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DEATH AND T CELLS: AN EVALUATION OF HOST CONTRIBUTIONS TO MURINE CYTOMEGALOVIRUS PATHOGENESIS AND EXTRINSIC CELL DEATH TO T CELLS

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By

Devon Livingston-Rosanoff
B.A., B.S., University of Washington, 2003

Advisor: Edward S. Mocarski, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Immunology and Molecular Pathogenesis.
2013

Abstract

DEATH AND T CELLS: AN EVALUATION OF HOST CONTRIBUTIONS TO MURINE CYTOMEGALOVIRUS PATHOGENESIS AND EXTRINSIC CELL DEATH TO T CELLS

Cytomegaloviruses (CMVs) establish a lifelong persistent infection in their hosts. They rarely cause disease in the immunocompetent but are a significant source of morbidity and mortality in immunocompromised hosts as well as following transplacental transmission during pregnancy. Due to strict species specificity, studies of pathogenesis or the immune response to CMV rely on murine CMV (MCMV). MCMV induces a potent T cell response that helps control viral replication. MCMV infection also activates innate immunity, including extrinsic apoptotic and programmed necrotic pathways. Caspase 8 (Casp8) is the apical caspase controlling extrinsic apoptosis while simultaneously suppressing programmed necrosis mediated by receptor interacting protein (RIP)1 and RIP3. In this dissertation, I describe work evaluating host contributions to MCMV pathogenesis and contributions of extrinsic cell death pathways to the antiviral T cell response.

Host factors involved in CMV pathogenesis remain poorly understood. I found that antiviral T cells mediate a lethal hepatitis during MCMV infection. Lethality is associated with increased cytokine production by hepatic CD8 T cells at day four. Thus, the antiviral T cell response contributes to or protects from MCMV disease depending on the context and antiviral T cells exert their effects in the periphery at earlier times that previously appreciated.

RIP3 mediates programmed necrosis and its inhibition is required for productive *in vivo* replication of MCMV, but its contribution to the immune response is unknown. I found that, at low doses, RIP3 is dispensable for the antiviral T cell response and control of infection. However, RIP3 helps to protect mice from a Casp8-dependent death following high dose inoculation. Thus, under certain conditions, programmed necrosis restricts extrinsic apoptosis and can protect from disease.

CD8 T cells lacking Casp8 undergo a RIP1/RIP3 mediated programmed necrosis upon TCR ligation, but functions of Casp8 in T cells outside of regulating this necrotic pathways remain unexplored. Utilizing *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, I found that Casp8 is required for homeostatic turnover of CD8 T cells, but is dispensable for a robust CD8 T cell response to MCMV. Thus, during an antiviral CD8 T cell response, extrinsic apoptosis is completely dispensable.

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Acknowledgements

Getting to this point has been a long journey, and there is no way I would be here without the help and support of many people. First, I would like to thank Ed for giving me the freedom to develop my own projects, and the guidance to make sure that I did not end up wandering in the wilderness completely alone. Your ability to constantly be interested and amazed by all areas of science is a joy to observe and always reminds me why I entered this field in the first place. I have learned more from working with you than I can express, and I am exceptionally grateful for the training you have given me. I would also like to express my gratitude to every member of the Mocarski lab, past and present. Your support, and the 3pm dance parties, helped in ways too numerous to count. I have learned so much from each of you about science and about life in general. For many reasons, I would not be here without the MD/PhD program at Emory, specifically the incomparable Mary Horton. No matter the problem, Mary was always there to listen and remind me that I could get through this.

Grad school would have been significantly (with $p<0.0000000001$) more difficult without my fellow compatriots Harry, Hiro, Taymour, Alyssa, Keli and Katie. For those of you ahead of me, you always reminded me that there was light at the end of the tunnel, even if it wasn't apparent, and for those of you at the same stage as me I could not have asked for a better group of friends.

Thank you to my dance and slayer family for helping me to maintain my sanity throughout this process. David, Alicia, Desiree, Rick, Jerry, Abbey and Lexi, you provided me with a place where I felt safe to explore areas way outside my comfort zone. Your unwavering support and enthusiasm have been wonderful. I hope, one day, to be able to repay you for all that you have given me.

To my parents, all I can say is thanks. You have always encouraged my interest in science, and in asking questions. Just knowing that you are always on my side and that your love and support were steadfast has helped more than I can say. I love you both very much.

Finally, my deepest gratitude goes out to my husband, John. Thank you for being the most supportive, understanding, patient, and loving partner. You are my best friend, and I don't have enough words to express how much you mean to me.

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Abbreviations

7-AAD: 7-Aminoactinomycin D
Ab: Antibody
Ag: Antigen
AIDS: Acquired immunodeficiency syndrome
ALPS: Autoimmune lymphoproliferative syndrome
ALT: Alanine transaminase
APC: Antigen presenting cell
APC (fluorophore): allophycocyanin
BFA: Brefeldin A
BMDM: Bone marrow derived macrophages
bp: base pair
Casp8: Caspase 8
CCR: CC Chemokine receptor
CD: Cluster of differentiation
CFSE: carboxyfluorescein succinimidyl ester
CMV: Cytomegalovirus
CTL: cytotoxic lymphocyte
d: Day
DAI: DNA-dependent activator of IFN-regulatory factors
DAPI: 4',6-diamidino-2-phenylindole
DAR: Division of Animal Resources
DC: Dendritic cell
DD: Death domain
DE: Delayed early
DISC: Death-inducing signaling complex
DKO: Double knock out
DMEM: Dulbecco's modified Eagle's medium
DNA: Deoxyribonucleic acid
Dpi: Day(s) post infection
DR: Death receptor
E: Early
EBV: Epstein-Barr virus
EDTA: Ethylenediaminetetraacetic acid
EGFR: Epidermal growth factor receptor
ER: Endoplasmic reticulum
FADD: Fas-associated death domain
FasL: Fas ligand
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
FLICE: FADD-like IL-1 converting enzyme (ICE)
FLIP: FLICE inhibitory protein
GalN: D-galactosamine
GFP: Green fluorescent protein
GI: Gastrointestinal

HAART: Highly active antiretroviral therapy
HCMV: Human Cytomegalovirus
HHV: Human herpesvirus
HIV: Human immunodeficiency virus
HPC: Hematopoietic progenitor cell
Hr: Hour
HSCT: Hematopoietic stem cell transplant
IB: Immunoblot
IE: Immediate early
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IM: Inflammatory monocyte
INIB: Intranuclear inclusion body
I.p.: Intraperitoneal
Kb: Kilobase
KLRG1: Killer cell lectin-like receptor subfamily G member 1
L: Late
LAMP-1: Lysosomal-associated membrane protein 1
LCMV: Lymphocytiv choriomeningitis virus
LD_{50}: Median lethal dose
LN: Lymph node
LPS: Lipopolysaccharide
MCK: Murine cytomegalovirus chemokine
MCMV: Murine Cytomegalovirus
M-CSF: Macrophage colony stimulating factor
MHC: Major histocompatibility complex
MHV-68: Murine herpesvirus-68
MIP-1α: Macrophage inflammatory protein 1α
MLKL: Mixed lineage kinase domain-like protein
MOMP: Mitochondrial outer membrane permeabilization
mRNA: Messenger RNA
ND: Not done
Neuts: Neutrophils
NFκB: Nuclear factor kappa B
NK: Natural killer
NOD: Non-obese diabetic
N.S.: Not significant
NSG: NOD-SCID-$\gamma_c^{-/-}$
PAMP: Pathogen associated molecular pattern
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PE: Phycoerythrin
PerCP: Peridinin chlorophyll protein
PFU: Plaque forming units
PKR: Protein kinase R

PMA: Phorbol 12-myristate 13-acetate
PMN: Polymorphonuclear cells (Neutrophils)
RFLP: Restriction fragment length polymorphism
RHIM: RIP homotypic interaction motif
RIP: Receptor interacting protein
SCID: Severe combined immunodeficiency
SOT: Solid organ transplant
STAT: Signal transducer and activator of transcription
T_{CM}: Central memory T cell
TCR: T cell receptor
T_{EM}: Effector memory T cell
TLR: Toll like receptor
TNF: Tumor necrosis factor
TNFR1: TNF receptor-1
TRIF: TIR-domain-containing adapter-inducing interferon-β
vIRA: viral inhibitor of RIP activation
vICA: viral inhibitor of caspase activation
WT: Wild type

CHAPTER 1.

Introduction

A) Betaherpesvirinae Background

Taxonomy, genome and structure

The betaherpesvirinae subfamily is made up of three genera: cytomegalovirus (CMV), muromegalovirus, and roseolovirus. Within these genera, different species have been identified including cercopithecine herpesvirus 5 (african green monkey CMV), cercopithecine herpesvirus 8 (rhesus CMV), human herpesvirus (HHV) 5 (human CMV (HCMV)), and pongine herpesvirus 4 (chimpanzee CMV) in CMV; murid herpesvirus 1 (murine CMV (MCMV)) and murid herpesvirus 2 (rat CMV) in muromegalovirus; and HHV-6 and HHV-7 in roseolovirus (1). Of these, HCMV, MCMV and HHV-6 are the prototypical viruses for each genus.

Like all herpesviruses, betaherpesvirinae are large double stranded DNA viruses enclosed within an icosahedral capsid embedded in a proteinaceous tegument and surrounded by an envelope expressing viral glycoproteins on its surface (2). The genomes range in size from HHV-6 at approximately 160kb, encoding up to 85 gene products, to HCMV and MCMV both at approximately 230kb, potentially encoding over 700 gene products (3-5). Of these gene products, 70 are conserved within betaherpesvirinae with 40 bearing core herpesvirus gene functions (4). For the most part, these conserved genes are arranged collinearly across the subfamily.

Tropism

Many different cell types are permissive to CMV, contrasting the other herpesvirus subfamilies, alpha and gamma, that are more limited in their cellular tropism (4). Cells derived from all three germ layers, including endothelial cells, fibroblasts,

epithelial cells, macrophages, dendritic cells, neurons, and hepatocytes, are susceptible to CMV infection and contribute to the pathogenesis and manifestations of CMV disease(6). Notably, lymphocytes are not permissive to CMV infection (7). In contrast to their broad cellular tropism, CMVs are species specific. For this reason, pathogenesis and *in vivo* studies of HCMV biology are difficult and the field relies upon animal models, such as MCMV and Rhesus CMV.

Viral life cycle

The viral replication cycle of CMV can be broken down into seven separate steps: 1. Attachment and entry, 2. Trafficking and uncoating, 3. Expression of immediate early (IE or α), early (E) and delayed early (DE or β) gene products, 4. DNA replication, 5. Late (L or γ) gene expression, 6. DNA encapsidation, and 7. Egress and release. Each step will be briefly summarized.

CMV can bind to many cells via glycoprotein (g)B engagement of heparin sulfate on the cell surface, however attachment does not automatically lead to entry (4). Glycoprotein complexes such as gB trimers, gH:gL dimers, gH/gL/gO trimers, or gH/gL/UL128/UL130/UL131 pentamers bind integrins and epidermal growth factor receptors (EGFRs) on the host cell surface helping to facilitate viral entry (8-11) Other host receptors that directly trigger and mediate viral entry remain unknown. In cells such as fibroblasts, CMV enters via direct fusion with the cell surface, although in other cells, such as endothelial cells, CMV enters following endocytosis (4). Following uncoating, the nucleocapsid traffics to the nucleus and viral DNA is released. IE (or α) gene expression follows the deposition of viral DNA in the nucleus, and, by definition, does

not require *de novo* DNA replication (12). Genes in this category include IE1 and IE2 (ie3 in MCMV) that are required for viral replication, the UL36-37 locus encoding multiple cell death suppressors (13, 14) as well as genes such as US3, TRS1 and IRS1 that modulate the cellular response to viral infection (4, 12). Once IE proteins have been made, early and delayed early (β) gene expression proceeds (15). Early genes have many different functions that are absolutely required for successful viral replication including direct participation in viral DNA synthesis, cleavage and genome packaging, and viral assembly. Viral DNA synthesis is initiated in a genomic region called *ori*Lyt and likely proceeds via a rolling circle mechanism on circularized genomes (16). Following replication of the viral genome, late (γ) genes are expressed. These genes encode proteins that aid in viral assembly and egress (4). Packaging viral DNA into the procapsid occurs through recognition of conserved *pac* sequences at either end of the genome by viral proteins and follows a process similar to that of bacteriophages (4, 17). Like other herpesviruses, CMV undergoes a complicated egress process involving primary envelopment at the nuclear membrane, de-envelopment, and finally, secondary envelopment at the ER/Golgi intermediate compartment leading to budding and the release of progeny virus (17).

Pathogenesis

Much of our knowledge of CMV pathogenesis comes from work with MCMV. MCMV is a natural mouse pathogen that establishes a latent or persistent infection similar to that observed in humans infected with HCMV. Infection is characterized by little disease in the immunocompetent with sporadic periods of reactivation. Many

principles of viral pathogenesis (18-20) and immune evasion (21-28) first identified in MCMV have been subsequently translated to HCMV (23, 29, 30).

Like the viral replication cycle, the *in vivo* life cycle of CMV also follows a prescribed pattern (Fig. 1.1). Primary infection initiates at the site of entry, in humans this is thought to be a mucosal surface that has come into contact with infected secretions, while in mice transmission occurs primarily through biting so the site of entry is not limited to mucosa. Upon viral replication at the entry point, the innate immune response is triggered. Viral replication leads to the production of type I interferons (IFNs) by infected cells (31). CMV employs many different mechanisms to inhibit the effects of these cytokines including inhibition of IFN production, signal transduction, and the activity of IFN responsive genes such as protein kinase R (PKR) (32). This enables continued viral replication within the infected cell and viral spread to neighboring cells. Host and virus production of additional pro-inflammatory cytokines (25, 26) leads to recruitment of inflammatory cells to the site of inoculation (33). Recruited monocytes transport viral Ag back to the draining lymph node to prime the adaptive immune response. Infected monocytes disseminate virus throughout the body leading to viremia, and the infection of secondary organs such as the spleen, liver, lungs, adrenal glands, kidneys, and salivary glands (28, 34-36). Upon infection of the secretory and excretory organs such as the salivary glands, kidneys, and gastrointestinal tract, viral shedding begins, completing the infectious life cycle. Viral shedding continues for months or even years (in humans) following primary infection with CMV (6). Unlike other herpesviruses, such as Epstein-Barr virus (EBV), with well characterized latent phases (37), latency in CMV is less well understood. Regardless, at some point following primary infection in an

immunocompetent host, replicating CMV becomes undetectable except during times of periodic reactivation or immunosuppression (6).

B) HCMV and Disease

HCMV epidemiology and disease in immunocompetent individuals

HCMV is a ubiquitous human pathogen. In the United States, 50-80% of adults are infected by the age of 40 (38). Transmission occurs via direct contact with infected secretions such as urine, saliva, semen, cervical secretions or breast milk (4). HCMV can also cross the placenta to infect the developing fetus. Like all herpesviruses, CMV establishes a latent infection for the life of the host with periodic reactivation and viral shedding. In immunocompetent individuals, primary infections and reactivations are rarely symptomatic. However, when symptoms are observed, the most common signs of CMV infection are elevated liver enzymes and mononucleosis-like symptoms (4, 39).

HCMV disease and treatment in the immunocompromised patient

HCMV disease is most commonly observed in immunocompromised individuals and can be a serious complication for transplant recipients and individuals infected with human immunodeficiency virus (HIV). In immunocompromised, the risk of disease is directly proportional to T cell levels. This has been most clearly observed in HIV+ individuals where CMV disease is classically observed when CD4 T cell counts drop below 50 cells/μL (39). Demonstrating the importance of T cells in restraining CMV replication and disease. CMV disease can present in any organ leading to gastroenteritis, pancreatitis, myocarditis, hepatitis, pneumonia, nephritis, adrenal insufficiency, bone marrow suppression, retinitis, meningitis, or encephalitis (39). Evidence of CMV infection is found in 30-92% of patients that undergo solid organ transplantation (SOT) making it the most common viral infection in this setting (40, 41). CMV in the SOT

recipient is associated with increased graft rejection, opportunistic infections, and mortality (42). Patients that receive lung, pancreas or intestine transplants have the highest risk of death due to CMV disease (6). Seronegative recipients that receive a seropositive organ are at the greatest risk for developing disease due to their lack of preexisting immunity (43). In contrast to SOT, for bone marrow (BMT) or hematopoietic stem cell transplantation (HSCT), seropositive recipients are at the highest risk because of their profound immunosuppression and high potential for reactivation due to the ablation that accompanies preparation for HSCT (44). CMV disease has been associated with BMT and HSCT since the initial development of successful BMT regimens in the 1970s (45). Historically, the disease burden in this population has been especially high: prior to the adoption of aggressive prophylaxis, between 60-80% of seropositive HSCT recipients would experience reactivation with ~33% developing symptomatic CMV disease (46). Even with current best practices (outlined below), CMV is still an important complication for HSCT, and once CMV disease develops, the prognosis remains poor (44). CMV pneumonia remainas common in this population, being observed in 15-20% of HSCT recipients and can have a case fatality rate up to 88% (39). Finally, wide-spread adoption of highly active antiretroviral therapy (HAART) has drastically decreased the incidence of CMV disease in HIV-infected individuals, yet it remains an important clinical problem (47). CMV retinitis, the most common manifestation of CMV disease in HIV+ individuals, remains on the list of acquired immunodeficiency syndrome (AIDS)-defining conditions (48) and CMV encephalitis continues to contribute to the mortality of HIV-infected individuals (49).

Treatment of CMV disease primarily relies upon the use of antiviral chemotherapy, most commonly ganciclovir, foscarnet and cidofovir. Ganciclovir is a guanosine analog that requires initial phosphorylation by the viral UL97 kinase followed by subsequent phosphoryliation to the active tri-phosphate form by host kinases. This tri-phosphate form of ganciclovir terminates viral DNA synthesis via the CMV DNA polymerase (39). The development of ganciclovir, and the orally available valganciclovir, revolutionized the treatment of CMV infections. Indeed, it was fortuitous that these drugs were licensed for clinical use as the AIDS epidemic, and thus the cases of CMV disease, exploded (50). Foscarnet is a structural mimic of pyrophosphate that also inhibits DNA synthesis and requires phosphorylation only by host, and not viral, kinases leading to potential activity in uninfected cells (39). Because of this, foscarnet has significantly greater toxicity potential and is primarily reserved for treating ganciclovir-resistant infections. Cidofovir is another nucleoside analog that does not require phosphorylation by viral kinases to be active and inhibit DNA synthesis (39). Due to its nephrotoxicity, cidofovir is not typically used as a first line agent in treating CMV disease (39).

In transplant recipients there are two strategies for preventing CMV disease: antiviral prophylaxis, administering antivirals to all transplant recipients, and pre-emptive therapy, only giving antivirals to patients with evidence of CMV replication (40, 51). In the SOT field there is still some debate over which approach is better. Prophylaxis is universally preferred in the HSCT setting, as these patients are exquisitely sensitive to CMV infections and complications (44, 46). In the case of HIV-infected individuals, prophylactic therapy is not advised, however once CMV disease is apparent, antivirals

are prescribed for the acute disease and then maintained to prevent disease recurrence (52).

Bolstering the immune response is also critical in preventing and treating CMV in the immunocompromised patient. In SOT, this entails closely tailoring immunosuppressive regimens to maintain the balance of an immune response capable of preventing reactivation while not rejecting the transplanted organ (51). Prior to engraftment in the HSCT patient, antivirals are really the only option, however, the expansion and transfer of autologous CMV-specific CD8 T cells has been successful in treating ganciclovir-resistant CMV infections in these patients (53). For HIV+ individuals, maintaining high T cell levels by the administration of HAART is the standard of care and prevents most disease (52).

Congenital and perinatal HCMV disease

Congenital CMV infection most often results from primary infection of the mother during pregnancy, although it can follow reactivation as well (39). The mechanisms underlying trans-placental transmission are not understood. In the United States, approximately 1:150 babies are born with a CMV infection, of these, almost 20% (over 5,000 children per year) are born with or will develop serious sequelae as a result of this infection (54). Congenital CMV is estimated to cause more disease than fetal alcohol syndrome, Down syndrome, or neural tube defects (54) and is the most common cause of nongenetic hearing loss (55).

The clinical manifestations of congenial CMV disease vary wildly. Sensorineural hearing loss, visual defects and mental retardation are the most common symptoms (56).

These sequelae may not develop for months or even years. However, the most severely affected babies exhibit clear signs of disease at birth most commonly petechiae, hepatosplenomegaly, jaundice, and microcephaly (39, 56). Prognosis for these severely affected children is poor, with few escaping permanent mental or hearing difficulties later in life.

Sadly, treatment options for congenital and perinatal CMV infection are extremely limited. Due to toxicity issues, antivirals are not administered during pregnancy and are only recommended for symptomatic infants born with disease affecting the central nervous system or life-threatening disseminated disease (55). Preventing primary infection in pregnant women, by encouraging strict hygiene routines after contact with potentially infected material, is currently the first and only line of defense (54).

C) CMV and the T cell response

Observations in the clinic have identified a crucial role for T cells in preventing and controlling CMV disease. Drops in T cell levels, and the CD4:CD8 ratios, are associated with CMV reactivation and disease in HIV+ individuals (39), while transfer of autologous CMV-specific CD8 T cells can treat disease in HSCT recipients (57, 58). In immunocompetent individuals, HCMV induces a strong T cell response. Over the lifetime of an HCMV-infected individual, the frequency of CMV-specific CD8 T cells increases (59). In the elderly, CMV-specific cells constitute up to 25% of the total CD8 compartment (60) and may contribute to deterioration in immune function associated with ageing (60-62). Due to strict species specificity, evaluating the T cell response to HCMV is limited to *in vitro* studies. By utilizing the natural mouse pathogen, MCMV, much has been learned about how anti-CMV T cell responses develop. Many of these findings have been verified in HCMV and have influenced clinical treatment (57, 58, 63, 64).

One important lesson from MCMV is that T cell responses to CMV do not develop in a vacuum; many other cells contribute and modulate this response. MCMV infection leads to robust innate and adaptive immune responses that work together to control viral replication and disease (Fig. 1.1). Following infection, cells produce IFNα/β stimulating the production of MIP-1α (18) which, along with virally produced MCK-2 (25, 26, 33), recruits inflammatory monocytes (IMs) and other monocytic cells that facilitate viral dissemination (18, 28, 33). IMs, in turn, recruit NK cells that are then able to target and kill infected cells, helping to control viral replication (18, 65). All of this occurs within the first three days following infection (65, 66). Meanwhile, T and B cells

are activated by antigen presenting cells (APCs) in secondary lymphoid organs. Acute antiviral CD8 T cell responses peak around seven dpi aiding in the control of MCMV infection (explored in much more detail below) (67, 68). Thus, while T cells are critical to controlling MCMV infection, it is important to remember that they perform their antiviral function within the context of a broader immune response.

In the following sections the development of the antiviral T cell response to MCMV are reviewed beginning with initial T cell priming and activation culminating with the development of memory cells.

Antigen presentation and T cell activation

Initiating and potentiating an antiviral T cell response requires three signals from professional APCs:presentation of viral antigen (Ag) in the context of a major histocompatability (MHC) molecule (signal 1), co-stimulation provided by the expression of CD80 or CD86 (signal 2), and finally, cytokine production (signal 3). Thus, T cell responses require the careful orchestration of many different signaling pathways within APCs. Macrophages and dendritic cells (DCs), the professional APCs primarily responsible for T cell priming and activation, are readily infected by MCMV *in vivo*, indeed, one study found 75% of splenic DCs are MCMV+ two days following i.p. inoculation (69). This gives MCMV the opportunity to directly interfere with pathways required for appropriate T cell activation in APCs.

The first step of Ag presentation, signal 1, is getting viral proteins inside the APC for further processing. This occurs either by phagocytosis and endocytosis for exogenous proteins or by translation within infected cells for endogenous proteins. Through

unknown mechanisms, the endocytic capacity of immature DCs is almost completely inhibited by MCMV infection *in vitro* (69). This may limit the capacity of infected APCs to present exogenous Ag to CD4 T cells via direct presentation, or to CD8 T cells via cross-presentation bus has not yet been evaluated *in vivo*. Within the cell, Ag processing is initiated by protealytic cleavage via the proteasome or immunoproteasome (70). Recruitment of the immunoproteasome by IFNγ can be blocked by the MCMV-encoded STAT2 inhibitor M27 (71). The next step in Ag processing and presentation, transport of peptide-loaded MHC-I molecules through the Golgi to the cell surface, is the most popular site for CMV interference (Table 1.1). m152 retains MHC-I molecules and NKG2D ligands in the Golgi (72, 73), while m06 targets MHC-I for lysosomal degradation (74). Finally, once at the cell surface, m04 complexes and interferes with MHC-I recognition (21, 75). Despite this, mutant viruses lacking m04, m06 and m152 do not impact the size or breadth of the CD8 T cell response (76, 77). This surprising result suggests that either MHC-I expression is not completely lost on infected APCs *in vivo*, or that CD8 T cell priming occurs predominantly through cross presentation. While studies have demonstrated that cross presentation can occur during MCMV infection (78, 79), our incomplete understanding of the pathways and cells responsible for this process has hampered evaluation of its importance. MCMV can also inhibit MHC-II expression and subsequent presentation to CD4 T cells through unknown mechanisms (80, 81).

Inhibition of signal 2, expression of the co-stimulatory molecules CD80 and CD86 on APCs, is widely documented in infected cells (69, 82). CD80 and CD86 are upregulated following the recognition of “danger signals” or pathogen associated molecular patterns (PAMPs) by APCs (70). During MCMV infection these signals

include TLR2 (83), TLR3 (7), TLR9 (84), and other cytosolic sensors such as DAI (85). CD80 is targeted to vesicles that are part of the endosomal/lysosomal pathways by m138 (86). Meanwhile, CD86 expression is downregulated by m147.5 (87). MCMV also prevents infected APCs from receiving co-stimulatory signals and becoming fully activated by inhibiting CD40 expression via m155 (88). Intriguingly, while three MCMV gene products have been identified that each interfere with signal 2 (86-88), only one of these genes has been evaluated for an effect on T cell activation. Blocking CD40 expression on APCs by m155 restricts CD4 T cell activation (88) indicating that fully activated APCs are required for the CD4 T cell response against MCMV.

Cytokines, such as IL-12 and IL-2, are the third and final signal leading to fully activated T cells. IL-12 production by APCs stimulates IL-2 production in T cells and IL-2, which can also be made by APCs, enhances T cell survival and proliferation (70). MCMV interferes with DC production of both IL-12 and IL-2 although the mechanism is unknown (69). MCMV infection also leads to production of IL-10, an anti-inflammatory cytokine that dampens the developing CD4 T cell response (89). Additionally, MCMV encodes a viral chemokine, MCK-2 (25, 26), that actively recruits inflammatory monocytes (IMs) (33), which, in turn, produce nitric oxide restricting T cell activation and expansion (28).

While MCMV is capable of disrupting each signal required for T cell priming and activation, the relative impact these disruptions on the *in vivo* T cell response appears relatively minor. In the end, the host overcomes all of these potential barriers and develops strong anti-MCMV T cell responses.

Acute T cell response to MCMV

The CD4 T cell response to MCMV has only recently been described, nevertheless, its importance during MCMV infection has long been appreciated. CD4 T cells, and not CD8 T cells, are required for viral clearance in both the salivary glands and lungs (90, 91). While the clearance mechanism remains unclear (92), IFNγ production is important (93). CD4 help is also required for the development of B cell and Ab responses against MCMV (94). During acute infection, MCMV induces a broad CD4 T cell response to proteins spread throughout the genome (95, 96). These cells are capable of producing many different cytokines including IFNγ, TNF, IL-2, IL-10 and IL-17 (95). However the relative importance of these cytokines during infection remains unevaluated. Co-stimulation, via CD80/CD86 molecules, enhances the CD4 T cell response and control of virus in the salivary glands (97). Additionally, as previously mentioned, CD4 T cells are more sensitive to the requirement that APCs be fully activated (88).

MCMV infection induces a robust acute CD8 T cell response. Between days four and five post infection, evidence of MCMV-specific CD8 T cell expansion is apparent in secondary lymphoid organs (Fig. 1.2). At the peak of this response, between days seven to ten, MCMV-specific T cells represent a significant proportion of the CD8 T cell compartment. The two immunodominant epitopes, IE1 and m164 (98) (Table 1.2), make up almost 10% of the total splenic CD8 population in $H2^d$ BALB/c mice (28, 68), while approximately 35% of splenic CD8 T cells respond to the top four MCMV epitopes in $H2^b$ C57BL/6 mice (67). These acute CD8 T cell responses develop independently of CD4 help (99, 100).

The function of an antiviral CD8 T cell is to recognize virally infected cells and kill them. For appropriate recognition, CD8 T cells require viral Ag to be presented in the context of an MHC-I molecule. As described in the previous section, MCMV has the capacity to downregulate MHC-I expression on infected cells. Consistent with the decrease in MHC-I expression on infected cells, lysis of infected cells by MCMV-specific CD8 T cells *in vitro* is inhibited by the expression of these immune evasion genes (101). Yet WT and mutant virus lacking the m04, m06 and m152 genes are both effectively cleared *in vivo* with similar kinetics (102). Thus, the down-regulation of MHC-I by MCMV does not effect overall control of viral infection by CD8 T cells. It has been demonstrated that, for at least one MCMV-specific epitope, inhibition of MHC-I expression prevents the presentation of Ag to CD8 T cells and the subsequent killing of target cells *in vivo* (79). In C57BL/6 mice, the majority of MCMV-specific CD8 T cells recognize a peptide derived from M45 (67) (Table 1.2), yet transfer of these cells does not protect immunosuppressed mice from MCMV infection unless the virus does not express the immune evasion gene m152 (79, 103). Interestingly, this only occurs with the D^b restricted epitope, CD8 T cells that recognize a D^d restricted epitope from the same viral protein are able to control infection after transfer indicating that MCMV does not simply inhibit presentation of M45-derived peptides (103). Some have speculated that differences in Ag presentation could be due to the apparent increased susceptibility of D^b molecules to lysosomal rerouting by m152 (104), however further evaluation of additional D^b restricted CD8 T cell epitopes is needed before this can be stated with certainty. Nevertheless, inhibiting MHC-I expression on potential target cells can be successful in avoiding recognition by specific CD8 T cells.

In addition to directly controlling MHC-I expression, MCMV exerts indirect control on this process by inhibiting the cellular response to type I IFNs. Upon viral infection, cells rapidly secrete IFNα/β to induce an antiviral state in neighboring cells and activate innate host responses via autocrine signaling (70). One of the direct effects of IFN is to upregulate MHC-I molecules and the presentation of endogenous Ag via activation of the immunoproteasome, amongst other measures (105). MCMV interferes with both the production of type I IFNs and their signal transduction within infected cells (32). However, *in vivo* consequences of these efforts to avoid CTL lysis have not been fully evaluated. Despite all of these measures to avoid CTL detection, CD8 T cells do kill MCMV-infected cells *in vivo* (106, 107), thus inhibition of MHC-I expression on infected cells cannot be complete.

CD8 T cells have two main ways of controlling viral infection: cytokine production and direct cytotoxic capacity. MCMV-specific effector cells secrete abundant levels of IFNγ and TNF, including many bifunctional cells that produce both cytokines (28, 99). Interestingly, these cells do not produce detectable levels of IL-2 in response to peptide stimulation (Fig. 1.3), contrasting the phenotype of effector cells from other acute viral infections, such as lymphocytic choriomeningitis virus (LCMV) (108). IFNγ production can aid in controlling viral levels, however this contribution is modest and does not appear to alter host susceptibility to MCMV infection (93, 106). TNF production is completely dispensable for limiting viral replication (109). In contrast, the cytotoxic potential of CTLs is absolutely required as mice lacking perforin are exquisitely sensitive to MCMV disease (106, 110). Perforin is a pore-forming protein found, along with serine proteases known as granzymes, in the granules of CD8 T cells. Upon TCR ligation in an

effector cell, perforin and granzymes are released into the immunological synapse. Direct release into the synapse enables the potent cytotoxic effects of these molecules to be delivered only to the target cell, shielding neighboring cells from collateral damage (111). Thus, perforin and granzyme killing requires direct contact between the CD8 effector and its target, unlike cytokines which, by definition, can work on distant cells. Upon its release, perforin forms pores in cellular or endosomal membranes allowing granzymes access to the cytosol leading to eventual death of the target cell (111). Granzymes kill cells via caspase dependent and independent mechanisms (112). This ensures that even MCMV-infected cells expressing numerous cell death suppressors (113-117) can be killed. Intriguingly, while granzymes clearly help restrain CMV viral replication, perforin is required to protect from lethal disease (110).

The relative contribution of CD8 T cells to host control of MCMV infection varies depending on mouse genotype. In BALB/c mice, CD8 T cells are absolutely required for constraining viral replication (106) and can protect from lethal challenge (100, 118). It is interesting to note that peak viral titers are observed between three and five dpi in the spleen and liver (35, 68) (Fig. 1.1), several days before the peak CD8 T cell response (Fig. 1.2). Given that, in the absence of CD8 T cells, virus is not controlled in these organs (106), it is likely that low levels of MCMV-specific CD8 T cells present prior to the peak response, are actively killing infected cells. Identification of functional antiviral CD8 T cells in both secondary lymphoid (spleen) and peripheral (liver) organs at three and four dpi would challenge the current assumption that antiviral CD8 T cell activity primarily occurs after clonal expansion.

The role of CD8 T cells during MCMV-infection in C57BL/6 mice is more complicated. MCMV encodes m157, an MHC-I homologue, which binds to the NK cell activating receptor Ly49H (119, 120). C57BL/6 mice, unlike BALB/c, express Ly49H, leading to robust activation and expansion of the NK cell compartment (121) that can control viral replication in the spleen, lungs, and liver (122) (reviewed in (123)). Under these conditions, the CD8 T cell response is dampened, potentially due to decreased Ag load (107). Due to this, many groups utilize m157-deficient virus to study CD8 T cell responses in C57BL/6 mice (99, 107, 124, 125). However, viral-specific CD8 T cells readily develop in C57BL/6 mice infected with WT MCMV (67) and exhibit cytokine and cytotoxicity profiles consistent with functional effector cells (see Ch. 3-5) (67). Studies in immunodeficient mice have demonstrated that CD8 T cells do help control viral replication in C57BL/6 mice. Depletion of CD8 T cells leads to increased viral titers in the spleen (107), suggesting that CD8 T cells aid NK cells in viral control. T cell deficient nude mice on the C57BL/6 background, with an intact NK cell compartment, exhibit increased susceptibility to MCMV infection (66) indicating that T cells can also protect from lethal disease in this genetic background.

Development of memory T cells and the memory response to MCMV

The development of memory T cells during MCMV infection coincides with the establishment of latent infection in the host. Latency is characterized by low or undetectable viral levels throughout the mouse except in salivary glands where active viral replication continues for weeks. Immunosuppression by steroids or lymphoid depletion can induce reactivation leading to active viral replication (126, 127). This

indicates the importance of an intact adaptive immune response in controlling latent infection. During latent infection, two different categories of memory T cells develop: those that are maintained at a constant, low level termed stable memory cells, and those that increase in number and frequency, termed inflationary memory cells (128). Notably, inflationary cells are also observed during HCMV infection (129). As was the case for the acute response, memory CD4 responses to MCMV have only recently been described and functional roles for these cells have yet to be elucidated. Memory CD4 cells do develop, with both stable and inflationary characteristics (95, 96)

Stable memory CD8 T cells develop following contraction of the T cell response and the resolution of acute infection (128, 130). These cells develop a central memory (T_{CM}) phenotype with high expression of the cytokine receptors CD127 (IL-7Rα) and CD122 (IL-15Rβ), and the activation marker CD27 (130, 131). CD62L, another traditional marker of T_{CM} (108), is usually expressed on these cells, although the level of expression varies (68, 131). Consistent with this phenotype, effector molecules, such as granzyme B, are down-regulated (130). Conflicting evidence exists as to whether CD4 help is required for the development of stable memory cells (99, 132). Neither viral replication nor the presence of viral Ag is required for the maintenance of these cells (131-133). These cells are capable of secreting cytokines upon Ag encounter and can kill target cells (68, 130, 131). Their *in vivo* protective capacity, however, varies. As previously mentioned, in C57BL/6 mice, the stable M45 epitope (Table 1.2) cannot protect immunosuppressed mice from disease due to the inhibition of Ag presentation in infected cells (79, 103), yet a different epitope of M45 recognized in BALB/c mice, that also develops into stable memory cells, is able to protect (103). While their role in the

context of an ongoing viral infection that induces both stable and inflationary CD8 T cells remains unclear, these stable cells exhibit all of the phenotypic and functional characteristics of competent memory cells.

The patterns and kinetics for inflationary memory cell development differ depending on epitope. Some epitopes expand during acute infection, such as m139 and M38 in C57BL/6 and IE1 and m164 in BALB/c mice (Table 1.2) (98, 128), while others do not, only inflating during memory time points, such as IE3 in C57BL/6 mice (98, 128). For the epitopes that do respond during acute infection, some contract following acute infection, m139 in C57BL/6 and IE1 in BALB/c mice, while others do not, M38 in C57BL/6 mice (68, 128). To date, no host or viral factor has been identified that contributes to these different kinetics. The capacity of these cells to inflate over time is remarkable, after one year of infection MCMV-specific CD8 T cells can make up 20-50% of the total CD8 population (68, 131). Unlike the T_{CM} phenotype of stable memory cells, inflationary cells develop an effector memory (T_{EM}) phenotype with low expression of CD127, CD27, and CD62L (68, 130, 131) while KLRG1 expression is high (131). Consistent with the T_{EM} phenotype, inflationary cells are found throughout the body, including in non-lymphoid organs (68, 130, 134).

While the mechanism by which MCMV drives memory inflation remains unknown, it is dependent on Ag presentation within the context of an MCMV infection. When an inflationary epitope from MCMV is encoded by another virus, such as vaccinia, T cells that recognize that epitope no longer inflate (68), conversely, a non-inflationary epitope from another virus can be made inflationary by insertion into the MCMV genome (135). Persistent low-level Ag exposure has long been thought to drive inflation, indeed,

inflationary cells are unable to divide or survive when transferred into a naïve host (131). However, recent work using antivirals and a replication-defective virus has added a layer of complexity to this hypothesis. When administered via foot pad inoculation, a route of inoculation that requires the recruitment of immune cells to facilitate dissemination, inflation does not occur in the absence of viral replication (133). Yet, when administered via i.p. inoculation, where viral particles are directly delivered to organs throughout the peritoneal cavity, including the spleen, via the lymphatic vessels (136), this replication-defective virus does induce inflation (133). Similar results were also observed when antivirals were administered. These results suggest that, while active viral replication is not required for memory inflation, the presence of viral DNA, and presumably viral Ag, is needed. Intriguingly, it is possible that a non-hematopoietic cell located outside the salivary gland, the location most likely to have persistent Ag, is responsible for presenting Ag and driving inflation (134, 137).

The host signals that contribute to memory inflation are not completely understood. Inflationary CD8 T cells absolutely require CD4 help for their development (99, 132). Yet they are less dependent on CD80/CD86 co-stimulation than stable memory cells (138). Stimulation by the TNFR family members 4-1BB and OX40 contributes to the development of inflationary cells (139, 140). With the exception of IL-10, which has been shown to restrict CD8 inflation (141), the contributions of cytokines to this phenomenon have not been elucidated. Memory inflation is at least partially maintained by the recruitment of naïve CD8 T cells (131), but these cells do not need to be recent thymic emigrants (137).

Inflationary CD8 T cells are fully functional during MCMV infection. They secrete multiple cytokines in response to Ag stimulation (130) and can kill target cells (131). Transfer of these cells into immunocompromised hosts has long been understood to protect from lethal infection and resolve infections that were present prior to cell transfer (29, 100, 142). This contrasts what would be expected during chronic viral infections, such as LCMV-Clone 13, where the constant exposure to high levels of Ag leads to T cell exhaustion and the inability to respond to Ag (108, 143). Differences in the relative levels of persistent Ag likely explain these seemingly discordant results with lower levels present during the latent MCMV infection. This suggests that a threshold exists for the T cell response to persistent Ag. Below this threshold, Ag continues to activate T cells while above it, Ag drives exhaustion. Better understanding the interplay between activating and exhausting signals T cell receive following persistent exposure to Ag could lead inform the development of vaccines capable of inducing long-lasting immunity as well as more targeted therapies for autoimmune disease.

Amazingly, the importance of memory CD8 T cells during latent infection is still not well understood. Depletion of lymphocytes (126, 127) leads to MCMV reactivation indicating the importance of T and B cells together in controlling chronic infection. While reactivation cannot be induced by depletion of CD8 T cells alone (127), MCMV-specific CD8 T cells are enriched at sites where reactivation commonly occurs (144), appear to maintain latency by sensing transcriptional reactivation (145), and a transient decrease in cell numbers is associated with reactivation (146). All of this suggests that CD8 T cells patrol for and kill reactivating cells.

Overall, the CD8 T cell response to MCMV infection has been well characterized over the past 25 years. Yet, potential impacts of T cells on MCMV pathogenesis remain unexplored. Work in this dissertation begins to address this question by examining the contributions of T cells to lethal MCMV disease. T cells are found to mediate a lethal hepatitis between days four and five in immunocompetent mice infected with a highly virulent viral variant. The hepatic Ag-specific CD8 T cells associated with lethality appear more functional, with evidence of increased cytotoxic capacity and cytokine secretion. These results demonstrate that T cells are critically involved in MCMV pathogenesis and can mediate disease at early time points in addition to their previously described protective function.

D) Programmed cell death pathways and T cells

Cell death is an important part of the T cell life cycle. Under naïve homeostatic conditions, T cells exist in a steady state with recent thymic emigrants replacing older cells that have died. In response to foreign Ag, T cells can expand tens of thousand-fold, making them the most proliferative somatic cell in the body (147). Following clearance of Ag, the T cell response contracts back down to levels only slightly higher than those found pre-Ag exposure. Having programmed pathways that are triggered during this period of massive cell death ensures that the resolution of the immune response does not contribute to additional disease such as autoimmunity or immunopathology. Two different pathways, intrinsic and extrinsic, are known to mediate T cell death. The intrinsic pathway is activated by intercellular stress signals, such as genotoxic stress, while extrinsic cell death is triggered by external ligands binding their death receptors, such as Fas or TNFR1, on the cell surface. Both cell death pathways activate a series of kinases and caspases that eventually lead to the ordered dismantling of the cell and maintenance of normal immune function.

The intrinsic death pathway relies on mitochondrial permeabilization to activate effector caspases leading to apoptosis (147). In T cells, this pathway is most clearly activated when pro-survival cytokines, such as IL-2 or IL-7, are removed from culture (148). Cytokine withdrawal death leads to activation of the Bcl-2 family member Bim. Bim then leads to Bak and Bax oligamerization, the formation of a mitochondrial pore, and mitochondrial outer membrane permeabilization (MOMP) culminating with the activation of effector caspases (147). Bim regulated death pathways are important during T cell development (148) by contributing to negative selection and killing self-reactive T

cells (149). During viral infection, Bim mediates T cell contraction, presumably activated by the decreased availability of cytokines following Ag clearance (150).

Extrinsic death signals can lead to apoptotic or programmed necrotic cell death. Both pathways converge on caspase (Casp)8, which is activated following death receptor signals (151). Casp8 activation leads to apoptosis via direct activation of effector caspases and/or mitochondrial amplification by cleavage of Bid (152). Receptor interacting protein (RIP)1 and RIP3 are also recruited following death receptor signaling, but their activity is normally restrained by Casp8 (151). In the absence of Casp8, death receptor ligation leads to a RIP1/RIP3-mediated necrosis (153-155). Interestingly, TCR signaling can also activate Casp8 via the Bcl-10/MALT1/CARMA complex (156). The importance of Casp8 in T cells was first revealed by studies in mice with conditional Casp8 deletion in T cells (*tCasp8*$^{-/-}$) (157-159). While thymic development appears intact, naïve *tCasp8*$^{-/-}$ mice have fewer T cells in the periphery (158) indicating that Casp8 provides a pro-survival signal under homeostatic conditions. RIP1/RIP3-mediated necrosis is unleashed in *Casp8*$^{-/-}$ T cells following TCR ligation leading to a profound immunodeficiency in *tCasp8*$^{-/-}$ mice (158-160).

The discovery that RIP3 deficiency rescues many of the defects observed in Casp8-deficient settings provides the opportunity to evaluate RIP3-independent functions of Casp8 in T cells. Utilizing MCMV, the contribution of extrinsic death pathways to the antiviral T cell response is explored in this dissertation. Both RIP3 and Casp8 are found to be globally dispensable for T cell development. RIP3 contributes to host susceptibility to high doses of MCMV but not to the generation of antiviral CD8 T cell responses and viral control at low doses. Casp8 helps control homeostatic CD8 T cell turnover, but is

completely dispensable for a long-lived CD8 T cell response to MCMV. Together, these studies demonstrate that extrinsic death pathways are not needed for functional antiviral immunity, and that programmed necrotic pathways contribute to host defense under certain circumstances.

E) Figures and Figure Legends

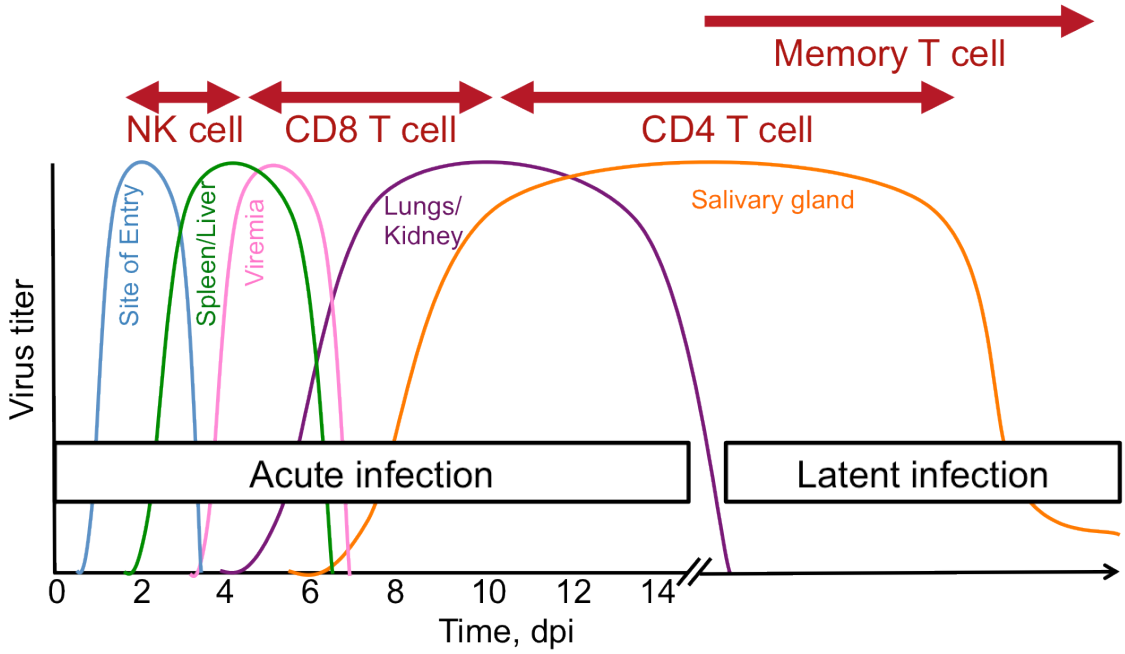


Figure 1.1: Schematic overview of viral replication and immune control during *in vivo* MCMV infection. Following i.p. inoculation the spleen and the liver are seeded within hours, however peak and clearance kinetics remain as indicated in the diagram.

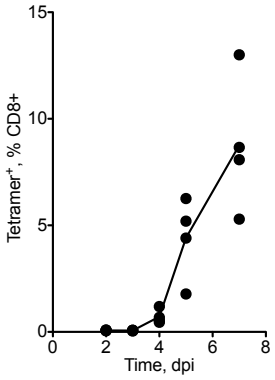


Figure 1.2: Development of Ag-specific CD8 T cell response. Frequency of M45-tetramer^{+} CD8 T cells. *Rip3*$^{-/-}$ mice were inoculated with 1×10^5 PFU v70 and spleens harvested on indicated days. Single cell suspensions were obtained, cells stained, and flow cytometry performed as previously described (161).

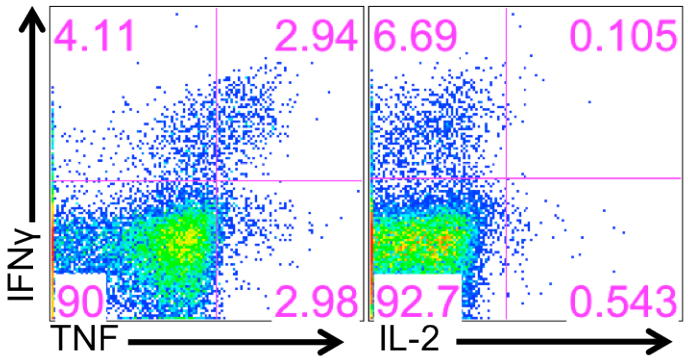


Figure 1.3: Cytokine production by MCMV-specific CD8 T cells. Flow charts showing cytokine production in splenic CD8 T cells at 7 dpi after 5 hr stimulation with M45-peptide in the presence of BFA. C57BL/6 mice were inoculated with 1×10^5 PFU v70. Representative of more than 5 mice.

F) Tables

Table 1.1: HCMV and MCMV interference with MHC expression and Ag presentation

Virus	Gene/Gene product	Function	Ref.
HCMV	pp65	Inhibits proteasome	(162)
	US6	Inhibits TAP	(163)
	US2	Dislocates MHC-I from ER	(164)
	US11	Dislocates MHC-I from ER	(165)
	US3	Restricts MHC-I movement into Golgi	(166, 167)
	IE/E product	Interferes with MHC-II expression	(162)
MCMV	M27	Blocks recruitment of immunoproteasome	(71)
	m152	Retains MHC-I and NKG2D ligands in Golgi	(72, 73)
	m06	Redirects MHC-I to lysosome	(74)
	m04	Binds MHC-I on cell surface	(75)

Table 1.2: Predominant MHC-I restricted epitopes during MCMV infection

Strain	ORF*	Amino acids	Sequence	MHC	Present in acute?	Inflates	Ref
C57BL/6	**M45**	985-993	HGIRNASFI	D^b	Yes	No	(67, 128)
	M57	816-824	SCLEFWQRV	K^b	Yes	No	
	m139	419-426	TVYGFCLL	K^b	Yes	Yes	
	M38	316-323	SSPPMFRV	K^b	Yes	Yes	
	IE3	416-423	GTVINLTSV	K^b	No	Yes	
	IE3	461-475	RALEYKNL	K^b	No	Yes	
	M86	1062-1070	SQNINTVEM	D^b	Yes	No	
	M97	210-217	IISPFPGL	K^b	Yes	No	
	m141	15-23	VIDAFSRL	K^b	Yes	No	
	M102	486-500	RLAHSSPRIFRRVRS	K^b	Yes	Yes	
BALB/c	**IE1**	168-176	YPHFMPTNL	L^d	Yes	Yes	(168, 169)
	m164	168-265	AGPPRYSRI	D^d	Yes	Yes	(98)
	M45	507-515	VGPALGRGL	D^d	Yes	No	(170)
	M04	243-251	YGPSLYRRF	D^d	Yes	No	(171)
	M83	761-769	YPSKEPFNF	L^d	Yes	No	(172)
	M84	297-305	AYAGLFTPL	K^d	Yes	No	(173)

* Bold indicates immunodominant epitope during acute infections.

CHAPTER 2.

Antiviral T cell response triggers cytomegalovirus hepatitis in mice.

The data presented in this chapter was published in the *Journal of Virology* as: Livingston-Rosanoff, D., Daley-Bauer, L.P., Garcia, AP., McCormick, A.L., Huang, J., Mocarski, E.S. (2012). "Antiviral T cell response triggers cytomegalovirus hepatitis in mice." J Virol 86(23): 12879-90. The content is reproduced here in whole with permission from the publisher. Data not generated by the Ph.D candidate is indicated in the figure legends.

A) Abstract

One common sign of human cytomegalovirus infection is altered liver function. Murine cytomegalovirus strain v70 induces a rapid and severe hepatitis in immunocompetent mice that requires the presence of T cells to develop. v70 exhibits approximately 10-fold greater virulence than the commonly used strain K181, resulting in a more severe, sustained and lethal hepatitis but not dramatically higher viral replication levels. Hepatitis and death are markedly delayed in immunodeficient SCID compared to immunocompetent BALB/c mice. Transfer of BALB/c splenocytes to SCID mice conferred rapid disease following infection, and depletion of either CD4 or CD8 T cells in BALB/c mice reduced virus-induced hepatitis. The frequency of CD8 T cells producing interferon γ and tumor necrosis factor in response to viral antigen was higher in settings where more severe disease occurred. Thus, virus-specific effector CD8 T cells appear to contribute to lethal virus-induced hepatitis, contrasting their protective role during sublethal infection. This study reveals how protection and disease during cytomegalovirus infection depends on viral strain and dose, as well as the quality of the T cell response.

B) Introduction

Human cytomegalovirus (HCMV) disease is associated with liver dysfunction. Hepatosplenomegaly and jaundice are two signs of systemic congenital disease (39, 56). Cytomegalovirus (CMV) disease in solid organ and hematopoietic allograft recipients is a leading cause of graft loss and mortality where elevated liver enzymes and hepatitis are common (39, 40, 174-176). In immunocompetent individuals, elevated liver enzymes accompany subclinical infection (177) as well as the natural disease, mononucleosis (39, 178) where hepatitis can be the presenting illness (179, 180). A better understanding of viral and host contributors to HCMV-induced liver damage and hepatitis in immunocompetent individuals will provide insights into potential therapeutic interventions as well as a foundation from which disease can be further studied in immunocompromised patients. HCMV exhibits strict species specificity (4) making the study of disease pathogenesis difficult. Murine CMV (MCMV) is a natural mouse pathogen that has unveiled principles of host immunity (13, 14, 116, 181), viral immune modulation (21, 23-27) and disease pathogenesis (18, 20) that have been translated to HCMV (23, 29, 30).

Like HCMV, MCMV causes a chronic, sub-clinical, systemic infection associated with elevated liver enzymes as well as histologic evidence of hepatic inflammation and damage (18, 34, 136, 182). While lethal MCMV disease in immunocompetent BALB/c mice is attributed to liver damage culminating in a severe hepatitis within the first week of infection (183), factors contributing to this disease have not been characterized. Disease is prevented by administration of antiviral drugs, revealing a critical contribution of ongoing viral replication (184). Unlike other forms of hepatitis, TNF is dispensable for

disease in BALB/c mice (185). Further elucidation of host and viral determinants of rapid hepatitis in immunocompetent mice may unveil mechanisms underlying liver damage during HCMV infection.

Inflammatory monocytes (IMs) are involved in MCMV-hepatitis and disease pathogenesis. IMs are recruited by host (MCP1/CCR2) and viral (MCK2) chemokine signals (25, 26, 186). In C57BL/6 mice IMs protect from lethal hepatitis by recruiting natural killer (NK) cells that control infection (18). In BALB/c mice, IMs restrict the antiviral CD8 T cell response leading to a delay in viral clearance from peripheral organs (28). In this setting, IMs may also be responsible for immunopathology, as has been shown in other viral infections (187, 188).

During sublethal MCMV infection in BALB/c mice, CD8 T cells control viral replication in the liver as well as in most peripheral organs (106); while CD4 T cells control infection in salivary glands (91). Immunity depends on the collaborative efforts of cytokine and cytolytic activity of CD8 T cells (106, 189) to protect mice from lethal challenge (100). Immunodeficient mice lacking T cells exhibit a delay in time to death and less severe hepatitis compared to immunocompetent mice (66, 190, 191), raising the possibility that this arm of host defense may also contribute to disease. Given that CD8 T cell responses contribute to hepatitis in humans infected with hepatitis viruses A, B and C, and Epstein Barr virus, in mouse models of hepatitis B infection (192, 193), and in mice infected with lymphocytic choriomeningitis virus (LCMV) (194), the potential contribution of antiviral T cell responses to MCMV-induced lethal hepatitis needs to be evaluated.

The disease potential of MCMV depends on the source as well as strain of virus. Virus isolated from salivary glands is more virulent than virus propagated in cell culture or isolated from other organs (195). For this reason, and because natural viral transmission is mediated by saliva, pathogenesis studies have relied on salivary gland-derived virus (18, 20, 66, 182, 183, 196, 197). The Smith strain of MCMV was subjected to serial propagation through salivary glands of Swiss-Webster mice (198) resulting in the isolation of strain K181. K181 is more virulent than Smith (199) and can cause lethal hepatitis in BALB/c (183) but not in C57BL/6 mice (unpublished observation). When strain K181 was subjected to sequential passage in Swiss-Webster mice (195), strain v70 was isolated (although this strain, like K181, has often been called "Smith") (65, 200). This strain was adopted for use in C57BL/6 mice based on its virulence characteristics (C.A. Biron and M.J. Selgrade, personal communication). Strain v70 has provided valuable insights into mechanisms of host response in C57BL/6 mice (19, 20, 65, 66, 197, 200-202) where control of virus is mediated by interferon (IFN) and IM recruitment of NK cells (18). In this strain, hepatitis results from poor control of viral infection and is associated with TNF production. In almost 20 years of study, v70 has not been directly compared to other MCMV strains or evaluated for disease potential in a common susceptible strain of mice such as BALB/c where CD8 T cells dominate host control and the potential for T cell-mediated pathology is greatest.

To identify host factors involved in lethal MCMV hepatitis, strain v70 was evaluated for its virulence potential in BALB/c mice using K181 as a reference. We show that BALB/c mice develop a rapid lethal hepatitis at a lower dose of v70 than K181, with

a 10-fold difference in virulence potential. In examining host factors involved in disease, we identify a potent antiviral T cell response as a contributor to v70-induced hepatitis.

C) Materials and Methods

Mice

Six to twelve week old mice were used in all experiments. BALB/c and CBySmn.CB17-Prkdcscid/J (SCID) mice were purchased from the Jackson Laboratory. MCP1$^{-/-}$CCR2$^{-/-}$ on a BALB/c background (33) and non-obese diabetic (NOD)/SCID/$\gamma_c^{-/-}$ (NSG) mice were bred and maintained in house. Mice were group housed, maintained on a 12:12 hour light:dark cycle, and fed rodent diet (LabDiet 5010, Purina Mills) *ad libitum.* All mice were maintained under specific pathogen-free conditions by the Division of Animal Resources at Emory University or the Department of Comparative Medicine at Stanford University. Experiments were conducted under protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care and the Emory University Institutional Animal Care and Use Committee.

Viruses

K181+ is a plaque purified isolate of K181 that has been previously characterized (34). Salivary gland-propagated v70 was kindly provided by C. Biron (Brown University) (66). v70+ was generated by three rounds of plaque purification on 3T3-Swiss albino fibroblasts (ATCC CCL-92) cultured in Dulbecco's modified mimimum essential medium supplemented with 10% fetal bovine serum and antibiotics (DMEM). Tissue culture stocks of K181+ or v70+ were grown in NIH-3T3 fibroblasts (ATCC CRL-1658). Viral stocks used in these studies were generated by inoculating BALB/c mice intraperitoneally (i.p.) with 1×10^3 PFU of salivary gland-propagated v70 or 1×10^6 PFU

of tissue culture-derived K181+ or v70+ as previously described (34). Organ sonicates (10% weight/volume in DMEM) were stored in single use aliquots at -80°C.

Infections

All experiments were carried out by i.p. inoculation using salivary gland-derived virus stocks. Mock infection was carried out using an equal volume of DMEM. Infected mice were monitored for development of disease by being weighed once daily and observed twice daily for signs of morbidity: piloerection, hunched posture, and lethargy. Imminent death was defined as loss of 20% initial body weight or development of severe lethargy (unresponsiveness to touch) established in a preliminary experiment using death as the endpoint. In experiments where mice were sacrificed at specific times, equal numbers of v70, v70+, or K181+ infected mice were evaluated at each time point.

Viral titers and serum chemistries

For quantification of viral titers, organs were placed in 1mL of DMEM, stored at -80°C until they were thawed and disrupted by sonication, and viral titers evaluated by plaque assay on 3T3-Swiss Albino fibroblasts as previously described (203). Blood was obtained by cheek bleeds or cardiac puncture. Automated serum chemistries were evaluated using a VetScan VS2 machine (Abaxis) by the Division of Animal Resources at Emory University.

Histology

Peripheral organs were isolated at the times indicated in text and figure legends. For histological analysis, organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E. The Division of Animal Resources and Yerkes Department of Pathology at Emory University performed all processing of histological samples following fixation. H&E stained slides were blinded and evaluated by a veterinary pathologist (A.G.). Immunohistochemistry was performed on paraffin-embedded tissues using the anti-IE1 monoclonal antibody (Ab) CROMA 101 (kindly provided by S. Jonjic, University of Rijeka, Croatia) as previously described (142). Bound Ab was detected using the Vectastain ABC kit (Vector Labs), following manufacturer's instructions and counterstained with Gill's hematoxylin solution #2 (Electron Microscopy Services). Images of histology were acquired using an Olympus Q Color 3 camera and an Olympus BX43 microscope.

Fluorescently-conjugated tetramer and Abs

PE-conjugated H-2L^{d}-IE1$_{168\text{-}174}$ tetramers were obtained from the NIH Tetramer Core Facility (Emory University). The Abs used were: Ly6C FITC (AL-21), IFNγ FITC (XMG1.2), CD3 PE (17A2), IL-17 PE (TC11-18H10), CD4 PerCP-Cy5.5 (RM4-5), TNF PE-Cy7 (MP6-XT22), B220 PE-Cy7 (RA3-6B2), CD49b APC (DX5), CD11b APC-Cy7 (M1/70), CD3 Pacific Blue (500A2), purchased from BD Biosciences; CD107a APC (1D4B), CD8 APC-Cy7 (53-6.7), purchased from BioLegend; and CD45 PE-Texas Red (30-F11), CD69 PE-Texas Red (H1.2-F3), and CD8 Pacific Orange (5H10), purchased from Invitrogen.

Characterization of leukocytes and flow cytometry

Single cell suspensions were isolated from spleen and liver as previously described (20, 204). In all instances 1×10^6 live cells, as evaluated by trypan blue (Cellgrow) exclusion, were prepared for flow cytometric detection of surface and intracellular antigens (Ag). For evaluation of T cell function, cells were incubated for 5 hours at 37^oC with 1×10^{-9}M IE1 peptide (JPT Peptide Technologies) (169) or 50ng/mL Phorbol 12-myristate 13-acetate (PMA) (Sigma) and 500ng/mL ionophore (Sigma) in the presence of GolgiStop (BD Biosciences) and CD107a Ab. Prior to incubating with lineage specific Ab, cells were incubated with 10% normal rat serum (Pel-Freez) and anti-mouse CD16/CD32 Ab (2.4G2; BD Pharmingen) to reduce non-specific interactions. For detection of intracellular cytokines the Cytofix/Cytoperm kit from BD was used according to manufacturer's instructions. Data were acquired using an LSRII flow cytometer (BD Biosciences) maintained by the Emory University Flow Cytometry Core and analyzed by FlowJo software (Tree Star). For all samples, live cells were gated on based on forward and side scatter properties followed by identification of leukocytes by CD45 expression. T cells were identified by expression of CD3 and further segregated into subsets based on expression of CD4 or CD8. All gates were established based on appropriate isotype and unstained controls.

Adoptive transfer and depletion

Splenocytes used in adoptive transfers were prepared by mechanically disrupting the spleens of naïve BALB/c mice through a metal strainer, isolation via a histopaque

1119 gradient (Sigma) according to manufacturer's instructions and filtration through a 40μm nylon screen. Viability was assessed on an aliquot of cells by trypan blue exclusion using a hemocytometer. 4×10^7 cells in a total volume of 250-300μL PBS were injected into tail veins of SCID mice. Mice were inoculated with MCMV one-day post-transfer of cells.

Rabbit anti-asialo-GM1 antisera (Wako) was administered in doses of 50μL in 200μL PBS i.p. one-day prior, one-day following infection and every three days thereafter. CD4 (GK1.5 - BioXcell) and CD8 (H35 – kindly provided by A. Lukacher, Emory University) (205) Abs were used to deplete T cell subsets from BALB/c mice. For CD4 depletion, 500μg of Ab was administered i.p. one-day prior and one-day following infection. For CD8 depletion, 500μg of Ab was administered i.p. on days -3, -1 and +1 relative to infection and maintained by weekly Ab injections. Prior to infection, blood was obtained from cheek bleeds in tubes containing lithium heparin (BD Biosciences), RBCs were lysed using ammonium chloride solution (0.15 M NH_4Cl, 10 mM $NaHCO_3$, and 1.0mM Na_2EDTA in H_2O, pH 7.4) and engraftment or depletion evaluated by flow cytometry. Ab-depletion of NK, CD4 or CD8 cells achieved $\geq$ 95% depletion based on flow cytometry analysis.

Statistics

All statistical analyses were performed using Prism software (GraphPad). For comparison of survival curves the Mantel-Cox test was employed. The Mann-Whitney test was used for all other comparisons. In all comparisons, a p value of ≤ 0.05 was considered significant.

D) Results

Comparison of v70 and K181+ in BALB/c mice

Many studies have employed salivary gland propagated v70 (18, 20, 65, 66, 200, 202, 206) because it is a virulent strain of virus. To determine viral and host factors that contribute to MCMV disease pathogenesis, v70 was compared to K181+ (34), evaluating the endpoint of lethal infection in BALB/c mice. The criteria used to define imminent death were: loss of greater than 20% body weight or severe lethargy (see Materials and Methods). Mice inoculated with 5×10^5 PFU of K181+ uniformly died between days three and eight (Fig. 2.1A); whereas, mice survived a lower dose (2×10^5 PFU) of this virus. A dose of 1×10^5 PFU of v70 was uniformly lethal by four dpi (Fig. 2.1B), and a dose of 5×10^4 PFU resulted in half the mice dying between days four and six (Fig. 2.1C). This, together with additional experiments not shown, allowed an estimation of the LD_{50} for v70 between 2×10^4 and 5×10^4 PFU; whereas, the LD_{50} for K181+ was estimated to be between 4×10^5 and 6×10^5 PFU. Both viruses exhibited a sharp cut-off for lethality (Fig. 2.1A), in line with expectations (183, 190, 195). These experiments established that v70 is highly virulent for BALB/c mice, showing an LD_{50} roughly ten-fold lower than K181+.

To determine whether differences in virulence were due to viral strain-specific factors, a coinfection experiment was performed (195). The pattern of lethality in BALB/c mice inoculated with 5×10^4 PFU of K181+ or v70 alone was compared to mice coinfected with 5×10^4 PFU of each strain given together (Fig. 2.1C). Coinfection resulted in death of 60% of the mice by day six, similar to v70 alone but significantly different from single infection with K181+, where all mice survived. This pattern

demonstrates that K181+ does not express a protective factor and is consistent with v70 encoding a dominant virulence factor contributing to lethal disease.

Disease severity and viral replication.

Strain-specific replication potential *in vivo* has been associated with the virulence of strain K181 when compared to Smith (199). To determine whether v70-associated virulence was associated with greater replication potential, we followed disease patterns and assessed viral titers in peripheral organs after inoculation with 1×10^5 PFU v70 or K181+. This was predicted to be a lethal v70 dose but sublethal for K181+ (Fig. 2.1A-B). Mice inoculated with either v70 or K181+ all showed signs of illness (weight loss and changes in appearance) beginning on day two and continuing on day three (Fig. 2.2A and data not shown). Important differences in disease were observed on days four and five, when mice infected with K181+ stabilized as mice infected with v70 continued to decline. By day five, eight out of ten mice infected with v70 had died, in contrast to those infected with K181+, where all survived. At no point did any of the infected mice develop neurological symptoms (photophobia, ataxia), consistent with previous studies (183). Viral titers were indistinguishable in organs assayed at day three, but, thereafter, followed a pattern implicating the liver as a target of disease. In the liver, viral titers declined more gradually during v70 infection than during K181+ infection, such that v70 was sustained in this organ at a modestly higher level as disease progressed and mice died (Fig. 2.2B top panel). Viral titers in other organs (spleen, lung, and kidney) followed organ-specific patterns that did not correlate with disease outcome because v70 and K181+ were indistinguishable (Fig. 2.2B bottom panels and data not shown). Although

infections resulting in lethal and sublethal disease exhibited similar patterns of viral replication overall, the sustained v70 titers in the liver suggests a relationship to disease and death.

To further evaluate the replication potential of these viral strains, we compared virus titers at a dose that was sublethal for v70 as well as K181+. After inoculation with 1×10^4 PFU of either virus strain, weight loss (Fig. 2.2C) and appearance (data not shown) were similar and did not change as dramatically as at the high dose (compare to Fig. 2.2A). Overall, peak titers in liver and spleen occurred over the same time frame (by three dpi) but remained orders of magnitude lower in mice infected with 1×10^4 PFU (Fig. 2.2D top left and middle panels) compared to animals receiving 1×10^5 PFU (Fig. 2.2B top panels), as expected from earlier evaluations (207). At the low dose, both v70 and K181+ titers declined in the liver with an identical pattern by day 10 (Fig. 2.2D top left panel). Consistent with high dose data, replication followed similar organ-specific patterns in spleen, kidneys, and lungs (Fig. 2.2D bottom panels and data not shown). The patterns of replication in salivary glands, the target of viral dissemination, were also identical, peaking near 10^8 PFU/g of tissue at 10 and 14 dpi (Fig. 2.2D right panel). Overall patterns of viral replication and clearance were remarkably parallel, suggesting that the disease process was responsible for sustaining viral levels in the liver at the high dose of v70.

Hepatitis underlies lethal disease

We next followed the development of disease in relation to serum chemistries and pathology. Again, a dose of 1×10^5 PFU of v70 or K181+ was employed to induce a

lethal (v70) or sublethal (K181+) infection. This dose resulted in a similar pattern of weight loss through three dpi (Fig. 2.3A), with weight loss in v70-infection progressing through day four, when two mice died, and day five, where remaining mice succumbed. In contrast, all mice infected with K181+ survived, with mice showing signs of recovery at days four and five. Blood was analyzed for serum chemistries and samples of liver, adrenal gland, gastrointestinal (GI) tract, pancreas and spleen were evaluated daily for histopathological damage from days two through five. These organs were chosen as they all become productively infected within the first five days of infection with MCMV (136, 183). Samples were collected from randomly selected mice at all time points except for days four and five when mice that had died were included.

At day three, serum alanine transaminase (ALT) levels were higher in sublethally infected compared to lethally infected mice (Fig. 2.3B). ALT levels subsequently decreased during sublethal infection and stabilized by day five. Over this time, ALT levels increased progressively in lethally infected mice, ultimately rising above peak levels observed in mice that survived. ALT levels may increase due to liver or kidney dysfunction. Kidney function was not altered based on serum BUN and creatinine levels (Fig. 2.3C and data not shown), indicating that elevated ALT reflected liver damage. The falling pattern of ALT during sublethal infection was consistent with recovery, whereas the rising pattern in lethally infected mice was associated with disease and death.

To directly evaluate hepatic pathology, liver sections were blinded and scored by a veterinary pathologist (AG). Virus-associated hepatic cytopathology was assessed by the presence of intranuclear inclusion bodies and multinucleated hepatocytes. Sublethally infected mice reached peak scores for necrosis and inflammation at day three (Fig. 2.3D-

E). The scores in lethally infected mice were parallel through day three but continued to rise at days four and five when mice succumbed. While less histopathological damage was evident at day two in lethally infected mice and ALT levels were lower at day three, this delay was not associated with v70-induced disease because hepatic viral titers were identical in lethally and sublethally infected mice at these times (Fig. 2.2B and data not shown). Taken together with hepatic viral titers (Fig. 2.2B) and lethality (Fig. 2.1 and 2.3A), liver pathology at days four to five correlated with disease outcome.

In contrast to the liver, there were no histopathological differences in other organs from sublethally or lethally infected mice. Adrenal glands had similar cytopathology (Table 2.1) and viral titers (Fig. 2.3F) as well as mild to moderate levels of inflammation and necrosis at days four and five (Table 2.1). Likewise, the small intestine showed mild inflammation that was maintained for the five-day experiment while the stomach and colon appeared normal (data not shown). In pancreatic sections, little pathology was observed and serum amylase levels, used as an indicator of pancreatic function, remained normal (data not shown). Coincident with the high levels of virus (Fig. 2.2B middle panel), spleens showed gross pathology associated with firm, dark surface areas that developed in both sublethally and lethally infected mice independent of virus strain. These were first apparent on day three and increased in size through day five when the entire organ appeared necrotic (data not shown). Gross evidence of necrosis was confirmed by histological evaluation, and was associated with moderate to severe lymphoid depletion and necrosis (Fig. 2.3G and Table 2.1), similar to an earlier report (208). Thus, splenic damage during MCMV infection is severe, but does not correlate with disease outcome.

This histological data agrees with earlier reports that concluded hepatitis underlies lethal MCMV infection in susceptible strains of mice (183, 196) and identifies a critical time period from days three to five when this disease develops, potentially setting the stage for contributions of the host immune response to hepatic damage.

IMs do not influence lethal disease in BALB/c mice

In evaluating the contribution of host responses to severe disease, we initially focused on IMs, known mediators of pathogenesis in MCMV and other viral infections (18, 28, 187, 188). Mice lacking MCP1 and/or CCR2 are impaired in their ability to recruit IMs from bone marrow (33, 186). BALB/c and $MCP1^{-/-}CCR2^{-/-}$ mice exhibited identical susceptibility to 1×10^5 and 1×10^4 PFU v70 (Fig. 2.4). Compromising CCR2-signaling and the subsequent mobilization of IMs from bone marrow did not increase susceptibility of BALB/c mice to lethal disease, consistent with earlier work (33). This result contrasts the protective role that IMs play in C57BL/6 mice (18) where these cells recruit NK cells to the liver, controlling viral infection and preventing disease. While IMs protect from lethal hepatitis in C57BL/6 mice, they do not contribute to protection from or promotion of disease in BALB/c mice.

Adaptive immune response contributes to disease

Having ruled out IMs, we next evaluated the contribution of cytotoxic lymphocytes to disease progression by inoculating NSG mice. NSG mice are on the BALB/c related NOD background and harbor the severe combined immunodeficiency (SCID) mutation and lack the common gamma chain of the IL-2 receptor resulting in a

lack of functional T, B, and NK cells and impaired cytokine signaling. When NSG mice were inoculated with 1×10^5 PFU of v70 or K181+, lethal disease developed between days nine and ten (Fig. 2.5A). A six-day delay in time to disease was evident in these immunodeficient mice compared to immunocompetent BALB/c mice inoculated with the same dose of v70 (see Fig. 2.1B), indicating a potential contribution of immune components to rapid hepatitis. Based on this pattern of delayed death in immunodeficient mice, we set out to identify the components of the immune response that predispose to rapid and severe disease in immunocompetent mice.

NK, T and B cell levels were assessed in the liver via flow cytometry at day five in BALB/c mice inoculated with a lethal dose of a plaque-purified derivative of v70, called v70+, or a sublethal dose of K181+. At this time point mice infected with v70+ were dying while those infected with K181+ were recovering (data not shown). There were no differences in total numbers or proportion of any leukocyte population in the liver (Fig. 2.5B and data not shown). We determined whether NK cell activation was altered during lethal and sublethal infection. No differences were observed in the NK cell activation markers IFNγ or CD69 (Fig. 2.5C). Additionally, depletion of NK cells using anti-asialo-GM1 did not affect v70-induced disease in BALB/c mice (Fig. 2.5D). While critical for controlling infection as well as disease in C57BL/6 mice (18, 209), our data reinforce the lack of NK cell contribution to either host defense or disease pathogenesis in BALB/c mice.

The contribution of the adaptive immune response to disease was assessed by inoculating SCID mice with 1×10^5 PFU of v70 or K181+. These mice are on the BALB/c background and lack functional T and B cells due to the SCID mutation. SCID

mice died in a pattern similar to NSG mice (Fig. 2.5E). Severe hepatitis observed in BALB/c mice infected with v70 was absent in SCID mice (Fig. 2.5F). v70-infected BALB/c mice also showed greater weight loss at day four (Fig. 2.5G). Viral titers at day four in BALB/c and SCID mice were identical, indicating that viral replication is not the primary driver of hepatitis (data not shown). These results implicate the adaptive T and B cell response in the rapid disease affecting immunocompetent mice.

To further investigate the contribution of immune cells to disease, we attempted to isolate splenocytes from BALB/c mice infected for four days with a lethal dose of v70, however, splenic necrosis (Fig. 2.3G and Table 2.1) prevented isolation of sufficient cells (data not shown). We then tested naïve cells, transferring 4×10^7 BALB/c splenocytes into SCID mice one day prior to infection with 1×10^5 PFU of v70. Mice that received splenocytes lost significantly more weight than unmanipulated SCID mice in a pattern similar to BALB/c mice (Fig. 2.5G). Three out of four SCID mice that received splenocytes died at day four similar to BALB/c mice (Fig. 2.5H), whereas control SCID mice succumbed between days six and ten. This data showed that BALB/c splenocytes contained a cell population that conferred disease on SCID mice. One SCID mouse that received splenocytes survived through day 12, reminiscent of the occasional survival of BALB/c mice subjected to a lethal dose of v70 (Fig. 2.2A). As expected, splenocytes from sublethally infected-BALB/c mice protected SCID mice from a lethal challenge one day after transfer (data not shown). Thus, the response mounted by naïve splenocytes contributes to disease or provides protection, depending on the dose of virus given.

Disease is dependent on T cells

We next sought to investigate whether an adaptive immune cell population was associated with rapid disease in immunocompetent mice. Given that both SCID and T cell deficient athymic *nude/nude* mice show a similar delayed pattern of death (191), we focused on T cells by depleting CD4 or CD8 cells from BALB/c mice followed by a lethal dose of v70. T cell-depleted mice exhibited less disease than control BALB/c mice, with depleted mice surviving the experiment (aside from one CD8-depleted mouse that died at day five) while control mice succumbed (Fig. 2.6A). Histological analysis at four dpi revealed the expected necrosis in immunocompetent mice that was uniformly absent from livers of CD4- or CD8-depleted mice (Fig. 2.6B). Thus, lethal hepatitis in immunocompetent mice required the combined activity of CD4 and CD8 T cell subsets.

T cell response parameters were evaluated in livers of BALB/c mice, comparing responses generated during lethal (v70) and sublethal (K181+) infections. At day four, a similar number of hepatic CD4 T cells were recovered from both groups of animals (Fig. 2.6C). There was a trend of fewer total CD8 T cells and fewer CD8 T cells recognizing the immunodominant viral IE1 epitope (as measured by tetramer staining) recovered from lethally infected livers (Fig. 2.6C-D), although the frequency of IE1-specific CD8 T cells was the same in both infections (data not shown). When liver sections from infected mice were stained for IE1 antigen, there were greater numbers viral Ag-positive cells in lethally than sublethally infected mice (Fig. 2.6E), consistent with the pattern of viral titers (Fig. 2.2B top panel) as well as the trend toward fewer CD8 T cells (Fig. 2.6C-D). We next compared the quality of hepatic T cell responses in lethally and sublethally infected mice, employing PMA/ionophore stimulation to activate T cells. We did not

observe any difference in IL-17-producing T cells (Fig. 2.6F), ruling out Th17 cells as major contributors to lethal hepatitis (210) in the context of MCMV infection. There was a trend of increased bifunctional T cells, secreting both IFNγ and TNF, within the CD4 and CD8 T cell populations from lethally infected mice (Fig. 2.6G). Results similar to PMA and ionophore were also observed with anti-CD3 and anti-CD28 Ab costimulation (data not shown). Extending this analysis to include Ag-specific CD8 T cell responses, we stimulated with IE1 peptide (169) and looked at functionality. Lethally infected mice generated a significantly higher frequency of bifunctional CD8 T cells (Fig. 2.6H-I). These cells also had increased surface levels of the degranulation marker CD107a (211) (Fig. 2.6J) consistent with increased effector phenotype. Overall, patterns of increased hepatitis and disease correlated with potency and functionality of hepatic CD8 T cells. Thus, the quality, rather than the quantity, of the T cell response correlated with MCMV-associated hepatic disease.

E) Discussion

To more fully understand host factors that contribute to disease, we investigated virus-induced pathology and identified differences in host response parameters correlating with disease potential. By employing a highly virulent MCMV strain, v70, we characterized lethal infection as follows: (i.) hepatitis underlies the rapid and severe disease that kills immunocompetent mice; (ii.) both CD4 and CD8 T cells contribute to disease; and, (2.) potent antiviral CD8 T cells normally associated with control of infection, predominate in the disease setting. It is difficult to dismiss the sustained viral levels in the livers of lethally infected mice as these levels may contribute to or be a result of disease pathogenesis. Indeed, it is possible that the recruitment of fewer CD8 T cells to the liver during lethal infections directly leads to the elevated titers. However the strikingly parallel replication and dissemination patterns observed when sublethal doses of v70 and K181+ were compared indicate that differences in replication potential are not at the root of disease pathogenesis. These studies establish that the increased virulence of v70, which has been utilized almost exclusively in virus-resistant C57BL/6 mice (18, 20, 65, 66, 197, 200, 202, 206, 212), applies to pathogenesis in virus-susceptible BALB/c mice. In these mice, T cells can control infection as well as mediate immunopathology that seems to be in a delicate balance with viral factors during the response to infection.

The rapid hepatitis observed in BALB/c mice is reminiscent of earlier observations of hepatic dysfunction during lethal MCMV infection (183). The liver, rather than other organs such as the adrenal glands, kidneys or GI tract, is the target organ underlying disease. Between days three and four, factors elaborated by the more virulent v70 strain tilt the balance towards progression to hepatitis. These virulence factors

interface with the T cell response as a partner in disease pathogenesis. Following i.p. inoculation, both liver and spleen are seeded within hours (136) and both are damaged in lethally infected mice. Hepatic damage leads to life-threatening illness; whereas, splenic damage is tolerated, even when severe (208). Our observations suggest that lethal doses of MCMV result in the dual insult of viral infection and pathologic anti-viral T cell responses that together result in lethal hepatitis.

The finding that IMs do not contribute to disease susceptibility in BALB/c mice contrasts observations in C57BL/6 mice where this axis contributes to NK cell recruitment that protects from lethal hepatitis (18). Unlike BALB/c, C57BL/6 mice express Ly49H, an activating NK cell receptor that recognizes virus-encoded m157, an MHC-I homologue that drives an overwhelming NK response (119, 120). When m157 or Ly49H are eliminated, MCMV infection in C57BL/6 resembles BALB/c mice with viral control mediated by a robust CD8 T cell response rather than NK cells (107, 122). Our understanding of disease pathogenesis in BALB/c mice therefore opens the door to future mechanistic studies of lethal T cell-dependent hepatitis utilizing existing mutant strains of mice on the C57BL/6 background in combination with an m157-deficient virus.

The contribution of T cells to disease in BALB/c mice is consistent with the delayed susceptibility of both NSG and SCID mice and is reminiscent of the behavior in T cell-deficient *nude/nude* mice (191). Like hepatitis viruses A, B, and C, as well as LCMV (192-194), MCMV induces disease that is dominated by T cell-mediated pathology rather than direct damage resulting from virus replication. Studies in these various systems have identified a contribution of virus-specific CD8 T cells, but no role for CD4 T cell responses, in the disease susceptibility of immunocompetent hosts. In our

study, depletion of CD4 or CD8 cells suggest that both T cell subsets work together to produce the conditions leading to lethal hepatitis. While CD4 T cells are unlikely to directly kill hepatocytes, these cells produce a wide range of cytokines that influence the immune response, including IL-17, a cytokine that has been associated with acute hepatitis by facilitating the recruitment of neutrophils (210). We did not observe any difference in IL-17 production by CD4 T cells or neutrophil recruitment during lethal or sublethal infection (Fig. 2.5B) suggesting this axis does not drive lethal MCMV hepatitis. Other CD4-derived cytokines can influence hepatitis via direct effects on hepatocytes (such as IFNγ and TNF) and modulation of CD8 T cells by supporting (IL-2 and IFNγ) or inhibiting (IL-10 and IL-4) survival and antiviral activity. Given that a stronger CD8 T cell response is associated with hepatitis, the CD4 T cell response may help increase potency of the CD8 T cells responding to lethal infection, similar to the role CD4 T cells play in maintaining CD8 effector memory in the periphery (99). The Th1 cytokines, IL-2 and IFNγ, and proinflammatory TNF contribute to the help CD4 T cells provide CD8 T cells. Although the frequency of IL-2, IFNγ, or TNF producing CD4 T cells did not vary during lethal compared to sublethal infection (data not shown), differences may emerge from further studies of the Ag-specific CD4 response.

In a pattern analogous to other viral hepatitides, virus-specific CD8 T cell responses appear to be involved in lethal MCMV hepatitis. Significant differences in hepatic CD8 T cell quality in mice infected with lethal or sublethal doses were only observed after co-culture with viral Ag, but not nonspecific stimulation. Broadly speaking, there are two ways that CD8 T cells may mediate protection or pathology: direct lysis of infected cells or indirect damage via secreted cytokines, such as IFNγ and

TNF. Studies in hepatitis B virus, in particular, distinguish between these two capacities with cytotoxicity associated with disease pathology and cytokines associated with protection (193). In LCMV-induced hepatitis, IFNγ, in particular, drives cytotoxic capacity in CD8 T cells, contributing to both protection and pathology (143, 213-215). Experiments distinguishing a protective CD8 T cell response from a pathologic one have not been performed. By utilizing two strains of MCMV with different disease potentials we directly compared a protective CD8 T cell response (mounted against K181+) to a pathologic response (mounted against v70). Interestingly, we found the pathologic CD8 T cells were characterized by increased cytotoxic potential and bifunctionality, characteristics that are typically associated with protection (143). Thus, it appears that a more intense response is not necessarily better, and can lead to pathology and even death. Further studies on the quality of infected cells and the T cells that respond to lethal and sublethal infections should provide insights into these different disease outcomes.

The presence of functional virus-specific CD8 T cells in the liver at four dpi was unexpected. In mouse models, such T cell responses are typically not detected in nonlymphoid organs prior to five dpi, and the responses peak between days seven to ten (128, 143). Studies have historically focused on peak responses, which may follow rather than precede disease. Here, an antiviral T cell response at day four seemed to be the crucial determinant in the outcome of infection. T cell correlates of protection and pathology may differ depending on the time post infection as a CD8 T cell response considered to be more protective at day seven (143) is associated with immunopathology at day four. Further study of these early T cell responses in MCMV and other viral

infections will likely lead to insights into disease pathogenesis and therapeutic interventions.

While our study focused on host contributions to disease, the comparison between v70 and K181+ show that viral factors are also important in disease pathogenesis. The dominance of v70 during co-infection suggests that v70 encodes a virulence determinant contributing to disease. Once v70 stock virus is fully characterized, future sequence analysis will seek to identify the viral factor(s) responsible for virulence differences. Our findings regarding the involvement of the host T cell response in lethal v70-infection leads to the expectation that the virulence factor likely targets either CD4 or CD8 T cells. MCMV is known to encode several genes that enhance or restrict the CD8 T cell response such as m04, m06, m129-m131 (MCK-2), and m152 (21, 28, 216). While less well understood, MCMV also modulates CD4 T cell responses through mechanisms interfering with MHC-II expression (80, 81) and T cell activation via downregulation of co-stimulatory molecules by m138 and m155 (86, 88). Any T cell modulation that occurs in v70-infected BALB/c mice appears subtle, as viral titers remain very similar to K181+ at low or high doses. Viral regulation of the T cell response may be limited to the liver as sustained hepatic titers at high doses of v70 was the only difference observed. Future studies focused on identification of v70 virulence determinants will enable better understanding of the mechanisms through which v70 induces a potent pathological CD8 T cell response.

Given that identification of protective anti-MCMV CD8 T cell responses have been followed by the recognition that anti-HCMV responses are similarly protective (30), the results revealed here suggest the possibility that anti-HCMV T cell responses may

mediate pathology in some settings, such as the liver dysfunction that accompanies infection (4, 39). Studies that dissect the contribution of anti-HCMV specific CD4 or CD8 T cell responses to disease are needed. Given that T cells are clearly involved in hepatitis during MCMV infection, evaluation of T cell function in immunocompetent patients with HCMV-hepatitis would be especially informative.

F) Figures and Legends

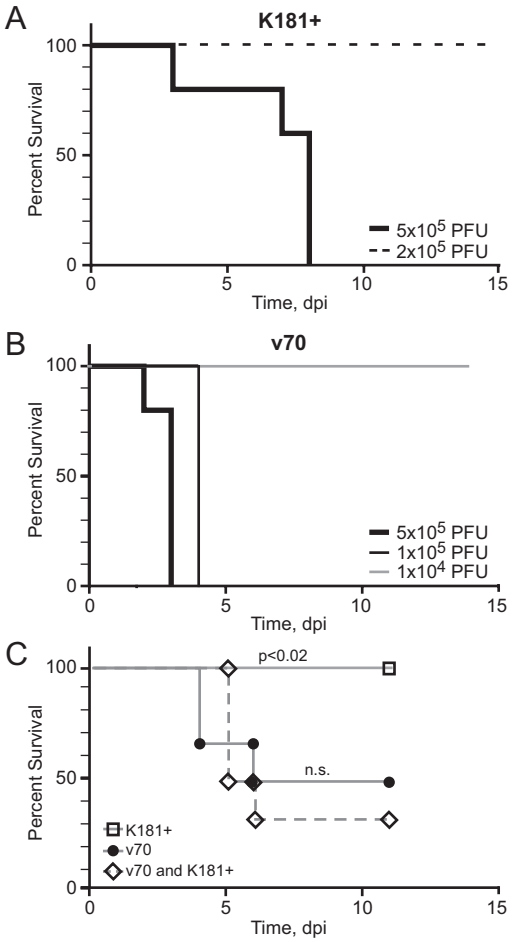


Figure 2.1: Kaplan-Meier plots showing percent survival following infection with v70 or K181+ in BALB/c mice. A-B. Infection with the indicated doses of K181+ (A) or v70 (B). C. Infections with a dose of 5×10^4 PFU of K181+ alone or v70 alone or a combined dose of 5×10^4 PFU of K181+ mixed with 5×10^4 PFU of v70 together. Mantel-Cox test was used to calculate p values by comparing singly infected mice to mice receiving the combined dose. Nonsignificant (n.s.): $p>0.05$. Six or more mice were used in each group. Data in panels A and B were generated by J. Huang.

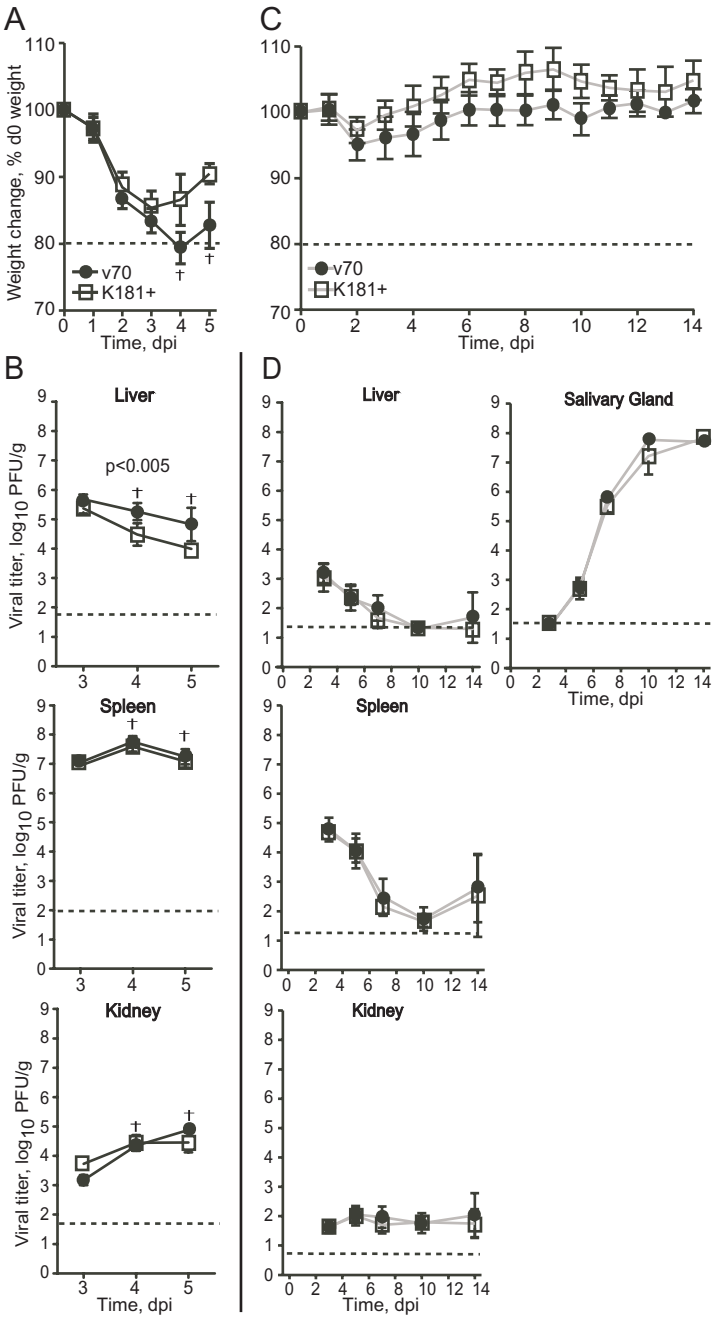


Figure 2.2: Disease and viral replication patterns at high and low doses. A. Weight loss in mice inoculated with 1×10^5 PFU of v70 or K181+ expressed as percentage of weight prior to infection (d0). Shown is the mean ± range. Dotted line indicates 20% weight loss. Seven and one v70-infected mice died at days four and five post infection, respectively. Data is representative of three independent experiments. B. Viral titers in

Figure 2.2 (cont.): indicated organs from mice in panel A. Five mice from each group were sampled at d3, seven at d4, and three at d5. Symbols represent mean of $\log_{10}$ of viral titers ± SD. Dashed line indicates limit of detection. C. Weight loss in mice inoculated with 1×10^4 PFU of v70 or K181+ as depicted in panel A. Data represents a single experiment. D. Viral titers in indicated organs from mice in panel C. Five mice were sampled from each group at indicated times. Graphed as in panel B. † indicates a time when v70-infected mice met euthanasia criteria. p value determined by Mann-Whitney t test.

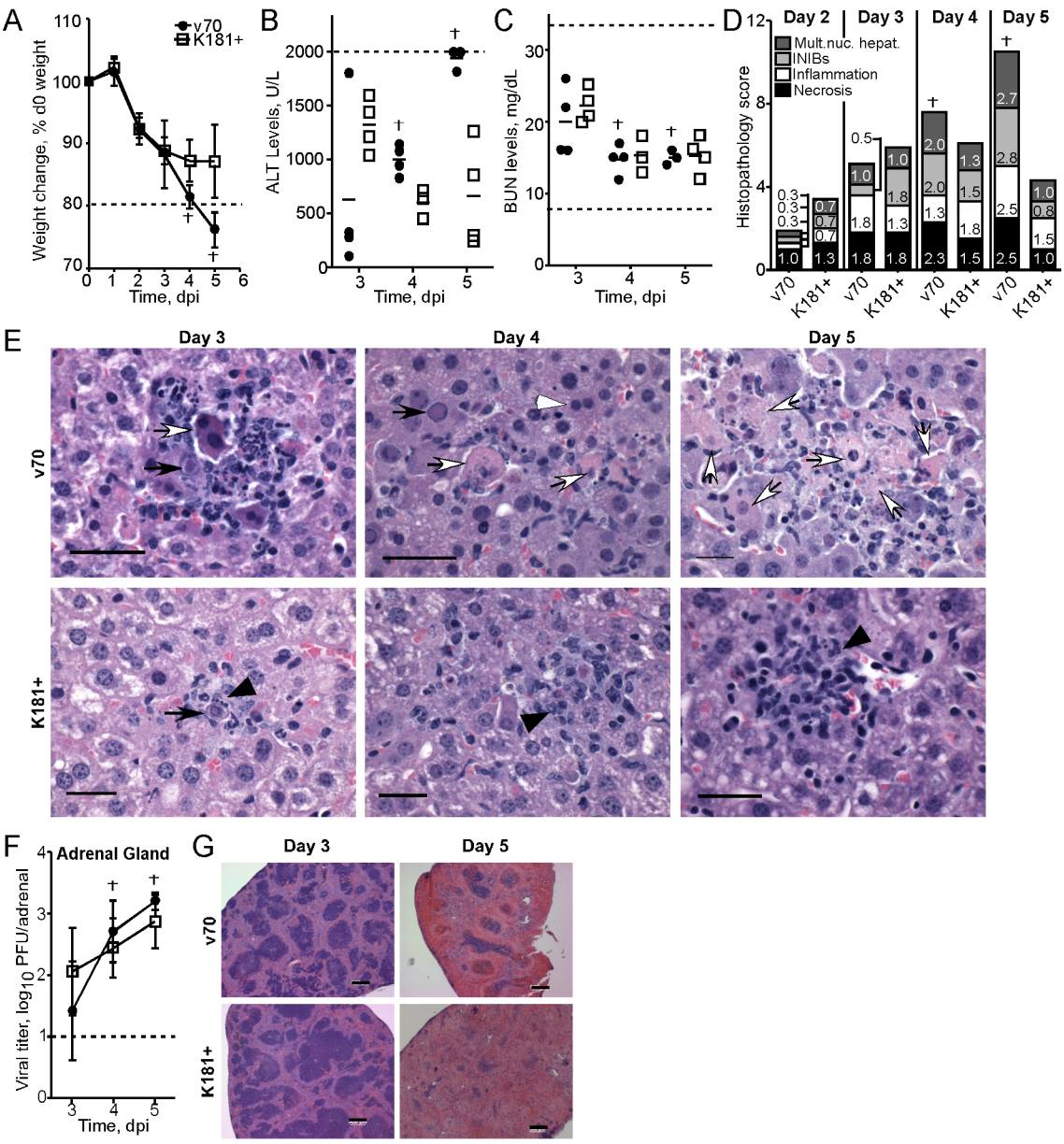


Figure 2.3: Evaluation of pathological changes during lethal and nonlethal infections. BALB/c mice were inoculated with 1×10^5 PFU of v70 or K181+. A. Weight loss as depicted in panel 2.2A with dashed line indicating 20% weight loss from d0. B-C. Evaluation of serum chemistries from mice in panel A. B. Serum ALT levels at indicated times; the upper limit of detection for this assay was 2000 U/L indicated by dashed line. On day five, two v70-infected samples were above the upper limit of detection. C. Serum

Figure 2.3 (cont.): BUN levels at indicated times. Dashed lines indicate normal BUN range. D-E. Evaluation of hepatic histopathology from mice in panel A. D. Scores of cumulative pathology on indicated days post infection for multinucleated hepatocytes, intranuclear inclusion bodies (INIBs), inflammation and necrosis using the following scoring system: 0: normal, no pathology; 1: mild, 1-3 abnormal areas, 2: moderate, 3-5 abnormal areas; 3: severe, >5 abnormal areas. Prior to evaluation, histological samples were blinded. Bars correspond to the mean score for each parameter. The height of each bar represents the total histological score (out of 12) that incorporates each individual pathology parameter. Three mice infected with each virus were evaluated at day two and four mice for each infection group at all other times. E. Representative images of liver histology at indicated time points from samples used to assemble panel D. White arrowheads indicate multinucleated hepatocytes, black arrows INIBs, black arrowheads inflammation, and white arrows necrosis. Scale bars indicate 250μm. Images are representative of two independent experiments. F. Viral titers in the adrenal gland from mice in panel A. are depicted at indicated time points as in panel 2C. G. Representative images of splenic pathology at indicated time points from mice in panel A. Scale bars indicate 200μm. † indicates a time point when v70-infected mice died. Data in panel D and arrows in panel E were determined by AP. Garcia.

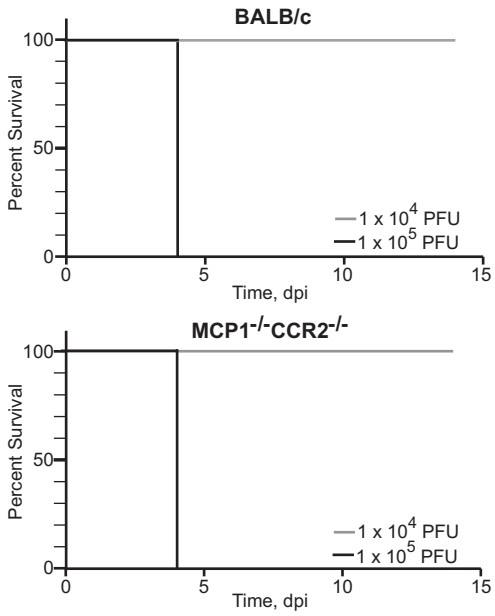


Figure 2.4: Kaplan-Meier plots showing the percent survival of BALB/c or MCP1$^{-/-}$ CCR2$^{-/-}$ mice infected with v70. Mice were inoculated with the indicated doses of v70. Three mice were administered the high dose and five the low dose. Data was generated by J. Huang.

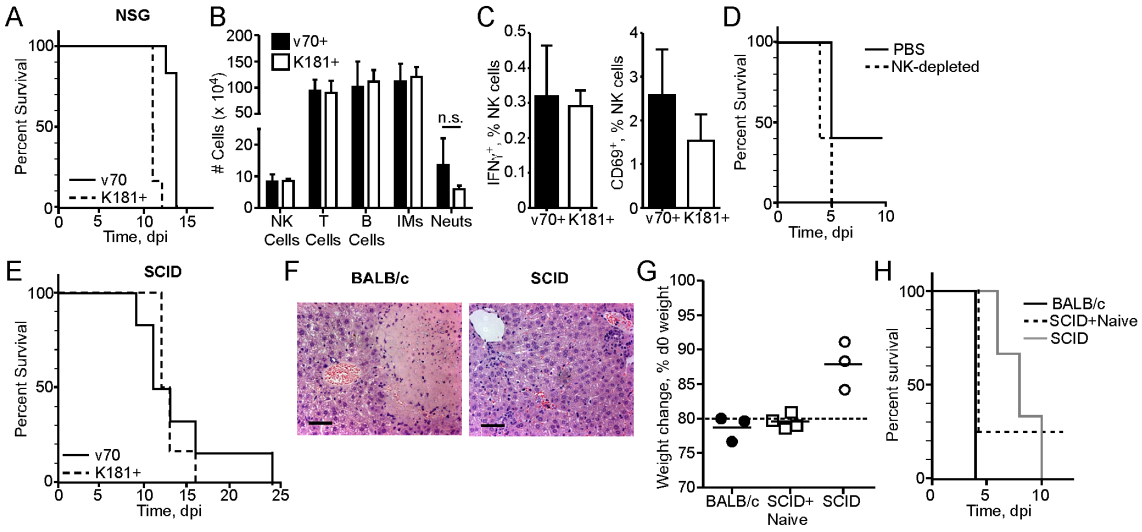


Figure 2.5: Evaluation of NK cells and adaptive immunity in disease. Mice were inoculated with 1×10^5 PFU of v70, v70+, or K181+. A. Kaplan-Meier plot showing percent survival of NSG mice inoculated with v70 or K181+; six mice per group. B. Total number of indicated hepatic leukocytes isolated at five dpi from BALB/c mice. Using flow cytometry, subsets were defined as follows: NK cells, $CD3^-CD49d^+$; T cells, $CD3^+CD49d^-$; B cells, $CD19^+$; IMs, $CD3^-Ly6C^{hi}CD11b^+$; Neutrophils (Neuts), $CD3^-Ly6C^{Int}CD11b^+$. Nonsignificant (n.s.; $p>0.05$) by Mann-Whitney t test. C. Frequency of IFNγ+ or CD69+ NK cells. NK cells identified in B. were evaluated directly *ex vivo* for intracellular IFNg or surface expression of CD69. Bars in B. and C. indicate mean ± SD of five mice in each group. D. Kaplan-Meier plot showing percent survival of BALB/c mice following administration of anti-asialo-GM1 or PBS control prior to inoculation with v70; five mice per group. E. Kaplan-Meier plot showing percent survival of SCID mice inoculated with v70 or K181+; six mice per group. F. H&E stained liver sections at four dpi from BALB/c and SCID mice inoculated with v70. Scale bars indicate 100µm. G. Weight loss at day four following v70 inoculation of BALB/c, SCID mice that received 4×10^7 bulk splenocytes from naive BALB/c mice one day prior to infection, or

Figure 2.5 (cont.) unmanipulated SCID mice. Line indicates mean; dashed line indicates 20% weight loss from d0. H. Kaplan-Meier plot showing percent survival of mice in panel E. All data is representative of two or three independent experiments except panels C and D. where a single experiment is shown.

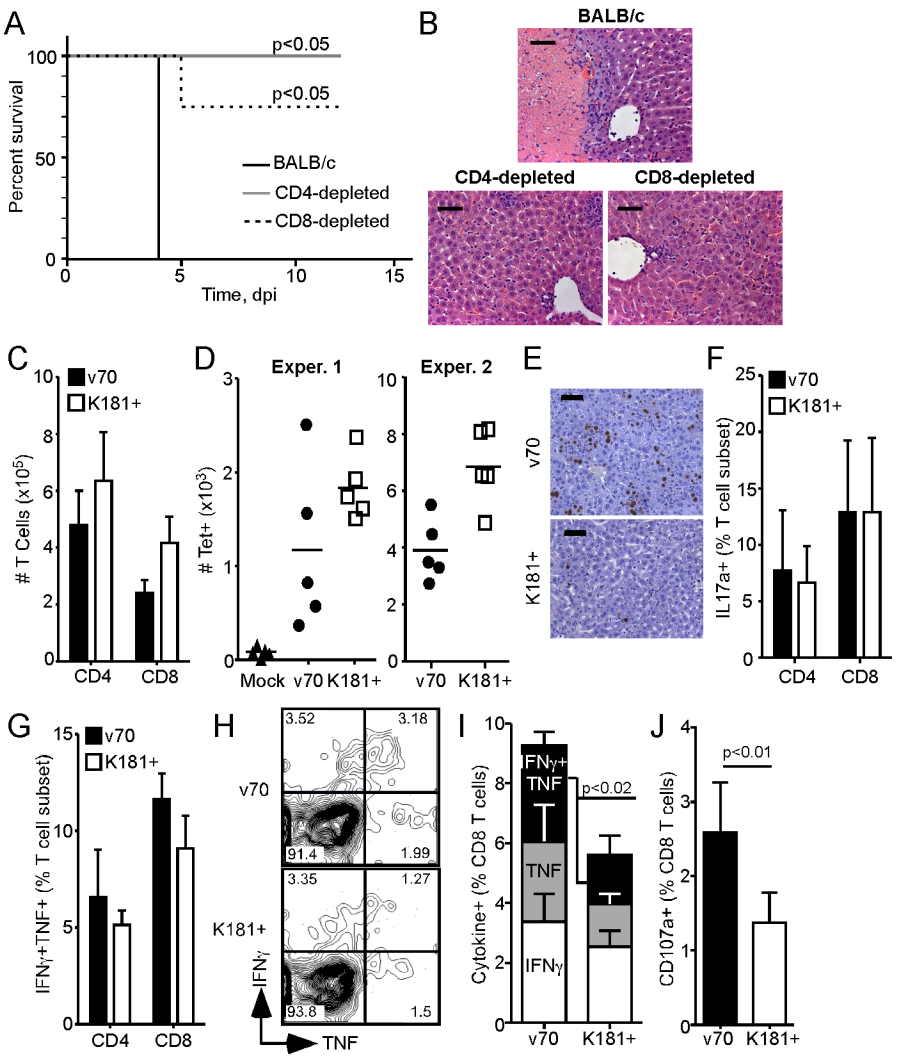


Figure 2.6: Evaluation of T cells in lethal disease. A. Kaplan-Meier plot showing percent survival of BALB/c mice depleted of CD4 or CD8 cells (4 mice per group) or left untreated (BALB/c - 3 mice) and inoculated with 1×10^5 PFU of v70. Mantel-Cox test was used to calculate p value by comparing depleted mice to untreated BALB/c mice. B. Representative H&E stained liver sections at four dpi from mice treated as in panel A. Scale bars indicate 100µm. C-J. Livers were harvested at four dpi from BALB/c mice inoculated with 1×10^5 PFU v70 or K181+. Bar graphs indicate mean of five mice per group ± SD. C. Total number of hepatic CD4 and CD8 T cells isolated. D. Total number of hepatic IE1-specific (tetramer+) CD8 T cells. Line indicates mean. Two independent

Figure 2.6 (cont.): experiments are shown. E. IE1 and hematoxylin-stained liver sections. Scale bars indicate 100μm. F. Frequency of hepatic CD4 or CD8 T cells producing IL-17a after a five-hour stimulation with PMA and ionophore. G. Frequency of hepatic CD4 and CD8 T cells producing both IFNγ and TNF after a five-hour stimulation with PMA and ionophore. H. Flow cytometric plots of CD8 T cells assessed for IFNγ and TNF production following IE1-peptide stimulation. I. Frequency of CD8 T cells producing IFNγ, TNF or both cytokines following IE1-peptide stimulation. J. Frequency of CD107a+ CD8 T cells during IE1-peptide stimulation. p values in panels I and J were calculated by Mann-Whitney test. All data is representative of two independent experiments.

G) Table

Adrenal	Virus[1]	dpi	INIB[2]	Inflammation	Necrosis	
	K181+	2	0.0	0.0	0.0	
	v70	2	0.0	0.0	0.0	
	K181+	3	0.0	0.0	0.0	
	v70	3	0.0	0.7	0.0	
	K181+	4	0.3	1.0	0.7	
	v70	4	0.5	0.0	0.5	
	K181+	5	0.5	0.5	0.5	
	v70	5	1.7	1.3	1.7	
Spleen	**Virus**	**dpi**	**INIB**	**Inflammation**	**Necrosis**	**Lymphoid Depletion**
	K181+	2	0.3	0.0	0.0	0.3
	v70	2	0.0	0.3	0.0	0.0
	K181+	3	0.5	0.8	0.3	0.0
	v70	3	0.0	0.3	0.0	0.0
	K181+	4	1.8	0.3	1.0	1.3
	v70	4	2.5	0.0	2.0	2.3
	K181+	5	2.0	0.3	1.8	2.5
	v70	5	2.5	0.5	3.0	2.9

Table 2.1. Pathology in adrenal glands and spleen. [1]BALB/c mice were inoculated with 1×10^5 PFU of indicated virus. [2] Values represent the average score for each parameter of three to four mice per virus at each time point. Prior to evaluation, samples were blinded and scores determined as in Fig. 2.3. Data in this table was generated by AP. Garcia.

CHAPTER 3.

Remarkably intact CD8 T cell immunity in the absence of extrinsic apoptosis and programmed necrosis

A version of this chapter has been submitted to the *Journal of Immunology* as: Livingston-Rosanoff, D., Daley-Bauer, L. P., Mocarski, E. S. "Remarkably intact CD8 T cell immunity in the absence of extrinsic apoptosis and programmed necrosis."

A) Abstract:

Caspase (Casp)8 is both the central mediator of extrinsic apoptosis and key pro-survival factor preventing programmed necrosis. TCR stimulation of Casp8-deficient murine or human T cells results in receptor interacting protein kinase (RIP)-dependent necrosis. When this potential is eliminated in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, lymphoid hyperplasia develops as mice age, reminiscent of Fas-deficiency. CD8 T cells from these mice respond robustly to TCR stimulation, and following CMV infection, develop normal effector functions as well as memory, with expected patterns of inflationary and noninflationary epitopes. Notably, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice control primary infection and undergo a robust CD8 T cell expansion upon secondary CMV challenge. These studies reinforce the contribution of Casp8 to homeostatic lymphocyte turnover previously ascribed to Fas-signaling, but clearly reveal that neither extrinsic apoptosis nor necrosis contributes to normal patterns of CD8 T cell immunity.

B) Introduction:

Extrinsic apoptosis mediates T cell homeostatic turnover and response to Ag in humans as well as mice (217). This pathway is most commonly initiated by death receptor signals, such as those triggered by TNFR1, culminating in the activation of executioner caspases driving apoptosis, cell death associated with membrane blebbing and DNA fragmentation. Caspase (Casp)8 is the crucial initiator caspase controlling extrinsic apoptosis in mice, with Casp10 function also contributing in humans (218). Recent investigations in mouse T cells (159, 160) reveal an interplay between Casp8 and a complex of receptor interacting protein kinase (RIP)1 and RIP3 downstream of TCR signaling that controls a novel death pathway first described in death receptor signaling (153-155). Signal transduction in either setting unleashes a novel RIP1/RIP3-mediated programmed necrotic cell death, termed necroptosis (151), when Casp8 is compromised. Mice with T cell-specific disruption of Casp8 (*tCasp8*$^{-/-}$) or death receptor adaptor Fas-associated death domain (FADD) (*tFadd*^{DD}*)* exhibit dramatic defects in T cell homeostasis (158, 219), with fewer naïve T cells overall and a preferential impact on the CD8 subset. Casp8-deficient T cells fail to proliferate in response to TCR activation following infection or antigen stimulation, and, instead, die via necroptosis (159, 160). The phenotype of *tCasp8*$^{-/-}$ and *tFadd*$^{-/-}$ mice manifests as a profound immunodeficiency, similar to Casp8-deficient humans (218).

Casp8-dependent extrinsic apoptosis contributes to T cell function based on wide-ranging studies of mice with defects in Fas (CD95, Apo-1) death domain (DD) signaling (109, 217, 220-223). Other death receptors, such as TNFR1 and TRAIL, do not directly contribute to T cell homeostasis (217); although, TNF receptor family members trigger

NF-kB signaling important in immunity (224). Fas-deficient mice exhibit defects in homeostatic maintenance of T cells dependent on environmental and commensal microbial Ag (217, 220). These mice develop lymphoid hyperplasia with $B220^{+}CD4^{-}CD8^{-}$ T cells. Similar accumulation of abnormal T cells occurs in the human disorder, autoimmune lymphoproliferative syndrome (ALPS) as a result of mutations in Fas or Casp10. Fas-signaling has been implicated in CD8 T cell contraction during chronic viral infections in mice (221, 222), as well as in the memory response to secondary challenge (223). Consistent with Casp8 activity downstream of Fas, CD8 T cells from transgenic mice expressing a poxvirus-derived Casp8 inhibitor also fail to mount an effective memory response (225). Recent observations revealing that RIP3 deficiency rescues the embryonic lethality of Casp8-deficient mice (204, 226), as well as the necrosis following TCR engagement in *tCasp8*$^{-/-}$ mice (160), open the way to identify RIP3-independent contributions of Casp8 to T cell biology.

Murine CMV (MCMV), a natural mouse pathogen, possesses genes encoding cell death suppressors that directly target Casp8 and RIP3 pathways to facilitate pathogenesis (85, 113, 116, 151, 227). Following acute infection, this virus establishes a chronic infection and induces a robust antiviral CD8 T cell response that collaborates with NK cells to control viral levels (107, 125, 128, 228). In addition to classic memory response patterns following initial contraction, where a stable memory pool is maintained, a subset of MCMV-specific CD8 T cells expand over time (68, 128). This memory inflation is also characteristic of human and other primate CMV infections (129), and appears to result from the unique pattern of persistent antigen in the CMV-infected host (133).

Here, we investigate the contribution of RIP3-independent Casp8 function in CD8 T cells and report that Casp8 contributes to homeostatic turnover, but is dispensable for CD8 T cell development, activation and participation in the control of MCMV infection.

C) Materials and Methods:

Mice, infections, and viral titers:

Casp8$^{+/+}$*Rip3*$^{+/+}$ (WT), *Casp8*$^{+/-}$*Rip3*$^{-/-}$ (*Rip3*$^{-/-}$), and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (DKO) mice were bred as previously described (204). These mice were subsequently back-crossed to C57BL/6 mice (Jackson Laboratory) for five or seven generations. Mice used in these studies were back-crossed five generations, except where noted in figure legends. All animal experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Emory University. Mice were between 6-18 weeks of age. Infections were carried out via i.p. inoculation with 1×10^5 PFU salivary gland-derived MCMV strain v70 (161). For challenge experiments, mice were inoculated with v70 and, after 70+ days, challenged with 1×10^6 PFU tissue culture-derived RM427+ (26). Viral titers were quantified by plaque assay on 3T3-Swiss Albino fibroblasts.

Characterization of lymphocytes and flow cytometry:

Single cell suspensions were prepared from spleen, blood and liver as previously described (128, 161). Lungs were perfused with 10 U/mL heparin (Abraxis) in PBS prior to harvest and leukocytes isolated by incubating diced lungs in 2mg/mL collagenase D (Sigma) at 37°C for 1 h followed by gradient centrifugation using Histopaque 1119 (Sigma). For evaluation of cytokine production, cells were incubated at 37°C for 5 h with 1×10^{-9}M M45 peptide (JPT Technologies) in the presence of GolgiStop (BD). Cells were stained and data acquired and analyzed as previously described (161).

Flourescently-labeled tetramers and Ab:

Tetramers were synthesized by the NIH Tetramer Core facility (Emory University). The Abs used were: B220 (RA3-6B2), KLRG1 (2F1), IFNγ (XMG1.2), IL2 (JES6-5H4), CD4 (GK1.5), CD4 (RM4-5), CD62L (MEL-14), TNF (MP6-XT22), CD3 (17A2), from BD Biosciences; CD127 (A7R34), CD8 (53-6.7), CD44 (IM7), from BioLegend; and CD45 (30-F11), CD8 (5H10) from Invitrogen.

In vitro proliferation:

Splenocytes were labeled with CFSE (Invitrogen) and 2×10^5CFSE$^+$cells were incubated with 5ug/mL CD3 (145-2C11) and 2ug/mL CD28 (37.51) Abs (BioLegend) at 37°C for 3 d. Proliferation was assessed by CFSE dilution and viability by 7-aminoactinomycin D (7-AAD; BD) staining using flow cytometry.

In vivo killing assay:

In vivo cytotoxicity assay was performed as previously described (125) with slight modifications. Briefly, target cells were prepared from naïve WT splenocytes that were incubated with 1μM M45 peptide (JPT Technologies) at 37°C for 1 h and labeled with 5μM CFSE (CFSE^{Hi}). Target cells were combined in a 1:1 ratio with peptide-free splenocytes labeled with 0.5μM CFSE (CFSE^{Lo}). 2×10^7 total cells (~1×10^7 each CFSE^{Hi} and CFSE^{Lo}) were injected via tail veins at 6 dpi. CFSE$^+$ splenocytes were analyzed 18 h post transfer. Percent specific killing was calculated as follows: $(1-[r_{naive}/r_{infected}]) \times 100$, where r = % CFSE^{Lo}/% CFSE^{Hi}.

Statistics:

All statistical analyses were performed using Prism software (GraphPad). ANOVA followed by Bonferroni's multiple comparison test and unpaired t tests were used for comparisons as indicated in figure legends and $p \leq 0.05$ was considered significant.

D) Results/Discussion:

Casp8 has no impact on naïve CD8 T cell functions

To assess the contributions of Casp8 in development and homeostasis without the confounding influence of unleashed RIP3 necrosis, CD8 T cells in naïve *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (DKO), littermate *Casp8*$^{+/-}$*Rip3*$^{-/-}$ (*Rip3*$^{-/-}$) and wild-type *Casp8*$^{+/+}$*Rip3*$^{+/+}$ mice were compared. On average, there were five times as many splenic CD8 T cells in 16- to 20-wk-old DKO mice than in either WT or *Rip3*$^{-/-}$ mice (Fig. 3.1A). Despite this increase in T cell numbers, CD4 to CD8 ratios were similar to WT in spleen and lymph nodes of DKO as well as *Rip3*$^{-/-}$ mice (Fig. 3.1B). Taken together with our previous description of normal thymic development in DKO mice (204), these data demonstrate that Casp8 is dispensable for naïve CD8 T cell survival in the periphery when RIP3 is eliminated. This is consistent with recent studies demonstrating that RIP3 deficiency restores survival in CD8 T cells from *tFadd*^{DD} mice (229) and the recovery of immune potential in *tCasp8*$^{-/-}$ mice (160). In all settings where Casp8 or FADD has been eliminated, CD8 T cell homeostatic defects arise from unleashed RIP3 death.

Elevated CD8 T cell numbers present in adult DKO mice (Fig. 3.1A) continue to increase with age (data not shown). This accumulation is reminiscent of the increased number of activated CD8 T cells in naïve Fas-signaling deficient mice where the failure to eliminate these cells contributes to lymphoid hyperplasia (217). DKO CD8 T cells were assessed for CD44 expression. T cells from DKO mice exhibited higher frequencies of CD44^{hi} cells compared to WT or *Rip3*$^{-/-}$controls (Fig 3.1C and 3.S1). Thus, like T cells from Fas-deficient mice, cells from naïve DKO mice show evidence of Ag experience as well as the increased activation that accompanies proliferation (230). These data are

consistent with the expected role of Casp8 in Fas-signaling to mediate apoptosis in homeostatic elimination of CD8 T cells.

To determine whether Casp8 contributes to TCR signaling in the absence of RIP3, the proliferation of naïve CD8 T cells was assessed following three days of CD3/CD28 stimulation. CD8 T cells from DKO mice proliferated with kinetics that were similar to controls (Fig. 3.1D and data not shown), demonstrating that TCR and co-stimulatory signaling pathways remained intact. Similar results were observed when bulk splenocytes or positively selected CD8 T cells were stimulated (data not shown). This again is consistent with results from *tCasp8*$^{-/-}$ studies where TCR engagement results in RIP3 necrotic death (160). Our results bolster current evidence that Casp8 provides a critical role in suppression of RIP3 pathways during T cell activation, but otherwise is dispensable for expansion of naïve cells.

In addition to greater CD8 T cell numbers, DKO mice accumulate abnormal $CD3^{+}B220^{+}CD4^{-}CD8^{-}$ T cells as they age, reminiscent of Fas signaling-deficient mice where these cells contribute to lymphoid hyperplasia (204, 217, 226). These abnormal cells retain TCR expression, and appear to arise from a failure in CD8 T cell homeostatic turnover (231). In DKO mice, this population of $B220^{+}$ T cells exhibited a dramatically reduced proliferative response to CD3/CD28 stimulation compared to CD8 T cells from the same mice (Fig 3.1D). These cells also failed to produce detectable IFNγ or TNF following TCR or PMA/ionophore stimulation (data not shown). The similarity to mice with deficiency in Fas ligand (FasL) or Fas DD signaling is striking, and further supports the interpretation that once embryonic lethality of *Casp8*$^{-/-}$ mice is controlled by the elimination of RIP3, the role of Casp8-dependent extrinsic apoptosis downstream of Fas-

deficiency becomes evident (204, 226). Based on these results, together with the current understanding of Fas deficiency (217), we anticipate that $B220^+$ T cells would not contribute to the T cell response. Thus, the significance of Casp8-mediated apoptosis in the homeostatic elimination of CD8 T cells, a process shown to be intrinsic to T cells (160), is clearly revealed so long as the confounding influence of RIP3 death on TCR signaling is absent.

T cells undergo cytokine withdrawal death, mediated by Bim, when pro-survival cytokines, such as IL-2, become limiting (147). This pathway predominates contraction following Ag clearance (148). To ensure that combined deficiencies of Casp8 and RIP3 did not disrupt this pathway, we evaluated the survival of naïve DKO CD8 T cells incubated in medium alone for three days. In the absence of pro-survival stimuli, DKO CD8 T cells showed a similarly high (~80%) proportion of dead cells as *Rip3*$^{-/-}$ controls (Fig. 3.1E), indistinguishable from WT T cells as well (data not shown). Furthermore, cytokine withdrawal death was similarly rescued by TCR stimulation in DKO and *Rip3*$^{-/-}$ T cells. These data demonstrate that cell death pathways activated upon cytokine withdrawal remain intact in the absence of Casp8 and RIP3, consistent with evidence that intrinsic apoptosis, mediated via Bim, controls this aspect of T cell survival (147). Thus, CD8 T cells lacking Casp8 and RIP3 are fully capable of responding to TCR and co-stimulatory signals *in vitro*, dying when stimulation is terminated.

Casp8 is dispensable for an acute antiviral CD8 T cell response

To establish whether Casp8 or RIP3 contributes to CD8 T cell activation *in vivo* we inoculated DKO, *Rip3*$^{-/-}$, and WT mice with MCMV and examined the CD8 T cell

response to the immunodominant M45-epitope (67) during acute infection. Similar frequencies of M45-specific CD8 T cells were observed in WT and DKO mice at five, seven and 14 dpi, with the peak at day seven (Fig 3.2A and data not shown), indicating that the antigen-specific T cell response did not become compromised by the elimination of extrinsic death pathways. A decrease in the frequency of Ag-specific CD8 T cells was observed at seven dpi in *Rip3*$^{-/-}$ mice when compared to WT; however this drop was not observed in subsequent experiments (data not shown). We also evaluated m139-specific CD8 T cells and did not observe any differences in DKO, *Rip3*$^{-/-}$, and WT mice (data not shown). To evaluate the functionality of the responding CD8 T cells, M45 peptide-induced cytokine production was assessed. Consistent with tetramer staining patterns, levels of IFNγ and TNF, as well as the frequency of bifunctional cells producing both cytokines, were similar in WT and DKO mice (Fig 3.2B-C). DKOs had higher frequencies of IFNγ^{+} and bifunctional CD8 T cells than *Rip3*$^{-/-}$ mice (Fig. 3.2B-C and data not shown). This is consistent with our earlier report that found a higher frequency of bifunctional CD8 T cells in DKO mice following TCR stimulation (204). These data suggest that in the absence of RIP3 pathways, Casp8 may constrain cytokine production in T cells. This effect, however, is fairly minor, as we have never observed differences in IFNγ or TNF production between WT and *Rip3*$^{-/-}$ cells (Fig. 3.2B-C and data not shown). In all genotypes, cytokine production after stimulation with M54, m139 or M38 peptides followed patterns similar to those observed with M45 (data not shown). All d7 T cell data has been reproduced with identical results in DKO mice back-crossed to C57BL/6 mice for seven generations. Finally, we evaluated the cytotoxic capacity of the acute CD8 T cell response *in vivo*. Regardless of genotype, killing of M45-pulsed targets was nearly

complete (Fig 3.2D). Specific killing levels of 99.6 ± 0.1% (mean ± SD) in DKO, 98.1 ± 2.1% in *Rip3*$^{-/-}$ and 99.5 ± 0.1% in WT mice were observed. Thus, cytotoxicity develops in the absence of extrinsic death pathways. To determine whether viral levels were controlled in these mice, splenic and hepatic viral titers were determined at seven dpi. At this time point CD8 T cells collaborate with NK cells to restrain MCMV levels. Mice depleted of CD8 T cells exhibit a 30 to 100-fold increase in viral titers (107). Virus was similarly controlled in all mice (Fig 3.2E-F), demonstrating that despite the absence of Casp8 and RIP3, APCs process and present Ag to CD8 T cells with sufficiently robust co-stimulatory and cytokine signals to produce fully activated antiviral effector cells. This confirms and extends our earlier studies showing MCMV infection is controlled in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (DKO) (204) as well as the study of lymphocytic choriomeningitis virus in *tCasp8*$^{-/-}$*Rip3*$^{-/-}$ and murine hepatitis virus in *tFADD*^{DD}*Rip3*$^{-/-}$ mice (160, 229). Additionally, extrinsic cell death pathways do not contribute to clearance of virus-infected cells. These data should dispel any remaining doubt as to the dispensability of Fas-FasL interactions, or any other death receptor, in the function of cytotoxic cells. Altogether, our results indicate that extrinsic apoptosis and programmed necrosis are dispensable for acute antiviral CD8 T cell responses. Our results add to the accumulating evidence that suppression of RIP3 pathways by a Casp8-FADD axis is absolutely crucial to antiviral CD8 T cell responses. Under conditions where necrotic death is shackled, Casp8-dependent extrinsic apoptosis does not contribute to T cell control of acute viral infection.

Casp8 is dispensable for CD8 T cell memory development and function

Next, the contraction and memory development of Ag-experienced CD8 T cells were evaluated at the peak of acute infection and at 49 dpi, when memory had been established in WT mice. The hallmark patterns of contraction (M45) and memory inflation (IE3) of Ag-specific CD8 T cells (128) were preserved in DKO mice (Fig 3.3A). This contrasts with previous studies focused on Fas-signaling in the context of Bim/Fas combined deficiency (221, 222). Our data establishes that extrinsic death signaling, whether triggered by Fas, TNFR1 or TRAIL receptor does not contribute to T cell contraction. Frequencies of M45-, m139-, and IE3-specific CD8 T cells were similar at this memory time point in all mice examined (Fig 3.3B and data not shown). The difference in frequencies of IE3-specific cells between WT and DKO mice was interesting, and may suggest a role for extrinsic death pathways in the inflationary response. However, contrasting results were obtained when the frequency of these cells at d49 was compared to day seven (Fig. 3.3A), where WT cells show a higher level of inflation. Future, more detailed, analyses of the inflationary response in DKO mice will address this issue. Furthermore, expression of CD44, KLRG1, CD62L, and CD127 remained indistinguishable in all genotypes (Fig 3.3C). Comparison with historical data suggests that neither Casp8 nor RIP3 impact the phenotype of memory cells. M45-specific cells express low amounts of KLRG1 and higher levels of CD127, while IE3-specific cells exhibit the opposite pattern, as reported (131). The patterns of contraction and memory inflation observed in spleen (Fig 3.3A) were preserved in liver and lung when evaluated by tetramer staining (Fig 3.S2). These data reveal that Casp8 is dispensable for the generation of normal CD8 T cell responses in the periphery and

establish that Casp8 does not contribute to the orchestration of a memory CD8 T cell response so long as RIP3-dependent pathways are absent.

Viral titers were also assessed at memory time points. When measured at 31 dpi, patterns of viral titers in the spleen (Fig 3.3D) and liver (Fig 3.3E) were variable without gross differences. Thus, Casp8 is dispensable for control of viral infection, consistent with earlier work in mice lacking Fas and TNFR1 expression (109). It is remarkable that DKO mice are not any more susceptible to viral infection than control mice. Whereas extrinsic death pathways most likely evolved to eliminate virus-infected cells and cut short viral infection (113, 116, 151, 227), large DNA viruses like MCMV adapted to these host defense mechanisms by expressing Casp8 and RIP3 suppressors that neutralize the impact of death pathways within virus-infected cells. These studies in DKO mice demonstrate that extrinsic cell death pathways do not contribute markedly in the cells that remain uninfected as they respond to virus infection.

The true hallmark of memory CD8 T cells is their ability to respond to secondary challenge, a capacity that is compromised during listeria infection of Fas-signaling deficient mice (223). To establish whether Casp8 contributes to the development of functional memory cells, mice infected with MCMV strain v70 for 75+ days were subsequently challenged with a different, *lac*Z-tagged strain of MCMV. Five days post challenge CD8 T cells from DKO mice expanded similarly to controls (Fig 3.3F) and challenge virus was not detected in any tissue examined (Fig. 3.3G and data not shown). Thus Casp8 apoptotic signals are dispensable for the generation of functional antiviral memory CD8 T cells. The complete control of challenge virus also suggests that immune compartments in addition to CD8 T cells, such as B cells, are intact and capable of

forming memory in the absence of extrinsic death signaling. Previous data implicating Fas signaling in memory CD8 T cell maintenance (223) may result from pathogen- or organ-specific impacts independent of Casp8 function. Taken together, these observations demonstrate that antiviral CD8 T cell-mediated clearance and recall proceeds independent of extrinsic apoptotic pathways.

Following TCR stimulation, Casp8-regulated pathways are activated in CD8 T cells (147, 217). Despite the fact that *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice retain the ability to control MCMV (204) the extent to which these mice mount an intact antiviral CD8 T cell response was unexpected. CD8 T cells are important for control of MCMV infection, functioning together with the dominant NK cells (107). In addition, B cells produce virus-specific Ab that mitigates levels of virus during infection (94, 232). The demonstration that extrinsic apoptosis and programmed necrosis are dispensable in host defense makes it tempting to speculate that human Casp8 deficiency-associated defects in NK, B and T cell function (233) result from uncontrolled RIP3 activation that eliminates these cells upon activation. A more formal evaluation of CD4 T, NK and B cell function in DKO mice in the future will address this area. Overall, once RIP3 necrotic pathways are neutralized, Casp8 function downstream of death receptor signaling does not contribute to the induction or function of adaptive immunity to this virus and invites further study of additional mouse pathogens. Casp8 facilitates homeostatic T cell turnover in naïve mice, but is completely dispensable for the functional antiviral CD8 T cell response that controls acute infection and provides long-term immunological memory in the virus-infected host.

E) Figures and Legends.

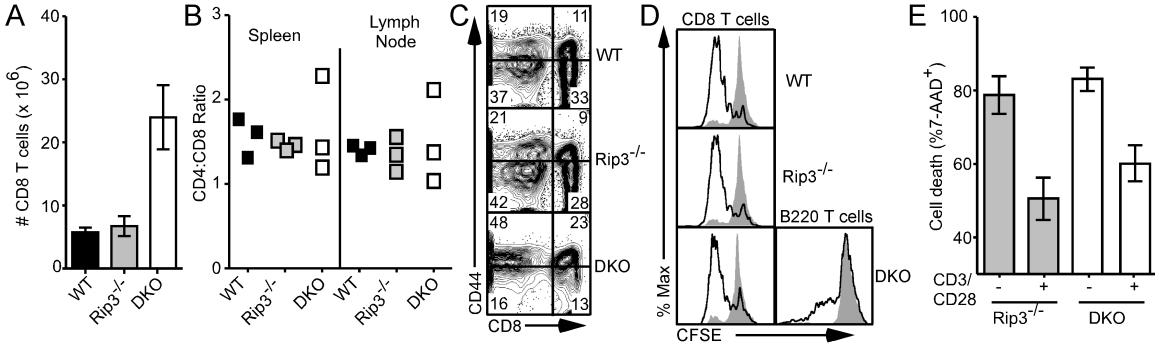


Figure 3.1: Naïve CD8 T cells are intact in mice lacking Casp8 and RIP3. A. Total number of splenic CD8 ($CD8^+CD4^-B220^-CD3^+$) T cells in 16 to 20 week-old WT (*Casp8*$^{+/+}$*Rip3*$^{+/+}$), *Rip3*$^{-/-}$ (*Casp8*$^{+/-}$*Rip3*$^{-/-}$), and DKO (*Casp8*$^{-/-}$*Rip3*$^{-/-}$) mice, calculated based on flow cytometric analysis. Bars indicate mean ±SD of 3 mice per group. B. Ratio of the frequencies of CD4 and CD8 T cells from spleen and lymph node based on flow cytometry. C. Flow profile of CD44 and CD8 expression on $CD3^+B220^-$ splenocytes. Numbers indicate percentage of cells in each quadrant. Data is representative of 3 mice. D. Histograms showing proliferation of splenic CD8 and B220 ($CD8^-CD4^-B220^+CD3^+$) T cells following CD3/CD28 Ab stimulation. Black line indicates CD3/CD28 stimulated cells and solid grey indicates cells incubated with medium alone for 3 days. E. Frequency of dead ($7\text{-}AAD^+$) splenic CD8 T cells following 3 day incubation with CD3/CD28 Abs or medium alone. Bars indicate mean ±SD of 4 or 5 mice per group. Mice used in panels D. and E. had been back-crossed for seven generations to C57BL/6. All data in this figure is representative of 2 or 3 independent experiments with 3 to 5 mice per group.

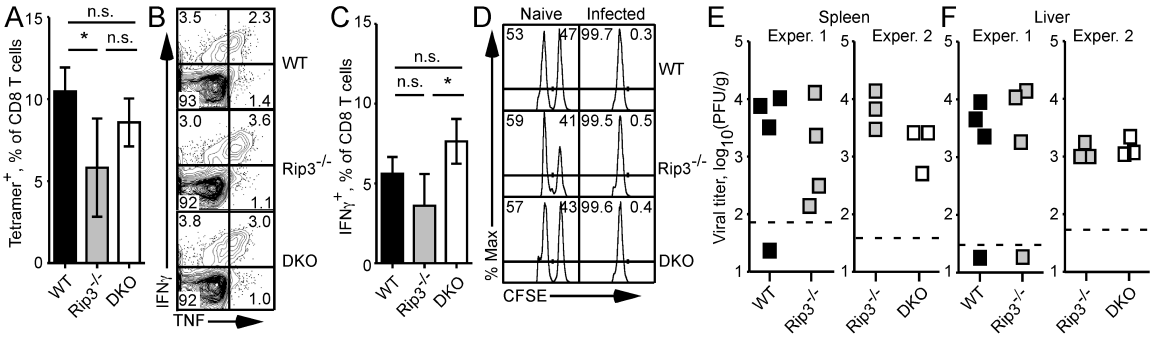


Figure 3.2: CD8 T cell response to acute MCMV infection remains functional without Casp8. Mice were inoculated with 10^5 PFU MCMV strain v70 and samples collected at 7 dpi. A. Frequency of M45 tetramer$^+$ splenic CD8 T cells. Bars represent mean ±SD, 5 mice per group. * indicates $p \leq 0.05$ and n.s. not significant by Bonferroni's multiple comparison test following ANOVA analysis. B. Flow cytometric plots of splenic CD8 T cells assessed for TNF and IFNg production following M45 peptide stimulation. Cell frequencies are indicated in each quadrant. C. Frequency of splenic CD8 T cells producing IFNg following M45 peptide stimulation. Bars represent mean ±SD, 5 mice per group. Statistics as in A. D. Histograms showing frequencies of nonpulsed CFSE^{Low} and M45-pulsed CFSE^{Hi} cells 18 h after adoptive transfer into naive or 6 dpi infected mice. Representative graphs of 3 infected mice are shown. Mice in this experiment had been back-crossed to C57BL/6 for seven generations. E. Splenic viral titers from two independent experiments. Dotted line indicates limit of detection. F. Hepatic viral titers as in E. All data in this figure is representative of 2 or 3 independent experiments with 4 or 5 mice per group except D where a single experiment is shown.

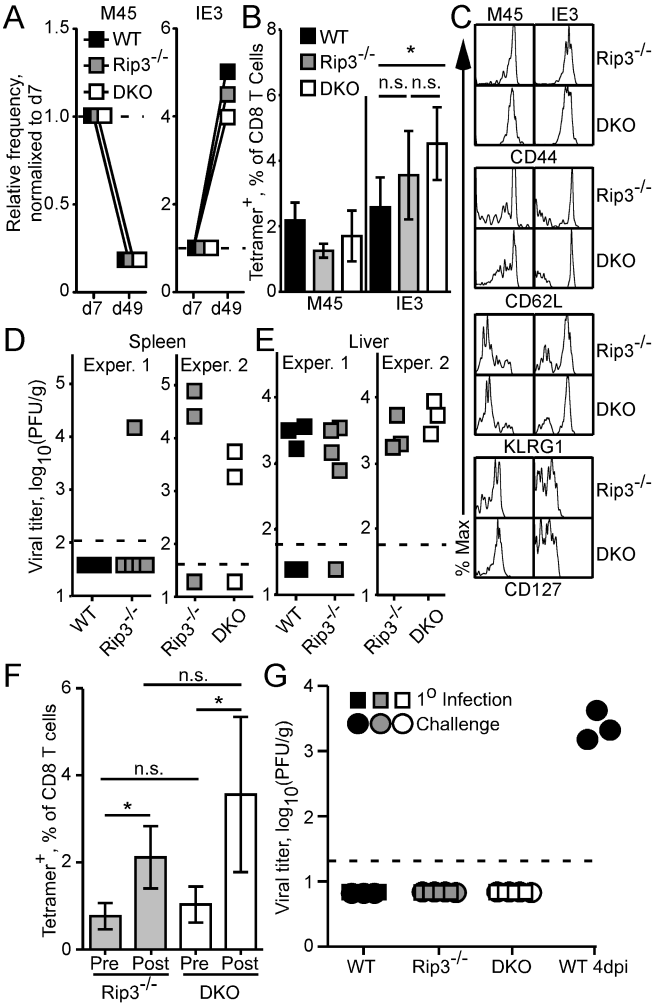


Figure 3.3: Generation of memory CD8 T cell response is independent of Casp8 signals. Mice were inoculated with 10^5 PFU MCMV strain v70. A. Relative frequencies of tetramer$^+$ splenic CD8 T cells at 7 and 49 dpi normalized to d7 frequencies. Symbols indicate mean ratio of 4 or 5 mice. B. Frequency of tetramer$^+$ splenic CD8 T cells at 49 dpi. Bars indicate mean $\pm$ SD of 5 mice. Statistics were calculated as in Fig. 2A. C. CD44, CD62L, CD127 and KLRG1 expression on tetramer$^+$ peripheral blood CD8 T cells at 118 dpi. Representative histograms of 5 mice. D. Splenic viral titers at 31 dpi graphed as in Fig 3.2E. E. Hepatic viral titers at 31 dpi, graphed as in D. F. Frequencies of tetramer$^+$ CD8 T cells in the peripheral blood prior to and following secondary

Figure 3.3 (cont.): challenge. Mice were initially inoculated with 10^5 PFU MCMV strain v70, at 118 dpi mice were bled and frequency of tetramer$^+$ CD8 T cells assessed by flow cytometry. Mice were then challenged with 10^6 PFU MCMV *lacZ*+ strain RM427+, and at 5d post challenge mice were bled and M45 tetramer$^+$ CD8 T cells were assessed. Bars indicate mean ± SD of 3 *RIP3*$^{-/-}$ or 4 DKO mice. * indicates $p<0.05$ and n.s. indicates not significant by t test. G. Splenic viral titers 4 days post challenge. Mice were inoculated as in F and challenged at 75 dpi. One group of naïve WT mice (WT 4 dpi) received only the challenge virus as controls. All data in this figure is representative of at least 2 independent experiments except panel F where a single experiment is shown.

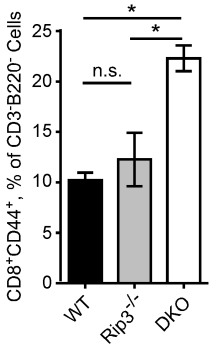


Figure 3.S1: Frequency of $CD8^+CD44^+$ T cells from naïve mice. Bars indicated mean ± SD. * indicates $p<0.05$ and n.s. indicates not significant by Bonferroni's multiple comparison test following ANOVA analysis.

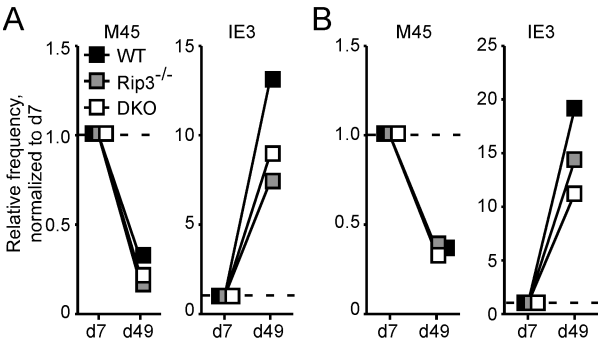


Figure 3.S2: CD8 T cell response in non-lymphoid tissues. A. Relative frequency of tetramer^{+} hepatic CD8 T cells at 7 and 49 dpi. Graphed as in Fig. 3.3A. B. Frequency of tetramer+ pulmonic CD8 T cells at 7 and 49 dpi. Graphed as in A. Pooled samples from 5 mice and a single experiment are shown.

CHAPTER 4

RIP3 contributes to host defense against murine cytomegalovirus infection.

Data presented as figures 4.5 and 4.6 in this chapter were originally published in Kaiser, W.J., Upton, J.W., Long, A.B., Livingston-Rosanoff, D., Daley-Bauer, L.P., Hakem, R., Caspary, T., Mocarski, E.S (2011). "RIP3 mediates the embryonic lethality of caspase-8-deficient mice." Nature 471(7338): 368-72. The content is reproduced here with permission from the publisher and W.J. Kaiser. Data not generated by the Ph.D candidate is indicated in the figure legends.

A) Introduction

Programmed cell death pathways are critical components of the innate antiviral immune response. Killing infected cells prevents viral spread and influences the development of adaptive immunity. The importance of programmed cell death to host defense is revealed by the numerous strategies viruses have evolved to circumvent this process (151, 162, 234). Cell death has historically been characterized as apoptotic or necrotic based on the appearance of the dying cell. Apoptosis is characterized by membrane blebbing and DNA fragmentation while necrosis is characterized by cellular swelling and membrane leakage. Programmed necrosis, or necroptosis, was first observed following death receptor signaling, such as Fas or TNFR1, in cells where caspase activity was compromised (235-237). Subsequent studies identified RIP1 and RIP3 as mediators of this death (153-155, 237). Ligation of death receptors at the cell surface leads to the recruitment of the adaptor molecule Fas-associated death domain protein (FADD), FLICE inhibitory protein (FLIP), caspase 8 (Casp8), RIP1 and RIP3. In the absence of Casp8 regulation, RIP1/RIP3 necrotic signals proceed via the kinase-like adaptor MLKL (238) leading eventually to death. These signals can also, under appropriate conditions, lead to the activation of NFκB and pro-inflammatory signals (239).

Regulation of RIP3 pathways is also required for T cell activation. Upon TCR ligation, RIP1/RIP3 are activated and, if Casp8 is absent, the cell undergoes necroptosis (159, 160). This aberrant RIP3-necrosis is responsible for the inability of mice with conditional deletion of Casp8 in T cells to mount any antiviral T cell responses and their profound immunodeficiency (158, 160).

Mice with germ-line disruptions in RIP3 are viable and fertile with intact immune compartments (240), indicating that, unlike extrinsic apoptosis (241), programmed necrotic pathways are dispensable for mammalian development. Nevertheless, RIP3 has the potential to be an important host factor involved in antiviral immunity. Currently, two viral infections are known to induce necroptosis: vaccinia and MCMV (116, 153). Vaccinia induces a TNF-dependent necrosis in infected tissues (242, 243). This necrosis is required for inflammatory cell recruitment and control of vaccinia infection. Recently, vaccinia-induced necrosis was shown to be dependent on RIP3 expression: *Rip3*$^{-/-}$ mice do not exhibit necrosis in infected tissues and are more susceptible to vaccinia infection (153). Induction of necroptosis during MCMV infection was initially appreciated following identification of a virally encoded RIP3 inhibitor, viral inhibitor of RHIM activation (vIRA) (115, 116). vIRA, binds to RIP3 via a RIP homotypic interaction motif (RHIM) (115) preventing the recruitment of RIP3 following death receptor signaling and inhibiting virally induced necrosis. Mutant virus lacking vIRA does not disseminate past the initial site of inoculation and cannot kill immunodeficient SCID mice (116, 244). Growth of vIRA-deficient virus is rescued in mice that do not express RIP3 (116). Modulation of RIP3-dependent pathways is clearly important in the infected cell during MCMV infection, but potential RIP3 contributions to the anti-MCMV host immune response during WT infections have not yet been elucidated.

In this study we evaluated the involvement of RIP3 in control of MCMV infection and the antiviral T cell response. We found that RIP3 is completely dispensable for viral control and an Ag-specific CD8 T cell response at all points during a low dose infection.

However, RIP3 helps to protect mice from Casp8 mediated death during high dose infection.

B) Materials and Methods

Mice and Infections

C57BL/6 (BL/6) mice were purchased from Jackson Labs. *Rip3*$^{-/-}$ mice (240), a gift from Francis Chan (University of Massachusetts) with permission from Genentech, and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (DKO) mice (204) were bred in house as previously described (116, 204). Mice used in all experiments were between 6-16 weeks of age. Experiments were conducted under a protocol approved by the Emory University Institutional Animal Care and Use Committee. Mice were inoculated via the i.p. route with salivary gland derived v70 prepared as previously described (161). Infected mice were monitored for development of disease by being weighed once daily and observed twice daily for signs of morbidity: piloerection, hunched posture, and lethargy. Imminent death was defined as loss of 20% initial body weight or development of severe lethargy (unresponsiveness to touch) established in a preliminary experiment using death as the endpoint. Viral titers were assessed by plaque assay on 3T3-SA fibroblasts.

Fluorescently conjugated tetramers and antibodies

PE-conjugated tetramers specific for M45, m139 and IE3 epitopes (67, 128) were generated by the NIH Tetramer Core (Emory University). The Abs used were: IFNγ FITC (XMG1.2), B220 FITC (RA3-6B2), Ly6C FITC (AL-21), CD4 PerCP-Cy5.5 (RM4-5), CD19 PerCP-Cy5.5 (1D3), TNF PE-Cy7 (MP6-XT22), CD49b APC (DX5), CD11b APC-Cy7 (M1/70), CD3 Pacific Blue (500A2), purchased from BD Biosciences; CD107a APC (1D4B), CD8 APC and APC-Cy7 (53-6.7), purchased from BioLegend;

CD4 PE (GK1.5) purchased from eBioscience; and CD45 PE-Texas Red (30-F11) purchased from Invitrogen.

Characterization of T cells and Flow cytometry

Single cell suspensions were isolated from thymus, bone marrow, spleen, and lymph nodes as previously described (245). 1×10^6 live cells, as evaluated by trypan blue (Cellgrow) exclusion, were prepared for flow cytometric analysis. To evaluate of T cell function, cells were incubated for 5 hours at 37^o C with 1×10^{-9} M M45, M57, M38, m139 or IE3 peptide (JPT Peptide Technologies) (131) in the presence of GolgiStop (BD Biosciences) and CD107a Ab. Cells were stained for surface and intracellular antigen as previously described (161). Data were acquired on an LSRII flow cytometer (BD Biosciences) maintained by the Emory University Flow Cytometry Core and analyzed using FlowJo software (Tree Star). Live cells were gated on based on forward and side scatter properties followed by leukocytes identification using CD45 expression. T cells were identified by expression of CD3 and further segregated based on expression of CD4 or CD8. Gates were always established based on appropriate isotype and unstained controls.

C) Results

RIP3 is dispensable for NK cell recruitment and early control of MCMV infection.

To evaluate potential contributions of RIP3 to the initial innate antiviral response, *Rip3*$^{-/-}$ and WT mice were inoculated with 1×10^5 PFU MCMV. NK cells dominate control of MCMV infection at these time points in the C57BL/6 background (209, 246). Splenic and hepatic viral titers were assessed at three and five dpi (Fig. 4.1). At both time points examined, viral levels were identical in *Rip3*$^{-/-}$ and WT mice (Fig. 3.1) Titers, in mice of either genotype, at day five were 10-100 fold lower than at day three, indicating that NK cells were actively clearing infected cells, as viral titers in mice depleted of NK cells do not decrease during this time period (246). These data demonstrate that RIP3 pathways are dispensable for NK cell recruitment, cytotoxic activity and for the clearance of virus-infected cells.

RIP3 does not contribute to the antiviral CD8 T cell response.

Following the strong NK cell response initiated immediately upon infection (65), CD8 T cells are recruited and aid in controlling MCMV infections (107). To assess potential contributions of RIP3 to the antiviral T cell response, recruitment of splenic Ag-specific CD8 T cells was assessed by tetramer staining at day seven (Fig. 4.2A). Three tetramers were employed: one that recognizes the immunodominant M45 epitope, another that recognizes the subdominant m139 epitope, and one that recognizes the IE3 inflationary epitope (67, 128). No difference was observed in the frequency of tetramer$^+$ CD8 T cells between genotypes at this time point (Fig. 4.2A). M45-specific cells made up ~12% and m139-specific made up ~6% of the total CD8 T cell population, while IE3-

specific cells did not expand notably over naïve levels (Fig. 4.2A) similar to previously published studies in WT mice (67, 128). We next evaluated the functionality of these cells. Cytokine production was assessed following peptide stimulation with each of four epitopes that were present in significant levels at d7: M45, M57, M38, and m139. The capacity of *Rip3*$^{-/-}$ and WT cells to produce IFNγ, TNF, or both cytokines was equivalent for all peptides tested (Fig. 4.2B-C). CD107a, or LAMP-1, expression during peptide stimulation was used as a marker of degranulation (211). Levels of this molecule were again similar across genotype (Fig. 4.2D). Consistent with the *ex vivo* studies, *Rip3*$^{-/-}$ CD8 T cells appeared fully functional *in vivo* as viral titers in the spleen and liver were indistinguishable from WT at day seven (Fig. 4.2E). Thus, RIP3 pathways are dispensable for generation of an acute CD8 T cell response and control of MCMV infection.

To evaluate potential contributions of RIP3 to T cell contraction and memory development, CD8 T cell responses were assessed at 14 and 31 dpi. Similar decreases in frequencies of M45-, M57-, M38-, and m139-specific CD8 T cells were observed between seven and 14 dpi by both tetramer staining and cytokine production (Fig. 4.2A and C). Memory cells also developed by 31 dpi with WT efficiency in *Rip3*$^{-/-}$ mice as measured by tetramer and ICCS. As was mentioned previously (Ch. 1), some MCMV-specific memory cells inflate over time, such as M38, m139, and IE3, while others are maintained at a low stable level, such as M45 and M57. Inflationary CD8 T cells developed similarly to WT in *Rip3*$^{-/-}$ mice, most clearly observed in the IE3-specific cells (Fig. 4.2A, right graph). Additionally, inflationary and stable memory cells from both genotypes developed with the expected (131) overall phenotype of CD44^{Hi}CD122^{Hi}, with

differences emerging in the $CD127^{Hi}$ expression on stable and $CD127^{Low}$ on inflationary cells (Fig. 4.2F). Together, these results indicate that RIP3 plays no role in an antiviral CD8 T cell response.

RIP3 is globally dispensable during MCMV infection.

Due to its broad cellular tropism, MCMV can infect most organs throughout the body. However, the kinetics and mechanisms of clearance differ for different organs. While CTLs constrain viral replication during acute time points in the spleen and liver (107, 246), CD4 T cells are required for control of infection in the lungs and salivary glands (91). Following acute infection, antiviral Ab and memory T cells help limit reactivation (127, 232). To evaluate possible contributions of RIP3 to these antiviral mechanisms, viral titers were assessed from acute through chronic infection in the spleen, liver, lungs, kidneys, and salivary glands (Fig. 4.3) At all time points, in all organs, viral titers in *Rip3*$^{-/-}$ mice were similar to WT. Viral titers peaked in the spleen at or prior to three dpi followed by clearance. Hepatic titers initially contracted between three and seven dpi, but increased at 14 dpi with eventual clearance by 49 dpi. Pulmonary titers peaked at day seven and were subsequently cleared. Viral titers in the kidney were routinely low or undetectable save for WT mice at 14 dpi where three out of five mice had virus present. Importantly, the kinetics of viral trafficking to the salivary gland, the gland of dissemination, were indistinguishable between the two genotypes. Thus, RIP3 is dispensable for viral dissemination and the generation of a functional antiviral immune response.

RIP3 contributes to host susceptibility to MCMV infection at high doses.

Finally, the potential contribution of RIP3 to susceptibility to infection was evaluated. After inoculation with 1×10^5 PFU, both *Rip3*$^{-/-}$ and WT mice developed mild hunching, piloerection, and weight loss between three to five dpi (Fig. 4.4A and data not shown). All of these mice recovered form the infection as evidenced by their weight gain beginning on day six. No mouse died over the course of the experiment (Table 4.1). Lethal disease was observed in *Rip3*$^{-/-}$ mice with doses above 1×10^5 PFU. To determine whether Casp8 activity contributed to the increased susceptibility of *Rip3*$^{-/-}$ mice we utilized the recently described *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice (204).

Prior to infection, the immune compartment of DKO mice was assessed to ensure that Casp8 deficiency did not contribute to leukocyte development of maintenance. No differences were observed between genotypes in the frequency of any cell type in primary lymphoid organs, bone marrow and thymus (Fig. 4.5A-B). While fewer neutrophils were recovered from the bone marrow of Rip3$^{-/-}$ mice (Fig. 4.5A), this difference could be due to inter-mouse variability or loss during processing as this was not observed in subsequent experiments. Between 60-70% of B cells isolated from the bone marrow expressed IgD on their surface regardless of genotype (data not shown) demonstrating that extrinsic death signals do not contribute to B cell development. The frequencies of thymic T cells expressing CD4, CD8, both or none of these markers were also similar in all mice (Fig. 4.5C). Together these data indicate that leukocyte development does not depend on Casp8. To assess homeostatic maintenance of lymphocytes, the cellular components of secondary lymphoid organs were assessed. Upon gross examination, it was apparent that Casp8 contributes to lymphoid homeostasis

as the spleen and lymph nodes from DKO mice were larger than Rip3$^{-/-}$ controls (Fig. 4.6A-B). Consistent with their increased size, spleens from DKO mice weighed, on average, five times more than controls (Fig. 4.6C) and lead to the recovery of six times as many cells (Fig. 4.6D). Both spleens and lymph nodes from DKO mice had increased frequency of T cells and decreased frequency of B cells compared to Rip3$^{-/-}$ and WT (Fig. 4.6E-F). However, B cells were present in the spleens of DKOs at levels similar to or higher than those found in controls (Fig. 4.6G). Approximately six times as many splenic T cells were recovered from DKO mice compared to controls. As reported in Chapter 3, the accumulation of B220 T cells contributes to the increased T cell numbers in the spleen and lymph nodes of DKO mice (Fig. 4.6H-J). Thus, in the absence of RIP3, Casp8 is dispensable for leukocyte development but helps to regulate the homeostatic turnover of naïve T cells.

WT, *Rip3*$^{-/-}$, and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were inoculated with 1.5×10^6 PFU (Table 4.1 and Fig. 4.7A-B). While all mice exhibited similar kinetics of disease with weight loss again peaking at five dpi (Fig. 4.7A), *Rip3*$^{-/-}$ mice lost more weight between days three and five than WT. This led to 80% of *Rip3*$^{-/-}$ mice succumbing on days four and five (Table 4.1 and Fig. 4.7B). No WT mouse died during this experiment demonstrating that *Rip3*$^{-/-}$ mice exhibit increased susceptibility to MCMV infection. *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were partially protected, with one mouse dying at day five (Table 4.1 and Fig. 4.7B). These results indicate that RIP3 pathways help protect mice from lethal MCMV infection by regulating Casp8.

D) Discussion

These data demonstrate that RIP3 is dispensable for the antiviral cytotoxic cellular response as well as control of viral infection at low doses. The increased susceptibility of *Rip3*$^{-/-}$ mice to high dose MCMV infection was unexpected, but consistent with an earlier report utilizing vaccinia (153). In this earlier study, *Rip3*$^{-/-}$ mice infected with vaccinia exhibited less necrosis and inflammation, but succumbed to the infection at a greater rate than WT controls. The authors speculated that necrosis was needed to recruit appropriate immune cells to control infection. Necrosis has been observed in MCMV-infected livers, spleens, pancreas, and adrenal glands during lethal infections (161, 183) and it is widely accepted that severe hepatitis drives lethal disease in WT mice (18, 161, 183), yet, contributions of necrosis to lethality have not been directly evaluated. Disrupting RIP3 signals could lead to two different outcomes that either individually, or combined could increase host susceptibility to MCMV infection. Without RIP3, programmed necrosis is shackled leading, potentially, to increased viral replication in cells that normally would have died by this pathway. Alternatively, removing RIP3 could unleash increased apoptosis, leading to overwhelming cell death and organ dysfunction. Given that mice lacking both Casp8 and RIP3 were partially protected from death following inoculation with a high dose, the later seems most likely. Overall, our data suggest that RIP3 regulates Casp8 function, and that this regulation is needed to prevent death. Future studies of high dose MCMV infections in *Rip3*$^{-/-}$ and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, including histological evaluations, will directly evaluate the contributions of RIP3 and Casp8 to antiviral host defense.

The potential contributions of TNF to the increased susceptibility of *Rip3*$^{-/-}$ mice also need to be evaluated. In vaccinia infection, TNF clearly aids viral clearance through the recruitment of immune cells (153, 242). The similarities between mice deficient in either TNFR1 or TNFR2 and RIP3 suggest that TNF is driving necroptosis *in vivo* that, in turn, recruits inflammatory cells. The role of TNF in MCMV pathogenesis is more complex. TNF is produced early during infection and can aid viral clearance (65, 109) but does not promote inflammatory cell accumulation (20). In fact, TNF contributes to hepatic damage during non-lethal infections (20). This hepatitis is potentially a result of TNF directly killing infected cells, possibly by inducing necrosis. In the absence of RIP3, TNF may drive a Casp8-dependent apoptosis thereby contributing to the increased susceptibility of *Rip3*$^{-/-}$ mice.

The kinetics of viral titers in the liver following low dose inoculation were interesting. The initial pattern observed in the first week of infection, high viral titers at two to three dpi followed by rapid clearance, has been reported in numerous studies (19, 66, 106, 209, 246) and is known to be due to NK and CD8 CTL activity (107, 246). The subsequent increase in hepatic titers at 14 dpi was novel. Prior work examining the immune response to MCMV infection in the liver of C57BL/6 mice has been limited to the first ten days of infection (19, 66, 106, 246). These data suggest that study of hepatic MCMV replication and the subsequent immune control after the first week of infection is needed.

Examination of viral titers from multiple organs over the course of infection combined with evaluation of Ag-specific CD8 T cells gave us a broad picture of RIP3 contributions to the NK cell and adaptive immune responses. Similar viral titers at day

three and five post infection (Fig. 4.1) strongly suggest that the NK cell response is intact in *Rip3*$^{-/-}$ mice as NK cells are required for viral control at this time in C57BL/6 mice (209, 246). *Rip3*$^{-/-}$ CD8 T cells were phenotypically and functionally indistinguishable from WT at all time-points (Fig. 4.2) indicating that, while regulation of RIP3 pathways is critical for T cell activation (160), these pathways are not involved in the antiviral T cell response. The lack of a difference in viral titers during latent infection (Fig. 4.3) demonstrates that B cell dysfunction in *Rip3*$^{-/-}$ mice is also unlikely. Finally, CD4 T cells appear capable of mounting an antiviral immune response as viral replication was controlled at levels similar to WT in both the lungs and salivary glands, organs that require CD4 T cells to constrain infection (91). While formal evaluation of the antiviral NK, CD4 T, and B cell responses in *Rip3*$^{-/-}$ mice are still needed, RIP3 necrotic pathways are dispensable for the generation of a functional multipronged antiviral immune response against MCMV.

E) Figures and Legends

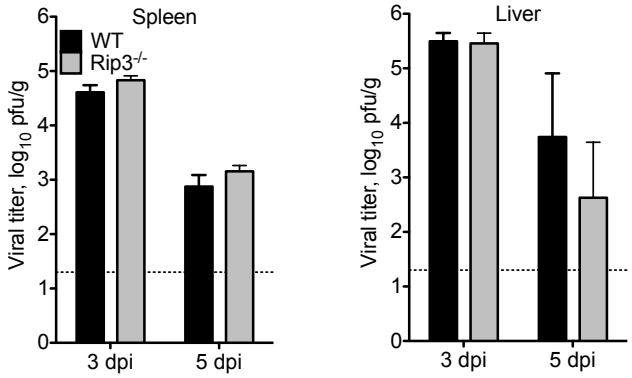


Figure 4.1: Evaluation of early viral titers. Mice were inoculated with 1×10^5 PFU v70. Splenic or hepatic samples for viral titers were taken on indicated days. Bars represent mean of $\log_{10}$ of viral titers ± SD of 4 mice. Dashed line indicates limit of detection.

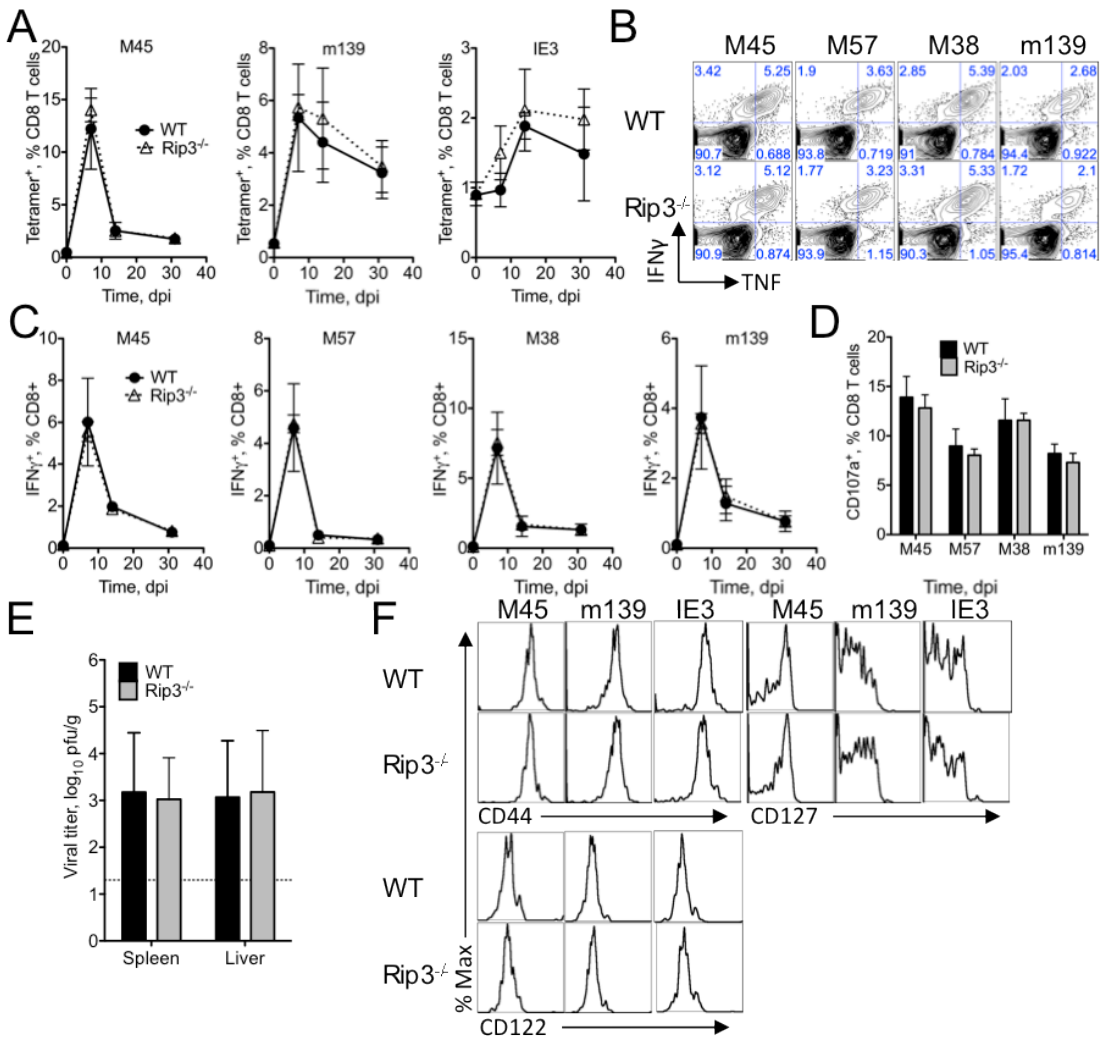


Figure 4.2: Evaluation of T cell response in *Rip3*$^{-/-}$ mice. Mice were inoculated with 1 × 10^5 PFU v70. Samples were taken at 0, 7, 14, and 31 dpi. A. Frequency of tetramer$^+$ splenic CD8 T cells. Symbols represent mean ± SD of 3 or 5 mice per group. B. Flow plots showing IFNγ and TNF production of splenic CD8 T cells following peptide stimulation at 7dpi. Frequencies of each quadrant are indicated. Representative of 5 mice. C. Frequency of total IFNγ$^+$ splenic CD8 T cells following peptide stimulation. Graphed as in A. D. Frequency of CD107a$^+$ splenic CD8 T cells at 7 dpi during peptide stimulation. Bars represent mean ± SD of 5 mice per group. E. Splenic and hepatic viral titers at 7 dpi. Graphed as in Fig. 4.1. F. Histograms showing expression of CD44,

Figure 4.2: (cont.): CD122 and CD127 on splenic tetramer^{+} CD8 T cells at d31. Representative of 5 mice.

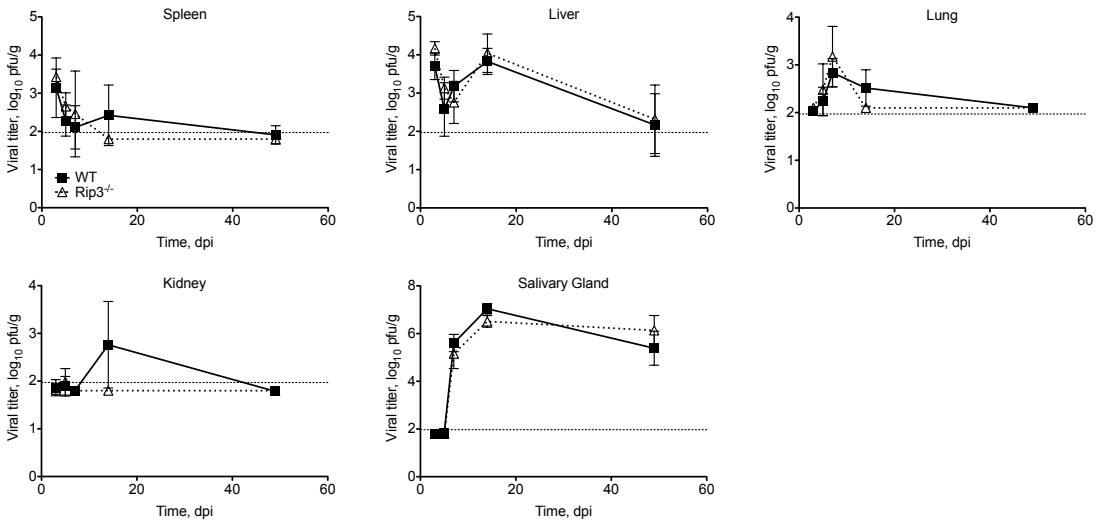


Figure 4.3: Evaluation of viral replication over the course of infection. Mice were inoculated with 1×10^5 PFU v70. Samples were taken from indicated organs at 3, 5, 7, 14, and 49 dpi. Symbols represent mean of $\log_{10}$ of viral titers ± SD of 4 or 5 mice. Dashed line indicates limit of detection.

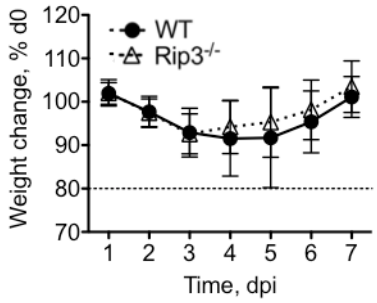
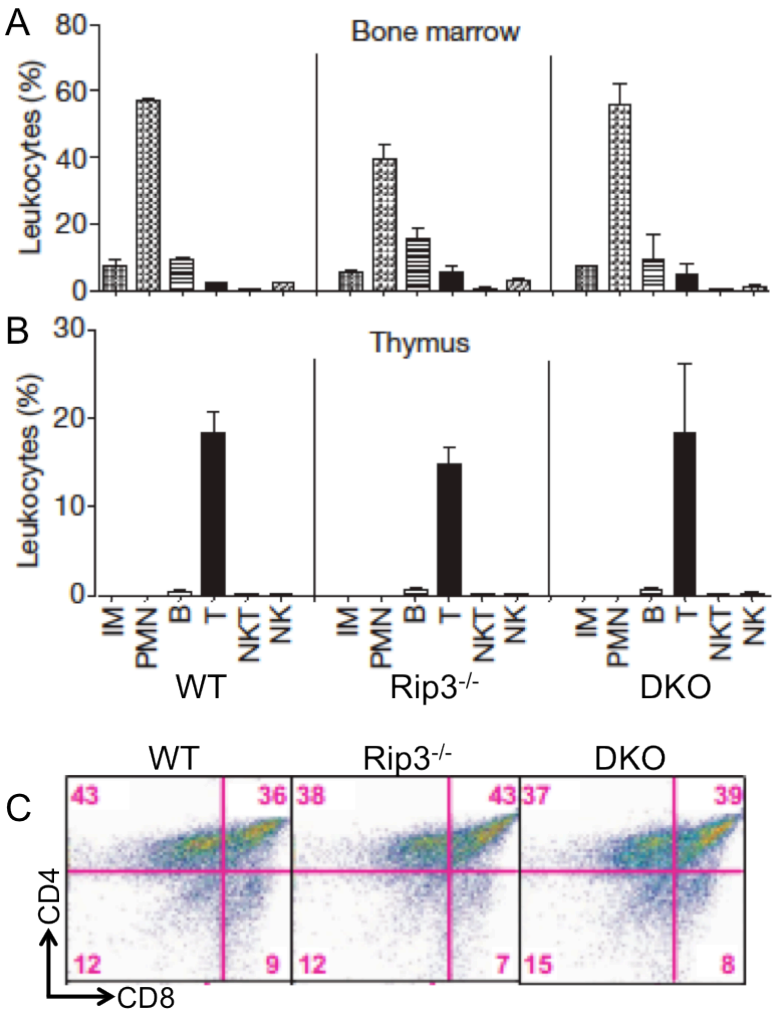


Figure 4.4: Susceptibility of *Rip3*$^{-/-}$ mice to low dose MCMV infection. Weight loss in mice inoculated with 1×10^5 PFU of v70 expressed as percentage of weight prior to infection (d0). Shown is the mean ± range of 4 mice per group. Dotted line indicates 20% weight loss. B. Weight loss of mice inoculated with 1.5×10^6 PFU of v70, graphed as in A. One and three *Rip3*$^{-/-}$ mice succumbed at days 4 and 5 respectively. One *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mouse died at day 5. C. Kaplan-Meier plot showing percent survival of mice in B.



Figures 4.5: Immune compartment of primary lymphoid organs in 16-week-old DKO mice. Live cells from bone marrow (A) and thymus (B) gated based on forward and side scatter properties, and stained for surface expression of CD19,CD3, CD49b, Ly6C and CD11b to define non-overlapping leukocyte (CD45) populations. The average and s.d. for three WT (left panels), four Rip3$^{-/-}$ (middle panels) littermate control and three DKO (right panels) mice showing levels of inflammatory monocytes (IM), polymorphonuclear leukocytes (PMN), B cells, T cells, NK cells and NKT cells. C. Flow plot showing CD4 vs. CD8 expression of CD3^{+} T cells in thymus.

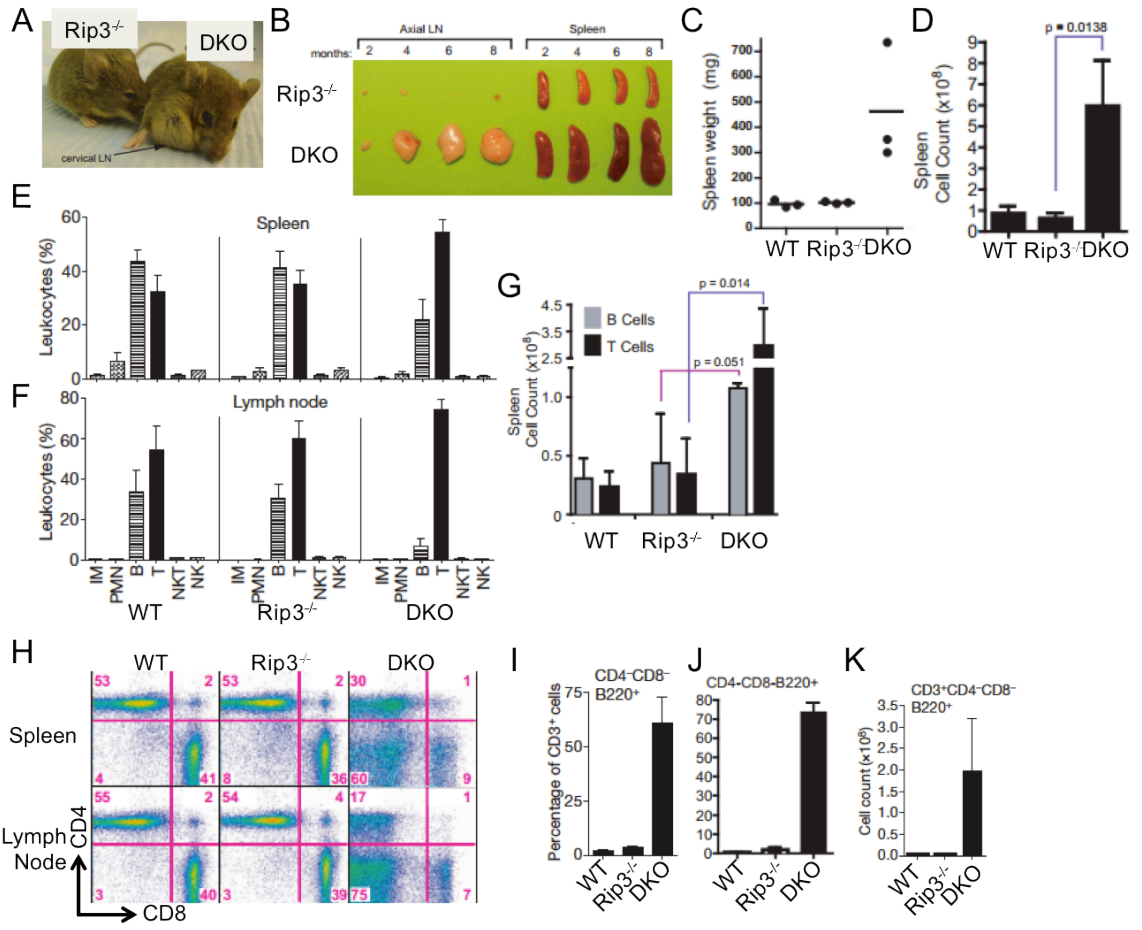


Figure 4.6: Immune compartment in secondary lymphoid organs. A. Photographs of six month-old Rip3$^{-/-}$ and DKO mice. The arrow indicates enlarged cervical lymph node (LN) present in the DKO mouse. B. Images of spleen and axial LNs from Rip3$^{-/-}$ and DKO mice of the indicated ages. C. Graph of weights of spleen from mice of the indicated genotype. Horizontal lines represent the mean. D. The numbers of cells recovered from spleens from mice with the indicated genotype. Statistical analyses were performed applying a two-tailed unpaired Student's t-test. E. Live cells from spleen graphed as in Fig. 4.5A. F. Live cells from cervical lymph nodes graphed as in panel E. G. The numbers of total B and T cells recovered from spleens from mice with the indicated genotype. Statistical analyses were performed applying a two-tailed unpaired Student's t-test. H. Flow plot showing CD4 vs. CD8 expression of CD3^{+} T cells in spleen

Figure 4.6 (cont.): (top) and lymph node (bottom) I. Frequency of splenic $CD3^+CD4^-CD8^-B220^+$ T cells. Bars indicate mean ± SD of 3 or 4 mice per group. J. Frequency of $CD3^+CD4^-CD8^-B220^+$ T cells from cervical lymph nodes graphed as in I. K. Total number of splenic $CD3^+CD4^-CD8^-B220^+$ T cells recovered. Graphed as in I. Panels A and B were generated by W.J. Kaiser.

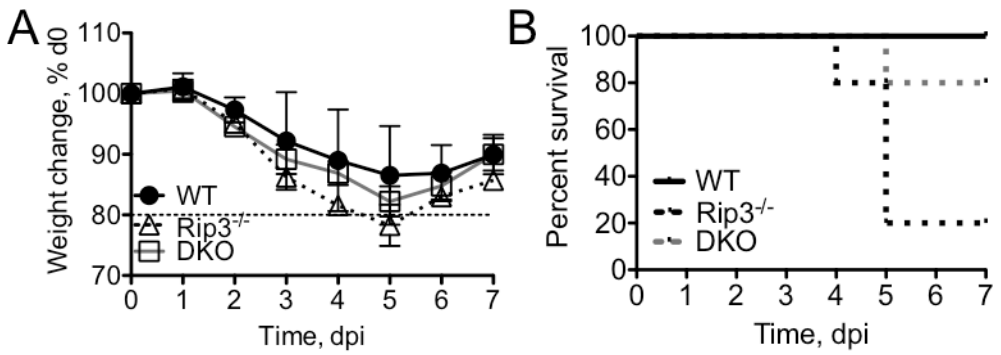


Figure 4.7: Susceptibility of *Rip3*$^{-/-}$ mice to low dose MCMV infection. A. Weight loss of mice inoculated with 1.5×10^6 PFU of v70, graphed as in Fig. 4.4. One and three *Rip3*$^{-/-}$ mice succumbed at days 4 and 5 respectively. One DKO mouse died at day 5. B. Kaplan-Meier plot showing percent survival of mice in A.

F) Table

Dose (PFU)[1]	WT[2]	*Rip3*$^{-/-}$	*Casp8*$^{-/-}$*Rip3*$^{-/-}$
1×10^5	0/5 (100%)	0/5 (100%)	0/5 (100%)
5×10^5	N.D.	4/4 (0%)	N.D.
1.5×10^6	0/5 (100%)	4/5 (20%)	1/5 (80%)
2×10^6	N.D.	4/4 (0%)	N.D.

Table 4.1: Susceptibility to lethal disease. [1] Mice were inoculated with the indicated doses of v70. [2] The number of mice that died, followed by percent survival, is indicated. N.D. indicates not done.

CHAPTER 5.

Discussion and Future Directions

Despite major improvements in treatment options over the past 30 years, CMV remains a major cause of disease and even death in transplant recipients and neonates infected congenitally. While antivirals are available, their toxicity profiles and the rise in resistant strains demonstrate the need for novel treatments. To develop better treatment modalities, a more complete understanding of CMV pathogenesis is needed. The strict species specificity of CMVs precludes the study of HCMV outside of tissue culture. While much has been learned from examining HCMV *in vitro*, critical questions remain related to pathogenesis and the host immune response that cannot be addressed without *in vivo* work. The utility of MCMV as a model for HCMV has only grown since the isolation of both viruses in the 1950s (247-250). The ease of generating mutant viruses combined with the availability of mice carrying germline mutations in thousands of genes allows for the manipulation of both the virus and the host. This is the true power of the MCMV system, the capacity to perturb and study both sides of the host-pathogen interaction.

By utilizing viral variants in conjunction with mice lacking genes that mediate extrinsic cell death pathways, the data presented in this dissertation addresses the interaction between death and T cells. In Chapter 2 differences in virulence between two different MCMV variants were characterized and then those differences were used identify contributions of T cells to lethal disease. These experiments found that T cells are critical mediators of CMV pathogenesis and demonstrated that antiviral T cells exert significant effects in the periphery earlier than previously appreciated. We next turned our attention to host extrinsic death pathways, pathways that are tightly controlled during MCMV infection (151, 234). This led to Chapters 3 and 5, a series of experiments that

utilized the virulent MCMV variant, v70, characterized in Chapter 2 to interrogate host-pathogen interactions and evaluate how cell death pathways contribute to antiviral immunity and function. Chapter 5 demonstrates that Casp8 helps to regulate homeostatic turnover in CD8 T cells, but does not contribute to a functional antiviral T cell response or viral control. Finally, in Chapter 4, we found that RIP3, a pro-necrotic protein whose inhibition is critical to viral replication within many cells (116), was dispensable for the host antiviral response, including T cells, and control of MCMV infection at low doses. Yet, RIP3 helped to protect mice from Casp8-mediated death at high doses.

Chapter 2 presents intriguing possibilities that in addition to protecting the host, T cell responses can contribute to CMV disease. The data in this chapter demonstrate that MCMV disease can be caused by the antiviral T cell response and that a viral isolate repeatedly selected for its ability to grow *in vivo* can drive this response. This suggests that some CTL responses may actually be beneficial for the virus. While MCMV has a long history of subverting the host T cell response, all currently described mechanisms blunt the antiviral T cell response. The immune evasion genes m04, m06, and m152 all work to hide infected cells from MCMV-specific CD8 T cells by interfering with MHC-I expression (21) and recruitment of IMs by host and viral chemokines (25, 26) constrains CD8 T cell responses through production of nitric oxide (28). Yet stimulation of an antiviral CD8 T cell response in observed by other herpesviruses. Direct viral enhancement of T cell responses have been observed during infection with the gamma-herpesvirus MHV-68 (251). MHV-68 drives expansion of a population of CD8 T cells that all express the Vβ4 TCR by encoding M1, a secreted protein that stimulates these cells similarly to a super-Ag. In the immunocompetent, this helps establish latency and

inhibit reactivation benefiting both the host and the virus by preventing disease in the host and aiding in viral persistence. However, in immunocompromised settings, such as IFNγR$^{-/-}$ mice, these expanded T cells lead to severe immunopathology. Thus, viral stimulation of the CD8 T cell response can be beneficial or pathologic depending on circumstances. Something similar may be occurring during v70 infection. Following low dose inoculation, enhanced T cell activity may help prevent host pathology while preserving latent infection. Yet at higher doses, such as those employed in Chapter 2, boosting the antiviral T cell response could raise the immunopathology potential by increasing the risk of damage to bystander cells.

As we only observed enhanced CD8 T cell quality in the liver, it is possible that the virus or host modulates T cell function depending on location. Given that T cells from naïve mice differ in phenotype and function based on anatomical location (70, 252), it would not be surprising to observe similar behavior during viral infection. All previous work examining the effects of MCMV gene products on the CD8 T cell response have focused on secondary lymphoid organs. A broader examination of the immune response to WT and viral mutants lacking known T cell modulators in peripheral organs such as the liver, gastrointestinal tract, and lungs, organs that are known to modulate T cell responses in the uninfected and that also happen to be the most common areas of CMV disease and reactivation (39, 70, 126, 252), will likely provide valuable insight into potential contributions of antiviral T cell responses to CMV disease.

In addition to host factors, viral factors also contribute to viral pathogenesis. Sequence differences must exist between v70 and K181+ as the increased virulence of v70 is maintained in a plaque purified isolate (Chapter 2 and data not shown).

Preliminary restriction fragment length polymorphism (RFLP) analysis did not reveal any large deletions or insertions in the v70 genome (data not shown). Based on this analysis, v70 appears to be a pool of viruses exhibiting gross RFLP similarities to both K181 and Smith necessitating further in depth sequence analysis. Sequence comparisons between K181 and the less virulent Smith have demonstrated that small sequence differences can lead to dramatic differences in virulence. While the viral gene(s) responsible for the increased virulence of K181 have not been identified, approximately 96% of predicted ORFs share more than 95% amino acid homology with Smith (253). Out of the remaining seven genes, only two share less than 85% homology and one of these has been ruled out as a virulence determinant (A. Redwood, personal communication). Thus we hypothesize that differences in relative viral gene expression or epigenetic modifications will contribute more to virulence differences than gene composition. Much work is needed to better understand the transcriptional and epigenetic profile of MCMV, although studies have begun to address these issues (254).

How these differences in virulence arose is likely buried in the long history of passaging MCMV pools, rather than plaque purified isolates, *in vivo*. Different CMV strains can co-infect the same cell *in vivo* (255), and, upon co-infection, likely recombine with serial passaging (256). Recently, a pool of Smith virus passaged *in vivo* for many years was plaque purified and sequenced (257). When aligned with previously published sequences of Smith (Accession # NC_004065) (258) and K181 (AM886412) (253, 259), this new isolate, Smith-SL (GU305914) (257), appears to be a chimera of both viruses (Fig. 6.1) most likely resulting from cross-over events. We speculate that a similar process likely occurred during the decades of *in vivo* v70 passage leading to its increased

virulence. Future sequencing of v70 and comparison to previously sequenced strains will address this.

The major question left unanswered by Chapter 3, is what about other lymphocytes, are they fully functional too? Preliminary data suggests that Casp8 may be important in CD4 T cell activation (data not shown), but further work is needed to fully evaluate this phenomenon. Hints that NK responses to MCMV infection do not require extrinsic death signaling exist in the lack of a difference in viral titers at any time-point. In the C57BL/6 background, NK cells are critical for early control of MCMV replication due to their robust activation via m157 expressed on infected cells (119, 120, 209). Without proper NK cell function, viral titers are 10-1000 times higher in the spleen and liver at early time points, indeed most C57BL/6 mice that lack NK cell function succumb to infection by day ten (106, 209). We did not observe any difference in viral titers between days three and seven following infection, nor did mice succumb to infection or exhibit signs of increased disease (Fig. 3.2 and data not shown). Thus, via indirect measures, NK cells appear intact in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice. However direct examination of the NK cell compartment in the presence and absence of MCMV infection are needed to formally address this question.

The contribution of Fas signaling to the cytotoxic capacity of CTLs has long been of interest to immunologists. Upon activation, CD8 T cells upregulate expression of FasL, thus it is logical to speculate that CD8 T cells are able to kill target cells via death receptor signaling. This theory has become deeply engrained in the field and continues to be touted in textbooks (70). Yet the experimental data supporting this hypothesis are murky. Studies of viruses as varied as influenza, murine hepatitis virus, herpes simplex

virus 2 (HSV-2), and west nile virus have all shown that Fas-mediated death can work with perforin to control infection (260-263). However, none of this work has identified an independent role for Fas signals in the CTL response. Additionally, all of these viruses rely primarily on the cytokines produced by T cells, specifically IFNγ, to control infection rather than the cytolytic capacity of CD8 T cells (260, 264, 265). MCMV, in contrast, relies almost entirely on the direct lysing of infected cells by CTLs to control virus (106, 266). The fact that mice lacking the ability to signal through Fas, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$, constrain MCMV infection as well as controls (Chapter 3) indicates that Fas-mediated killing of virus infected cells does not contribute to control of viral infection in settings where the cytotoxic effects of CD8 T cells dominate. Thus, in all systems that have been studied to date, Fas signals are not directly involved in CTL control of viral infection.

Chapter 4 demonstrates that Casp8 regulation of RIP3 necrotic pathways, is, in some ways, more important to mammalian development than its pro-apoptotic functions. Without Casp8, development is halted at embryonic day 10.5 (241) and hematopoietic cell development is blocked both *in vitro* and *in vivo* (267). Additionally, mice with targeted Casp8 deletions in specific immune cell types reveal a profound crippling of both innate and adaptive immunity (157, 158, 267, 268). Casp8 expression is needed for M-CSF differentiation of macrophages (267). While Casp8 is dispensable for lymphocyte development (158, 268), naïve T cells require Casp8 to survive in the periphery (158). Casp8 is also essential for B and T cell activation (158, 268). Remarkably, as the data in Chapter 4 demonstrate, in all of these different systems, the primary role of Casp8 is to regulate RIP3-necrotic pathways. Abnormalities do develop in the T cell compartment of

Casp8$^{-/-}$*Rip3*$^{-/-}$ mice, with an accumulation of abnormal T cells reminiscent of the phenotype of mice lacking Fas signals, yet all other defects observed in Casp8$^{-/-}$ settings appear to be due to unleashed RIP3 activity. While extrinsic death pathways are clearly stimulated during development and activation of the immune compartment, these signals are not required for an intact and functional immune response.

Controlling RIP3-mediated necrosis is demonstrably critical for the viral life cycle of MCMV (115, 116). Therefore it was remarkable that no aspect of the host immune response relies directly on RIP3 to control viral infection when compared to WT at low doses (Chapter 4). Hints at RIP3 contributions to the antiviral T cell response were only revealed when *Rip3*$^{-/-}$ mice were compared to *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice (Fig. 3.2). These data suggest that RIP3 may help to regulate a slight dampening in cytokine production mediated by Casp8 activity in effector cells. If true, the effect is very small as no difference in the apparent function of *Rip3*$^{-/-}$ CD8 T cells was observed when compared to WT *in vitro* or both WT and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ *in vivo* (Fig. 3.2). It is possible that infection with a non-lethal dose of MCMV did not perturb the system enough to allow detection of measurable differences due to the subtle effects of RIP3. These effects may contribute to the increased susceptibility of *Rip3*$^{-/-}$ mice to high dose infection (Fig. 4.4). Casp8-dependent deficiencies in *Rip3*$^{-/-}$ T cell function may be augmented following inoculation with a high dose of virus. This could contribute to the increased susceptibility of *Rip3*$^{-/-}$ mice to MCMV. The observation that *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice are protected from death at higher doses supports the hypothesis that RIP3 regulates Casp8-mediated pathways in some circumstances.

Although the rescue of embryonic lethality and development of competent immunity is remarkable in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, defects attributable to the lack of extrinsic death pathways are still apparent. Gross examination of adult *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice reveals a profound lymphadenopathy, and further characterization identifies the accumulation of abnormal B220^{+} T cells as a major contributor to the enlarged lymphoid organs (Chapters 3 and 4). In phenotypic and functional assays, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ B220^{+} T cells appear to mimic the B220^{+} T cells present in mice deficient in Fas signals (217). That *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice appear to phenocopy the lymphoproliferative phenotype in mice lacking Fas signals (217) is striking, and suggests that the primary trigger of Casp8 activation during T cell homeostasis comes through the Fas death receptor. While specific steps leading to the accumulation of these B220 T cells in mice lacking Fas signaling remain unclear, the development of these cells appears to be driven by exposure to environmental Ag and commensal bacteria (220). This suggests that B220 T cells may arise due to a defect in oral tolerance. The importance of Fas signals in the development of oral tolerance has been known for almost 30 years (269), yet no one has evaluated whether this may contribute to the abnormal T cell population that arises. We hypothesize that B220 T cells develop from cells that were tolerized to environmental Ag, but could not die appropriately due to the lack of Fas signals. Assuming that the B220^{+} T cells in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice arise through a similar mechanism, we would predict that *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice would be impaired in their ability to develop oral tolerance. Future studies on these lines would improve our understanding of the signals required for tolerogenic responses.

Figure and Legend

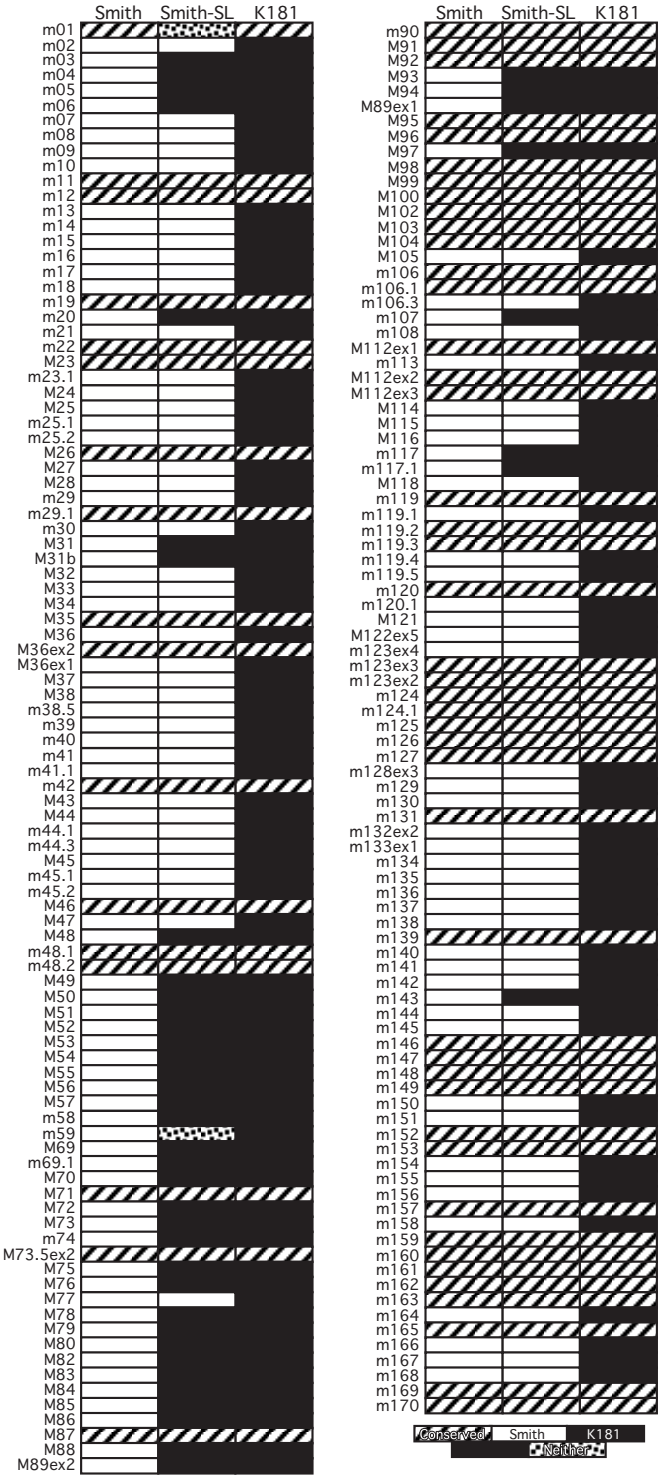


Figure 6.1: Summary of sequence variation between three published MCMV strains by ORF.

Figure 6.1 (cont.): Smith, Smith-SL and K181 were aligned using ClustalW software. ORFs are coded according to their similarity to the reference strains Smith or K181.

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