either B6 or BALB/c mice.

Discussion

This study was undertaken in order to determine whether the recently discovered mouse pathogen, MNV, had an impact on a mouse model of MCMV infection. MNV is a common contaminant in mouse colonies around the world, and while there is much concern regarding this emerging pathogen, there have been no reports to date on its impact on other infectious agents or models. There is a degree of variability in our *in vivo* MCMV model that has always been puzzling. The variation segregates by experimental day and often by cage. Mice infected with the same dose on different days may have MCMV titers in their salivary glands that differ by up to two logs at 21 days p.i. Even more alarming is that two cages of mice infected with the same dose on the *same* day may have similar MCMV titers in their salivary glands within a cage, but rather different between cages.

This kind of variability could be explained by inadvertent infection with another agent. Upon learning that sentinel mice in our colony were MNV seropositive, I was keen to see whether MNV was the non-random cause of our experimental variability. It is important to note that the experiments performed for this study were solely concerned with the outcomes of MCMV infection; I did not address the impact of MCMV on MNV-1 pathogenesis. In order to

assess the effect of MNV-1 on MCMV infection, two well-characterized outcomes of infection were measured: the MCMV-specific CD8 T cell response and MCMV viral titers.

I focused primarily on the impact of MNV-1 infection occurring around the time of MCMV infection, which I thought would most likely to affect our experimental outcomes. However, I did not detect an impact of MNV-1 on acute MCMV infection or on persistent infection of the salivary glands. Furthermore, acute MNV-1 infection did not drive detectable reactivation of latent MCMV. I can cautiously conclude that MNV-1 does not have a large impact on the course of MCMV infection in immunocompetent mice and is unlikely to be a major contributor to the experimental variation within our model system. The cause of our experimental variation remains to be determined; possible explanations that have not yet been addressed include adventitious infection with a currently unknown agent (it is unlikely that MNV will be the last such agent to be identified), differences in commensal flora between commercial mice and those bred in our facility, and the impact of the estrus cycle (as we predominantly use female mice).

There are possible caveats to this conclusion, which I base on experimental infection with two laboratory-passaged isolates of MNV-1. A growing number of MNV-1 isolates have been identified that possess different characteristics regarding persistence, etc., and it has also been reported that it does not take many generations for the phenotype of a strain to change quite drastically (Mumphrey et al., 2007; Thackray et al., 2007; Bailey et al., 2008). The strains that have spontaneously contaminated our mouse colony on the West coast may have very different characteristics than those propagated for use in this study. Nevertheless, I used high dose infection with two very different MNV strains without detecting an impact on the course of MCMV infection.

I did reproducibly detect a small, but significant, impact of MNV-1 infection two days prior to MCMV infection on the size of the CD8 T cell response to the most immunodominant epitopes in both BALB/c and B6 mice. Curiously, there was no impact on the magnitude of the

CD8 T cell response to subdominant peptides, nor were there any changes in the hierarchy itself. As described in the introduction, evidence from knock-out mice indicates that MNV control depends on innate immune cytokines. The characterization of innate immune control of MNV is incomplete, but it is known that MNV-1 infection induces a strong serum type I IFN response in 129/SvJ mice, which peaks at 24 hours p.i. (Thackray et al., 2007). The CD8 T cell response is known to be regulated in part by inflammatory cytokines, including type I IFN, and it is possible that the impact of MNV on the cytokine milieu present at the time of CD8 T cell priming may be responsible for its impact on the CD8 T cell response to MCMV. This impact was rather small and would not alter the interpretation of most of our experiments. Nevertheless, it should be kept in mind, particularly for studies on immunodominance and the size of the CD8 T cell response.

Despite the modest difference in the CD8 T cell response with MNV exposure, prior or concomitant MNV infection did not reproducibly impact MCMV viral titers in either BALB/c or B6 mice. The MCMV titers were the same regardless of the timing of infection, the time points at which virus titers were measured, and the organs examined. The virus titer results strongly suggest that the immune response induced by MNV infection does not generally dampen the immune response to MCMV. In BALB/c mice, the organ titer results also suggest that MNV exposure did not enhance the immune response to MCMV, because the MCMV viral loads were not decreased in mice infected with MNV. MNV also does not induce reactivation of MCMV-BAC in either BALB/c or B6 mice. I hypothesized that MNV infection could induce reactivation because MNV infects macrophages and DCs, both cell types shown to harbor latent MCMV (Pollock et al., 1997; Koffron et al., 1998; Wobus et al., 2004). The results show, however, that this is not the case, and while MNV infection is presumably stimulating significant cytokine production, it could be an insufficient amount, the wrong cytokines, or in the wrong compartment to induce reactivation.

That MCMV viral titers did not change in the face of a decreased CD8 T cell response could be explained by a few things. First, the impact on the CD8 T cell response was modest and

is likely not biologically relevant. Second, the relationship between the antigen-specific CD8 T cell response and viral burden is complicated by many factors—most importantly, redundancy of the immune system. Finally, it is possible that MNV *does* impact MCMV viral titers but at time points or physiological sites not measured in this study, although I consider that unlikely given the breadth of scenarios measured.

This study cannot predict the impact that MNV might have on other infectious models. MNV-1 is much more likely to impact studies using immunodeficient mice, since it is lethal for mice severely impaired in IFN responses (Karst et al., 2003). Further investigations are needed before the overall impact of this agent is clear. In the meantime, MNV remains a non-random variable that is superimposed on our experiments. Mice purchased from commercial vendors are free from MNV, whereas strains bred in-house at many facilities may be contaminated. In particular, immunodeficient mutant strains are more likely to be chronic carriers (Wobus et al., 2006). Given these considerations, even without direct evidence of the impact of MNV on experimental models, it is likely that many facilities will decide to eradicate MNV, as some already have. Eradication of MNV from most facilities will require embryonic rederivation of mouse strains that cannot be purchased commercially. Since it is highly unlikely that MNV is the last "adventitious" murine infection that will be discovered, a side benefit of rederivation is that it will likely remove other as yet undiscovered agents in addition to MNV.

As I conclude that MNV exposure does not explain the aspect of variability we see in our model, the cause of variability remains to be determined. The prospect of a contaminating infection altering our results has led my laboratory to think more, however, about the SPF environment in which our mice are housed and how that may impact the questions we are asking. In the following and final discussion chapter of this dissertation, I will include an analysis of our model itself and how it could be changed to try to more closely model the true immunobiology of MCMV infection.

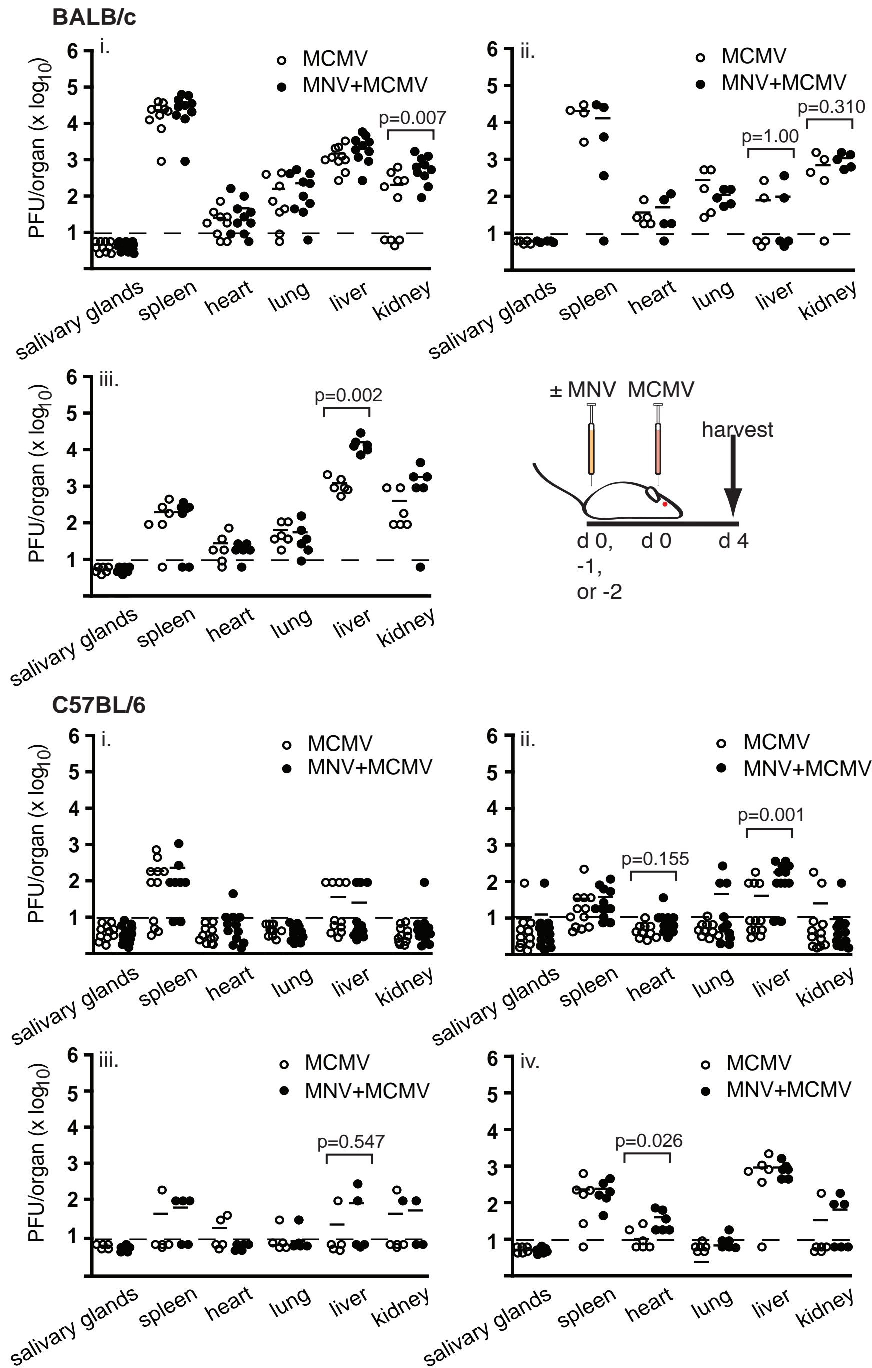


Figure 5.1. MNV infection does not alter the magnitude of MCMV-BAC infection. BALB/c or C57BL/6 mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2, -1, or 0. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV-BAC. Four days later (d 4), mice were sacrificed. Salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid N_2. MCMV titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 or 12 for each group. Roman numerals (i-iv.) represent similar experiments performed on different days. MNV was given on day 0 in: BALB/c i.; C57BL/6 i. MNV was given on day -1 in: BALB/c ii.; C57BL/6 ii., iii. MNV was given on day -2 in: BALB/c iii.; C57BL/6 iv. All mice were MNV-seronegative. All p-values comparing organ virus titers ± MNV infection were > 0.100, unless otherwise noted. In each case where there was a significant difference between organ virus titers, there is an example provided from a similar experiment where no significant difference was found.

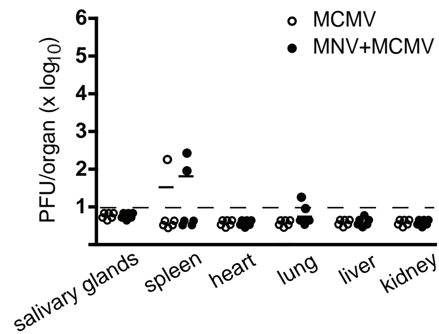


Figure 5.2. MNV infection does not alter the magnitude of MCMV-K181 infection. C57BL/6 mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV-K181. Four days later (d 4), mice were sacrificed. The salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid nitrogen. MCMV titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. All mice were MNV-seronegative. All p-values comparing organ virus titers with and without MNV infection were > 0.100.

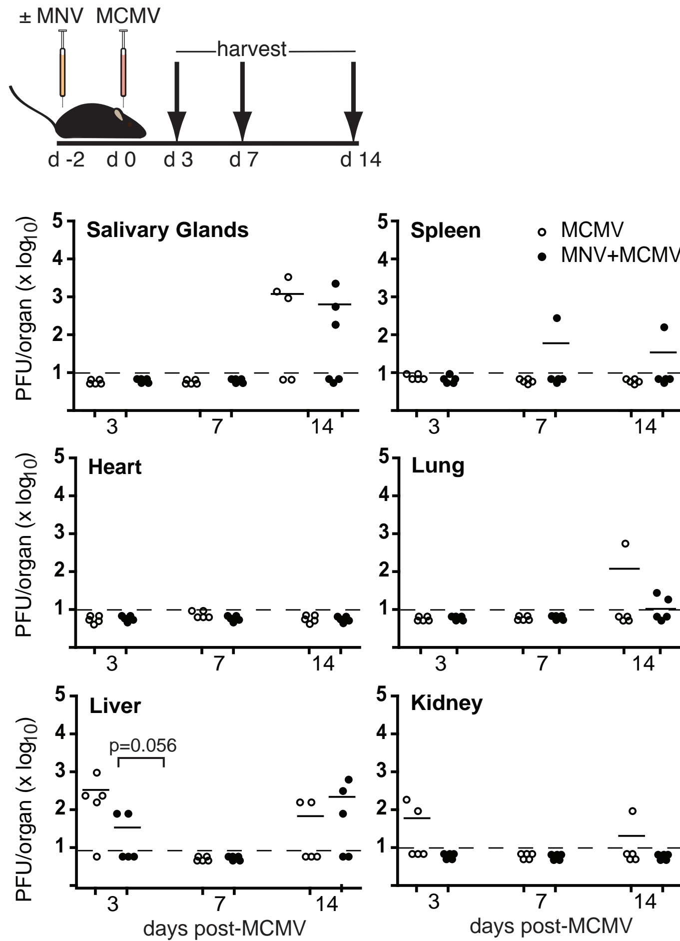


Figure 5.3. MNV infection does not alter the kinetics of MCMV infection. C57BL/6 mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV-BAC. Three, 7, and 14 days later mice were sacrificed. The salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid nitrogen. MCMV titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. All mice were MNV-seronegative. All p-values comparing organ titers with and without MNV infection were > 0.200, unless otherwise noted.

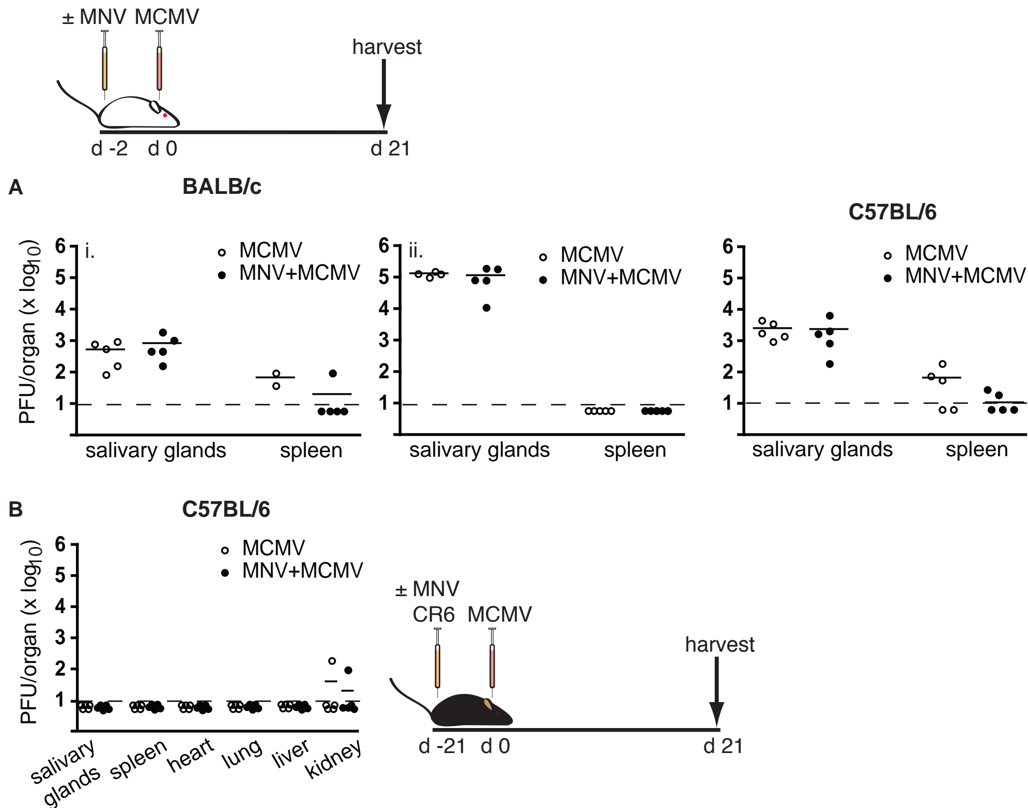


Figure 5.4. MNV infection does not impact the peak of MCMV titers in the salivary glands. A) C57BL/6 or BALB/c were infected p.o. with 3x10^7 PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with 5x10^8 PFU MCMV-BAC. B) C57BL/6 mice were infected p.o. with 10^5 PFU MNV-1 CR6 on day -21 and infected i.p. with 5x10^5 PFU MCMV-BAC on day 0. Twenty-one days later, mice were sacrificed. The salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid nitrogen. MCMV titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. Roman numerals (i.-ii.) represent identical experiments performed on different days. All mice were MNV-seronegative except A) 12/15 MNV-infected mice were MNV-seropositive and B) 5/5 MNV-infected mice were MNV-seropositive.

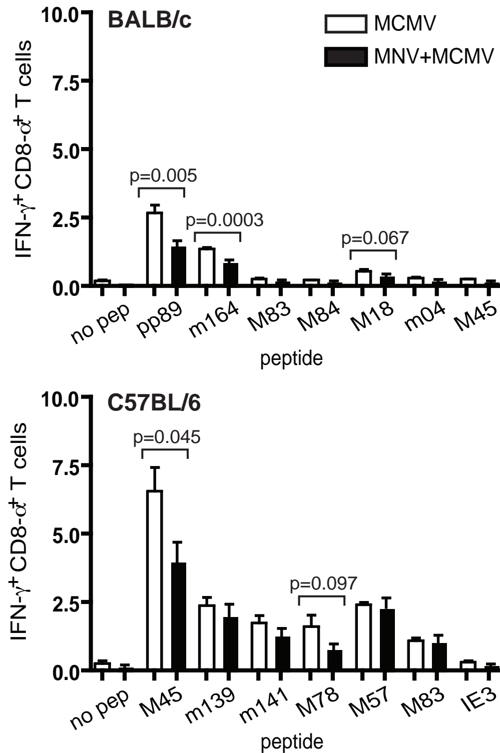


Figure 5.5. MNV infection impacts the immunodominant CD8 T cell response to MCMV. BALB/c or C57BL/6 mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV-BAC. A week later (d 7), the spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. Error bars indicate SEM. n=6 for each group. All mice were MNV-seronegative. All p-values comparing an epitope-specific response with and without MNV infection were > 0.100, unless otherwise noted.

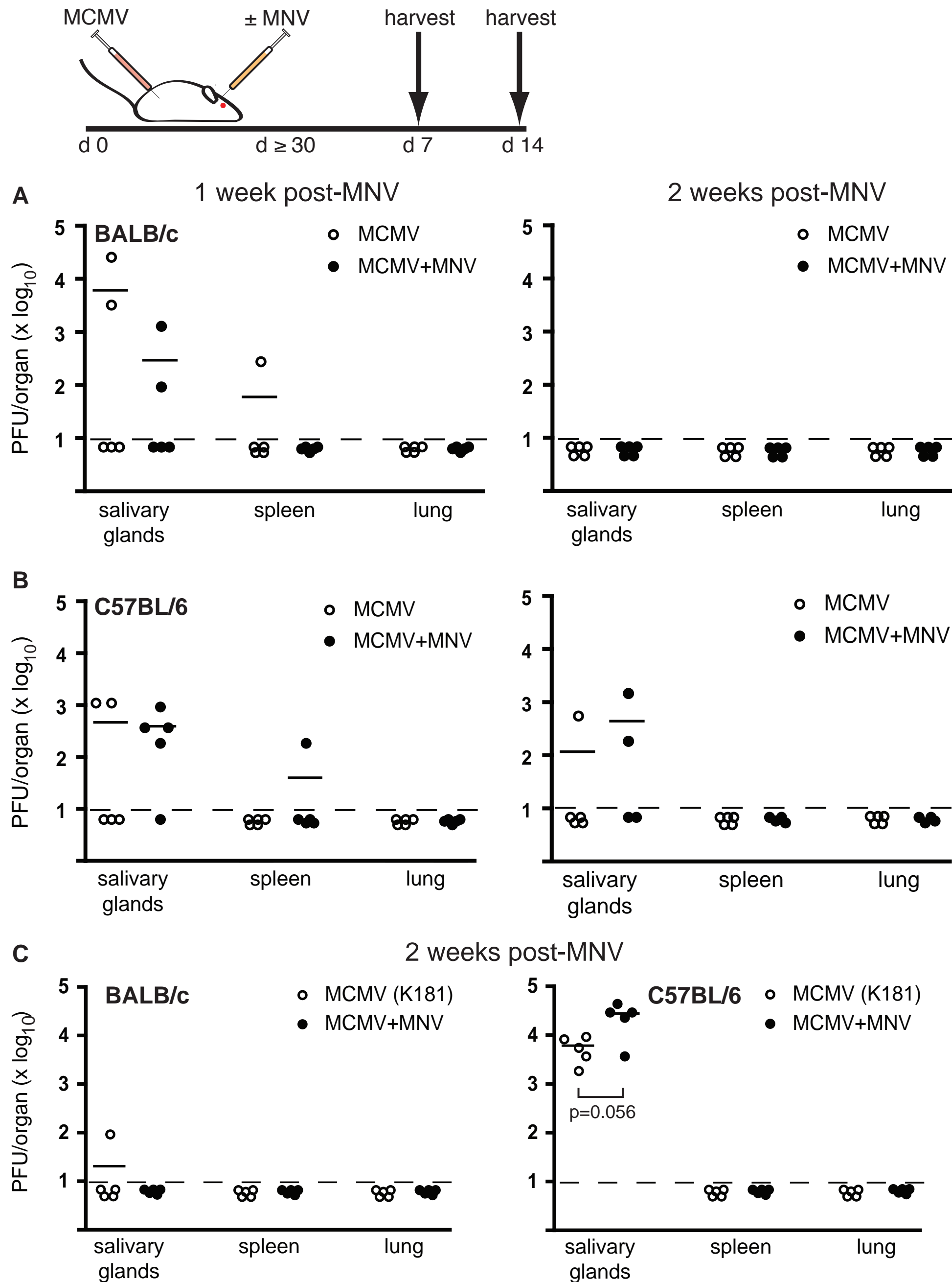


Figure 5.6. MCMV-BAC does not reactivate in response to MNV infection. A) BALB/c or B) C57BL/6 mice were infected i.p. with $5x10^5$ PFU MCMV-BAC on day ≥ 30. On day 0, mice mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3. Either 1 (left panels) or 2 (right panels) weeks later, mice were sacrificed. C) BALB/c (left) or C57BL/6 (right) were infected i.p. with $5x10^5$ PFU MCMV-K181 on day ≥ 30. On day 0, mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3. Two weeks later, mice were sacrificed. The salivary glands, spleen, and lung were harvested into 1 mL DMEM-complete. MCMV titers in these organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. All mice were MNV-seronegative. All p-values comparing organ virus titers with and without MNV infection were > 0.400, unless otherwise noted.

Chapter 6: DISCUSSION

I approached this work wanting to better understand the CD8 T cell response to MCMV infection in immunocompetent mice. CD8 T cells have been shown to be required for viral control in the bone marrow transplant model of MCMV infection, but it is important to consider, in this case, the lack of immune system redundancy. The experiments in that model were central to showing that CD8 T cells are required to control MCMV infection in some situations, but it is a situation of force. If the layers of redundancy are removed, can CD8 T cells control MCMV? Yes. What if the layers of redundancy are intact—then what is their necessity? This is far less clear.

I focused my work on the fact that MCMV encodes MHC class I immune evasion genes, the products of which robustly inhibit CD8 T cell-mediated killing of infected cells *in vitro*. There was also evidence *in vivo* than a mutant virus lacking the MHC class I immune evasion gene *m152* was better controlled than a wild type revertant virus in the bone marrow transplant model (Holtappels et al., 2004). These data, along with the fact that these genes are selected for by evolution, led me to expect that the immune evasion proteins would have a demonstrable impact on the CD8 T cell response *in vivo*. For my dissertation work, I committed to identifying the role of these proteins as a “counterattack” mechanism in immunocompetent mice.

To my surprise, I discovered that the MHC class I immune evasion proteins do not have a global phenotype *in vivo*, except for the approximately 10-fold growth advantage they provide in the salivary glands of BALB/c mice, as first reported in Lu *et al.* (Lu et al., 2006). By focusing almost exclusively *in vivo*, I also uncovered a phenomenon that our laboratory has consistently, but sporadically, observed—one of considerable variation in our *in vivo* model. We usually avoid this by choosing conditions where we see relative consistency, but when we investigate other conditions (*e.g.* low dose infection, viral titers in B6 mice), variability becomes a problem. Those

findings have turned this dissertation into not simply an analysis of the T cell response to MCMV infection, but an analysis of our model itself.

Taken together, my experimental findings tell us that:

• The magnitude and immunodominance hierarchy of the acute, wild type MCMV-specific CD8 T cell response is impacted by non-MHC genes. B6 and 129 mice share their MHC haplotype, but B6 mice mount a stronger CD8 T cell response and it is driven by different epitopes. B6 H-2^{bxd} mice respond to both H-2^{b}- and H-2^{d}-restricted epitopes in acute infection, while BALB/c H-2^{bxd} mice respond primarily to H-2^{d}-restricted epitopes. Finally, CD$40^{-/-}$ mice have a much more robust CD8 T cell response than BALB/c mice (Chapter 3).

• The magnitude and immunodominance hierarchy of the chronic, wild type MCMV-specific CD8 T cell response is impacted by non-MHC genes. In chronic infection, B6 H-2^{bxd} and BALB/c H-2^{bxd} mount similar responses, both driven by H-2^{d}-restricted epitopes. The CD8 T cell response also equalizes in chronic infection of CD$40^{-/-}$ and BALB/c mice (Chapter 3).

• Despite nearly complete prevention of CD8 T cell-mediated cytotoxicity of MCMV-BAC-infected cells *in vitro*, MCMV-BAC-infected cells do induce IFN-γ production from MCMV-specific CD8 T cells, suggesting that direct antigen presentation does occur in the face of the MHC class I immune evasion genes (Appendix A).

• The MHC class I immune evasion proteins do not impact the magnitude or immunodominance hierarchy of the acute or chronic CD8 T cell response in all mouse strains tested (B6, 129, BALB/c, and BALB/c CD$40^{-/-}$), using high dose, i.p. infection (Chapter 3).

• Low dose infection of MCMV via more natural routes of infection does not reveal an *in vivo* role for the MHC class I immune evasion proteins in acute or chronic infection of adult B6 or BALB/c mice or of BALB/c neonates (Chapter 4).

• My ability to detect small, significant differences was hampered by a significant amount of experiment-to-experiment variability—particularly viral titers in the salivary glands and spleen (Chapters 4 and 5).

• The experimental variability we see in our model is not explained by MNV contamination of our mouse facility (Chapter 5).

The MHC class I immune evasion genes may not function by inhibiting antigen presentation to CD8 T cells

These results lead to two very obvious questions: 1) Why are the MHC class I immune evasion genes encoded and conserved? 2) What is the source of variation in our model? There are at least five possibilities as to why these genes evolved: 1) they have a function other than/in addition to MHC class I downregulation, 2) they allow for better establishment of initial infection, 3) they allow the virus to survive and reactivate in the host after the adaptive immune response has developed, 4) they allow for more efficient transmission, and/or 5) they allow mice to be superinfected.

While it remains a formal possibility that the MHC class I immune evasion proteins have a secondary, unrecognized function(s), none of the suggested candidates appear to be involved. For example, m152 has a known second function; it downregulates the NKG2D ligand, retinoic acid early-inducible gene (RAE)-1. NKG2D is an activating NK cell receptor that is also expressed on antigen-experienced CD8 T cells. RAE-1 stimulates NKG2D, so it has been proposed that downregulation of RAE-1 may inhibit CD8 T cell co-stimulation. However, Pinto *et al*. showed that blocking NKG2D only has a small impact on CD8 T cell cytotoxicity *in vitro* (Pinto et al., 2007).

We have recent data from our laboratory that, while it does not suggest a new function for the proteins, it has changed our view regarding the *impact* of the MHC class I immune evasion proteins. My experiments showed that MCMV-BAC-infected cells could stimulate IFN-γ production from CD8 T cells (see Appendix A). Simultaneously, Pinto showed *in vitro* that while CD8 T cells could not kill MCMV-BAC-infected cells as measured by a ^{51}Cr-release assay, the T cells could release granzyme B (Pinto, 2006). Therefore, the infected cells were somehow

resistant to apoptosis. In its immune evasion armamentarium, MCMV also encodes anti-apoptotic genes, so infected cells could be resistant to apoptosis due to expression of those genes (Brune et al., 2001b; Jurak et al., 2008; Mack et al., 2008). In any case, resistance to apoptosis is not complete, as Δm04+m06+m152-MCMV encodes the anti-apoptotic genes, but Δm04+m06+m152-MCMV-infected cells can be lysed *in vitro*. Pinto went on to show that MHC class I downregulation led to decreased non-cognate MHC class I and that this led to the decrease in lysis of MCMV-BAC-infected cells (Pinto, 2006). She postulates that non-cognate peptide is necessary for sustained TCR signaling, which is required to maintain the immune synapse. Therefore, with impaired TCR signaling, the synapse is lost, and the effector molecules released cannot actually kill their target cells.

We may simply have been fooled by what seemed to be such an obvious purpose of these proteins. It was assumed, by myself and others, that the mechanism of immune evasion involved impairing antigen presentation to CD8 T cells, thus, impairing viral detection. The proteins do robustly downregulate MHC class I, but we now have evidence that the purpose of this downregulation is for something other than decreasing antigen presentation to CD8 T cells. This could certainly explain why I did not detect any differences in the MCMV-specific CD8 T cell response in my *in vivo* studies. If antigen is being presented normally in the presence of the MHC class I immune evasion genes, we would expect the same CD8 T cell response to be primed and maintained in Δm04+m06+m152-infection. Pinto's findings could also explain why there were no differences in MCMV viral titers, because while the integrity of the CD8 immune synapse may be damaged, redundant immune mechanisms could also control viral replication.

Why are the MHC class I immune evasion genes conserved?

While Pinto's finding elucidates the mechanism by which CD8 T cells are prevented from killing MCMV-BAC-infected cells *in vitro* and is a significant step for the field, it does not in itself explain why the MHC class I immune evasion genes are conserved. In the absence of

specific evidence for a newly identified function of the immune evasion proteins, there are four remaining arguments for why the genes are conserved: 1) they allow for better establishment of infection, 2) they help the virus survive and reactivate in the host once the adaptive immune response has developed, 3) they allow for more efficient transmission, and/or 4) they allow mice to be superinfected.

The first possibility can essentially be ruled out by my and others' data. As shown in Chapter 3, infection is clearly established with both MCMV-BAC and Δm04+m06+m152-MCMV at high infecting doses. While the results were widely variable at low doses, infection was established in a similar proportion of mice with both viruses (see Chapter 4). It is not surprising that evasion of the adaptive immune system would have little impact on the initial days—or seeding—of infection, as infection is established before the CD8 T cell response is fully generated. The acute CD8 T cell response is also virtually identical and remains so at late times post-infection. My finding that IFN-γ is produced in response to MCMV-BAC-infection *in vitro* (Appendix A) suggests that antigen presentation is occurring. Pinto's results support this and suggest an explanation as to how CD8 T cells can be primed even with downregulated MHC class I.

The second, and biologically more likely, argument for the conservation of the MHC class I immune evasion proteins is that they allow reactivating virus to escape immune control and form active foci of reactivation. Once the virus is latent, or persisting at a very low level, the MHC class I immune evasion genes may allow reactivating virus to escape memory CD8 T cell control. The sites of and cell types of reactivation (*e.g.* lung monocytes or endothelial cells) may be unique from the primary sites of acute infection. Notably, we know that expression and/or function of the MHC class I immune evasion proteins is somewhat cell-type-specific (Hengel et al., 2000; LoPiccolo et al., 2003; Pinto et al., 2006; Streblow et al., 2007). LoPiccolo *et al.* reported that *m04* plays a more prominent role in macrophages than fibroblasts (LoPiccolo et al., 2003). The rat CMV homolog of *m152*, *r151*, is highly expressed in the salivary gland ductal

cells of infected rats (Streblow et al., 2007). Therefore, the MHC class I immune evasion proteins may not simply function as effectively at the sites of acute infection and may be better able to exert their power in reactivating cells and tissues. The viral titer data from our laboratory does not support this theory, however (see Chapter 4 and M. Gold, unpublished observation). While we have not formally shown that the virus detectable in immunocompetent mice beyond four weeks p.i. is reactivating virus, the amount of virus detected at late times p.i. is clearly not impacted by the presence of the MHC class I proteins.

Furthermore, if both MCMV-BAC and Δm04+m06+m152-MCMV establish latency during the first days of infection, which it appears they are able to do equally based on similar viral loads and CD8 T cell responses, it may be impossible for the immune system to eradicate them (Reddehase et al., 1994). Reddehase *et al.* reported that after infection of BALB/c mice with MCMV-Smith, no virus was detected by plaque assay in the lungs at one week p.i., but at one year p.i., the lungs harbored more viral genomes than the salivary glands. It would be particularly difficult for the immune system to eradicate virus if the viral genome can be maintained and even replicated in host cells without expression of viral proteins, invisible to the immune system until it reactivates. Maintaining a true latent pool of virus would occur without any viral protein expression; maintaining replicative latency, however, occurs in the presence of viral protein expression (*e.g.* IE1/pp89). There is evidence that MCMV proteins *are* visible to the immune system before full viral reactivation and that CD8 T cells are important in terminating further viral gene expression. IE1/pp89 epitope-specific CD8 T cells have been shown to maintain replicative latency by decreasing the prevalence of IE1/pp89 transcripts in latently-infected lungs (Simon et al., 2006). Yet, the three known MHC class I immune evasion proteins are not expressed until the E phase. If the balance between latency and reactivation is dealt with by the immune system in the IE phase of gene expression, that further detracts from the argument that the MHC class I immune evasion proteins allow reactivating virus to escape CD8 T cell control.

A third possible role for the MHC class I immune evasion proteins is that they allow for more efficient transmission of the virus. In the wild, virus reactivation leads to replication in the salivary glands and virus transmission. Transmission can be considered perhaps the most important selective pressure on a virus, as the ability to transmit determines its ability to propagate itself over time. In immunocompetent mice, it is in this organ that the only evidence of increased viral fitness occurs due to expression of the MHC class I immune evasion proteins. MCMV-BAC has at least a one log growth advantage over Δm04+m06+m152-MCMV in the salivary glands in high dose infection of BALB/c mice (C. Doom, data not shown; Lu et al., 2006). As described in Chapter 4, there is no statistical advantage to MCMV-BAC in low dose infection, but the data suggests that the immune evasion proteins provide a survival advantage to the virus in the salivary glands. In this scenario, the MHC class I immune evasion proteins would allow for effective dissemination and increased viral fitness by impairing the cytotoxic function of CD8 T cells enough to impact control of virus in this organ, but not enough to disrupt its global equilibrium in the host.

The MHC class I immune evasion proteins may specifically target the acinar cells of the salivary glands to prevent effective CD8 T cell control (Streblow et al., 2007). Although the mechanism is not known, it does seem that acinar cells are peculiarly resistant to CD8 T cell control—in contrast to the lungs and liver, CD8 T cells have no impact on MCMV-Smith virus replication in these cells (Jonjic et al., 1989; Cavanaugh et al., 2003; Campbell et al., 2008). However, the fact that Δm04+m06+m152-MCMV grows more poorly in the salivary glands suggests that CD8 T cells do exert some control of those infected cells. Infected acinar cells may simply be resistant to apoptosis, but in that case, however, CD4 T cells should also have difficulty controlling MCMV in the salivary glands, which they do not. CD4 T cells are required to control virus in the salivary glands in early infection, and this has been shown to be dependent on IFN-γ (Jonjic et al., 1989; Lucin et al., 1992). While the MHC class I immune evasion proteins do not seem to completely block IFN-γ production from CD8 T cells, production is impaired compared

to Δm04+m06+m152-MCMV-infected cells (see Appendix A). It appears that in the salivary glands, the MHC class I immune evasion proteins prevent effective cytolysis of MCMV-BAC-infected cells and the IFN-γ they produce is not enough to effectively control virus. I have not tested, however, whether MCMV-BAC-infected acinar cells of the salivary glands can stimulate IFN-γ production from CD8 T cells.

The final argument for the existence of the MHC class I immune evasion genes is also related to transmission; they may allow for superinfection with other viral strains. As nicely put in a commentary by Stephen Hedrick, "A zebra doesn't have to outrun the lion, just the slowest member of the herd" (Hedrick, 2004). We know that superinfection not only occurs in the wild, but may be the norm (Gorman et al., 2006). The many identified strains of MCMV and the incidence of natural superinfection points to a constant pressure on one strain to outcompete another. It has been suggested that the MHC class I immune evasion genes' main role may be to allow infection with multiple strains of CMV (Gold et al., 2004; Cicin-Sain et al., 2005). Recent, strong experimental evidence for this has idea has been obtained in rhesus macaques using infection with a recombinant CMV (K. Fruh and C. Powers, personal communication). I attempted to establish a superinfection model, as described further in Appendix C, but I was unable to demonstrate superinfection of BALB/c mice.

How should we study MCMV infection *in vivo*?

My thesis work raises a number of important questions about our model itself. Our model specifically seeks an understanding of MCMV infection in immunocompetent animals, because we want to study the normal immunobiology, not the exceptions when CMV causes disease. We are interested in the relationship of MCMV with its host and the balance between immune system control and countermechanisms. MCMV causes many known problems when that balance is lost and immune control is impaired. But there are fascinating questions about the magnitude of the T

cell response, memory T cell generation and maintenance, and immunosenescence that should be addressed in an immunocompetent host. Unexpectedly, the CD8 T cell response may, in fact, *be* the major clinical problem that CMV causes, because of its correlation with immunosenescence.

We have primarily studied MCMV infection in inbred B6 mice using high dose, i.p. infection with the MCMV-BAC virus. This model was modified from other models where very high dose infection is routinely used. At the time, there was no perceived drawback to using a high dose and it provided a reproducible and characteristic CD8 T cell response, which was important for answering some of our laboratory's early questions about the antigen specificity of the response. The conclusions from my work follow two themes: there is little impact of the MHC class I immune evasion genes *in vivo* (see Chapters 3 and 4; Gold et al., 2002; Munks et al., 2007) and there is significant variability in our model. These conclusions raise two important issues regarding evolutionary pressure and the artificiality of our model.

In asking about the biological significance of the MHC class I immune evasion genes, and why they are conserved, it is important to consider the environment in which they are actually evolving. There are a number of key differences between that environment and the environment in our model: 1) wild mice are not infected with laboratory-passaged MCMV-Smith or a close variant, 2) wild mice are infected naturally with MCMV and often re-infected, 3) wild mice are not genetically identical, and 4) wild mice are not pathogen naïve; they are exposed to many different immune system stimulants.

One of the strengths of studying MCMV is that it is a natural mouse pathogen. It was discovered as a contaminant of laboratory mice, although it is no longer a common contaminant of mouse facilities. A new study on the prevalence of infectious agents in mouse colonies across North America and Europe reports that MCMV has a prevalence of only 0.04% (Pritchett-Corning et al., 2008). Although there appears to be some difference in the ability of the original Smith strain and the current Smith strain to transmit among mice, MCMV-Smith does not lack any large regions of its genome when compared to recent MCMV isolates, unlike laboratory and

clinical strains of HCMV (Smith et al., 2006, Smith et al., 2008). Despite many years as a laboratory strain, MCMV-Smith and recent isolates have very similar sequences, perhaps because MCMV-Smith was (and is) routinely passaged through mice.

It can be argued that salivary glands-derived virus may be more virulent and thus, natural. Infecting with a wild MCMV isolate or a more virulent laboratory strain would have been preferred for most accurately modeling natural infection, but that comparison is not yet possible, as no MHC class I immune evasion mutant of a wild isolate has been made. I was restricted to using BAC-derived MW97.01 as the wild type control, because the Δm04+m06+m152-MCMV was made using the MW97.01 strain. It is essential, not only for a potential repeat of these experiments, but also to studying MCMV infection *in vivo*, that one or more of the wild, recently-isolated strains of MCMV, along with a Δm04+m06+m152 mutant, is generated on a BAC-background.

Another difference between natural infection (whether in the wild or in the laboratory) and our model involves the way in which mice are exposed to MCMV. In Chapter 4, I tried to mimic more natural infection conditions, while still working within the constraints of an animal model. Although the conditions were more natural, they still relied on the artificial use of inbred, SPF mice. There are known differences in the immune responses of one inbred strain versus another, such as the T_H1-polarized response in B6 mice versus the more T_H2-polarized response in BALB/c mice. These genetic differences have led to strain-specific responses in various infection models (*e.g. Leishmania*) and have made it difficult to truly project experimental findings without repeating experiments across various mouse strains. I tested two strains of mice in the natural infection experiments to attempt to control for mouse strain-specific differences.

The final difference—that laboratory mice are immune naïve—may ultimately be the most important. The movement to control pathogen exposure in laboratory animals began in the 1950s. It started in order to control infectious disease outbreaks, particularly *Mycoplasma pulmonis*, which would spread through colonies and decimate the population, curtailing or ruining

many experiments in progress (Weisbroth, 1999). Some of the contaminating agents have since been shown to impact experimental results, and even more cause problems in immunocompromised animals (Rowe et al., 1959; Riley et al., 1960; Biggart and Ruebner, 1970; Bonnard et al., 1976; McKisic et al., 1993). As early as the 1930s, James Reyniers had developed a steam-sterilized isolator, in which embryos could be rederived and reared. As mentioned in Chapter 5, rederivation will get rid of many pathogens, including unrecognized ones, which is probably why many commercial vendors were MNV-free even before MNV had been discovered.

Given the history of pathogen contamination, it is easy to understand why such strict methods have been put in place to keep mouse colonies immune naïve. There had been a movement to develop completely germ-free mice until it was realized that these mice had abnormal intestinal histology since they were not colonized by commensal bacteria (Weisbroth, 1999). A cocktail of non-pathogenic bacteria was developed in order to colonize mice so that they would undergo "normal" intestinal and immune system development (Schaedler et al., 1965). These practices were very effective at reducing the incidence of unwanted infection, however, the contaminating, but often asymptomatic, murine viruses were unappreciated until the 1980s. The frequency of murine viruses mentioned in the title of *Laboratory Animal Science* articles jumped from one in 1980 to five in 1985 to 11 in 1990 (Weisbroth, 1999). It was somewhere around this time that use of the term "specific pathogen free" became common, in part because the research community was just realizing that an emerging iceberg of contaminating viruses existed.

The fact that laboratory mice do not receive any of the daily immune system challenges that wild-living mice do has serious implications regarding the "normal" development of their immune systems and how they deal with an infection. Mice in our colony are responding to MCMV in a relative vacuum and this is the response we measure in an effort to understand physiological MCMV infection. The true immunobiology of MCMV infection, however, is in the context of different microbial flora, constant danger signals, and likely frequent, low-grade inflammatory responses that could significantly influence how the immune system responds to

MCMV and how often it reactivates. The presence of other infections or immune system stimulation certainly influenced the evolution of the virus and the MHC class I immune evasion genes. Therefore, when I think about our model, I think there are two possibilities for why the MHC class I immune evasion genes do not have a stronger phenotype. The first is that we use SPF mice that are not (generally) fighting anything other than MCMV infection, and the second is that we use heavily passaged virus isolates that may trigger a very different immune response than wild strains.

The topic of immune naïveté of laboratory mice may also be a major contributor to the second theme of my work: experimental variability. At first glance, it is easy to assign the variability to the fact that we use an intact animal model, and a certain amount of wobble would be expected, particularly compared to *in vitro* systems. However, we use inbred animals that have identical genetic backgrounds and are housed in the same facility under SPF conditions. We have assumed these conditions should absolutely minimize variation, save experimenter error.

Within a mouse strain, we virtually never see variation in the immunodominance hierarchy itself—though we do see experiment-to-experiment variation in the maximum size of the CD8 T cell response. Since the variation we have is generally one of relative magnitude, it has not altered our conclusions. It has occurred without garnering much attention, because it can be explained by technical variation or error, such as differences in when exactly the organs are harvested p.i., differences in antibody staining during ICCS, and differences in compensation and gating during FACS analysis. In Chapter 5, however, I showed that MNV exposure can lead to a decrease in the CD8 T cell response. So, it is possible that adventitious infections have caused some of the variability our laboratory has seen in the magnitude of the CD8 T cell response.

The variability I and others have most commonly seen has been in virus load, as measured by plaque assay. As described in Chapter 4, the variability can be as extreme as all mice in one group having detectable virus, while all mice in another group with identical treatment having no detectable virus. The variability in viral titers may be due to the assay we

use; measuring viral load by qPCR would be more sensitive, but comes with the significant drawback of not measuring infectious virus. I think the variability is more likely due to fluctuations within our animal colony. SPF facilities become contaminated with known and emerging pathogens with some frequency. The variability in MCMV organ titers may be due to sporadic infections brought into the facility. While I have ruled out, under the conditions tested, that MNV is *not* the cause of this variability, there could be an as yet unidentified infectious cause.

In addition to pathogen contamination of our facility, factors such as which rodent vendor is used can have significant consequences on mouse colonies. C57BL/6 mice from Jackson East compared to Jackson West have vastly different sensitivities to the cecal ligation and puncture model of sepsis (J. Gold, personal communication). The commensal flora of an animal can also impact the development of an immune response (Ivanov et al., 2008), as well as altering the response to a specific infection (Tanaka et al., 2007). Mice from different sources are sure to have variations in bacterial flora. Therefore, while I have not been able to specifically identify the cause of this variation, I conclude that there is an unknown and uncontrolled-for variable, which impacts viral control of MCMV in laboratory mice.

Conclusions

For our laboratory's interest in characterizing the CD8 T cell response to MCMV infection, our model has proven to be quite robust. Mouse models are known to be artificial systems, but even in spite of this, the benefits have been long established. It would be impossible to ask the questions our laboratory is interested in if we did not control the environment in which we asked them. However, the necessity to use such an artificial system may mean that some questions may not be able to be asked, which requires us to find a different way to ask them and to understand and discuss the limits of the model in making interpretations.

Based on the herpesviruses near obsession with manipulating the MHC class I molecule, based on the evolutionary conservation of the MHC class I immune evasion genes, and based on the recent data implicating low MHC class I levels and low non-cognate peptide with impaired TCR signaling (A. Pinto, unpublished observation), I believe MCMV's MHC class I immune evasion genes exist for the purpose of immune evasion. And I believe that their protein products do it through the downregulation of MHC class I, and not solely through an, as yet, unidentified mechanism. For understanding the evolutionary conservation of the MHC class I immune evasion genes and their impact *in vivo*, though, I think we have answered the question as completely as we can with the tools and reagents currently available. In MCMV-susceptible laboratory mice, the MHC class I immune evasion proteins convincingly, although mildly, allow the virus to grow to higher titers in the salivary glands, suggesting they are conserved for enhanced transmission and/or for virus-to-virus competition. For further study into the true physiological role of the MHC class I immune evasion proteins and of the physiological response to MCMV infection, I think using wild virus isolates and mice that are not entirely immune naïve will bring the most relevant and immediate advances to the field.

Appendix A. The MCMV MHC I class immune evasion genes only have a modest impact on the ability of CD8 T cells to secrete cytokines, in contrast to their profound impact on cytolysis

Introduction

Our laboratory has tested the impact of the MCMV MHC class I immune evasion genes on CD8 T cell recognition *in vitro* by asking whether T cells could recognize and kill virus-infected cells, using ^{53}Cr-release assays. Amelia Pinto used the MCMV peptide panel to show that IC21 cells or primary macrophages infected with Δm04+m06+m152-MCMV could be killed by MCMV-specific CTL lines, while cells infected with MCMV-BAC categorically could not (Pinto et al., 2006). Her experiments tested the cytotoxic effector function of CD8 T cells but had not tested the other function—cytokine secretion. In what was to be a routine follow up, I set out to recapitulate Pinto's data by measuring IFN-γ release from CD8 T cells incubated with MCMV-BAC- or Δm04+m06+m152-MCMV-infected cells.

Results

The MHC class I immune evasion genes do not prevent CD8 T cell cytokine production

Primary DCs were chosen as the first cell type to investigate in combination with two T cell specificities (M45 and m139). The M45 and m139 specificities were chosen because these peptides generate reliable, high-efficiency T cell lines and because there were existing mutant viruses lacking each of the genes from which these peptides are generated, ΔM45-MCMV and Δm139-m141-MCMV. Graded numbers of DCs were pulsed with 10^{-6} M peptide or infected overnight with MCMV-BAC- or Δm04+m06+m152-MCMV in triplicate, in the presence of PAA. CTL were added and incubated for seven hours before the supernatants were harvested and IFN-γ was measured by sandwich ELISA. The first set of experiments, depicted in Figure A1,

revealed a surprising finding and showed an immediate contrast with Pinto's findings. M45- and m139-specific CTL produced IFN-γ in response to incubation with *both* MCMV-BAC and Δm04+m06+m152-MCMV-infected cells. IFN-γ production was more robust in response to Δm04+m06+m152-MCMV infection, however. The amount of IFN-γ produced in response to Δm04+m06+m152-MCMV-infected DCs was equivalent to the amount of IFN-γ produced in response to CTL pulsed with peptide; CTL incubated with MCMV-BAC-infected DCs produced an approximately 10-fold lower amount of IFN-γ.

A variety of MCMV-BAC-infected cell types stimulate IFN-γ production

To ask whether this was a general observation or something specific to primary DCs or MCMV itself, the experiments were repeated using different cell types and mutant viruses to control for antigen-specificity. When primary DCs infected with Δm139-141-MCMV were incubated with m139-specific CTL, there was IFN-γ production at the highest DC:CTL ratios, suggesting that the cytokine production was not entirely antigen specific. When primary macrophages were tested, MCMV-BAC was even more capable of stimulating CTL to produce IFN-γ and was essentially equivalent to Δm04+m06+m152-MCMV (Figure A2). Again in these primary cells, there was non-antigen-specific IFN-γ production at the highest macrophage:CTL ratios. In order to compare a primary cell type with an immortalized cell line, the macrophage-like cell line, IC21, were next examined. MCMV-BAC-infected IC21 stimulated IFN-γ production, but only at the highest IC21:CTL ratio used. A possible explanation for the IFN-γ produced in response to MCMV-BAC-infected cells could be that virus infection cause secretion of IL-12 and IL-18 from the DCs or macrophages, and that these cytokines then caused the CD8 T cells to secrete IFN-γ in an antigen-non-specific manner. However, all IFN-γ production in response to infected IC21 was antigen-specific, as Δm139-m141-MCMV-infected cells did not stimulate IFN-γ release.

The last cell type tested was fibroblasts, both primary MEFs and immortalized K41 cells (Figure A3). Primary MEFs infected with MCMV-BAC, Δm04+m06+m152-, or Δm139-m141-MCMV all stimulated some level of IFN-γ production from m139-specific CTL. The amount of IFN-γ secreted by CTL was the highest in response to Δm04+m06+m152-MCMV, but nearly all of the IFN-γ could be attributed to non-specific production because the amount produced by Δm139-m141-MCMV infection was significant at all MEF:CTL ratios tested. When transformed K41 cells were infected, their pattern of IFN-γ stimulation was very similar to that seen for primary MEFs, although there was greater variation.

The cell type producing IFN-γ and antigen specificity

The ELISA data suggests that MCMV-BAC infected *primary* cell types might be more likely to induce IFN-γ production than their transformed cell type counterparts, except for fibroblasts whereby MCMV-BAC-infected transformed K41 cells also robustly induced IFN-γ secretion. This difference may be due phenotypic changes undergone by cells as a result of transformation or passage. Importantly, primary cells more directly mirror the environment *in vivo*, where CD8 T cell-generated IFN-γ is known to help control MCMV infection (Lucin et al., 1992; Hengel et al., 1994; Lucin et al., 1994; Orange et al., 1995). The instances when MCMV-BAC infection resulted in robust IFN-γ production were also the instances when non-specific IFN-γ production was detected, however. In the experiments where MCMV-BAC-induced IFN-γ was very subtle and only seen at the highest APC:T cell ratios, there was usually very little evidence of non-specific IFN-γ produced.

To further investigate the role of non-specific IFN-γ production, some of these experiments were repeated using ICCS to determine whether CD8 T cells were actually the cells making the IFN-γ measured by ELISA. IFN-γ is produced by various other cell types, including γ/δ-T cells, CD4 (T_H1) T cells, and NK cells. The CTL lines were grown under CD8 T cell-

supporting conditions and generally, around 95% of the live IFN-γ$^+$ cells were also CD8-α$^+$, and approximately 90% of those had low granularity characteristic of CD8 T cells (data not shown).

Figure A4 shows ICCS analysis of CD8 T cells responding to infected IC21s, K41s, or primary MEFs. Unlike in the ELISA, only peptide-pulsed IC21s stimulated IFN-γ production from CD8-α$^+$ cells; neither Δm04+m06+m152-MCMV- nor MCMV-BAC-infected IC21s stimulated cytokine production. Infected primary MEFs also did not stimulate as much IFN-γ from CD8-α$^+$ cells by ICCS as the ELISA results had predicted. Δm04+m06+m152-MCMV-infected cells stimulated some cytokine production by both m139- and M45-specific CD8-α$^+$ cells, but it was much less than the cytokine production stimulated by peptide pulsing. K41 cells were the only cell type tested that stimulated a similar pattern of IFN-γ production from CD8-α$^+$ cells by ICCS as seen by ELISA.

Discussion

Upon investigating the cytokine effector function of CD8 T cells, I expected to see the same phenotype seen in the cytotoxicity assays, anticipating that only the CTL incubated with Δm04+m06+m152-MCMV-infected APCs would produce IFN-γ. Instead, both MCMV-BAC- and Δm04+m06+m152-MCMV-infected cells were able to stimulate IFN-γ from CTL, indicating either that functional antigen presentation resulting in an effector response by primed CD8 T cells occurs even in the presence of the MHC class I immune evasion genes *or* that MCMV infection itself leads to the release of cytokines. The cells infected with Δm04+m06+m152-MCMV were generally quantitatively better at stimulating IFN-γ production; it took between three- and 10-fold more MCMV-BAC-infected cells to stimulate the same amount of IFN-γ as it took Δm04+m06+m152-MCMV-infected cells. This finding was cell type-dependent, and there was quite a high degree of variability—both between experiments using the same cell types and occasionally within experiments—as shown in Figures A2-3. However, the experiments did

provide evidence that some CD8 T cell IFN-γ release in response to MCMV-infected cells could be accounted for by non-antigen-specific mechanisms, such as a response to IL-12 and IL-18. There was antigen-dependent IFN-γ release in response to MCMV-BAC-infected cells (Figure A2, IC21), but I did not conclusively demonstrate that this was produced by CD8 T cells (see Figure A4).

DCs

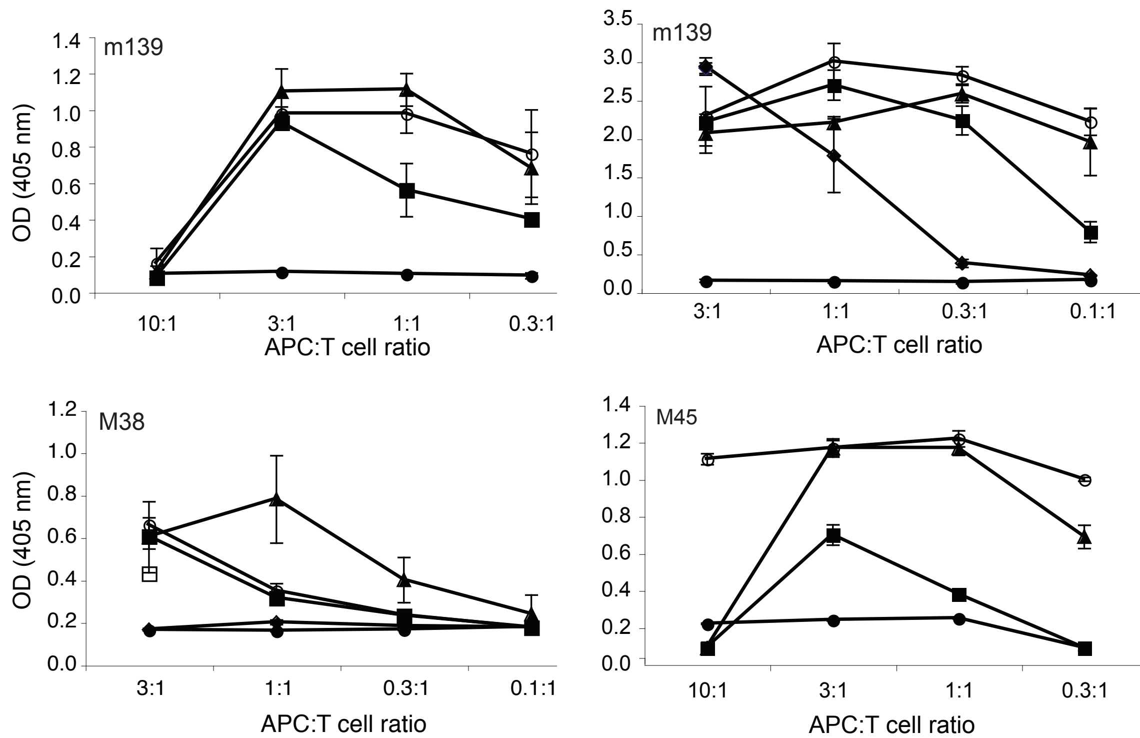
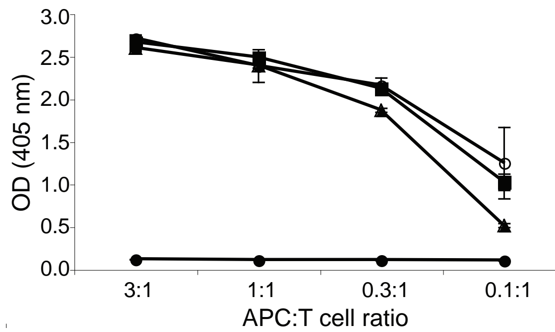
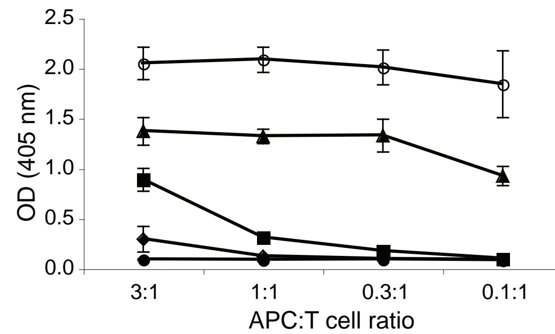


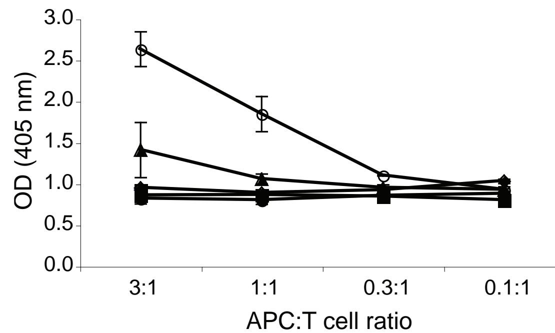
Figure A1. MCMV-specific CTL produce IFN-γ in response to DCs infected with either MCMV-BAC or Δm04+m06+m152-MCMV. Primary bone-marrow-derived DCs were left untreated (●), pulsed with 10^{-6} M peptide (○), or infected overnight with MCMV-BAC (■), Δm04+m06+m152-MCMV (▲), or Δm139-141-MCMV (♦) in the presence of PAA. Short-term, polyclonal CTL lines were grown against the indicated peptide and used between day 10-12. CTL were incubated with DCs for 10 hours; supernatant was harvested and analyzed by sandwich ELISA for IFN-γ. Error bars indicate SEM. □: CTL+peptide only. OD: optical density. APC: antigen-presenting cell. DC: dendritic cell.

Macrophages



IC21s





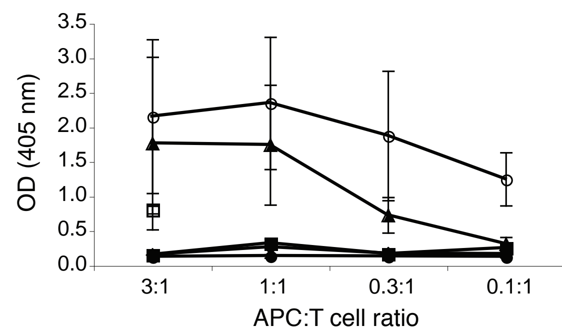
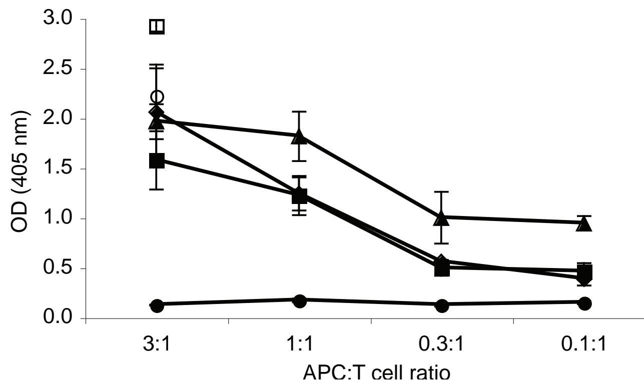


Figure A2. m139-specific CTL produce IFN-γ in response to macrophages infected with either MCMV-BAC or Δm04+m06+m152-MCMV. Primary bone-marrow-derived macrophages or IC21s were left untreated (●), pulsed with 10^{-6} M peptide (○), or infected overnight with MCMV-BAC (■), Δm04+m06+m152-MCMV (▲), or Δm139-141-MCMV (♦) in the presence of PAA. Short-term, polyclonal CTL lines were grown against m139 and used between day 10-12. CTL were incubated with cells for 10 hours; supernatant was harvested and analyzed by sandwich ELISA for IFN-γ. Error bars indicate SEM. □: CTL+peptide only. OD: optical density. APC: antigen-presenting cell.

Primary MEFs



K41 cells

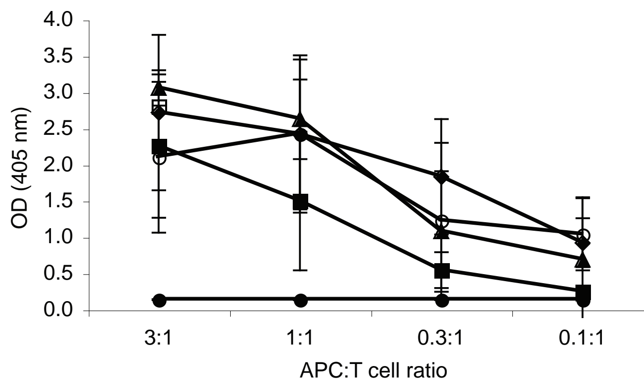
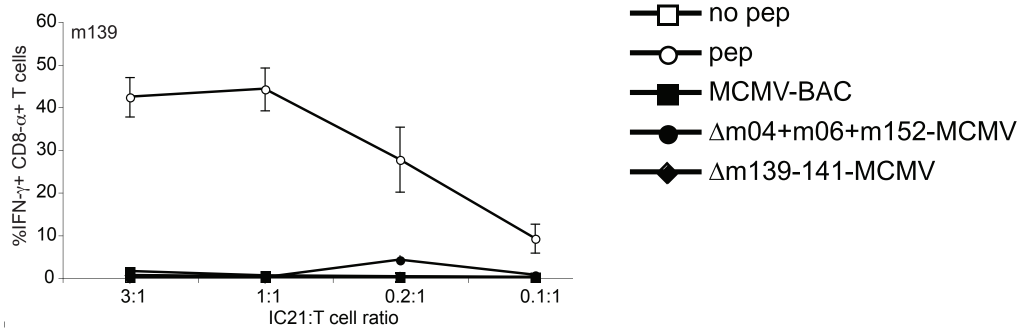
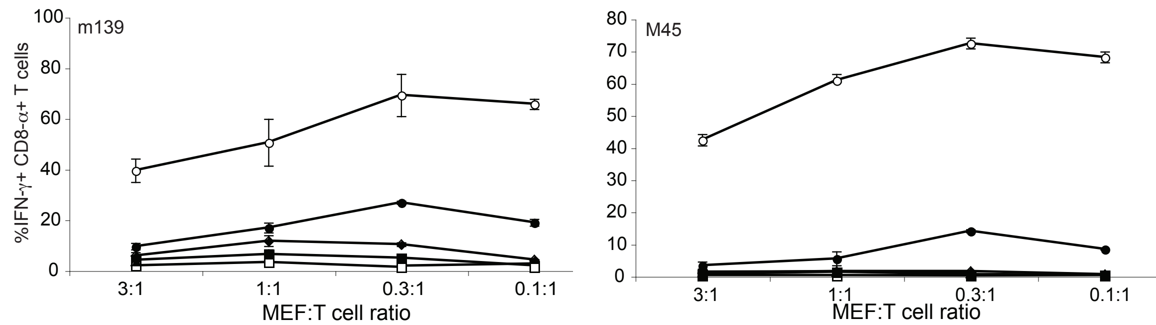


Figure A3. m139-specific CTL produce IFN-γ in response to fibroblasts infected with either MCMV-BAC or Δm04+m06+m152-MCMV. Fibroblasts were pretreated overnight with recombinant IFN-γ to increase cell surface MHC class I. The cells were left untreated (●), pulsed with 10^{-6} M peptide (○), or infected overnight with MCMV-BAC (■), Δm04+m06+m152-MCMV (▲), or Δm139-141-MCMV (♦) in the presence of PAA. Short-term, polyclonal CTL lines were grown against m139 and used between day 10-12. CTL were incubated with cells for 10 hours; supernatant was harvested and analyzed by sandwich ELISA for IFN-γ. Error bars indicate SEM. □: CTL+peptide only. OD: optical density. APC: antigen-presenting cell. MEF: murine embryonic fibroblast.

IC21 cells



Primary MEFs



K41 cells

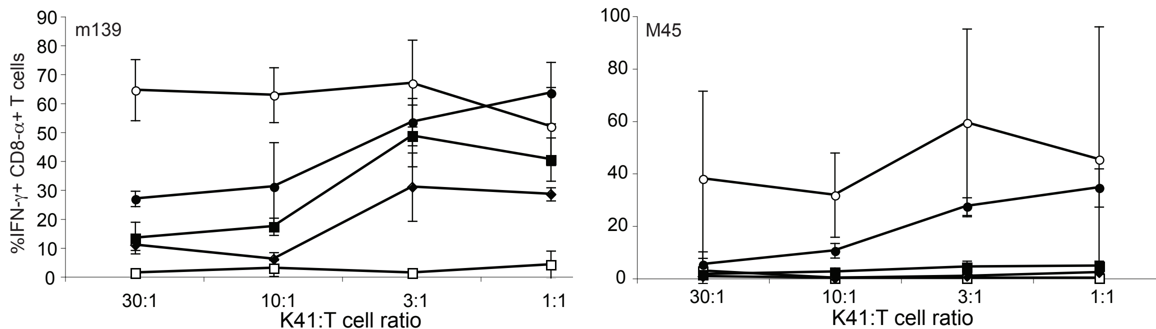


Figure A4. MCMV-specific CTL produce IFN-γ in response to K41 cells infected with either MCMV-BAC or Δm04+m06+m152-MCMV. The indicated cell types were left untreated, pulsed with 10^{-6} M peptide, or infected overnight with MCMV-BAC, Δm04+m06+m152-MCMV, or Δm139-m141-MCMV in the presence of PAA. Short-term, polyclonal CTL lines were grown against m139 or M45 and used between day 10-12 as effector cells. CTL were incubated with the cells for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8-α+ T cells was measured by flow cytometry on an LSR II and analyzed by FloJo software. Error bars indicate SEM.

Appendix B: Characterization of ΔgL-MCMV

A problem in demonstrating cross-presentation with viral models is that live viruses will continue to replicate and infect neighboring cells. We were fortunate to acquire a replication-deficient MCMV mutant, which could facilitate studying cross-presentation in the context of viral infection. Jane Allan engineered a virus lacking the essential envelope glycoprotein gL (ΔgL-MCMV). The virus is propagated on a complementing cell line transfected with gL. The virions contain gL and are able to infect cells *in vivo*. Those cells will replicate the virus and produce new virions, which, lacking gL, are unable to infect any further cells (J. Allan, personal communication). The ΔgL virus is grown and passaged in 3T3 fibroblasts that have been stably transfected with gL, which provides the complementing protein to allow the virus to grow and be infectious. This method of growing the virus for preparing stocks was inefficient, as the maximum titer we could achieve was ~10^4 PFU/mL. In order to increase the efficiency of gL expression, I generated a replication-deficient adenovirus (Ad) that expresses gL with which we could infect 3T3s (see Chapter 2 for details). Using Ad-gL-infected 3T3s to complement ΔgL-MCMV generated ΔgL-MCMV stocks with titers at least two logs higher than using the original method (data not shown).

Upon receiving the ΔgL mutant, we also wanted to characterize the magnitude and immunodominance hierarchy of the CD8 T cell response and determine whether the kinetics of the peak response were altered. I infected B6 mice with ΔgL-MCMV (i.p., 10^4 PFU) or a wild type revertant (gL-rev-MCMV), and measured the MCMV-specific CD8 T cell response at six, seven, and eight days p.i. As shown in Figure B1, the magnitude of the response peaked in both ΔgL-MCMV and gL-rev-MCMV infection at seven days p.i., however, the overall magnitude of the response to ΔgL-MCMV was smaller than the response to gL-rev-MCMV. This was not surprising as ΔgL-MCMV cannot go through further rounds of replication once cells are infected, so the amount of available antigen should be much lower in ΔgL-MCMV infection. The

immunodominance hierarchy was nearly identical in response to acute ΔgL-MCMV and gL-rev-MCMV infection. This was expected, as infected cells should be able to both directly- and cross-present antigen just as in wild type MCMV infection. Therefore, phenotypically ΔgL-MCMV behaved just as MCMV-BAC does and is capable of eliciting CD8 T cell recognition.

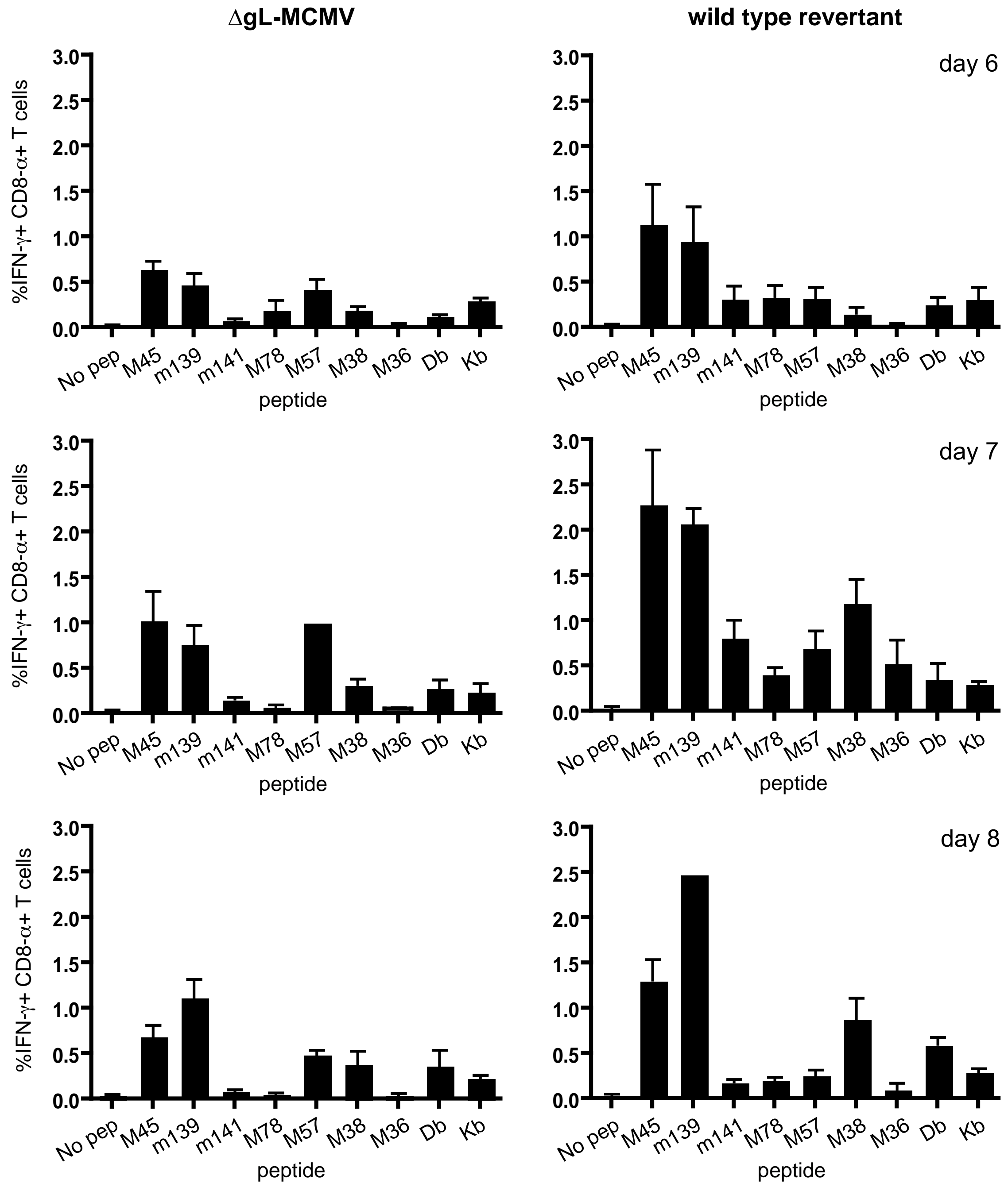


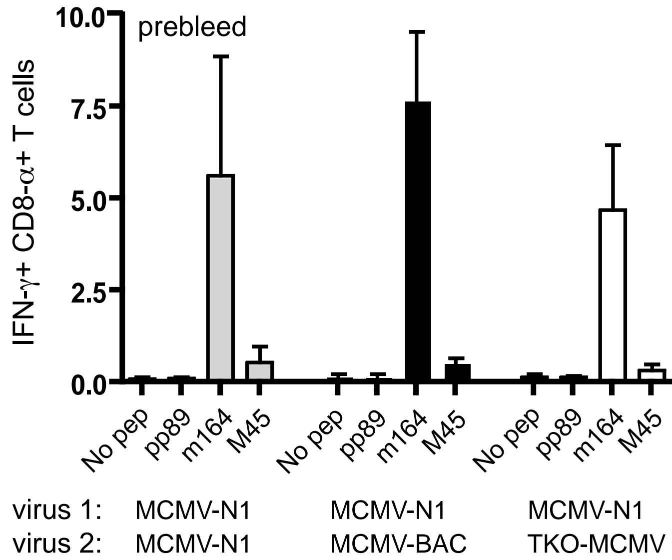
Figure B1. The kinetics and magnitude of the CD8 T cell response to ΔgL-MCMV infection of C57BL/6 mice. C57BL/6 mice were infected i.p. with 10^4 PFU of ΔgL-MCMV (left) or a wild type revertant (right). At the indicated day p.i., spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. n=3 for each group. Error bars indicate SEM.

Appendix C: Establishing a model of superinfection of MCMV

Because both humans and mice have been shown to be superinfected with CMV (Chou et al., 1989b; Baldanti et al., 1998; Boppana et al., 2001; Farroway et al., 2005; Gorman et al., 2006), I wanted to establish a model of superinfection to ask whether the MHC class I immune evasion proteins impact the ability of the virus to superinfect mice. I did not expect to be able to detect virus plaques from the superinfecting virus, and hence looked for a new CD8 T cell response to a very immunodominant epitope as evidence of superinfection. To test for superinfection, I used a recent wild isolate, MCMV-N1, with a known mutation in the IE1/pp89 epitope. The MCMV-N1 IE1/pp89 epitope has the sequence YLDFMPPNL in place of the highly immunodominant, Y**PH**FMP**T**NL L^d-restricted epitope from MCMV-K181 and -Smith IE1/pp89 (Lyons et al., 1996). Because of this difference, CD8 T cells primed against the MCMV-N1 IE1/pp89 epitope would not generate a response when stimulated with the IE1/pp89 epitope from MCMV-Smith. Therefore, if mice were first infected with MCMV-N1, followed by MCMV-Smith, any CD8 T cell response generated to the MCMV-Smith epitope would be evidence of superinfection.

BALB/c mice were infected i.p. with 10^6 PFU of MCMV-N1. Two months later, mice were challenged i.p. with 10^6 PFU MCMV-N1, MCMV-BAC, or Δm04+m06+m152-MCMV. The mice were bled just before superinfection and their MCMV-specific CD8 T cell response to MCMV-N1 was assessed. As expected, the T cells from infected mice did not generate IFN-γ in response to the MCMV-Smith IE1/pp89 epitope (see Figure C1). Because the IE1/pp89 epitope is so immunodominant, I expected it to prime a new response if the mice were superinfected. By four weeks after superinfection, however, none of the mice had generated a CD8 T cell response to the MCMV-Smith IE1/pp89 epitope, suggesting that superinfection had not occurred. I concluded that this model was not useful for testing the hypothesis that Δm04+m06+m152-MCMV would be impaired in superinfection. It is unclear why this model did not show any

evidence of superinfection.



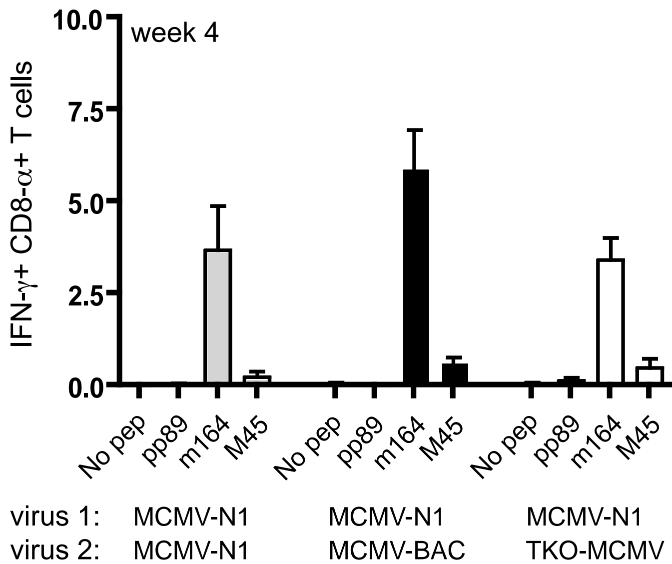


Figure C1. There is no evidence of MCMV superinfection of BALB/c mice. BALB/c mice were infected i.p. with 10^6 PFU of MCMV-N1. Two months later, mice were bled for their baseline MCMV-N1-specific CD8 T cell response (left) before being superinfected i.p. with 10^6 PFU of either MCMV-BAC or Δm04+m06+m152-MCMV. Mice were bled one month after superinfection (right). B) C57BL/6 mice were infected i.p. with 10^6 PFU of either MCMV-BAC or Δm04+m06+m152-MCMV. After all bleeds, peripheral blood mononuclear cells were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ+ CD8+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. n=4 for each group. Error bars indicate SEM. TKO: Δm04+m06+m152-MCMV.

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