Characterization of CD8 T cells in a Replication-Deficient Murine Cytomegalovirus Infection

By

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LIST OF ABBREVIATIONS

Ag: Antigen
ADCC: Antibody-dependent cellular cytotoxicity
AT: Adoptive transfer
B cell: Bursa (matured) cell
β2-m: β2-microglobulin
CD: Cluster of Differentiation
CNS: Central nervous system
CTL: Cytolytic T Lymphocyte
DC: Dendritic Cell
DNA: Deoxyribonucleic acid
DT: Diphtheria toxin
DTR: Diphtheria toxin receptor
GPCMV: Guinea pig CMV
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
ERAAP: Endoplasmic reticulum amino-peptidase
FACS: Florescence activated cell sort
gB: Glycoprotein B
gL: Glycoprotein L
GM-CSF: Granulocyte monocyte colony-stimulatory factor
HCMV: Human Cytomegalovirus
HC: Heavy chain
ICS: Intracellular cytokine staining assay
IFN-α: Interferon alpha
IFN-β: Interferon beta
IFN-γ: Interferon gamma
Ig: Immunoglobulin
IL: Interleukin
IV: Intravenous
IP: Intraperitoneal
KIR: Killer cell Ig like receptor
LT: Lymphotoxin
mAb: Monoclonal Antibody
MCMV: Murine Cytomegalovirus
MHC: Major Histocompatibility Complex
MPEC: Memory precursor effector cell
NHANES: National Health and Nutrition Examination Survey
NK: Natural Killer
NKC: Natural kill cell complex
PI: Post-infection
PFU: Plaque forming unit
TAP: Transporter associated with antigen processing
T cell: Thymus (matured) cell
T_h: T helper

OAS: Oligoadenylate Synthetases
SLEC: Short-lived effector cells
TCR: T cell receptor
Th: T helper cell
TNF-α: Tumor Necrosis Factor alpha
TK: Thymidine kinase
WT: Wild-type

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ABSTRACT

Development of attenuated viruses is important to the therapeutic vaccine strategy against viral infections, as the goal of a live-strain vaccine is to provide the most robust immune response in the least pathogenic package. In this thesis the goal is not to develop a new CMV based vaccine, but to understand the mechanism that drives a CD8 T cells response in a replication-deficient murine cytomegalovirus (MCMV) infection. Murine cytomegalovirus infection, like Human CMV, produces a robust chronic immune response, with the pool of Ag-specific CD8 T cells to CMV as high as 10% in the blood. What is less known is the acute CD8 T cell response to HCMV, as primary infection is often benign and unidentified in immune-competent individuals. Using the MCMV model we are able to identify the acute CD8 T cell response and track changes of these responses into the chronic stage of infection. The role of viral replication is important to the development of the antiviral CD8 T cell response as it produces viral antigen and initiates an inflammatory response (which provides co-stimulation) that is essential for the priming of naïve CD8 T cells. Therefore, blocking viral replication will reduce both available antigen and inflammation. My initial hypothesis of the CD8 T cell response to a replication-deficient MCMV was that the response would be weaker in comparison to a replication-competent infection. Surprisingly, the acute CD8 T cell response in a replication-deficient MCMV infection was increased and these antigen-specific CD8 T cells formed a greater memory population. The goal of this thesis is to characterize this CD8 T cell response and find a mechanism that helps explain this elevated response.

In order to investigate the role of viral replication in the CD8 T cell response to MCMV infection, we infected mice with a recombinant MCMV (MCMV-TK), where viral replication can be inhibited when mice are treated with antiviral drug Famcyclovir. MCMV-TK can replicate to measurable levels of titers, comparable to wild-type MCMV. However, Famcyclovir renders MCMV-TK replication-deficient as it acts as a DNA chain terminator during viral genome synthesis, thus producing undetectable titers at 3 days post-infection.

Infection of Famcyclovir treated C57BL/6 mice with a MCMV-TK resulted in priming of CD8 T cells and an enhanced T cell response at day 7 post-infection. This was a 2–3 fold enhancement of the CD8 T cell response in comparison to a replication-competent infection. Investigation of the dendritic cell (DC) numbers during acute infection show that conventional DC (cDC) numbers decline in a replicating infection, while cDC numbers are preserved in a replication-deficient infection. Re-introducing inflammation, specifically type I IFNs, into mice with a replication-deficient infection lowers CD8 T cell responses to wild-type levels and decreases DC numbers. This work demonstrates a mechanism in which the type I IFN inflammatory response during acute MCMV infection causes a decrease in cDC numbers, and this decrease in cDCs results in less proliferation of CD8 T cells that are dependent on cross-presented antigen.

CHAPTER 1: INTRODUCTION

1.1 Overview

The scope of this thesis is the CD8 T cell response in murine Cytomegalovirus (MCMV) infection in C57BL/6 mice. CD8 T cells are a type of lymphocyte that differentiates during infection to kill infected cells. These cells have an ability to form a memory to prevent reinfection from the same pathogen or prevent reactivation from a latent infection. Like all herpes viruses, Human CMV (HCMV) establishes a life-long infection. It infects 70% of the human population worldwide and can cause severe pathogenesis in individuals with weak immune systems (e.g., newborns, HIV patients, transplant patients, and the elderly). CMV infection in immunocompetent individuals is benign, mostly due to the memory CD8 T cell response that prevents reactivation. CMV is species-specific and has co-evolved with mammals. Human CMV and murine CMV share only 42.5% genome homology (overlapping ORFs), but the T cell response in the latent stage of infection is similar. Therefore, information gathered from studying MCMV CD8 T cell responses can reasonably serve HCMV research.

This thesis started from a surprising finding in the CD8 T cell response to a replication-deficient MCMV infection. The CD8 T cell response to two of the four most immunodominant epitopes was elevated in acute infection of C57BL/6 mice with replication-deficient virus. This result was surprising since the prevailing paradigm is that the magnitude of CD8 T cell response is dependent on the amount of antigen and co-stimulation provided during priming. Typically, an attenuated infection induces a weaker CD8 T cell response (less pathogen recognition = less host response). In fact, in a previous study of MCMV infection in BALB/c mice provided support for this paradigm,

since higher viral titers were associated with greater CMV-specific CD8 T cell responses (Andrews et al., 2010). Therefore, my goal was to understand a mechanism that leads to an elevated—perhaps better—immune response in our C57BL/6 model and gain further insight into the function of CD8 T cells during a viral infection. Our laboratory generated a recombinant virus that is rendered replication-deficient with an antiviral drug. This virus enabled us to investigate relationship between viral titers and the CD8 T cell response.

I found the relationship between the inflammatory response and acute infection and antigen presenting cells (APCs) to be important in dictating the magnitude of the CD8 T cell response to MCMV infection. In particular, I defined how the addition or subtraction of type I IFNs in acute infection could modulate the antigen-specific CD8 T cell response. However, not all epitopes during acute MCMV infection were affected by such inflammation. Type I IFNs depleted cross-presenting APCs (versus direct-presenting) and adversely affected the proliferation and differentiation of those CD8 T cells that are dependent on cross-presented antigen. The type I IFN modulation of cross-presenting DCs may explain why a selected group of MCMV epitopes is enhanced in the immune response to a replication-deficient infection.

In essence, this thesis is about using MCMV infection as a tool to study the basic science of T cell viral immunity. However, it is possible that information gained from this current work could be used to influence the study of immune therapy.

1.2 Cytomegalovirus

1.2.1 Classification

Herpesviridae are a large group of DNA viruses that persist as life-long infections. The root word herpein is Greek for "to creep", which refers to the virus' persistence in the infected host. These are ancient viruses that have been established for over 200 million years and have co-evolved with their hosts (McGeoch et al., 1995), a fact that has resulted in many species-specific herpesviruses. Herpesviruses are divided into 3 subgroups based on genome sequence and biological function: the Alphaherpesviridae, the Betaherpesviridae, and the Gammaherpesviridae. All herpesviruses share characteristics of a linear dsDNA genome encoding 100–200 genes and a virion structure that encapsulates the genome within an enveloped icosahedral capsid (triangulation number 16). Cytomegalovirus belongs to the Betaherpesviridae subfamily, which does not establish latency in non-hematopoietic cells. In comparison, alpha- and gammaherpesviridae viruses establish latency in neurons and B cell lymphocytes, respectively.

Cytomegalovirus has a narrow host range; humans and mice are the natural hosts of HCMV and MCMV, respectively. Cytomegalovirus infects an array of non-hematopoietic cells during acute infection. Infection of leukocytes includes dendritic cells and macrophages, but B and T cells are not infected. It is not completely known which cells host the CMV reservoir during latency, but HCMV is found in the hematopoietic stem cells and myeloid precursor cells (Hahn et al., 1998).

1.2.2 Genome

Cytomegaloviruses have the largest genomes of all other members of the Herpesviridae family (Davison et al., 2003). HCMV and MCMV have approximately 230 and 235 kilobasepairs (kbp), and 192 and 170 open-reading frames (ORFs), respectively (Murphy et al., 2003; Rawlinson et al., 1996). There is approximately 42.5% homology between HCMV and MCMV genomes, with 78-shared ORFs.

Like all herpes viruses, cytomegalovirus uses transcriptional feedback to regulate gene expression. Gene expression is categorized in three stages: immediate-early (IE), early (E), and late (L). IE genes are the first genes to be expressed, occurring during the first six hours post-infection (pi), as they are not dependent on the translation of other gene products. IE genes are important in viral replication as these genes transactivate E genes. Of the three IE genes in MCMV, only IE3 is essential for viral replication, while the depletion of IE1 or IE2 genes limit viral growth (Angulo et al., 2000; Greaves and Mocarski, 1998). The E genes are expressed 4–18 hours pi and encode non-structural proteins only after IE expression. These genes are necessary for the initiation of genomic DNA synthesis. E genes also encode proteins that modulate the immune response. In particular, m152 is an immune evasion gene that causes down regulation of the MHC class I molecule to escape T cell recognition (Del Val et al., 1989). In addition, E gene expression works to inhibit the expression of the IE genes. L gene translation occurs after the replication of the DNA genome, around 12 hours pi. The L genes encode proteins that package the genome and complete the structure of the virion.

1.2.3 Infection rate and transmission

Data from the National Health and Nutrition Examination Survey (NHANES III) show HCMV seropositivity in the United States from 1988–2004. The total population ≥ 6 years old were 58.9% seropositive. Specifically, 6–11 years old were 36.2% seropositive and seropositivity increased to 90.8% in those older than 80 years old (Bate et al., 2010), indicating that seropositivity gradually increases with age. Other demographic factors, including gender, race, and socioeconomic status, were also associated with seropositivity. The virus is disseminated through bodily fluids (i.e., blood, saliva, urine, breast milk, semen, and vaginal fluids), and is therefore easily transmitted during close physical contact. Infection typically occurs early in life; however, the longevity of an individual allows for increased opportunity to come into contact and be infected with HCMV.

1.2.4 Target populations

Cytomegalovirus infection has occurs worldwide, yet massive mortality is not associated with this disease. Immunocompetent individuals can live long lives after infection with a strong humoral and T cell response to limit viral spread and contain the virus during the chronic stage. However, individuals with weak immune systems are susceptible to CMV-related disease, including transplant patients under immunosuppressive drug therapy who lose adaptive immunity, HIV-positive individuals who have an immune degenerative disease, and neonates/newborns who do not have a

fully formed immune system (Boppana et al., 1995; Britt, 1996; Musiani et al., 1988; Stagno et al., 1986). The complications commonly associated with adult CMV infection are pneumonia, retinitis, and colitis.

The NHANES study also revealed data that particular groups of the US population are more likely to contract HCMV infection. For example, females have an increased risk—the CMV seroprevelence in females is 55%, compared to 45% in age-matched males (Bate et al., 2010). Congenital HCMV is the most contracted viral infection for infants, with approximately 1% of all newborns infected. While only 5–10% of the infected newborns are symptomatic, 50–90% of infected newborns go on to develop life-long illness associated with CMV infection. Disabilities from congenital infection include auditory defects, blindness, cerebral palsy, and mental retardation (Revello et al., 2002). Currently, screening for CMV in women is not common practice and has not been endorsed by the American College of Obstetricians and Gynecologists (Carlson et al., 2010). Detection of CMV is through a blood screen (an ELISA) for IgM and IgG anti-CMV antibodies. These tests are accessible, relatively affordable, and accurate only if the test is performed after 3 weeks from initial infection. Mothers who are seropositive before pregnancy are less likely to vertically transmit the virus to the fetus than mothers who become seropositive right before or during pregnancy (Munro et al., 2005; Stagno et al., 1986), as buildup of immunity (e.g., antibodies, T cells) limits viral replication and protects against viral spread to the fetus. HCMV can be transmitted through breast milk, although the benefits of breastfeeding outweigh the risk of transmission. Individuals and mothers who care for young children besides newborns are

also at a higher risk of CMV infection. Cytomegalovirus is shed in the urine: therefore daycare centers and preschools are at-risk environments.

Human CMV can cross the placenta to infect a fetus any time during pregnancy; however, infection at less than 20 weeks of gestation increases the chance of symptomatic CMV pathogenesis of the newborn. Since CMV is species-specific, the study of congenital CMV infection is limited to a comparable animal model. Interestingly, murine and rat CMV are not good models of fetal CMV infection, as they cannot cross the placenta to infect in utero (Johnson, 1969). Rhesus CMV can infect through the placenta, but the cost of and access to non-human primates is a major limitation to further study. Guinea pig CMV (GPCMV) is the ideal model of congenital infection since it is able to pass through the placenta and parallels human in utero infection (Bia et al., 1983). However, limitations the GPCMV infection model is that it does not induce CNS disease as seen in HCMV infection and, in addition, there are an incomplete characterization of the GPCMV genome and immunological reagents (Reddehase, 2013; Woolf et al., 2007).

1.2.5 Viral infection in mice

By far the most used laboratory strains of MCMV are the Smith and K181 strains. MCMV-Smith was first proprogated from a laboratory mouse infected with this wild isolate through colony contamination. K181 is a variant of the Smith strain, isolated from the salivary glands of mice after serial passage in vivo. It is a more virulent strain of Smith strain virus (Misra and Hudson, 1980). To facilitate production of recombinant

viruses, a bacterial artificial chromosome (BAC) of Smith strain was created (Wagner et al., 1999). In this thesis I infected mice with recombinant MCMV-TK and delta (d)m157-MCMV. Both of these viruses are BAC derived. These viruses are listed in the table below.

Table 1: Laboratory viruses

Virus	Isolated
Smith	Salivary gland derived
K181	Variant of Smith strain
MW97.01	BAC derived, Smith strain with the HindIII E' fragment of from K181
MCMV-TK	MW97.01 mutant, HSV-TK in m157 locus
dm157	MW97.01 mutant, disrupted m157 ORF

MCMV is administered to laboratory mice via intravenous (iv), intraperitoneal (ip), subcutaneous, or foot pad injections. These different routes of infection can initiate both local and systemic immune responses. In our laboratory, we primarily inject virus by ip injections. This method causes a systemic spread of the virus throughout the body and infects multiple organs. Within the organs, MCMV can infect stromal cells, epithelial cells, endothelial cells, macrophages, and dendritic cells during acute infection (Hsu et al., 2009; Jordan and Takagi, 1983). However, cytomegalovirus does not infect lymphocytes. HCMV and MCMV infection also spreads to the acinar and glandular epithelial cells of the salivary gland. The salivary gland is an important organ in dissemination of the virus from host to host, as the virus is spread through saliva (Henson and Strano, 1972).

1.2.6 Antiviral drug treatment

Antiviral drug therapy against HCMV infection is limited to five approved drugs to treat immunocompromised patients: Ganciclovir, Foscarnet, Cidofivir, Acyclovir, and Fomivirsen (Biron, 2006). With the exception of Fomivirsen, all of these drugs target viral replication to inhibit viral DNA polymerase, or act as a DNA chain terminator during viral genome replication. Fomivirsen is an antisense RNA that inhibits the translation of the essential immediate early 2 gene of CMV. Fomivirsen is approved for local treatment of CMV retinitis. Foscarnet is a pyrophosphate analog that inhibits viral DNA polymerase by attaching to the pyrophosphate-binding site and prevents the cleavage of pyrophosphate from the terminal nucleoside triphosphate in a growing viral

DNA chain. Ganciclovir, Cidofivir, and Acyclovir are nucleoside analogs that are activated by endogenous kinases or through viral kinases. Cidofivir functions by binding to the viral DNA polymerase and blocks the progression of viral genome replication. Because Cidofivir is activated by endogenous kinases, it also has efficacy in other viral infections. Ganciclovir and Acyclovir are nucleoside analogs that are efficiently activated by virus-transcribed kinases and weakly by endogenous kinases. These drugs are nucleoside analogs that block the function of the viral polymerase and ultimately act as a DNA chain terminator.

In my research for this thesis, I used the antiviral drug Famcyclovir to limit viral replication in a recombinant MCMV-TK. Famcyclovir is a prodrug to Pencyclovir, which belongs to the family of nucleoside analog drugs. Like Acyclovir, Famcyclovir is phosphorylated by HSV-1 thymidine kinase. Phosphorylation of Famcyclovir causes the incorporation of this molecule into a viral genome undergoing replication and prevents subsequent binding of nucleotides, thus inhibiting the complete synthesis of a viral genome. Early inhibition of MCMV-TK replication is achieved with Famcyclovir treatment prior to infection. Viral titers are undetectable at 3 days post-infection with this drug regimen (Snyder et al., 2011).

1.3 Immunology

1.3.1 General T cell Biology

1.3.1.1 Overview

T cells are a population of lymphocytes within the adaptive arm of the immune system. T cells are bone-marrow-derived cells that mature in the thymus, hence the term

"T cells". Like B cells, T cells are a diverse population, expressing polymorphic receptors that are able to recognize antigens from many different pathogens and foreign antigens. The T cell receptor (TCR) is responsible for the recognition of peptides bound to a major histocompatibility complex class I or class II molecule. In the thymus, T cells undergo positive and negative selection, in which T cells that are inert or over-reactive to self-antigen are excluded from the population. T cells are divided into two main subsets by the expression of surface glycoproteins: CD4 and CD8. The CD8 molecule is non-polymorphic, unlike the TCR. It binds as an essential accessory molecule to the MHC class I molecule and aids the stability of the TCR-MHC class I interaction, while the CD4 molecule binds as an accessory molecule to the MHC class II molecule (Kaplan et al., 1989). The CD8 molecule is important not only to the stability of the TCR-MHC interaction, but also to the intracellular orchestration of bringing tyrosine kinase, Lck, to the TCR to mitigate intracellular signaling (Veillette et al., 1988). When naïve CD8 T cells are primed in the acute stage of an infection (details of priming are discussed in the following sections), they become activated, proliferate to large numbers and undergo differentiation. Activation of CD8 T cells causes changes to their surface marker phenotype, loss of L-selectin (CD62L), and upregulation of CD44 (Kaech et al., 2002a). These changes to cell surface molecules are also true for CD4 T cells. $CD62L^{lo}$ and $CD44^{hi}$ expression helps define effector CD8 T cells. These cells migrate to sites of inflammation and infection. The re-expression of CD62L helps define memory CD8 T cells, and these cells egress to secondary lymphoid tissues (Weninger et al., 2001). Differentiation transforms CD8 T cells into effector cells that are able to kill target cells and secrete cytokines. When the pathogen is controlled or cleared, Ag-specific CD8 T

cells form a long-lasting memory population that is able to respond to a secondary challenge without the need for co-stimulation (in particular, CD28) if TCR stimulation is high, although T cell activation is tunable and must meet a threshold in order to become activated (Viola and Lanzavecchia, 1996). Co-stimulation in memory responses lowers this threshold and aid in the recall response (Boesteanu and Katsikis, 2009).

1.3.1.2 T cell receptor

Activation of the TCR transduces an intracellular signal and initiates the differentiation of a naïve T cell or a rapid secondary response from a memory cell. The structure of the TCR complex recognizes both the MHC molecule and bound peptide. The TCR is a heterodimer consisting of an α and β chain, which define the prominent CD8 T cell population, or a γ and δ chain (gamma-delta T cell). The α/β TCR has a constant and variable region. The variable region contains three complementary-determining regions (CDRs); CDR1 and CDR3 recognize the peptide-MHC class I complex, and the CDR2 recognizes the conserved region of the MHC class molecule.

Associated with the TCR is the CD3 complex, which consists of six transmembrane chains. The CD3ε/δ and CDγ/ε flank the TCR but the CD3ζ/ζ chains are associated with the TCR transmembrane domains and transduce intracellular activation signals (Janeway 2007).

1.3.1.3 MHC class I

A CD8 T cell responds to viral antigen only if it is presented on a major histocompatibility complex (MHC) class I molecule. MHC class-I is expressed by most

nucleated cells, and is encoded by the MHC complex on chromosome 6 and 17 in humans and mice, respectively (Pamer and Cresswell, 1998). MHC class I is made of two subunits that consist of a polymorphic heavy chain (HC) and a nonpolymorphic light chain β2-microglobulin (β2-m). Within the HC polypeptide are three functional units: α1, α2, α3. The α3 domain is an extracellular domain, but it extends as a transmembrane protein that anchors the MHC class I molecule to the cell surface. The β2m domain binds primarily to the α3 domain and provides stability to the MHC complex. The α1 and α2 domains of the HC are protein structures that contain alpha helices on top of β-pleated sheets, which in turn create a binding groove where a viral peptide can associate. The binding groove accepts peptides of 8–10 amino acids in length and contains defined binding regions that allow the C- and N-terminus of peptides to bind deep into the groove. The amino acid sequence of the peptide determines the strength of binding to the MHC class I complex. Peptides contain "anchor" positions—positions of the amino acid sequence that bind to particular pockets with greater affinity in the binding groove. For example, the murine MHC class I molecule H-2Kb binds with greater affinity to peptides that contain tyrosine or phenylalanine at position 5 of an 8-amino-acid-long peptide (Johansen et al., 1997).

1.3.1.4 MHC class I pathway

In the cytosol, viral proteins must be processed into smaller peptides for correct and stable binding to MHC molecules. The proteasome is a large cylindrical complex that degrades polypeptides in smaller peptides. The proteasome consists of 14 structural and 6 catalytic subunits. The catalytic subunits are two β1, two β2, and two β5. It is considered a multicatalytic protease since the barrel-shaped 20S complex uses the proteolytic active

sites to break the bonds between amino acids in an ATP-independent fashion. Polypeptides are typically tagged with ubiquitin in the cytosol, which labels them to be sent to the proteasome. The end products are peptides of 8–40 amino acids in length.

In the absence of an infection, cells express the constitutive proteasome. The primary function of the constitutive proteasome is to process self-antigens that are not needed or damaged. During an infection, the inflammatory response to the pathogen produces an alternative proteasome, called the immunoproteasome, with altered catalytic subunits: β1i (LMP2 or Psmb9), β2i (MECL1 or Psmb10), and β5i (LMP7 or Psmb8) (Coux et al., 1996). The conversion to the immunoproteasome is IFN-γ dependent (Tanaka, 1994), although there is a low expression of the immunoproteasome in the absence of an infection. Peptides produced by the immunoproteasome are highly immunogenic, as immunoproteasome deficient mice are severely deficient in MHC class-I epitope expression (Kincaid et al., 2012).

From the cytosol, the peptides get transported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) protein, a heterodimeric, transmembrane, ATP-dependent protein (Pamer and Cresswell, 1998). TAP is essential for transporting these peptides to the ER, and once in the ER, peptides of lengths longer than 10 amino acids are further processed and trimmed by the endoplasmic reticulum amino-peptidase (ERAAP) molecule (Rock et al., 2004) to achieve the optimal 8–10 amino acid peptide length. The ER is where peptides associate with MHC class I to form a stable complex.

However, before the MHC class I molecule can bind to a peptide, it must go through a series of dedicated steps to stabilize and conform to a structure that is able to

bind to the peptide. First, before the binding of β2m and peptide, the three α-domains are flanked by the chaperone protein, Calnexin, to help stabilize the conformation before β2m binds. Second, with the binding of β2m, Calnexin disassociates from the α domains, thus allowing chaperone proteins, Calreticulin and ERp57, to bind. The initiation of the MHC class I peptide-loading complex is the third step; the MHC class I, β2m, and chaperone proteins move to associate with the Tapasin protein, which bridges the MHC class I complex with TAP. At this step, the MHC class I molecule and the TAP protein form the MHC class I peptide-loading complex. Fourth, the peptide associates with the MHC class I molecule, and together they disassociate from the peptide-loading complex. From here, the peptide-MHC class I complex (pMHC) proceeds through the Golgi via vesicular transport until it reaches the cell surface.

1.3.1.5 Cross-presentation

There are two major mechanisms for presenting antigen via MHC class I to CD8 T cells: direct presentation by infected cells and cross-presentation by uninfected APCs. The MHC class I pathway described in the previous section is a direct presentation of viral peptides. Cross-presentation is an alternative pathway to present viral antigen via MHC class I in which exogenous protein (e.g., viral debris) is phagocytosised. Because cross-presentation is performed by uninfected APCs, they are not subjected to immune evasion mechanisms that are associated with particular viral infections (e.g., CMV encoded genes that downregulate MHC class I from the cell surface). Therefore, cross-presentation is an important mechanism for the activation and priming of CD8 T cells. In MCMV infection, the priming of CD8 T cells is mostly performed by cross-presenting APCs (Busche et al., 2013).

Cross-presentation can traffic acquired antigen in two distinct pathways: TAP-independent and TAP-dependent. In the TAP-independent pathway, phagocytosis of cellular or viral debris packages the protein into a phagosome inside the cell. Within the phagosome, the protein is processed into a peptide by protease cleavage (via Cathepsin S) (Rock and Shen, 2005). Since the protein is in a phagosome it is not exposed to the cytosol or proteasome activity. Therefore, this pathway is a TAP-independent method to process optimal peptides to be loaded on the MHC class I molecule. However, the mechanism of peptide loading to the MHC class I molecule is not clearly defined.

The TAP-dependent pathway of cross-presentation is similar to the process of the classical MHC class I processing, described above for direct presentation (Lin et al., 2008). Phagosomal release of viral polypeptides into the cytosol allows for proteasomal cleavage and TAP-mediated transport out of the cytosol; however, the pathway to load the peptide onto the MHC class I molecule to the surface is also unclear. There are two generally accepted TAP-dependent pathways to bring a peptide to a MHC molecule. TAP can export peptides to the ER, which can then load with the MHC class I molecule. Alternatively, TAP can export peptides back into a phagosome where it can associate with MHC class I (Rock and Shen, 2005).

1.3.2 Dendritic Cells

Paul Langerhans was the first to identify a subset of human skin cell to be neuron-like based on their similar morphology. A century later, Steinman and Cohn identified a similar population of cells in the peripheral lymphoid tissue of mice and classified them

as “dendritic” cells (DCs) by their long, finger-like morphology (Steinman and Cohn, 1973). DCs are a bone-marrow-derived, heterogeneous population. They make up only a small percentage of human peripheral blood and mouse spleen, yet they have profound effects on coordinating the innate immune and antiviral T cell response to viral infections. Ultimately, DCs act as a reconnaissance leukocyte population, sampling the environment to communicate messages back to lymphocytes by the presentation of peptide on their cell surface. Steinman called DCs “nature’s adjuvant” for their ability to initiate and coordinate immunity to infections. My evaluation of DCs in this thesis is intended to understand what role DCs play in the enhanced CD8 T cell response to a replication-deficient MCMV infection.

In humans and mice, the diversity and function of DCs are still being resolved. DCs can be classified into subgroups depending on tissue location, cytokine production, expression of co-stimulatory molecules, and morphology (Steinman et al., 1997). In mice, DC populations can be segmented into 4 subsets. The first subset is Langerhans cells, which are present in skin tissue. The second and third subsets are myeloid-derived cells, distinguished by the expression of CD8α and CD11b (CD8α+ versus CD8α-CD11b+). These myeloid cells are termed “conventional” DCs (cDCs), as they resemble the dendritic cells first discovered by Steinman. The fourth DC subset is the plasmacytoid DCs (pDCs), which are defined by their plasma-cell-like morphology, intermediate expression of CD11c, and their ability to secrete very large amounts of type I IFNs in response to infections (Colonna et al., 2002; Kadowaki et al., 2000).

Plasmacytoid DCs are mostly found in the blood and lymphoid tissues. They have a narrow expression of toll-like receptors, just TLR7 and TLR9. In contrast, cDCs are

distributed throughout the body and express a wide array of TLRs (Merad et al., 2013). Despite upregulating MHC and co-stimulatory molecules after being activated, pDCs are not considered good APCs as they do not present antigen as well as their cDC counterpart. The role of pDCs in immunity is mostly attributed to their release of type I IFNs to limit pathogen virulence and to help with the differentiation of CD8 T cells.

Dendritic cells are termed "professional" APCs due to their ability to present antigen via MHC class II, in addition to MHC class I. Furthermore, activated DCs highly express co-stimulatory molecules. Presentation of self-antigen by resting DCs induces anergy and tolerance of CD8 T cells (Steinman et al., 2000), and thymus-resident cDCs aid in negative selection during T cell maturation (Proietto et al., 2008). Conventional DCs highly express CD11c and MHC class II molecules. Both subsets of cDCs are able to take up soluble antigen; however, the CD8+ cDCs are more efficient than pDCs at presenting processed exogenous antigen on the cell surface via MHC class I (Iyoda et al., 2002; Schnorrer et al., 2006). Thus, CD8+ cDCs are most actively involved in cross-presentation. All nucleated cells express MHC class I and can present antigen to CD8 T cells, but the ability to rapidly increase the MHC and co-stimulatory molecules makes cDCs an important immune population as they function as a sentinel to alert the adaptive immune system to an infection.

1.3.3 Type I Interferon

1.3.3.1 Overview

Interferons (IFNs) are a family of soluble proteins discovered in the 1950s as proteins that exhibit protection against viral infections. There are three classes of IFNs: type I, type II, and type III. Type III IFNs are comprised of a family of IFN-λ proteins. They are widely distributed with limited tissue specificity and are not highly expressed in hematopoietic cells. Type II IFNs consists of a single gene product, IFN-γ. IFN-γ is a well-defined cytokine with regulatory properties including inhibiting viral replication. NK and T cells produce IFN-γ in response to a viral infection. Type I IFNs are a family of proteins that bind to a common IFNα/β receptor. In humans, type I IFNs consist of IFN-α, IFN-β, and IFN-ω. However, the IFN-α and IFN-β molecules are the prominent cytokines of this class. In both humans and mice, the IFN-α family consists of more than 12 IFN-α genes, while the IFN-β family contains a single gene (van Pesch et al., 2004).

With the appropriate stimulation, most hematopoietic and non-hematopoietic cells can produce type I IFNs. Depending on the location and activation of non-hematopoietic cells, they can provide a very important role in the antiviral response. The proprogation of type I IFNs is via a positive feedback loop because the release of IFN-β binds to IFNα/β receptors to initiate the transcription of IFN-α, which then continues to bind to the receptor (Stark et al., 1998). However, hematopoietic cells are the main source of type I IFNs, since pDC produce 10^2–10^3 times more type I IFNs than any other IFN-producing cells (Siegal et al., 1999). In this thesis I am focusing the role of type I IFNs and their role in regulating the adaptive immune response to MCMV infection.

1.3.3.2 IFNα/β receptor and signal transduction

All type I IFNs bind to a common heterodimer, IFNα/β receptor. The IFNα/β receptor is composed of two chains, IFNAR1 and IFNAR2, which are associated when bound to IFN-α or IFN-β. IFNAR1 is associated with the Janus family kinase Tyk2; and IFNAR2 is associated with Jak1, STAT1, and STAT2 (Signal Transducer and Activators of Transcription). Binding of IFNα/β brings together the chains of the receptor to activate Jak1 and Tyk2, which result in the heterodimerization of STAT1 and STAT2. Activation of STAT1-STAT2 causes its release from the receptor and migration to the nucleus (Stark et al., 1998). Once inside the nucleus, STAT1-STAT2 associates with transcription factor IFN regulatory factor 9 (IRF9) to form the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISRE) to express IFN stimulatory genes (ISGs) (Platanias, 2005).

1.3.3.3 Production of type I IFNs

Type I IFN is typically produced when intracellular or cell surface receptors recognize pathogens. Recognition of pathogens is most often in a form of pathogen-associated molecular patterns (PAMPs). PAMPs are distinct motifs of nucleic acid or molecules uniquely different from the host and specific to a pathogen. The receptors that recognize PAMPs are pathogen-recognition receptors (PRRs). Toll-like receptors (TLRs) and RIG-I like receptors (RLRs) recognize viral nucleic acid. Other PRRs can recognize an array of foreign molecules from pathogens (e.g., LPS, fungal metabolites). These receptors are expressed on the surface, endosomal, or in cytosolic compartments of cells to detect pathogens on initial contact or during replication (Akira and Hemmi, 2003;

Akira and Takeda, 2004; Loo and Gale, 2011). In particular, TLR3 is an endosomal transmembrane receptor that recognizes double-stranded RNA (e.g., from a replicating virus) or a synthetic molecule poly(I:C). Activation of TLR3 initiates a cascade of signaling proteins beginning with the adaptor molecule TRIF, which then associates with TBK1. TBK1 in turn activates IRF3, which migrates into the nucleus to transcribe high amounts of IFN-β. RIG-I and Mda5 are RNA helicases that recognize mitochondria-localized adaptor molecule IPS-1 or MAVS through their caspase recruitment domain (CARD). This complex then associates and activates the IRF3 molecule, which then leads to IFN-β induction (Kawai and Akira, 2006; Loo and Gale, 2011).

In MCMV infection, the nucleic acid material from the dsDNA genome, as well as genomic intermediates (e.g., ssDNA), is recognized by TLR3, TLR7, and TLR9. This recognition initiates the production of type I IFNs during acute infection (Krug et al., 2004; Tabeta et al., 2004; Zucchini et al., 2008).

1.3.3.4 Functions of type I IFNs

Type I IFNs are pleiotropic proteins that initiate multiple intracellular signaling pathways and ultimately activate hundreds of genes (Platanias, 2005). Therefore, the function of type I IFNs is broad as they defend against many viral and bacterial infections (Biron, 2001; Muller et al., 1994), as well as suppressing and activating the immune cells.

Type I IFNs directly impact the ability of a virus to replicate by initiating mechanisms to inhibit protein synthesis or to degrade viral RNA. For example, mice deficient in the IFN-α/β receptor have elevated viral titers and early mortality in LCMV, VSV, and MCMV (van den Broek et al., 1995). Two major products of type I IFN signaling are protein kinase R (PKR) and 2'–5' Oligoadenylate Synthetases (OAS).

These products lead to the inhibition of viral protein synthesis and degradation of viral mRNA, respectively.

In addition to blocking viral replication, they enhance antiviral immunity. In particular, type I IFNs increase the primary antibody response and maturation of antigen presenting cells (APCs) (Stark et al., 1998). These APCs provide additional co-stimulatory molecules and MHC class I expression to elicit the adaptive CD8 T cell response. In particular, type I IFNs upregulate co-stimulatory molecules (e.g., CD80 and CD86) and increase expression of MHC. Type I IFNs have a regulatory role in cell growth and differentiation of lymphocytes, including macrophages (Lin et al., 1998), dendritic cells, and CD8 T cells. As noted below, type I IFNs are a Signal 3 molecule (Mescher et al., 2006). CD8 T cells express the IFNα/β receptor, thus type I IFNs directly act on these cells to influence the generation of Ag-specific CD8 T cells (Gil et al., 2006; Kolumam et al., 2005a). However, type I IFNs can induce apoptosis in cells. In particular, type I IFNs affect the numbers of bystander CD8 T cells during a viral infection by depleting these cells to clear space for Ag-specific CD8 T cells to proliferate and develop (McNally et al., 2001). Thus, while inducing a reduction of the CD8 T cell population, the overall anti-viral CD8 T cell response to an infection benefits from the extra "space" generated by the actions of type I IFNs.

1.3.4 CD4 T cell response in viral Infection

CD4 T cells have significant supporting roles in the generation of the adaptive immune response. CD4 T cells promote affinity maturation and antibody class switching

by B cells, and they enhance the antigen presentation and co-stimulatory molecules on DCs. Most CD4 T cells are T-helper (T_h) cells, although some maybe cytotoxic cells. Within the T_h group there are multiple subgroups that are defined by their function, expression of transcription factors, and release of cytokines. These subgroups of T_h cells vary in location and function, and help with the activation and maturation of CD8 T cells indirectly. T_h cells provide "CD4 help" by transmitting a signal (e.g., CD40-CD40L, CD27-CD70 interactions) to B cells or APCs (Bennett et al., 1998; Feau et al., 2012; Schoenberger et al., 1998). This in turn helps APCs activate CD8 T cells during priming. CD4 T cells also secrete IL-2 that promotes proliferation of CD8+ T cells. Th_1 CD4 T cells also have direct antiviral function by secreting cytokines such as IFN-γ and TNF-α that inhibit viral replication.

1.3.5 Humoral Response in viral Infection

The humoral response is important in the defense against viral infection, viral spread, and prevention against pathogenesis. Antibodies can either be surface-bound on B cells or soluble. They can bind directly to virions or to antigens expressed on the surface of an infected cell. Class switching from one isotype to another (e.g., IgM to IgG) can occur, as isotypes can determine the function of the antibody. The binding of antibodies directly on pathogens protects host cells from infection by preventing pathogen-cell interaction. These antibodies are termed "neutralizing antibodies."

1.3.6 CD8 T cell priming

Priming involves two primary signals that a T cell must receive to move from a naïve state into an activated state where it can proliferate and differentiate into effector and memory cells. Through the TCR, a naïve CD8 T cell must recognize cognate antigen when it is presented in a MHC class I complex on the surface of an antigen-presenting cell (most often from a dendritic cell). This T cell recognition of antigen is termed Signal 1. The secondary signal a naïve CD8 T cell must receive is a verification signal, which tells a CD8 T cell that recognition of the foreign peptide is valid. This secondary signal, termed Signal 2, is co-stimulation generally described as being B7.1 and B7.2 (CD80 and CD86) molecules from a DC to the CD28 molecule on the T cell. Ligation of CD28, along with TCR-MHC class I binding, allows for chromatin remodeling, transcription, and translation of genes in activated CD8 T cells. One result of co-stimulation is the production of IL-2 and the induction of the high affinity IL-2 receptor (CD25) on the cell surface of the CD8 T cell (Janeway 2007). All together, this promotes differentiation and proliferation of T lymphocytes after priming. Signal 2 is essential to CD8 T cell priming as T cells that receive Signal 1 without Signal 2 are destined to be anergic, unresponsive to subsequent activation signals.

Signal 3 is an additional co-stimulatory event not necessary for priming, but recognized as an important stimulatory component in driving CD8 T cell proliferation and memory differentiation. The role of signal 3 on the differentiation of CD8 T cells was described in OT-1 transgenic mice crossed to IFNα/β receptor KO mice (Curtsinger et al., 2005). These mice were stimulated in vitro with OVA/B7 microspheres in the presence or absence of IFN-α or IL-12. IFNα/β receptor KO CD8 T cells proliferated, but the clonal expansion and survival of these CD8 T cells was poor in comparison to CD8 T cells

expressing a functional IFNα/β receptor. Stimulation in the presence of an IL-12 neutralizating antibody was also detrimental to the development and survival of CD8 T cells, indicating redundant signal 3 pathways and the importance of signal 3 to the differentiation of CD8 T cells. Signal 3 co-stimulation is via type I IFNs, IL-12, IL-21, as well as signaling through TNF receptor family proteins (e.g., OX-40, 4-1BB, and CD70).

Differentiation of CD8 T cells in an acute viral infection can occur through an antigen-dependent or antigen-independent pathway (Badovinac and Harty, 2002), meaning that CD8 T cell differentiation can develop without the continuation of antigen after initial priming. This characteristic suggests that CD8 T cell differentiation is imprinted at initial priming events – meaning that CD8 T cells will develop a differentiation program without the additional stimulation. Badovinac et al. show that a mouse infection with Listeria monocytogenes, which is cleared with antibiotics at 24 hours pi, has the same kinetics of expansion and contraction as an active LM infection (Badovinac and Harty, 2002), although the antibiotic treatment attenuates the size of the CD8 T cell response. The existence of cytokines adds another layer to this process, as some cytokines help to condition T cells to expand, contract, or differentiate. However, the functions of cytokines in a viral infection are not completely understood. Perforin and IFN-γ have been reported to have a role independent from their anti-microbial function. Specifically, infection of perforin- and IFN-γ-knockout mice results in the expansion of the CD8 T cell response and changes the immunodominant epitopes to acute Listeria monocytogenes infection (Badovinac et al., 2000). Type I IFNs are another inflammatory cytokine released in response to a viral infection. They interfere with fitness of the virus but can also have effects on APCs' maturation and direct binding to CD8 T cells for

differentiation.

1.3.7 Antigen dose and the size of the CD8 T cell response

The amount of antigen presented on the surface of an APC affects the magnitude of the CD8 T cell response to a viral or bacterial infection. Wherry et al. related the cytotoxic T cell response to the density of epitope expression on the surface of an APC by using a recombinant vaccinia virus that expresses stratified amounts of presented GP-33 peptide (Wherry et al., 1999). Their results show that increasing the levels of antigen drives greater Ag-specific CD8 T cell responses. Both Badovinac et al. and Joshi et al. extended this work, as increasing or decreasing the antigen load during a *Listeria monocytogenes* (LM) infection alters the CD8 T cell response (Badovinac et al., 2002; Joshi et al., 2007). They show that mice infected with LM expressing LLO antigen or GP-33 (antigen from LCMV) generate robust Ag-specific CD8 T cells. Administration of ampicillin 1-day pi completely clears the bacterial infection, and in consequence, the peak acute CD8 T cell response to these antigens is decreased. Overall, these reports show that the size of the CD8 T cell response to infection (viral or bacterial) is dependent on the amount of antigen produced during acute infection.

1.3.8 CD8 T cell priming and differentiation

All T cells are antigen-specific. However, priming and differentiating of CD8 T cells into effector and memory cells changes their function and response to the recognition of a foreign peptide. After naïve CD8 T cells receive a priming event of

signal 1 and 2, they progress through a series of phases that differentiate them into an effector and memory population. Priming causes T cells to become activated, expand (proliferate), contract (die), and remain as a small but stable memory population (Kaech et al., 2002b). As a result, it is generally accepted that a greater number of differentiated long-lasting memory CD8 T cells that are specific against a particular pathogen will provide better immunity. Therefore, understanding the factors that drive differentiation and the magnitude of Ag-specific CD8 T cells is important to the field of vaccine development.

T-bet is an important transcription factor that differentiates naïve T cells into type 1, IFN-γ producing cells. T-bet is classically known as a CD4 T cell transcription factor that drives differentiation of naïve CD4 T cells into Th_1 cells (Szabo et al., 2000). In comparison, the transcription factor GATA-3 drives the differentiation of CD4 T cells into an IL-4 producing Th_2 cells. T-bet also has the remarkable ability to induce Th_2 cells to differentiate into Th_1 cells (Szabo et al., 2000). More recently, T-bet has been discovered to play an important role in the differentiation of CD8 T cells into IFN-γ producing cells during a vial infection, as well as into specific effector cell subsets (Joshi et al., 2007). In particular, T-bet drives CD8 T cells into a short-lived effector cell (SLEC) population, expressing killer cell-like receptor group member-1 (KLRG1) and a low expression of the IL-7 receptor (CD127). This finding was discovered in T-bet-/-, T-bet-/+, and T-bet+/+ mice, where increasing the T-bet expression lead to a greater SLEC phenotype (Joshi et al., 2007). Functionally, SLECs are effector cells that are cytolytic, turn over faster, have a weaker proliferation capacity, and die after the infection is cleared. In contrast to SLEC are the memory precursor effector cells (MPECs). MPECs

are KLRG1^{lo}CD127^{hi}, have a greater proliferative capacity, and give rise to the long-lived memory population. Using the cell surface marker of KLRG1 and CD127 is a useful tool to measure the differentiation status of CD8 T cell during and after the priming stage in both acute and chronic infection.

1.4 Immunity to CMV

1.4.1 Overview

Research on CMV is important not only for the prevention of CMV pathogenesis, it also provides an opportunity to study the antiviral immune response. For instance, since populations acquire CMV at different stages in life, research can address questions related to how the immune system ages, responds to co-infection, and forms long-lasting immunity. Research on primary HCMV infection is difficult since most infections are undetected, and testing for CMV seropositivity is mostly restricted to at-risk groups (e.g., immune-compromised individuals, patients of reproduction health clinics). In the research that formed the basis for this thesis I investigated the CD8 T cell response to an acute MCMV infection. While little is known about the acute CD8 T cell response to HCMV infection, the chronic response is well established. A hallmark of chronic HCMV infection is the massive frequency of CD8 T cells that are devoted to recognizing CMV—~10% of total CD8 T cells are antigen-specific. MCMV is similar to HCMV in this regard, as it also elicits a massive chronic antigen-specific response. While it is difficult to compare the acute CD8 T cell response of MCMV to HCMV infection, there is good reason to believe that many factors that affect the priming and differentiation of naïve CD8 T cells to memory T cells can be related back to HCMV.

It is well established that individuals with weak or declining immune systems are susceptible to CMV infection and pathogenesis, although the immunological determinants that predispose individuals to disease is not completely understood. However, antibodies, innate cells (Natural Killer cells), T lymphocytes (CD4 and CD8), and antiviral cytokines (Type I IFNs) have all shown significant contributions to the attenuation and control of CMV replication and pathogenesis.

1.4.2 Cytokine response

Orange and Biron (Orange and Biron, 1996) characterized the systemic cytokine response to MCMV infection in mice. The cytokine response in MCMV infection happens predominantly very early during acute infection, before T cells become activated, indicating that initial recognition and viral-replication are strong initiators of this response. The release of cytokines occurs in waves. There are cytokines that are detected hours or days pi, and the release can peak and crash rapidly or be sustained over a period of time.

Orange et al. discovered that acute MCMV infection in C57BL/6 mice induces strong IFN-α/β, IFN-γ, TNF-α, and IL-12 levels in the serum (Orange and Biron, 1996). These responses peaked around 2–3 days pi and, interestingly, all of these cytokine responses, except IFN-γ, were NK and T cell independent. The early IFN-γ response was dependent on NK cells and neutralizing IL-12 completely negated the IFN-γ response. Following this work, other reports have identified the cytokines IL-6, IL-1, IL-10, IL-18, and hormone corticosterone as being immunoregulatory during acute infection (Lee et al.,

2009; Ruzek et al., 1997; Ruzek et al., 1999). The peak responses of these cytokines typically occur around 36–40 hours pi and the kinetics of these cytokines are not consistent. In particular, IL-6, TNF-α, and IL-18 have longer responses as measured in the serum, lasting a few days at elevated levels. This is in contrast to type I IFNs, which have a biphasic response, or IL-10, which has short and marginal response (Lee et al., 2009).

In MCMV acute infection, the kinetics of type I IFNs is a biphasic response (Schneider et al., 2008). Specifically looking at IFN-α levels, an initial response occurs at 8 hours pi and decreases at 24 hours pi until a secondary IFN-α response occurs at 36–48 hours pi. The initial acute IFN-α response to MCMV was considered a TLR response to MCMV PAMPs (Delale et al., 2005). However, this conclusion has reassessed, at least in the spleen, where the initial IFN-α response is due to the ligation by lymphotoxin-β (LTβ) on B cells of the lymphotoxin receptor-β (LTβR) on the stromal cells (Benedict et al., 2001). In contrast, the second peak of the IFN-α response is TLR dependent. MCMV infection of TLR9-/- shows a normal first peak of IFN-α and an attenuated secondary response (Tabeta et al., 2004).

Contrary to reports that type I IFNs are beneficial to the CD8 T cell response in other viral infections (Curtsinger et al., 1999; Curtsinger et al., 2005; Kolumam et al., 2005b), a more recent report indicates that NK cell control of MCMV replication causes type I IFN levels to be lowered and CD8 T cell responses to be enhanced (Robbins et al., 2007; Stadnisky et al., 2011). Therefore, understanding the kinetics and levels of type I IFNs released in a MCMV infection and how blocking viral-replication of MCMV affects the type I IFN and CD8 T cell response is a primary focus of this thesis.

1.4.3 Role of Dendritic cells

One of the most important functions of DCs is to prime CD8 T cells during the acute phase of an infection or after a vaccination. DCs are susceptible to infection and have the ability to prime naïve CD8 T cells via direct presentation. However, the quality of DC priming is severely reduced, as the infection lowers the expression of MHC class I and co-stimulatory molecules (e.g., CD80 and CD86). Thus, infected DCs have a suppressive interaction with CD8 T cells by supplying low levels of signal 1 and signal 2 (Andrews et al., 2001). Immune evasion genes can partially explain the reduced expression of MHC class I molecules. MCMV expresses immune evasion genes m06 and m152, which associate with the MHC class I molecule to redirect the molecule to a lysosome for degradation or retention of the MHC molecule in the ER-Golgi compartment, respectively (Reusch et al., 1999; Ziegler et al., 1997). In addition to a lower expression of MHC class I and co-stimulatory molecules, MCMV infection also causes DCs to have a dysfunction in the secretion of cytokines. In particular, stimulation of infected DCs with LPS causes a reduced IL-12 and IL-2 response in vitro, in comparison to naïve DCs (Andrews et al., 2001).

Cross-presenting cDCs have been recognized as the preeminent APC to prime naïve CD8 T cells (Busche et al., 2013; Snyder et al., 2010; Torti et al., 2011). Because cross-presented cells are non-infected APCs, these cells are not susceptible to immune evasion genes mechanisms or CMV-induced suppression.

1.4.4 NK cell response

NK cells are effector lymphocytes that are able to kill tumor and virally infected cells. They are bone-marrow-derived and stem from the same lymphoid cell progenitors as B and T cells, although they do not share the same cellular marker phenotype. Importantly, they do not express a polymorphic T cell or B cell receptor. NK cells have the ability to recognize cells that lack MHC class I in a process described as identifying "missing self" (Karre et al., 1986). Thus, the NK cell response is particularly important in HCMV and MCMV, as both viruses encode immune evasion genes that downregulate MHC molecules to evade T cell immunity (Doom and Hill, 2008; Powers et al., 2008). Furthermore, the NK cell response to MCMV is important during the acute stage of the infection, as the CD8 T cell response to infection is not immediate.

While it is not completely known how NK cells protect against HCMV, individuals without NK cells or NK function are susceptible to herpes infections (Biron et al., 1989). However, the expression of activation and inhibitory receptors on the cell surface of NK cells dictate NK cell functionality (Lanier, 1998). Humans and mice express a family of receptors that activate or inhibit NK cell effector function—KIR and Ly49 receptors, respectively. Ly49 is a family of genes under the Natural Killer cell complex (NKC) on chromosome 6 that codes for activation or inhibitory receptors (Scalzo et al., 1995). In particular, NK cells in C57BL/6 express activation receptor Ly49H that identifies the MCMV protein m157. m157 is a MHC class I-like molecule, although it does not associate with β2m. m157 is expressed on the cell surface of an infected cell and ligation to Ly49H causes an NK cell response to kill infected cells, which subsequently lowers viral titers (Arase et al., 2002; Smith et al., 2002). Despite the

similarity of m157 to MHC class I, immune evasion genes do not affect the surface cell expression (Tripathy et al., 2006). Most laboratory strains of MCMV express m157 (e.g., Smith, K181), however, most wild isolates do not. Furthermore, most strains of mice do not express Ly49H. In particular, C57BL/6 express Ly49H, but BALB/c mice do not.

A second resistance locus was mapped to H-2^k mice, independent of the m157-Ly49H interaction. This resistance is caused by the D^k MHC class I molecules on infected cells interacting with Ly49P and Ly49G2 molecules on NK cells{Stadnisky et al., 2011, #14377; Xie et al., 2009, #9756}. Ly49P is an NK cell activating receptor that recognizes MCMV gp34 in association with D^k. Ly49G2 is also a NK cell receptor where the ligand is not clearly known. However, mice that lack D^k or Ly49G2 are more susceptible to MCMV infection. Table 2 is a list of mice resistant and susceptible to MCMV infection based on NK cell effector function.

Table 2: Resistant and Susceptible mouse strains to MCMV

Mouse	**H-2**	**Resistance**
MA/My	k	H-2^k
C3H	k	H-2^k
CBA	k	H-2^k
BALB.K	k	H-2^k
C57BL/6	b	ly49H
C57L.M-H2k(R7)	k	H-2^k
Mouse	**H-2**	**Susceptible**
BALB/c	**d**	**ly49H null**
C57L.M-H2k(R2)	**k**	**Lack activating H-2^k**
IFNα/βR -/-		**Lack IFNα/β receptor**

1.4.5 T cell response in MCMV infection

1.4.5.1 Overview

During the first few days of MCMV infection, as the virus replicates and spreads, the innate immune mechanisms are the first to challenge the virus. In C57BL/6 mice, type I IFNs and the NK cell response limits viral replication and spread of the virus in the host. However, the innate immune response to primary infection does not prevent latent infection. Infectious virions can be detected by day 3 pi at the same time as the splenic architecture is remodeled—specifically, the integrity of the defined B and T cell zones of the follicle is destroyed (Benedict et al., 2006). After day 3 pi, viral titers begin to lower and the Ag-specific CD8 T cell response is detected in BALB/c mice (Robbins et al., 2007). From day 3–8 pi, antigen-specific CD8 T cells clonally expand to form peak responses (Figure 1). After peak responses are achieved in the acute stage of infection, the CD8 T cell response to some epitopes contracts and forms a stable memory population. These Ag-specific CD8 T cells are termed "non-inflationary." Three peptide epitopes that elicit non-inflationary CD8 T cells in acute MCMV infection of C57BL/6 mice are M45, m141, and M57. In our laboratory we previously identified that Ag-specific T cells of C57BL/6 mice are a heterogeneous population, which consists of cells forming distinct patterns of memory differentiation into the chronic stage of infection (Munks et al., 2006a). Some MCMV epitopes increase in number after the acute stage—resisting the contraction—and are termed "inflationary" epitopes. The inflationary epitopes to which responses can be seen in acute infection include m139 and M38. IE3 is also an inflationary epitope, but Ag-specific CD8 T cells defined by IE3 are poorly

detected during the acute phase of infection. However, these cells dominate during latency (Munks et al., 2006a). Table 3 is a list of the epitopes of C57BL/6 mice during acute infection.

In BALB/c mice, non-inflationary epitopes are not clearly defined. Holtappels et al. have clearly defined that the IE1 (pp89) and m164 are the immunodominant epitopes in the acute phase of infection and during latent infection (Holtappels et al., 2000; Holtappels et al., 2002).

A hallmark of CMV infection is the number of Ag-specific CD8 T cells in latent infection. In both humans and mice, Ag-specific CD8 T cells occupy up to 10% of the total CD8 T cell population in the blood (Gillespie et al., 2000; Karrer et al., 2004; Munks et al., 2006b; Sylwester et al., 2005). These Ag-specific CD8 T cells dominate the antiviral immune response to latent infection. Reactivation or low-level antigen presentation is thought to drive the memory CD8 T cell pool; however, our laboratory also shows the inflationary memory T cell pool is maintained by continuous recruitment of short-lived effector cells (Snyder et al., 2008).

MCMV non-inflationary CD8 T cells follow the general differentiation pattern of activation, expansion, contraction, and formation of memory. As noted earlier, MCMV inflationary epitopes deviate from this program and are unique in that they progress during the acute and chronic phase of infection until a level of homeostasis is achieved (Munks et al., 2006a). In this thesis I will present data that show non-inflationary epitopes, after initial activation (priming), expand 2- to 3-fold more after infection with a non-replicating MCMV versus a replicating virus.

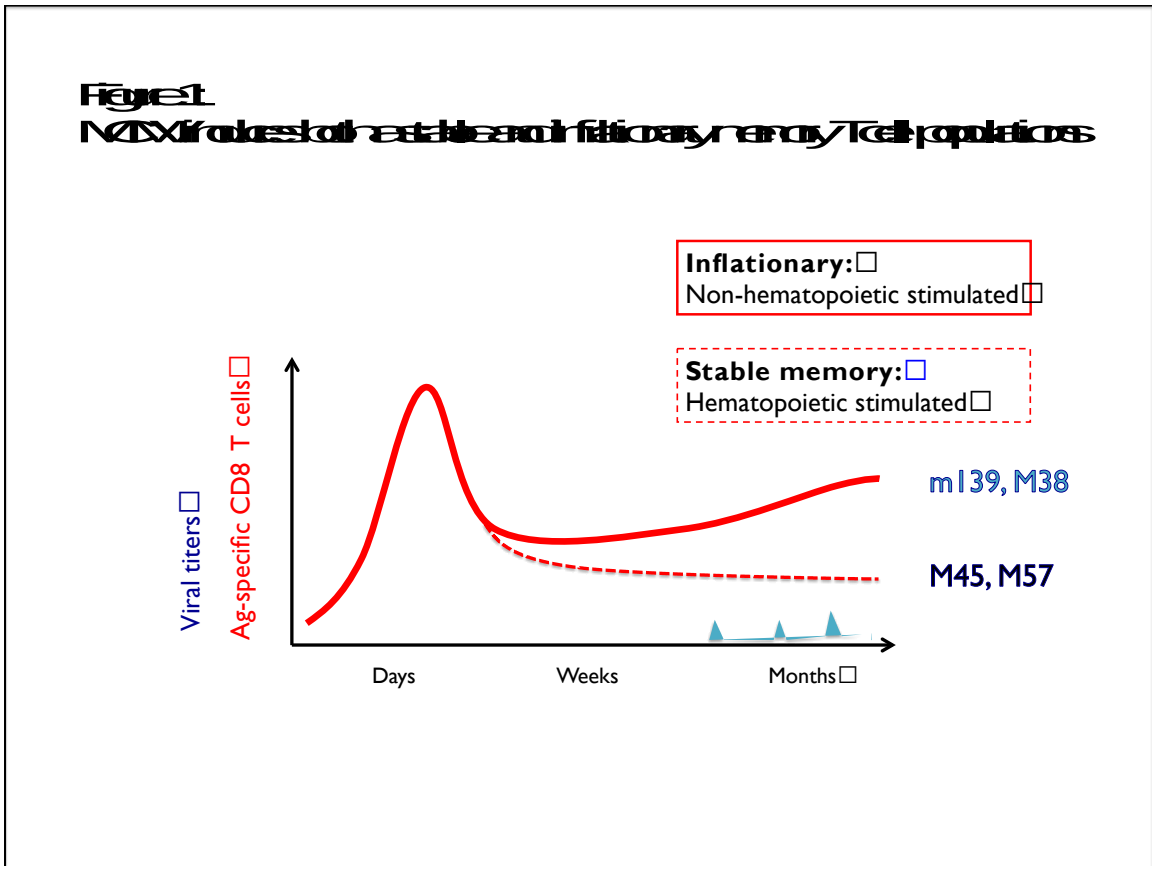


Table 3: Acute epitopes of MCMV infection in C57BL/6 mice

ID Rank	ORF	Restriction	a.a.	Peptide	Homolog	Function
1	**M45**	Db	985-993	HGIRNASFI	UL45	Ribonucleotide reductase
2	m139	Kb	419-426	TVYGFCLL	US22	US22 family homolog
3	**M57**	Kb	816-824	SCLEFWQRV	UL57	Major DNA binding protein
4	**m141**	Kb	16-23	VIDAFSRL	US24	US22 family homolog
5	M38	Kb	316-323	SSPPMFRV	UL38	

BOLD are non-inflationary epitopes

1.4.5.2 CD4 T cell Response

Interestingly, CD4 help is not necessary in the generation of the acute CD8 T cell response to MCMV, as CD4 null mice show no impairment of acute CD8 T cell responses at day 7 pi (Snyder et al., 2009). However, CD4 T cells do support the chronic (inflationary) CD8 T cell response, indicating that CD4 T cells play an active role in supporting long-term MCMV control (Snyder et al., 2009).

In primary infection, CD4 T cells have a direct role in suppressing viral-replication in the salivary gland. MCMV titers are reduced in the spleen and lungs within a week of infection, while peak titers are measured in the salivary gland 3 weeks pi and are persistently detected in the chronic phase (Bukowski et al., 1984). Depleting CD4 T cells from mice that are infected with MCMV leads to an increase of viral titers in the salivary gland, while titers in the lung and spleen show little to no change (Jonjic et al., 1989). Further work from Lucin et al. describes that CD4 Th1 cells, which release IFN-γ in response to infection, are an important T lymphocyte group to control viral replication in the salivary gland (Lucin et al., 1992). Overall, CD4 T cells provide a supporting role in the adaptive immune response to MCMV infection. However, in the salivary gland, the response from CD4 T cells is imperative to the control of viral replication and spread of the virus. Currently it is unclear why CD8 T cells are not the primary effector cells in the salivary gland but the Lu et al. showed that they could control infection of MCMV lacking immune evasion genes (Lu et al., 2006).

1.4.6 Humoral response

Glycoprotein B (gB) is a highly conserved protein in herpes viruses. It is necessary for viral replication but is also important for viral entry into a cell as it facilitates membrane fusion during cell-to-cell spread (Hobom et al., 2000; Isaacson and Compton, 2009). Glycoprotein B is one of many proteins that are antigenic to initiate an antibody response. It is expressed on the surface of the virion and the antibodies against gB are well characterized (Britt et al., 1990; Marshall et al., 1992), revealing five antigenic domains (Potzsch et al., 2011). Neutralizing antibodies block virion binding to new cell targets and limit the viral spread of the virus. Despite the humoral countermeasure to CMV, it does not prevent the establishment of latency, but passive immunity can still offer a layer of protection by reducing viral loads (Farrell and Shellam, 1991; Shanley et al., 1981).

Currently there is a need for a vaccine against CMV infection. The vaccine target populations are those who are immune-deficient and pregnant women. Congenital CMV infection is the biggest viral threat against newborns. Therefore, inducing a humoral response prior to pregnancy could help prevent infection of mothers before pregnancy. Furthermore, Ig molecules can cross the placenta during pregnancy, and neutralizing antibodies could help by reducing the pathogenicity of acute infection. Clinical trials are currently under review to test neutralizing antibodies against CMV (Griffiths et al., 2013).

1.4.7 Impact of NK cells and viral load on the CD8 T cell response

As discussed earlier, NK cells have an imperative role in the control of MCMV infection. Their antiviral function is to kill infected cells, while they also produce cytokines to modulate the immune response. The activation marker Ly49H has been central to the understanding of the innate immune response to MCMV infection. Research into Ly49H has brought to light that the NK cell-mediated control of acute MCMV infection also regulates the generation and functionality of Ag-specific CD8 T cells. Below is a review of the recent papers that describe this relationship.

Earlier in this thesis I described how NK cell activating receptor Ly49H recognizes MCMV encoded protein m157 to kill infected cells. Robbins et al. were first to show that NK cell activation via an m157-Ly49H interaction during acute MCMV infection affected both the inflammatory cytokine response and CD8 T cell response (Robbins et al., 2007). Their model of controlling NK cell activation was to infect wild-type BALB/c (Ly49H null) and BALB/c-Cmv-1^{r} transgenic mice (Ly49H expressing) with Smith strain of MCMV. Smith-MCMV expresses m157; however, wild-type BALB/c mice do not express the Ly49H receptor on NK cells. Therefore, BALB/c mice produce high titers 3–4 days pi. On the other hand, BALB/c-Cmv-1^{r} mice express Ly49H on NK cells and kill infected cells expressing m157, which causes an attenuated infection. Their data show that lowering viral titers decreases the inflammatory cytokine response—in particular, the release of type I IFNs from pDCs. Although not stated in their research, this cytokine response is most likely due to TLR activation. They also show that the NK cell control of MCMV enhances the IE1-specific CD8 T cell response—at day 3 pi, the IE1-specific CD8 T cells are significantly greater in BALB/c-

Cmv-1^r mice than BALB/c mice. However, the enhanced IE1-response is not sustained, as day 7 pi responses were equal between both groups of mice.

Andrews et al. follow up on this work by using a similar model of MCMV infection in BALB/c mice and BALB/c-Cmv-1^r mice (Andrews et al., 2010), although in their report they used a K181 strain of MCMV—a virulent Smith strain. The data by Andrews et al. confirm that the NK cell effector responses drive down viral titers and inflammatory cytokine responses in the blood. They found that the peak IE1 CD8 T cell response was greater in mice that do not express the Ly49H activating NK cell receptor. Andrews et al. measure antigen-specific T cell functionality through an in vivo killing assay. This assay was performed by pulsing splenocytes with or without IE1 peptide and injecting them intravenously into infected mice. The ratio of pulsed cells to unpulsed cells at day 3 post-transfer determines the percentage of killing. Their result was in the opposite direction to Robbins et al. However, it is difficult to compare the findings from Robbins et al. and Andrews et al., as they use different assays to measure antigen-specific IE1 T cells—tetramer staining versus a killing assay (respectively). However, Andrews et al. show in vitro that the residual antigen from an infection correlated with the generation of CD8 T cells. In this experiment, transgenic (Tg)-HA CD8 T cells are incubated with splenic DCs harvested from mice infected with MCMV-K181-expressing HA antigen at days 4 and 6 pi. BALB/c (Ly49H null) mice are able to induce HA-specific CD8 T cells to proliferate. In contrast, DCs from Ly49H expressing BALB/c mice do not proliferate HA-specific CD8 T cells at either 4 or 6 days pi. This fact strongly suggests that the lack of Ly49H NK cell control allows for the virus to replicate and increase the viral load,

which causes DCs to decrease in number while also presenting antigen longer, and the sustained antigen presentation induces a better priming event to Ag-specific CD8 T cells.

Stadnisky et al. also identify a requisite role of NK cells to the resistance of MCMV infection in H-2^k restricted mice, independent of Ly49H (Stadnisky et al., 2011). They previously showed that MHC class I D^k is a cognate ligand for Ly49G2 receptor on NK cells (Xie et al., 2009). The combination of Ly49P, which recognizes MCMV gp34-MHC class I, and Ly49G2 on infected cells, activates NK cells to kill. In turn, MCMV infection of MHC class I D^k disparate mice can distinguish between high and low viral loads and affect the corresponding CD8 T cell response in these infections (Stadnisky et al., 2011). They use novel strains of C57L mice, which are H-2b that contain either resistant (R7) or a susceptible (R2) H-$2D^k$ allele from MA/My mice. Stadnisky et al. show mice that express MHC class I D^k have lower viral titers and restore the cDC compartment at day 3.5 pi. This result is consistent with previous reports. However, they show that at day 6 pi CD8 T cells (M45 and m139-specific) are increased when viral loads were lower in mice. The enhanced M45 CD8 T cell response was not sustained at days 12 and 18 pi, as the frequency of M45 and m139-specific CD8 T cells in both R7 and R2 mice were not significantly different. Thus, this result differs from Andrews et al. in that a higher virus load leads to an increased CD8 T cell response. However, infection in their model is very virulent, with very high viral titers and low T cell response in susceptible mice, again making a direct comparison very difficult.

CHAPTER 2: THE INFLAMMATORY TYPE I IFN RESPONSE IN ACUTE MCMV INFECTION MODULATES EFFECTOR AND MEMORY FORMATION OF NON-INFLATIONARY ANTIGEN SPECIFIC CD8 T CELLS.

2.1 Abstract

Murine cytomegalovirus (MCMV) is a β-herpesvirus that causes, like HCMV, as much as 10% of the total memory CD8 T cell pool to be CMV-specific in latent phase of infection. While the characterization of these memory Ag-specific CD8 T cells is well established, the factors that drive MCMV-specific responses are not completely understood. Therefore, it is important to understand the early priming events of naïve CD8 T cells, as well as the continued interaction with antigen-presenting cells, after initial priming, to drive differentiation of CD8 T cells into effector and memory cells. Using an infection model in which we can inhibit viral replication of MCMV with antiviral drug treatment, we observed increased antigen-specific CD8 T cell responses at the acute and chronic phase of infection. We show that blocking viral replication reduces the inflammatory cytokine response resulting in preservation of conventional dendritic cells and a consequent enhancement of the MCMV-specific CD8 T cell response.

Results

Blocking viral replication enhances a subset of CD8+ T cell responses to MCMV

To identify changes in the effector CD8 T cell population between a replication-competent versus replication-deficient MCMV-TK infection, we measured the expression CD43 on CD8 T cells at day 6 pi (Figure 2-1A). CD43 is expressed upon activation of CD8+ T cells and thus serves as a surrogate measure for the total CMV-specific CD8+ T cell response. The frequency and absolute number of CD43+ CD8 T cells were greater in Famcyclovir treated mice, although the difference was not significant. While CD43 expression provides data for the generation of effector CD8 T cells, it does not provide a high-resolution account of the MCMV-specific CD8 T cell response between Famcyclovir treated and untreated mice. We therefore used MHC tetramers to measure both the frequency and absolute numbers of CD8 T cells specific for the M45, m139, M57, and M38 epitopes (Figure 2-1B). We also performed an ICS to identify the functional response of antigen (Ag)-specific CD8 T cells (Figure 2-1C,D, and E). We expected the Ag-specific CD8 T cell response in Famcyclovir treated mice to be inhibited, as blocking viral-replication reduces the antigenic load. Strikingly, the frequency and absolute numbers of M45 and M57 tetramer-specific and IFN-γ+ CD8 T cells were significantly increased in mice where virus replication was inhibited by Famcyclovir.

Responses to the immunodominant epitopes m139 and M38 were unchanged in frequency, as were the acute subdominant epitopes (Figure 2-1E). However, although M139-specific responses were not increased as a proportion of CD8+ T cells, the absolute

numbers of m139 tetramer+ CD8 T cells in the spleen were significantly increased in mice treated with Famcyclovir (Figure 2-1B). The total number of CD8 T cells in the spleen in Famcyclovir treated mice was also significantly increased over both untreated and naïve mice (Figure 2-1F), which shows that blocking viral-replication increases the total CD8 T cell pool during acute MCMV infection, and perhaps limits the splenic lymphopenia commonly associated with acute infection. The increased number of total CD8 T cells in Famcyclovir treated mice probably accounts for the increase in numbers of m139-specific T cells in the spleens of Famcyclovir treated mice, but it does not lead to a proportional increase in all MCMV-specific responses. Instead, only M45 and M57-specific CD8 T cells are consistently increased, which suggests that there is a selective mechanism to enhance these responses.

To gain further insight into the extent of this phenomenon, we examined the impact of Famcyclovir on the H-2^d restricted T cell response of BALB/c mice (Figure 2-1F). In BALB/c mice, blocking viral-replication with Famcyclovir did not increase the frequency of CD8 T cell responses. In contrast, responses to the immunodominant epitopes pp89 and m164 were decreased and subdominant epitopes were unchanged.

We also wondered whether CD4+ T cell responses to MCMV would be affected by inhibiting viral replication (Figure 2-1H). The acute CD4 T cell responses at day 7 pi were not affected by Famcyclovir treatment. The response to one epitope (M25) was increased, but the difference did not reach statistical significance.

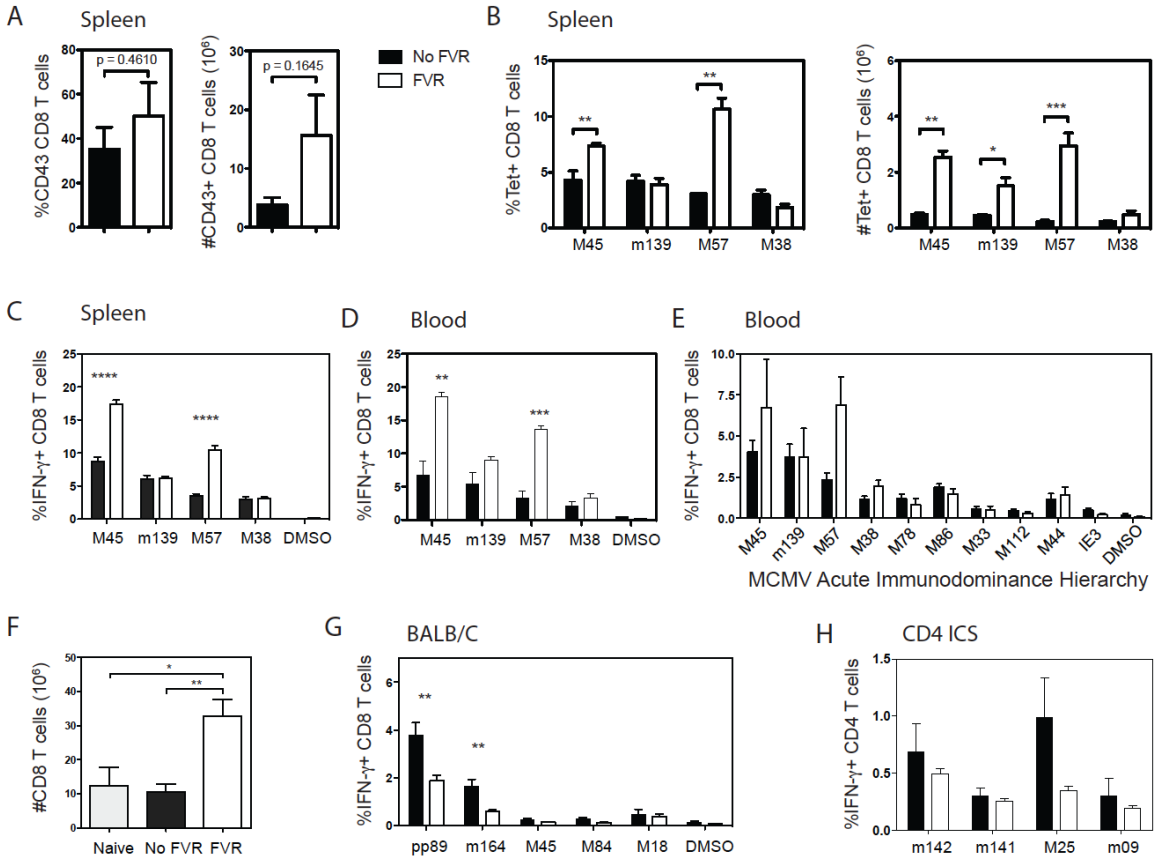


Figure 2-1: Inhibiting virus replication expands the T cell response to MCMV
C57BL/6 mice were pretreated with Famcyclovir-fortified water and infected with 2*105 PFU of MCMV-TK 3 days later. (A) At day 6 pi the frequency of CD43+ CD8 T cells was measured in the spleen. (B) At day 7 pi the frequency and absolute numbers of tetramer+ CD8 T cells were measured in the spleen. An ICS assay was performed to measure the frequency of IFN-g+ CD8 T cells in the spleen (C) and blood (D-E). (F) The absolute numbers of CD8 T cells were measured in the spleens of infected and naive mice. (G) BALB/c mice were infected with MCMV-TK and an ICS was performed to measure splenic IFN-γ + CD8 T cells. (H) C57BL/6 mice were infected with MCMV-TK to measure for IFN-γ+ CD4 T cells. White bars represent mice without Famcyclovir treatment and black bars represent mice under Famcyclovir treatment. Graphs represent the average with the SEM and significance as determined by a student's t-test (*p<0.05,**p<0.005, ***p<0.0005,****p<0.0001).

Impact of virus replication on expansion and contraction of Ag-specific populations

We previously reported that early treatment with Famcyclovir leads to undetectable MCMV-TK viral titers by three days post-infection (Snyder et al., 2011). Since clearance of the virus occurs rapidly, we wanted to investigate changes in the differentiation kinetics of CD8 T cells in Famcyclovir treated mice. We infected groups of Famcyclovir treated and untreated mice and harvested spleens at days 2, 4, and 6 days pi (Figure 2-2A). Our data show the absolute numbers of CD43+, PD-1+, and CD62L- CD8 T cells from days 0–4 pi were consistent across both groups of mice, indicating a small increase over this range of time. At days 4–6, the absolute numbers of these populations dramatically increased in the Famcyclovir treated group, while the numbers in untreated mice remained constant or only slightly increased. Measuring the absolute numbers of CD8 T cells during acute infection also shows a consistent number of cells between treated groups, with a sharp increase in Famcyclovir treated mice from days 4–6 pi. This data indicates that the increase in the CD43+, PD-1+, and CD62L- population is a result of the expanded CD8 T cell population. However, the day 6 pi peak numbers of these respective groups varies, indicating that as a whole, the CD8 T cell population in Famcyclovir treated mice is diverse. Interestingly, the kinetics of the CD69+ CD8 T cells population formed a different pattern. Famcyclovir treated mice reached ~10^6 at day 2 pi and stayed constant through day 6 pi. In contrast, the CD69+ CD8 T cells in mice without Famcyclovir expanded rapidly to ~$4*10^6$ cells, remained constant through day 4 pi, and decreased at day 6 pi. Unlike the other markers, which reflected TCR-mediated activation, CD69 can be expressed on cells in response to cytokines. Thus, the kinetics of

the CD69+ population is likely a response to the amount of viral load during the infection.

Since the total population and differentiated subsets of CD8 T cells increased at day 6 pi, we measured the proliferation of Ag-specific CD8 T cells. At day 6 pi the frequency of tetramer+Ki67+ was not significantly different in mice with Famcyclovir treatment (Figure 2-2B). In contrast, the absolute numbers of M45 and M57 tetramer+ cells expressing Ki67 was enhanced in Famcyclovir treated mice, while the m139 and M38-tetramer+ cells were unchanged.

The M45 and M57-specific CD8 T cells are particularly affected by the inhibition of viral-replication. We next measured the phenotype of these cells to better understand whether there are distinct changes in their differentiation that causes them to expand to greater numbers. Using the markers KLRG1 and CD127, we could divide differentiated CD8 T cells into distinct populations based on antigen and inflammation exposure (Joshi et al., 2007). Short-lived effector cells (SLEC) are phenotypically $KLRG1^{hi}CD127^{lo}$ and are driven by exposure to antigen and inflammation (Joshi et al., 2007; Prlic et al., 2006). Conversely, memory precursor effector cells (MPEC) are phenotypically $KLRG1^{lo}CD127^{hi}$; these cells give rise to long-lived memory cells. Because M45 and M57-specific CD8 T cells proliferate more during acute infection but are exposed to lower levels of antigen and inflammation, we wondered if these cells had a greater MPEC phenotype. As shown in figure 2-2C, the treatment of Famcyclovir caused a significant increase in the frequency of tetramer+ cells (M45, M57, m139, and M38) that displayed a MPEC phenotype and a decrease in the SLEC phenotype.

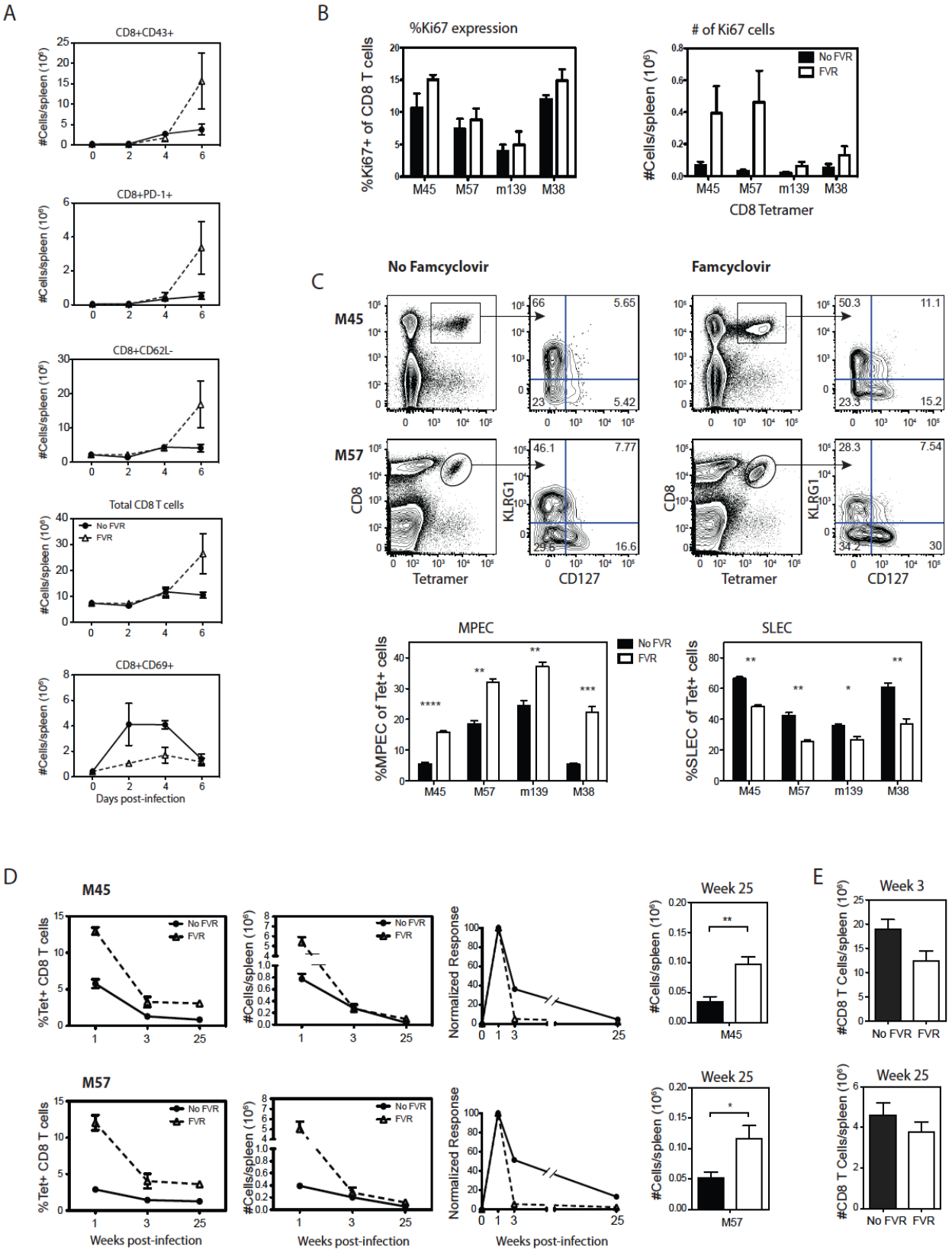
In acute viral infection naïve CD8 T cells become primed, clonally expand until peak numbers are reached, and contract down to 5–10% of peak levels to establish long-lived memory populations. We were therefore interested to know if the expanded frequency and numbers of M45 and M57-specific CD8 T cells measured in acute infection would translate into higher numbers in the latent stage of MCMV-TK infection. Tetramer+ cells were measured at weeks 1, 3, and 25 pi (Figure 2-2D). We found that the expanded numbers of M45 and M57 measured at week 1 pi were also elevated in the latent stage of infection, as numbers at week 25 were significantly increased in mice under Famcyclovir treatment. The absolute numbers of M45- and M57-specific CD8 T cells at week 3 pi were not significantly greater in Famcyclovir treated mice than in untreated mice, which led us to investigate the rate of contraction of these responses by measuring the normalized absolute numbers of these specific CD8 T cells over time (Figure 2-2D). This result clearly shows that M45- and M57-specific CD8 T cells from Famcyclovir treated mice contracted faster than from mice without Famcyclovir treatment. The faster contraction of these responses was unexpected since these Ag-specific CD8 T cells expressed a greater MPEC phenotype versus untreated mice. The absolute numbers of CD8 T cells were measured at weeks 3 and 25 and they showed no significant increase between Famcyclovir treated groups (Figure 2-2E). Together, these data show that blocking MCMV-TK viral-replication with Famcyclovir increased differentiation of CD8 T cells (e.g., expression of CD43+, PD-1+, CD62L-), increased the MPEC phenotype, and initiated a higher memory set-point during the latent phase of infection. However, the unusually strong contraction rate of M45 and M57 specific CD8

T cells from the peak acute response and the resulting lower than predicted number of residing memory cells from MCMV-TK infection deserves further investigation.

Figure 2-2: Impact of virus replication on expansion and contraction of Ag-specific populations

C57BL/6 mice were either treated or untreated with Famcyclovir before infection with MCMV-TK, and then spleens where harvested days to weeks post-infection.
(A) Phenotype (CD43, PD-1, CD62L, and CD69) tetramer+ CD8 T cells on days 0, 2, 4, and 6 pi. (B) Proliferation of tetramer+ CD8 T cells was measured by the expression of Ki67 on day 6 pi. (C) Contour plots of tetramer+ and effector/memory populations (CD127, KLRG1). Graphs show the frequency of MPEC and SLEC at day 7 pi. (D) Splenocytes were harvested at weeks 1, 3, and 25. Line graphs show the frequency and numbers of M45 and M57-tetramer+ CD8 T cells and the normalized response of tetramer+ cells at weeks 1, 3, and 25 pi. Bar graphs show the numbers of tetramer+ cells at week 25 pi and the numbers of CD8 T cells at week 3 pi. Graphs represent the average with the SEM and significance as determined by a student t-test (*$p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p<0.0001$).

Figure 2-2: Imact of virus replication on expansion and contraction of Ag-specific populations



Blocking viral-replication of MCMV-TK preserves the splenic architecture

A hallmark of MCMV pathogenesis is the destruction of the splenic architecture during acute infection (Benedict et al., 2006). Modulation of lymphoid chemokines by MCMV infection influences the compartmentalization of B and T cell zones in the white pulp of a splenic follicle. Thus, it is plausible that the regulation of secondary-lymphoid structures is a factor in the duration and quality of APC-CD8 T cell interaction during priming, and influences the generation of the CD8 T cell effector and memory response. We therefore wondered if blocking MCMV replication would prevent the destruction of the splenic architecture and allow for a better acute CD8 T cell response. Figure 2-3 shows tissue sections at day 3 pi in mice infected with MCMV-K181 or MCMV-TK (treated or untreated with Famcyclovir). In a cross-sectional view of a follicle from a naïve mouse, the T cells form a round mass, with the B cell zone forming a concentric ring around the T cell zone (Figure 2-3A). In a virulent strain of MCMV (K181), the B cell zone does not confine the T cell zone and the integrity of the follicle is lost (Figure 2-3B). MCMV-TK is derived from the somewhat attenuated Smith BAC. Infection with MCMV-TK caused some disruption of splenic architecture with T cells not in a defined zone but dispersed into the encapsulating B cell zone (Figure 2-3C). Figure 2-3D shows that blocking viral-replication with Famcyclovir preserved the splenic architecture, with distinct T and B cell zones similar to the naïve mouse. These pictures show that while MCMV-TK did not disrupt the splenic architecture as profoundly as K181 virus, it still caused some disruption, whereas infection in the presence of Famcyclovir caused no disruption. Thus, APC-T cell interaction during acute infection could be impaired in MCMV-TK.

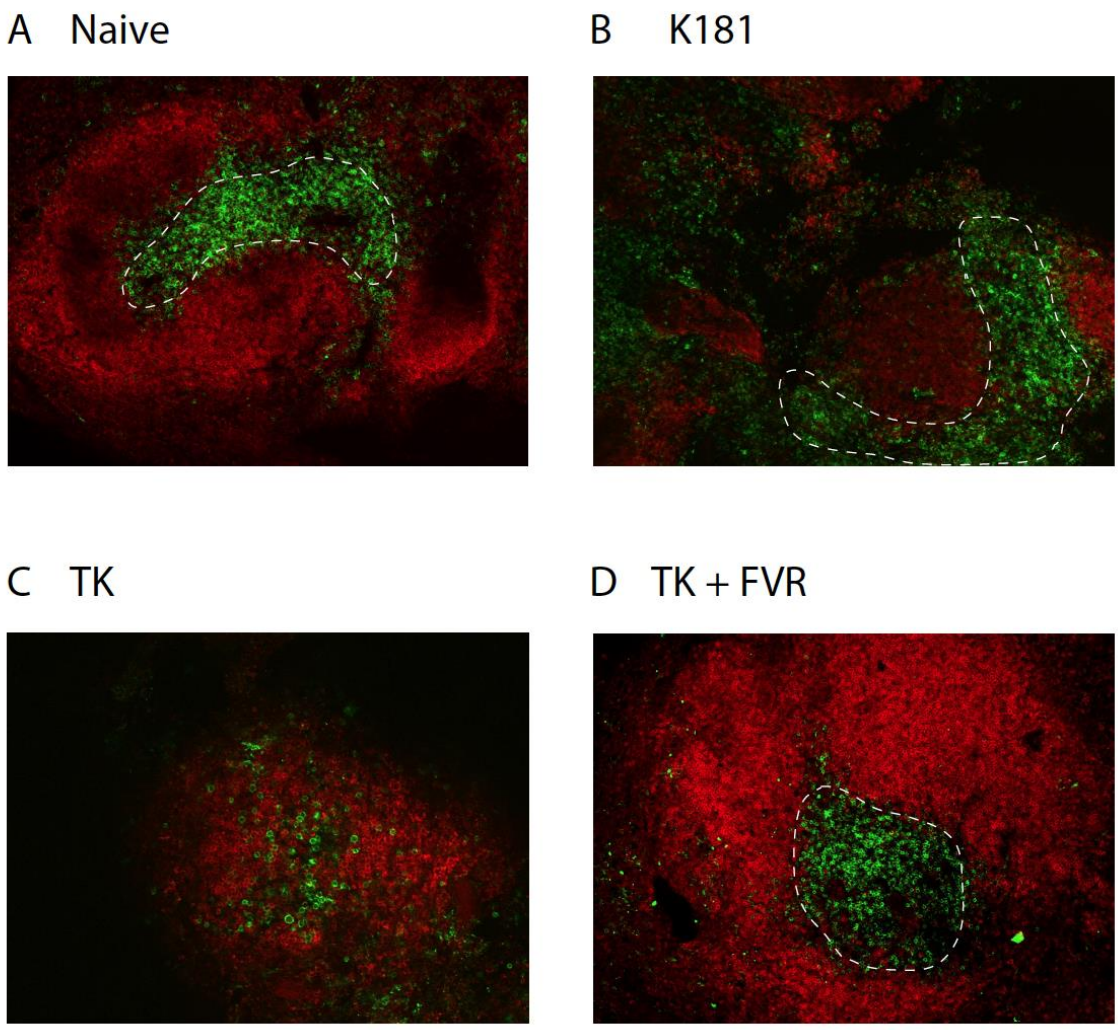


Figure 2-3: Mice treated with Famcyclovir have a preserved splenic architecture

C57BL/6 mice were infected with K181 or MCMV-TK (+/- Famcyclovir). At day 3 post-infection spleens were harvested and cut into 500um sections and stained for T (CD8, Green) and B (B220, Red) lymphocytes. (2-2A) Naive mouse. (2-2B) K181 infected. (2-2C) MCMV-TK infected. (2-2D) MCMV-TK infected with Famcyclovir (FVR). All mice were approximately 12-24 weeks old. Shown is 1 of 2 experiments performed. White dotted line describes the T cell zone of the splenic follicle.

CD8 T cell responses to MCMV-TK and co-infection with replication-competent viruses

Blocking viral-replication of MCMV-TK with Famcyclovir had the unexpected result of increasing M45- and M57-specific CD8 T cell responses. We reasoned that this could occur in two ways: (1) Famcyclovir could be acting as an adjuvant, specifically boosting the response, or (2) the uninhibited viral replication could be inducing a suppressive mechanism to diminish CD8 T cell responses in Famcyclovir-treated mice. To determine which of these possibilities was correct, we performed a co-infection experiment in which mice were infected with a MCMV-TK and a wild-type virus that was similarly deficient in m157 but largely resistant to Famcyclovir (MCMV-dm157). Figure 2-4A shows that the co-infection with MCMV-dm157 removed the ability of Famcyclovir to increase the frequency of M45 and M57 tetramer-specific CD8 T cells in MCMV-TK infection. We also stimulated splenocytes with peptide and measured the expression of degranulation marker CD107a (LAMP-1) on CD8 T cells (Figure 2-4B). Our data show that Famcyclovir treatment significantly increased the frequency of CD107a+ CD8 T cells in MCMV-TK infected mice, while this enhanced population was negated in a co-infection with MCMV-dm157. Thus, the presence of replicating virus inhibits the full CD8+ T cell response.

However, in a co-infection with wt-BAC (MCMV expressing m157), the M45- and M57-specific responses retained the enhanced frequency of these cells with Famcyclovir treatment (Figure 2-4C). It is not completely known why co-infection of MCMV-TK with wt-BAC produces an elevated CD8 T cell response when co-infection with dm157-MCMV does not (please refer to the discussion section).

We next performed an analysis of peripheral IFN-α levels after 2 days of infection with MCMV or wt-BAC. This work was done on frozen plasma samples, based on data from Figure 2-5. There the IFN-α levels were drastically different between MCMV-TK infected mice treated or untreated with Famcyclovir. Therefore, we were interested in measuring the IFN-α levels between an m157 expressing virus and an m157-null virus. Figure 2-4D is a graph of the peripheral IFN-α measured from mice infected with wt-BAC, MCMV-TK, or co-infected with these viruses. The IFN-α response was significantly reduced in MCMV-TK infection with Famcyclovir treatment. Not surprisingly, the wt-BAC, which is controlled by NK cells, induced a lower IFN-α response compared to the MCMV-TK infection. However, the IFN-α response in wt-BAC-infected mice treated with Famcyclovir was also reduced, indicating that Famcyclovir can reduce an inflammatory cytokine response in a non-TK expressing virus. This is not completely surprising, because Famcyclovir does have some impact on wt-MCMV replication. Unfortunately, we do not have analysis on the IFN-α response from dm157-MCMV.

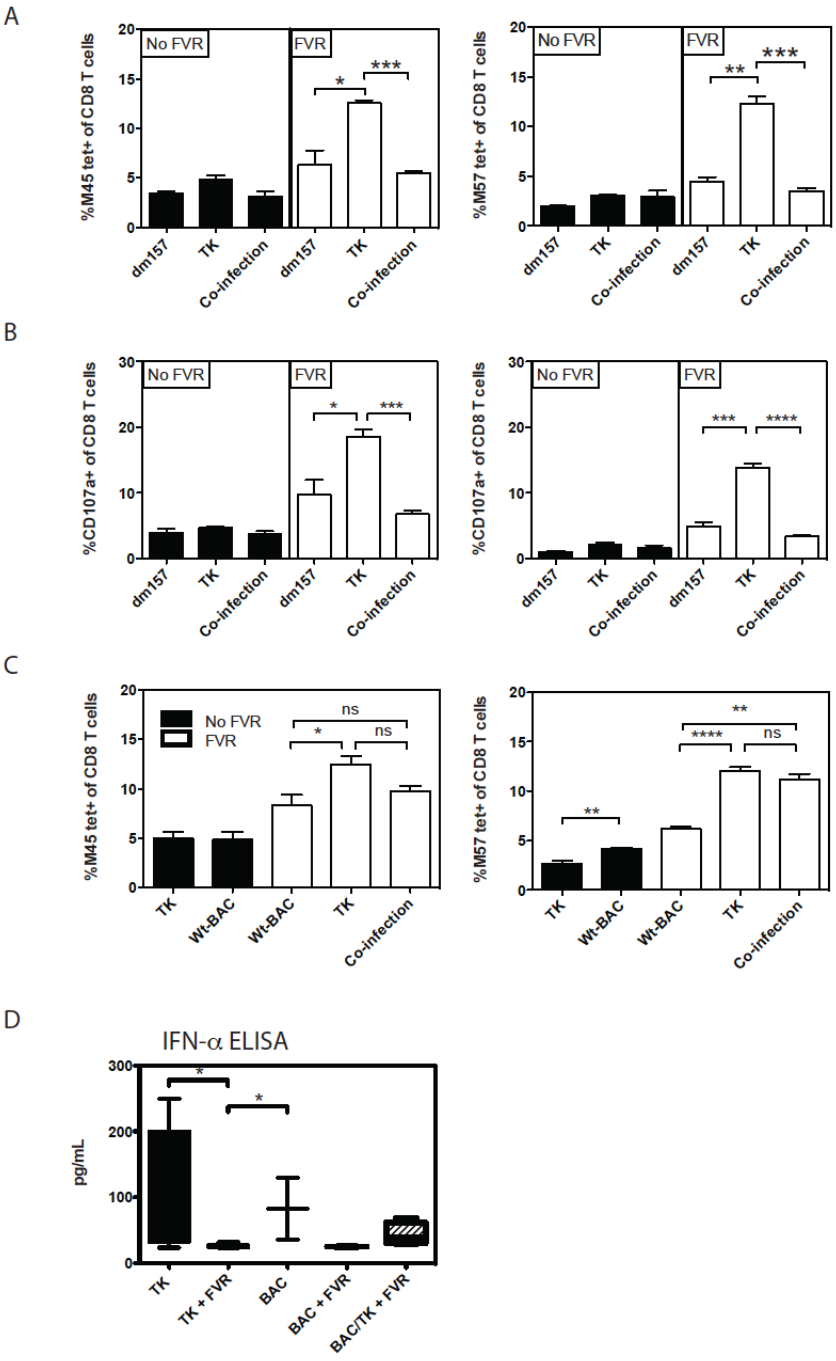


Figure 2-4: Co-infection of MCMV-TK and MCMV-dm157 decreases M45- and M57-specific responses C57BL/6 mice were infected with a MCMV-TK, MCMV-dm157, wt-BAC, or MCMV-TK co-infected with either dm157-MCMV or wt-BAC virus. Spleens were harvested at day 7 pi to measure Ag-specific T cell. (A) The frequency of M45 and M57-tetramer specific CD8 T cells and (B) CD107a expression in M45- and M57-tet+ CD8 T cells after 6 hours of peptide stimulation were measured in MCMV-TK/dm157-TK co-infection. (C) The frequency of M45 and M57-tetramer specific CD8 T cells were measured in MCMV-TK/wt-BAC co-infection. (D) Plasma was collected at day 2 pi and analyzed by an IFN-a ELISA. (E) Graphs represent the average with the SEM and significance as determined by a student's t-test (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001).

Inflammatory cytokines are abrogated with Famcyclovir treatment

Inflammatory response to a viral infection can act directly to limit the viral load during an acute infection, as well as initiate the maturation of antigen presenting cells and the differentiation of T cells. To evaluate the impact that blocking viral replication has on the inflammatory cytokine response to acute MCMV infection, we examined the circulating levels of pro-inflammatory cytokines. Plasma was collected at multiple time points between days 0–6 pi and then subjected to a single or cytokine array ELISA. Robust inflammatory cytokine responses were detected predominantly in mice not treated with Famcyclovir (Figure 2-5). Other reports have identified IFN-α and IL-12 as potent cytokines induced early in MCMV infection (Andrews et al., 2010; Robbins et al., 2007; Stadnisky et al., 2011). In our model, we noticed that IFN-α peaked at 8 and 48 hours pi, but the second peak was completely negated with Famcyclovir. The IL-12 response was less clear as there was variation at each time point between the groups of mice. IFN-g peaked at 2 days pi, TNF-a peaked at 4 days pi, and IL-6 peaked at 2–4 days pi. With Famcyclovir treatment, blocking viral replication abrogated these pro-inflammatory cytokine responses to low or near undetectable levels. Interestingly, IFN-α levels were comparable between treated or untreated mice at 8 hours pi. This early IFN-a response is likely not a result of viral-replication, but rather lymphotoxin (LT) a/b receptor B cells interacting with MCMV infected stromal cells expressing LT a/b (Benedict et al., 2001; Schneider et al., 2008). Overall, MCMV-TK induced a robust inflammatory cytokine during acute infection. Blocking viral replication with Famcyclovir ablated most of these responses to near baseline levels. These data are consistent with the hypothesis that the inflammatory response early during infection (before day 2 pi) is sufficient to help prime

CD8 T cells, and the inflammatory response at or after day 2 pi may provide a suppressive mechanism for specific CD8 T cell responses.

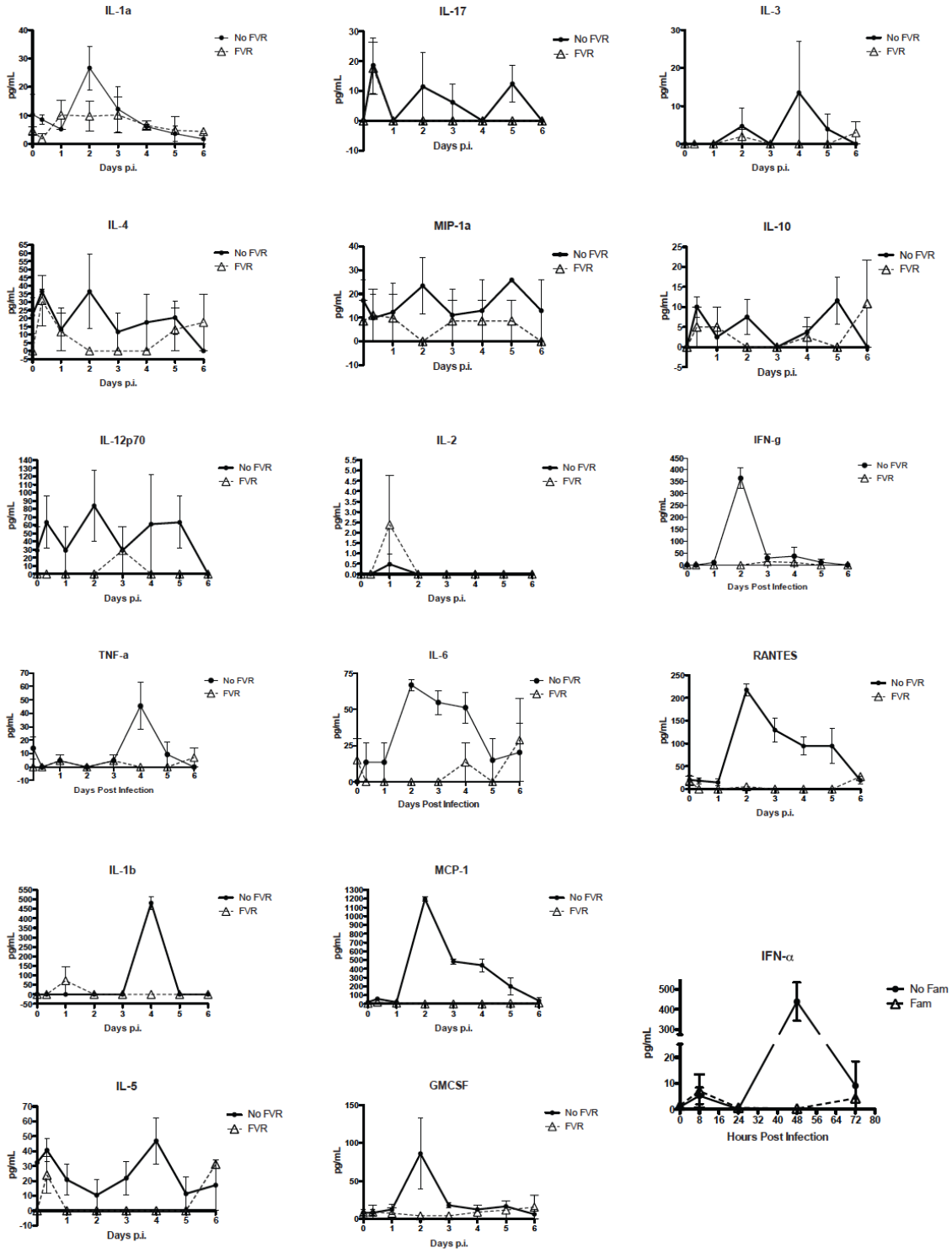


Figure 2-5: Famcyclovir Treatment Abrogates Acute Inflammatory Cytokines
Plasma was collected at multiple time-points post-infection and subjected to a single-plex IFN-α or multiplex cytokine detection assay. IFN-α samples were collected at times 0, 8, 24, 48, and 72 hours pi. All other cytokine responses were measured days 0-6 pi.

DCs are preserved with Famcyclovir treatment

Recent reports have shown dendritic cell numbers are impacted by how robust the NK cell host response is to MCMV acute infection. In particular, DC cells in acute MCMV infection are targets of infection, which results in reduced expression of co-stimulatory molecules and cell death (Andrews et al., 2001). However, the control of infection mediated by NK cells lowers viral titers and prevents the loss of DCs. To test, in our model, if Famcyclovir treatment also affected dendritic cell numbers, we quantified splenic dendritic cell numbers on days 0, 2, 4, and 6 pi (Figure 2-6A). Without Famcyclovir treatment, pDC numbers remained close to baseline levels at all time-points, conventional CD11b DC numbers declined by day 2 pi and remained steady from days 4–6 pi, and conventional CD8a DC numbers declined by day 2 pi and remained below baseline through day 4 pi. With Famcyclovir treatment, pDC numbers increased from baseline levels from days 0–4 pi, conventional CD11b DC numbers markedly increased from days 2–4 pi and returned to baseline numbers at day 6 pi, and conventional CD8a cDC were elevated from days 2–4 pi. These findings indicate a significant difference between cDC subsets in a replicating versus non-replicating MCMV infection. Blocking viral replication preserved or increased cDC subsets. We thought that preservation of conventional DCs could be responsible for the impact of Famcyclovir on the CD8 T cell response to MCMV.

Enhanced CD8 T cell responses are dependent on DCs

Dendritic cells have a strong ability to present antigen and co-stimulatory molecules to T cells. DCs are needed for the acute responses to MCMV, but once the response is primed, are not involved in driving the inflationary response (Andrews et al., 2001; Torti et al., 2011; Holtappels et al., 2002). CD8+ conventional dendritic cells are critical for cross-presentation of pathogen antigens, and the acute response to MCMV is driven by cross-presented antigen (Snyder et al., 2010; Torti et al., 2011). However, it is still not unclear whether dendritic cells play a role in enhancing the clonal burst size of CD8+ T cells after the initial priming. Since conventional DCs are reduced in the presence of replicating virus, we considered that this loss could be responsible for the muted CD8+ T cell response to cross-presented antigen. Therefore, we examined whether the ablation of dendritic cells after 2 days of infection, mimicking the impact of infection on DC populations, would alter the M45- and M57-specific CD8 T cell response. We infected mice that express the CD11c-Diphtheria Toxin Receptor (DTR) transgene and littermate controls, and administered diphtheria toxin (DT) at day 2 pi (Figure 2-6B). Diphtheria Toxin Receptor is not naturally expressed in mice; however, CD11c-DTR mice express the diphtheria toxin receptor under the CD11c promoter, and in these mice administration of diphtheria toxin ablates CD11c+ dendritic cells (Jung et al., 2002) (in data not shown).

A possible problem of DT administration into CD11c-DTR infected mice is the depletion of Ag-specific T cells. We have previously reported that CD11c expression is upregulated on MCMV Ag-specific CD8 T cells (Munks et al., 2006b). To address this issue, we performed an adoptive transfer of purified wild-type CD8 T cells (not

susceptible to depletion by DT) on mice prior to infection. We observed (in data not shown) the donor CD8 T cell response was similar to the endogenous response to MCMV-TK infection with DT administration. That is, early administration of DT did not affect the CD8 T cell response at day 7 pi. Figure 2-6C shows the total (donor and recipient) CD8 T cell response to MCMV-TK infection. With Famcyclovir treatment, CD11c-DTR negative mice showed significant enhancement in the frequency of M45- and M57-specific CD8 T cells, congruent to the response in wild-type C57BL/6 in Figure 2-1. Remarkably, CD11c-DTR positive mice did not show enhanced frequency of M45 and M57-specific CD8 T cells. This result indicates that the ablation of DCs after initial priming decreased the peak CD8 T cell response of MCMV-TK infection. Together, these data strongly suggest that the preservation of the CD11c APC compartment is necessary for the enhancement of the M45 and M57 Ag-specific CD8 T cell response in a replication-deficient MCMV infection.

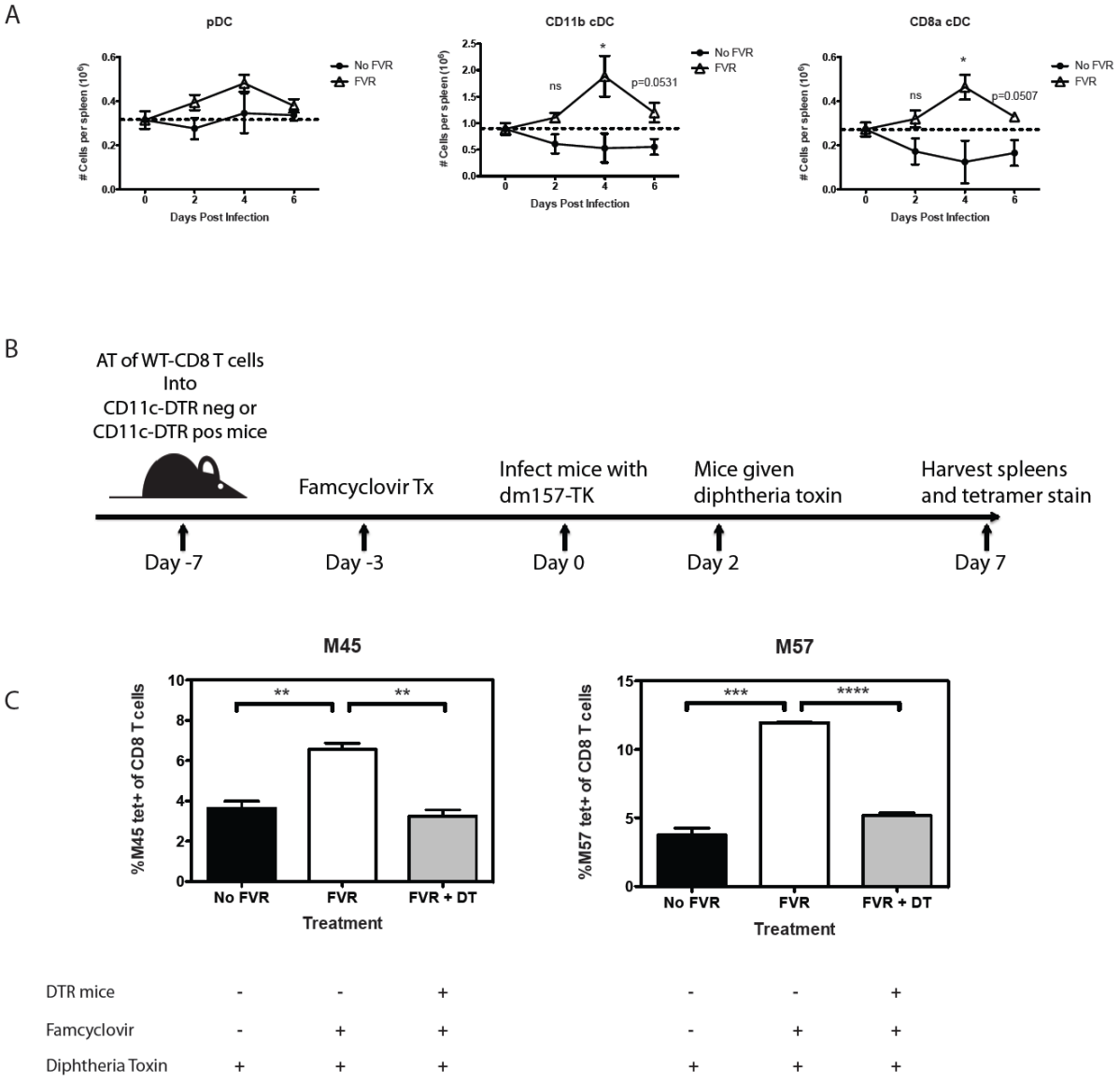


Figure 2-6: Dendritic cell populations are reserved early in Famcyclovir treated mice
(A) Splenic conventional DCs (CD8a+ and CD11b+) and plasmacytoid DCs (pDCs) were quantified from days 0-6 pi. (B) The experimental setup of the treatment of Famcyclovir and diphtheria toxin (DT) administration to CD11c-DTR+/- mice. (C) The frequency of M45 and M57-specific CD8 T cells from CD11c-DTR mice and littermate control mice infected with and without FVR and DT. Graphs represent the average with the SEM and significance as determined by a student t-test (**p<0.005, ***p<0.005).

Induced inflammation negates the enhanced M45 and M57-specific response in Famcyclovir treated mice

Because the broad inflammatory cytokine milieu in acute MCMV infection was reduced in Famcyclovir-treated mice (Figure 2-5), we wondered whether inflammation, without increased viral replication, could prevent the enhanced M45 and M57 specific T cell populations, and if it would decrease cDC numbers. It has been previously reported that an MCMV stimulates TLR3 (Tabeta et al., 2004). Therefore to mimic an MCMV-mediated inflammatory response in Famcyclovir treated mice, we administered TLR3 agonist Poly(I:C) (Figure 2-7A). We found that the treatment of Poly(I:C) at days 1 and 2 pi significantly negated the enhanced frequency day 7 pi M45 and M57 specific CD8 T cell populations. However, the M38-specific population was unaffected by Poly(I:C) treatment. Thus, similar to wild-type infection, the inflammatory response induced by Poly(I:C) suppressed a M45- and M57-specific CD8 T cells.

Since Poly(I:C) is known to induce type I IFN, Figure 2-7A shows that the IFN-α level at day 2 pi was significantly increased in mice treated with Poly(I:C) versus mice under Famcyclovir treatment without Poly(I:C). However, the IFN-α induced by Poly(I:C) was not increased to the same level as mice without Famcyclovir treatment. To determine whether type I IFN was responsible for the ability of Poly(I:C) to suppress T cell responses, we investigated the impact of blocking type I IFN signaling by using neutralizing antibody MAR1-5A3 against the IFNα/β receptor. The addition of MAR1-5A3 significantly restored the enhanced M45 and M57-specific response in Poly(I:C) treated mice (Figure 2-7B). Furthermore, we proceeded to administer MAR1-5A3 mAb

to MCMV-TK infected mice without Famcyclovir treatment. Neutralizing the IFN-α receptor caused these mice to be come very ill (e.g. lost weight, staggered movement, and severe lymphopenia). These mice were in poor condition and were euthanized prior to day 7 pi. These mice became ill likely from the unfettered control of MCMV.

Previous work by Andrews et. al. has indicated that MCMV viral replication increases circulating levels of IFN-α, and that this correlates with reduced dendritic cell numbers during the acute phase of infection (Andrews et al., 2010). In Figure 2-6A, we show that cDCs are decreased in mice infected with a replication-competent infection. We therefore hypothesized that neutralizing the IFNα/β receptor with MAR1-5A3 would restore the cDC population in mice treated with Poly(I:C). Figure 2-7C shows pDC and cDC numbers from the spleen at day 4 post-infection. As in Figure 2-6A, the conventional DC numbers were significantly reduced in mice without Famcyclovir treatment, while Famcyclovir treatment preserved or increased all measured DC subsets. However, the administration of Poly(I:C) into Famcyclovir-treated mice reduced the cDC (both CD11b+ and CD8a) subsets. Strikingly, with the administration of MAR1-5A3 mAb, these subsets were restored in Famcyclovir treated mice with Poly(I:C) administration. These results clearly show that an inflammatory response reduced Ag-specific CD8 T cell populations, and that neutralization of IFN-a allowed these Ag-specific CD8 T cell responses to reach maximum numbers.

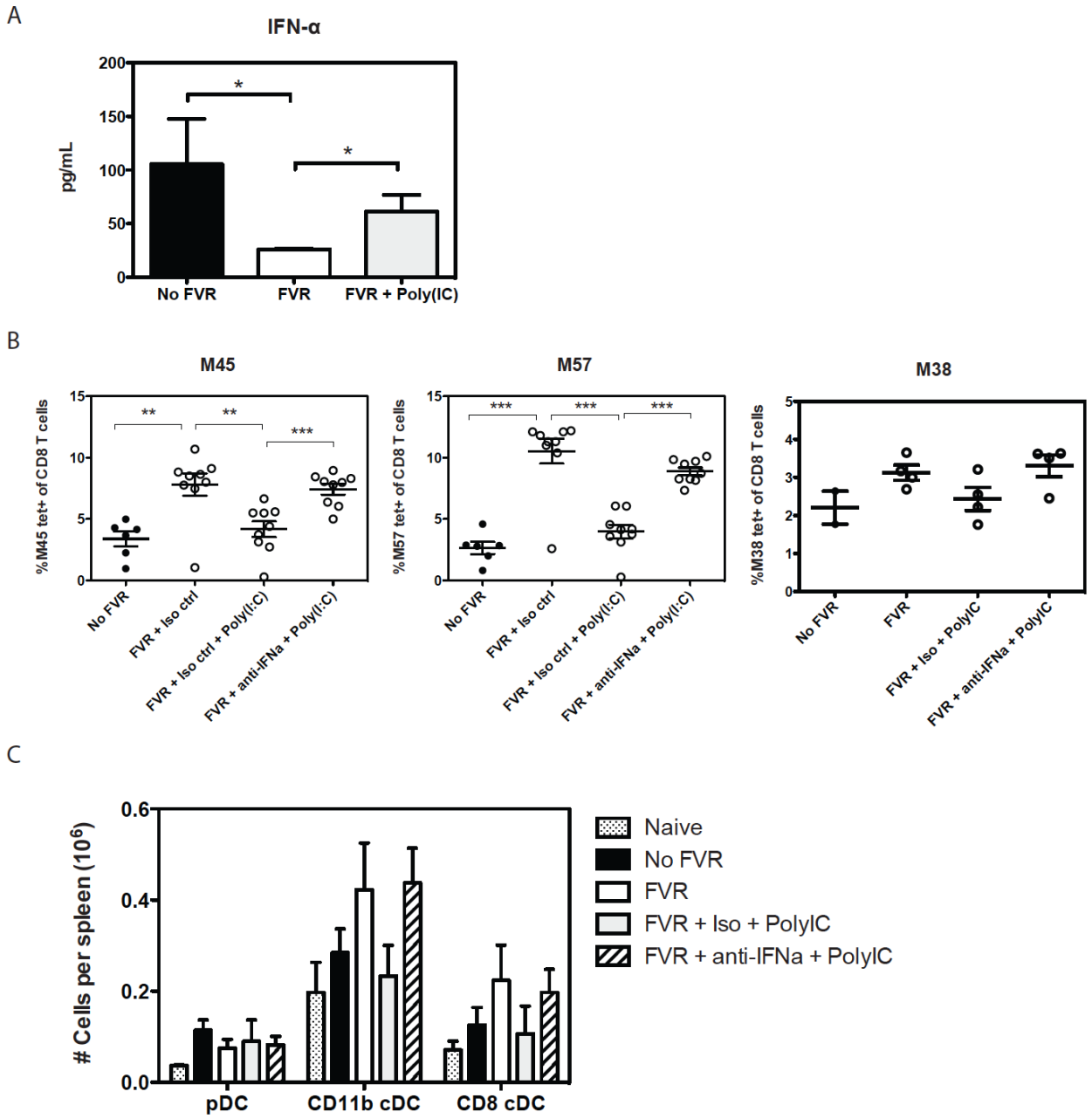


Figure 2-7: Administration of Poly(I:C) reduces CD8 T Cell responses in Famcyclovir treated mice
Infected mice were administered neutralizing IFNα/β receptor mAb (MAR1-5A3) or Isotype control i.p. at 12 hours pi. Poly(I:C) was administered i.p. at day 1 and 2 pi. (A) IFN-α levels were measured from the plasma of mice with +/- Famcyclovir treatment and Famcyclovir treatment + Poly(I:C). (B) The frequency of M45, M57, and M38-tetramer specific CD8 T cell populations were measured at day 7 pi. (C) The absolute numbers of splenic DCs were quantified from the infected mice treated with Famcyclovir and Poly(I:C). Graphs represent the average with the SEM and significance as determined by a student t-test (*p<0.05, **p<0.005, ***p<0.005).

Summary

The CD8 T cell response to MCMV infection is generally segregated into two groups: non-inflationary and inflationary. In the first week of infection, naive CD8 T cells become primed, clonally expand, reach peak responses, contract, and develop into memory T cells. A unique characteristic of MCMV infection is the development of both non-inflationary and inflationary memory CD8 T cells. The memory CD8 T cell pool forms after Ag-specific cells peak during the acute phase of infection. Non-inflationary CD8 T cells settle into a small memory pool that exists during the latent phase of infection. The inflationary pool of CD8 T cells expands during the latent phase of infection and commands as much as 10% of the total population of CD8 T cells. Our previous work has characterized the acute and chronic phase CD8 T cell response in C57BL/6 mice to BAC-derived MCMV infection, indicating a robust CD8 T cell response in both phases of infection (Gold et al., 2004; Munks et al., 2006a; Munks et al., 2006b; Munks et al., 2007; Snyder et al., 2010). Until recently, it was not fully understood how limiting the virulence of MCMV would affect the CD8 T cell response. Our investigation indicated that limiting viral spread in a primary infection does not prevent generation of memory CD8 T cells (Snyder et al., 2011). In conjunction with this work, we also learned that blocking viral-replication a few days after primary infection does not inhibit the formation of memory CD8 T cells. However, the acute CD8 T cell response where viral-replication is blocked early after initial infection was uncharacterized. We hypothesized that blocking viral-replication after initial infection would lead to a decrease in the effector CD8 T cell response in acute phase infection and limit the formation of memory CD8 T cells in the chronic phase of infection. In order to

study the acute CD8 T cell response to a replication-deficient MCMV infection, we used an infection model where the genomic replication of a recombinant MCMV is inhibited by drug therapy. Our laboratory previously described this model in C57BL/6 mice using the recombinant virus called MCMV-TK (Snyder et al., 2011). Famcyclovir completely inhibits replication of MCMV-TK both in vitro and in vivo. Specifically, mice infected with MCMV-TK in the continuous presence of Famcyclovir have undetectable virus at day 3 post-infection, the time of peak titers in the absence of the drug. In this study, we are able to compare replication-competent MCMV-TK infection to a replication-incompetent MCMV-TK infection and investigate the impact of viral-replication on the development of antigen-specific CD8 T cells. Surprisingly, we found that completely blocking MCMV replication during acute infection had the paradoxical effect of enhancing certain antigen-specific CD8 T cell responses. This effect was directly attributable to a reduction in type I IFN and the increased survival of cDCs when viral replication was blocked, since inducing a type I IFN response (via Poly(I:C)) or depletion of DCs reduced the magnitude of the CD8 T cell response. Antibody blockade of the IFNα/β receptor restored the enhanced CD8 T cell response and preserved the cDC compartment. These data show that type I IFN-dependent loss of cDCs during acute viral replication can limit the magnitude of anti-viral CD8 T cell populations.

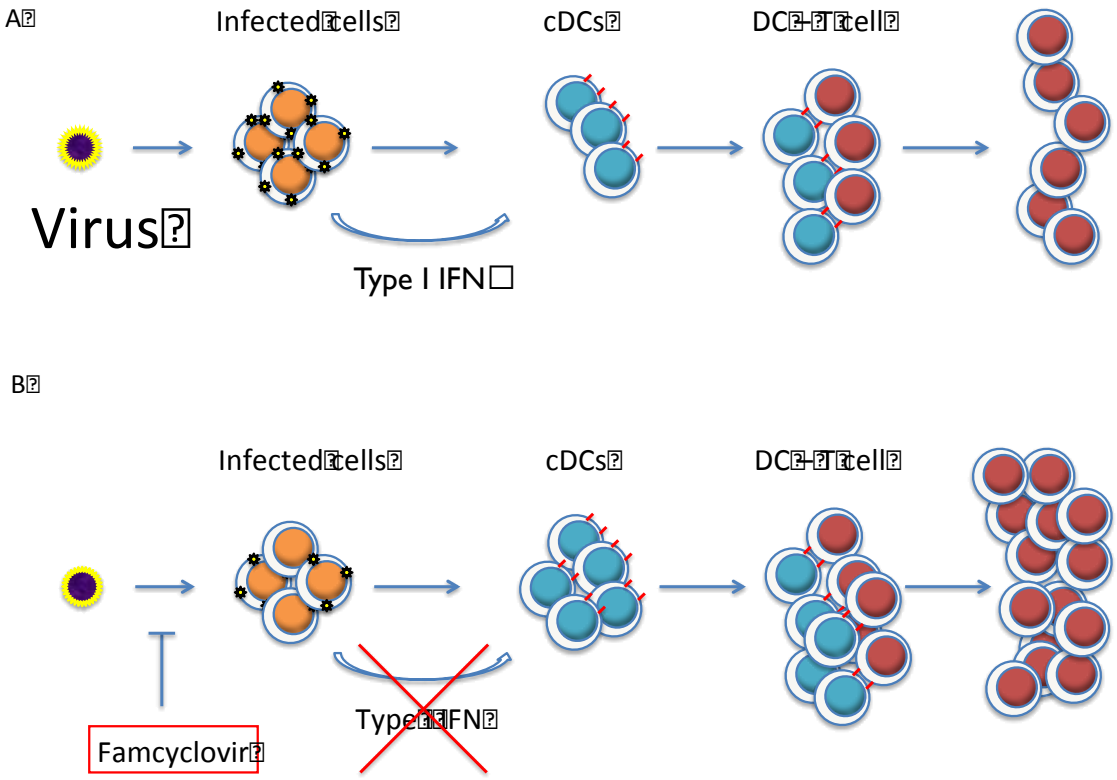
CHAPTER 3: DISCUSSION

Proposed model of CD8 T cell responses in an MCMV-TK infection

These studies demonstrate an unexpected role of viral replication—as a key factor in the generation of an antiviral immune response. Decreasing the pathogenic load typically correlates to a weaker CD8 T cell response, as seen in Listeria infection with ampicillin treatment (Badovinac et al., 2002), as well as our data (see Figure 2-2G) of MCMV-TK BALB/c mice with Famcyclovir treatment. In contrast, treating MCMV-TK infected C57BL/6 mice with Famcyclovir reduces the number of infected cells, decreases the inflammatory response (e.g., type I IFNs), preserves the cross-presenting DC populations, and enhances the non-inflationary M45- and M57-specific response at day 7 pi (Figure 3-1A and B). Furthermore, inducing inflammation via Poly(I:C) negates the enhanced CD8 T cell response measured in Famcyclovir-treated mice, while neutralizing the IFNα/β receptor restores the elevated CD8 T cell responses and preserves the splenic cDC compartment. Thus, we propose that the type I IFN response during acute MCMV infection is a key factor shaping both the proliferation of Ag-specific cells and the formation of memory cells in the chronic phase of infection.

There are recent conflicting reports regarding the acute CD8 T cell response when MCMV viral-replication is limited early after infection (Table 4). Some groups report that the

Figure 3-1: Model of blocking viral infection for enhancing adaptive immune during acute MCMV-TK infection



Blocking viral replication reduces the type I IFN response, preserves cDCs, and supports T cell proliferation and differentiation.

Table 4

The effect of controlling viral load and the subsequent adaptive immune response

Group	Viral load	CD8 T cell response	Mouse
(Robbins et al., 2007)	low	high IE1 (early, 4dpi)	BALB/c
(Andrews et al., 2010)	low	low IE1 (7dpi)	BALB/C
(Stadnisky et al., 2011)	low	high M45, M57, m139 (7dpi)	C57L.M-H2k
(Mitrović et al., 2012)	low	low M45, m139 (7dpi)	C57BL/6

increase in viral load of MCMV leads to better CD8 T cell responses, while others report the opposite result (Table 4).

Our results are in agreement with Stadnisky et al., who find increased CD8 T cell responses with lower viral loads, but differ from Andrews et al., who find enhanced CD8 T cell responses are derived from a robust replicating MCMV infection (Robbins et al., 2007; Stadnisky et al., 2011). The discrepancy between our data and the work of Andrews et al. may be due to the heterogeneity among between different strains of mice. Their data show a decreased acute IE1 response when the viral load is decreased. In fact, our experiments in BALB/c mice followed the same pattern that Andrew et al describe: the IE1 response was decreased in Famcyclovir-treated MCMV-TK-infect mice (Figure 2-1G). IE1 is defined as an inflationary epitope in MCMV infection, where responses progressively increase and dominate the chronic CD8 T cell response (Holtappels et al., 2000). In contrast to the BALB/c results, we found that replication-deficient infection in C57BL/6 mice results in enhanced M45- and M57-specfic CD8 T cell responses. This complements the results of Stadnisky et al. as they show increased M45-specific CD8 T cells in MHC-1 D^k disparate mice (Stadnisky et al., 2011; Xie et al., 2009).

We previously identified more than 15 epitopes during acute MCMV infection and up to 4 differentiation patterns of C57BL/6 Ag-specific CD8 T cell responses that progress into chronic infection (Munks et al., 2006a). The M45- and M57-specific CD8 T cell responses from C57BL/6 are non-inflationary, where peak levels are reached during acute infection and then contract during the chronic phase of infection. To align our data more closely with the results found in the BALB/c model, we investigated the inflationary m139 and M38 response from infected C57BL/6 mice and found there was

no attenuation or significant enhancement of these responses when Famcyclovir blocked viral replication (Figure 2-1). Comparing BALB/c (H-2d restricted) IE1 responses with the C57BL/6 (H-2k restricted) M45 and M57 responses is difficult, since inflationary and non-inflationary CD8 T cells have been reported to display disparate patterns of differentiation. We found, MCMV-TK infected BALB/c mice with Famcyclovir treatment, the results support the data from Andrews et al.—the IE1 response is elevated in a robust replicating infection. Thus, combining our work here with that of others shows that the CD8 T cell response cannot be labeled or defined by a single epitope. Instead there is differential regulation of non-inflationary and inflationary response to viral-replication, which will be important for the description of CD8 T cell responses in future models.

Mitrovic et al. study a model of MCMV infection in C57BL/6 mice using wild-type MCMV that expresses viral protein m157 and a m157-null virus. In their research, viral replication resulting in either low or high viral load is predominantly controlled by NK cell Ly49H interaction with viral protein m157. This model, as well as the model used by Robbins et al. and Andrews et al., limits the viral load during acute infection. In our C57BL/6 model, we also infect C57BL/6 mice with an m157-null virus; however, we completely inhibit viral-replication through antiviral drug therapy early after infection (Snyder et al., 2011). Similar to previous reports (Robbins et al., 2007; Stadnisky et al., 2011) and our data in Figure 2-6, Mitrovic et al. also show that there is a greater inflammatory cytokine response early in the infection and a decrease in cDCs around day 4 pi. Interestingly, they show the CD8 T cell responses are of greater frequency in both inflationary m139-specific and non-inflationary M45-specific responses after a week of

infection, which indicates that both the inflationary and non-inflationary responses are capable of being regulated by changes in viral-replication. They argue that despite a decrease in the cDC population, the increase of viral-load provides more antigen used for CD8 T cell priming. However, results from their IFN-γ ELISPOT assay show only the m139 response is elevated, with the M45 and M57 responses being equal to respective responses from a wild-type MCMV infection. The level of viral replication in these reports is regulated by the m157-Ly49H interaction or MHC D^k recognition of the virus. In either case, viral load is lowered but not eliminated. We believe our model is unique because we can investigate and characterize the CD8 T cell responses from an MCMV infection that can be completely inhibited/eliminated early after initial infection, which provides a CD8 T cell response primed by the first encounters with antigen and the inflammatory response.

A recent report by Torti et al. describes the importance of CD8α DCs in cross-presenting viral antigen in MCMV infection (Torti et al., 2011). They use Baft3-/- mice that lack CD8α and CD103 DCs, which are the DC subsets most responsible for cross-priming. Acute infection with MCMV in these mice shows more impairment of the priming of non-inflationary than the inflationary Ag-specific CD8 T cells. However, in the chronic phase of infection, the inflationary CD8 T cells are still able to differentiate into robust memory responses, presumably through direct presentation from chronically infected cells. This result implies that cross-presentation is important to the priming of all MCMV-specific CD8 T cells, but the non-inflationary CD8 T cells are more sensitive to changes of cross-presenting cells than the inflationary CD8 T cells—meaning that the loss of cross-presenting cells lowers the non-inflationary CD8 T cell responses to MCMV

infection. Since the enhanced CD8 T cells measured in our replication-deficient infection model were the only non-inflationary M45 and M57 epitopes, we believe that these elevated CD8 T cells are dependent on cross-presenting cells.

The Batf3-/- model is elegant, as it specifically removes the CD8a cDC population from the priming of CD8 T cells. However, one downside to using the Batf3-/- mouse is that cDCs are completely absent during the earliest stage of infection, and CD8α cDCs could be very important to the initial priming that occurs during the first few days after infection. Using a Batf3 conditional knockout mouse would be ideal, as depletion of these DCs could occur at multiple timepoints during acute infection and would provide a timeframe within which cDCs are necessary for the development of expanded CD8 T cells.

Using CD11c-DTR mice (Jung et al., 2002), we administered diphtheria toxin to deplete CD11c DCs after two days of infection. We show, unlike previous reports, which only correlate the reduction of DC populations to robust CD8 T cell responses, that a depletion of DCs after 2 days of priming negates the enhanced M45- and M57-specific CD8 T cell populations in a replication-deficient infection. Interestingly, the M45- and M57-specific CD8 T cell numbers were not lower than the numbers from a replication-competent infection, which shows that the first 2 days of priming is sufficient to drive clonal expansion of these Ag-specific T cells. However, these data also show the importance of DCs after initial priming in driving the enhanced CD8 T cell responses in a replication-deficient infection.

Implications of type I IFNs

Mice co-infected with both a replicating-deficient and a replication-competent virus did not form enhanced CD8 T cell responses (Figure 2-4). This led us to hypothesize that the inflammatory response during infection was modulating the Ag-specific CD8 T cell response. We investigated type I IFNs, as they are cytokines that can mature APCs to upregulate MHC and co-stimulatory molecules, as well as provide co-stimulation directly to CD8 T cells (Mescher et al., 2006; Stark et al., 1998).

Type I IFNs may also be important in the regulation of the cDC compartment during acute infection (Robbins et al., 2007; Stadnisky et al., 2011). Both Robbins et al. and Stadnisky et al. show that decreased viral load coincides with a decreased peripheral IFN-α, which correlates with a preserved cDC compartment. Robbins et al. go on to show that exogenous IFN-α administration decreases cDCs during infection (Robbins et al., 2007). In our model, we show that administration of Poly(I:C) to mice under Famcyclovir treatment negated the enhanced M45- and M57-specific populations. We used Poly(I:C) since it is a known adjuvant that induces IFN-α (Alexopoulou et al., 2001) and stimulates through TLR3, which is a known PRR for MCMV (Tabeta et al., 2004). We believed it would mimic a broad inflammatory response similar to that of a natural infection (Figure 2-7) (Matsumoto and Seya, 2008). Because Poly(I:C) induces a broad inflammatory response, we were able to single out the effect of type I IFNs by using the monoclonal antibody MAR1-5A3, which blocks the IFNα/β receptor. This antibody has previously been used to neutralize IFN-α signaling in West Nile Virus and LCMV with great efficacy (Pinto et al., 2011; Teijaro et al., 2013; Wilson et al., 2013). The addition of MAR1-5A3 to Famcyclovir-treated mice under Poly(I:C) administration restored the

enhanced M45 and M57-specific CD8 T cell response and the cDC populations. Ideally, we would like to have used MAR1-5A3 in MCMV-TK-infected mice without Famcyclovir, but MCMV is lethal in IFNα/β receptor knockout mice, and infection with MAR1-5A3 caused mice to lose a significant amount of weight and they were sacrificed during the first week of infection. Our data clearly show that the non-inflationary M45- and M57-specific CD8 T cells are dependent on DCs after the first two days of priming, although in this study we have not determined what type of support the DCs are providing to Ag-specific CD8 T cells during this time period. Andrews et al. have indicated that viral-replication leads to more infected DCs that can present antigen throughout acute infection and enhance CD8 T cell response. Although we have the opposite result– that viral replication leads to decreased CD8 T cell responses, the underlying explanation may still be related to the number of Ag-presenting DCs available to drive the response. The responses measured by Andrews et al. were driven by directly infected DCs, whereas ours are driven by cross-presenting DCs. In our model, blocking viral-replication preserves the cross-presenting cDCs that are presenting antigen non-inflationary Ag-specific CD8 T cells. However, this hypothesis needs further investigation.

More recently, Signal 3 has been identified as an aid to the differentiation of CD8 T cells by providing co-stimulation (Curtsinger et al., 1999; Curtsinger et al., 2005; Curtsinger and Mescher, 2010). Type I IFNs are a major signal 3 cytokine. The presence of an IFN-α in conjunction with or after priming of naïve CD8 T cells is important to the differentiation of these cells. Curtsinger et al. describe the stimulation of naïve CD8 T cells with an artificial APC (microsphere with antigen and B7 molecule) as poor, but with

the addition of IL-12 or type I IFNs, clonal expansion and effector function increase (Curtsinger et al., 1999; Curtsinger et al., 2005). This indicates that priming and differentiation process is dynamic, where multiple signals could be required at different time points to drive CD8 T cell differentiation.

During viral infections, the type I IFN response at the acute stages can be large and a persistent viral infection can drive constant production of this protein. Recently, the impact of type I IFN response in chronic infection has been described in clone-13 LCMV infection, where high levels of type I IFN induce immune suppression (Teijaro et al., 2013; Wilson et al., 2013). Neutralization of type I IFN signaling (via αIFNR1 mAb) helps to control and clear the virus, although the mechanism of suppression is unknown. The clearance of clone-13 LCMV is CD4 T cell-dependent as antibody depletion of CD4 negates the effect of αIFNR1 mAb treatment. These reports show that type I IFNs have various ways to activate or suppress many different cell types. Here, we focused on the early type I IFN events (< 3 days pi) during MCMV-TK infection that has an impact directly opposite to that predicted by a signal 3 response. We believe that type I IFN response initiates a suppressive mechanism to lower certain CD8 T cell responses.

Another remaining question is why the host or immune system would produce type I IFNs that decrease the DC populations and lower T cell responses. One of type I IFN's main function is to mature DCs and CD8 T cells. If this were its only functions, then it may follow that there should be robust CD8 T cell responses to infection. Alternatively, an overpopulation of these T cells might occur. The immune system has built-in mechanisms that serve as a "checks and balances" to regulate an overt response that would be detrimental to the host, such as pro-inflammatory (e.g., IL-1β, TNF-α) and

suppressive (TGF-β, IL-10) cytokines, cytotoxic cells (e.g., NK and T cells), and suppressive cells (e.g., Tregs and myeloid-derived suppressor cells). As stated above, the type I IFN response to MCMV is biphasic; the first at 8 hours pi and the second at 36–48 hours pi. I am not aware of any reports that distinguish between the function of each of these waves, but it is postulated that the first wave occurs from infected cells. Benedict et al. show infected stromal cells release type I IFNs in response to initial MCMV infection (Benedict et al., 2001), which may serve to alert the immune system of an infection, as well as provide activation of APCs and T cells to aid in priming.

We show that these two type I IFN waves are not the same size—the 8-hour IFN-α response is smaller than the second (Figure 2-5). Perhaps the first (weaker) IFN-α response aids primarily in activating localized DCs and CD8 T cells for priming (benefiting the host), while the second (greater) IFN-α response exists to help systemically limit viral growth and deplete cDCs (benefiting the host and the virus). The depletion of cDCs may serve two purposes—to eliminate infected cDCs early during infection in order to limit viral-replication and viral spread, or to remove APCs cells that prime and differentiate Ag-specific T cells into strong effector and memory populations. Perhaps taming the non-inflationary CD8 T cells prevents development of overzealous effector CD8 T cells that can harm the host. Or perhaps reducing these non-inflationary T cells will provide more space in lymphoid tissue for inflationary CD8 T cells to establish.

Differentiation of Ag-specific CD8 T cells in MCMV-TK infection

A hallmark of MCMV infection is the generation of a large Ag-specific CD8 T cell pool during latency. These Ag-specific CD8 T cells are described as "inflationary" cells, which are formed early or late in infection. A key characteristic of these cells is that they increase over time until a level of homeostasis is reached, resisting contraction. In comparison, "non-inflationary" cells are a CD8 T cell response that peaks in acute phase of infection and contracts by ~90% to form a stable memory population. In this thesis, I screened 10 epitopes that identify both immunodominant and subdominant responses. From this screen, only m139, M38 and IE3 are inflationary epitopes. Interestingly, only non-inflationary M45- and M57-specific CD8 T cells are enhanced when viral replication is inhibited with Famcyclovir treatment. It is interesting to compare this observation to that of Robbins et al. and Andrews et al., who found that the inflationary IE1 response is enhanced when the viral load is higher. Non-inflationary epitopes in BALB/c mice have not been elucidated, as both IE1 and m164 are inflationary epitopes.

A remaining question is why non-inflationary M45- and M57-specific CD8 T cell responses are enhanced with Famcyclovir treatment while inflationary m139 and M38 are not. Throughout most of this thesis I focus only on the immunodominant CD8 T cell responses in acute MCMV infection. These responses are large and easy to detect, especially through intracellular cytokine staining (ICS) or by cell-surface tetramer staining. Most of the data in this thesis are FACS based, but I believe a more sensitive assay could reveal other non-inflationary epitopes that are enhanced when viral replication is blocked. However, due to the weak response (< 1% of total CD8 T cells) of subdominant CD8 T cells, it is very hard to identify changes in their responses (above background) through ICS or tetramer staining. Thus, it would be interesting to analyze

subdominant epitopes in a more sensitive assay. An enzyme-linked immunosorbent spot (ELISPOT) assay is more sensitive than a FACS-based assay, as detectable responses to simulation can be found in less than 500 lymphocytes.

It is possible that gene regulation differentiates the amount of antigen available during an acute infection. M45, M57, m139, and M38 are all E genes. M45 (UL45, Ribonucleotide reductase homolog) and M57 (UL57, major DNA binding protein) are packaged in the virion and both aid in viral replication. m139 (UL22 homolog) also aids in viral replication and M38 (UL38 homolog) is an anti-apoptotic protein, Interestingly, M45 and M57 proteins are packaged in the virion, while m139 and M38 are not (Kattenhorn et al., 2004). It is not clear if the levels of these proteins packaged in the virion have a direct effect on the CD8 T cell response during an acute infection, especially in mice with Famcyclovir treatment as de novo antigen is limited to the first round of replication.

We propose that non-inflationary M45- and M57-specific CD8 T cells form better responses than CD8 T cells because these cells are predominantly primed by cross presentation. Our results provide support for hypothesis. In our model, cross-presenting cDCs decrease in a replication-competent infection and depletion of all DCs leads to a weaker M45- and M57-specific population. At day 7 pi, Ag-specific CD8 T cells from Famcyclovir-treated mice express a greater MPEC than untreated mice (Figure 2-2C). This data helps to identify changes of Ag-specific CD8 T cells between Famcyclovir-treated and untreated infections. However, both inflationary and non-inflationary MCMV-specific CD8 T cells have a greater MPEC phenotype in Famcyclovir-treated mice—indicating that the MPEC phenotype does not determine the M45 and M57

enhanced response in Famcyclovir-treated mice. Figure 2-2B clearly shows that the Ki67+ M45- and M57-specific CD8 T cell population is greater in Famcyclovir treated mice. This data may indicate that there is a mechanism that is causing these cells to proliferate over all other Ag-specific CD8 T cell populations, or it may indicate that there is a suppressive mechanism that is driving down m139- and M38-specific populations. We do not have data that detect the cell death and apoptosis of Ag-specific CD8 T cells between Famcyclovir-treated and untreated mice. In Appendix 5-3, we show that regardless of Famcyclovir treatment the total CD8 T cell population expressing cell death markers 7AAD+AnnexinV+ is significantly higher than in naïve mice. However, mice with Famcyclovir treatment have a significantly lower 7AAD+AnnexinV+ CD8 T cell population than untreated mice. This data does not definitively address why m139 and M38 are not enhanced in Famcyclovir-treated mice but it does provide an inference that cell death is a factor in the frequency or number of MCMV-specific CD8 T cells generated during acute infection. One future direction of this research includes is to identifying the cell death and apoptosis status of Ag-specific CD8 T cells.

It is important to note that in this research we cannot detect Ag-specific responses (via ICS or tetramer staining) before day 5 pi, which limits our ability to identify Ag-specific CD8 T cell differentiation leading up to the peak response/expansion at day 7 pi. It would be interesting to identify if there is a difference between the M45/M57-specific and the m139/M38-specific CD8 T cells at the early priming stage.

PD-1 expression in MCMV-TK infection

What is not fully understood is the expression of exhaustion molecules on these Ag-specific CD8 T cells. In Figure 2-2D, all immunodominant T cell responses analyzed show a high expression of programmed cell death-1 (PD-1). PD-1 is an exhaustion marker expressed on CD8 T cells that have been exposed to chronic inflammation and antigen (Sharpe et al., 2007). It is also an activation marker on CD8 T cells that identifies antigen-experienced CD8 T cells. Ligation of PD-1 with cognate ligands suppresses effector T cell function. Therefore, it is surprising that Ag-specific cells in Famcyclovir-treated mice express high levels of PD-1, as they are exposed to lower levels of inflammation and antigen. This phenomenon may mean that CD8 T cells in Famcyclovir-treated mice are seeing antigen even after viral-replication has been blocked—perhaps the preservation of cDCs causes an increased duration of presented antigen to CD8 T cells. Benedict el al. has reported that MCMV-infected DCs stunt the proliferation of CD8 T cells via a PD-1/PDL-1 interaction (Benedict et al., 2008). Although we have not compared the number of infected DCs between Famcyclovir-treated and untreated mice during acute infection, it is fair to assume that mice without Famcyclovir treatment would have more infected DCs. The PD-1/PDL-1 interaction in these mice could explain why Ag-specific CD8 T cells are not proliferating as much as CD8 T cells from Famcyclovir-treated mice.

The role of exhaustion in this model is unclear. It is possible that the increased inflammatory cytokine response and antigen exposure could cause CD8 T cells to

upregulate exhaustion markers (i.e., Lag3, 2B4, and PD-1) (Blackburn et al., 2009). However, despite the fact that PD-1 expression on M45- and M57-specific CD8 T cells is higher in Famcyclovir-treated mice, they proliferate more and expand into a greater acute stage T cell population, which indicates that identifying PD-1 expression is less important than identifying the ligand (e.g., B7-H1, B7-H2) expression during MCMV-TK infection. Future studies would analyze the role of the identification of PDL-1 in both the acute and chronic stages of infection, as well as neutralization of both B7-H1 and PD-1 molecules via monoclonal antibody therapy.

The role of DCs after initial priming

Our investigation led us to question the role of dendritic cells in this model, as DCs are professional APCs that prime naïve CD8 T cells and are sensitive to type I IFN-induced activation. We report in Figure 2-1 that without Famcyclovir treatment the viral load is high and the type I IFN response is increased, yet the conventional DC (cDC) numbers are lower, and the M45- and M57-specific response is lower. To address if there is a link between the strong type I IFN response and decreased cDC numbers, we measured DC numbers in mice given TLR3 adjuvant Poly(I:C) and neutralizing IFNα/β receptor antibody (Figure 2-7C). The results show that blocking the IFNα/β receptor restores the elevated M45- and M57-specific responses and the cDC population in Famcyclovir-treated mice at day 7 pi. A subsequent logical experiment involved infecting Famcyclovir-untreated mice with MCMV-TK and injecting neutralizing IFNα/β receptor antibody 1 day pi. Neutralizing early type I IFN responses during an acute MCMV

infection allows the virus to escape the antiviral effects of type I IFN. These mice have higher titers, lose weight rapidly, lack movement, and have severe lymphopenia. They are too sick to survive a week of infection and are very similar to IFNα/β receptor-/- mice when infected (personal observation).

We found that DCs are essential in our model of enhancing the CD8 T cell response during acute MCMV infection. We show the importance of preserving DCs after initial priming by infecting CD11c-DTR mice and depleting the DC compartment at day 2 pi with diphtheria toxin administration. One downside to using a CD11c-DTR mouse to deplete DCs is that all CD11c DCs are susceptible to diphtheria toxin elimination. Therefore, we cannot definitively conclude that cDCs are driving the enhanced M45- and M57-specific CD8 T cell responses in a replication-deficient infection. However, my work in this thesis shows that preservation of cDCs after initial priming is necessary for the enhancement of cross-presented Ag-specific CD8 T cells.

If the preservation of cDCs is truly needed for the expansion of M45- and M57-specific CD8 T cells in Famcyclovir-treated mice, then one of the questions left unanswered is what type of assistance the cDCs are providing to these cells after initial priming. The persistence of antigen or co-stimulation may act as another signal to help prime and drive more proliferation in these non-inflationary CD8 T cells. A next step in this project would be to identify co-stimulatory molecules on T cells (e.g., 4-1BB and OX40) and ligands on DCs that differ between Famcyclovir-treated and untreated mice. This study should be in conjunction with a study of the exhaustion markers to identify any suppressive mechanisms. An experiment that would support the theory that the preservation of DCs is necessary would involve an adoptive transfer of a CFSE labeled

Tg-M45 or Tg-M57 CD8 T cell into infected mice after 2 days pi and an analysis of whether these cells proliferate more in mice treated with Famcyclovir verses untreated mice. We currently do not have any of these transgenic mice. Alternatively, another way to raise the number of M45- and M57-specific cells is to point that they could be detected very early in infection would be to sort out M45 and M57 memory CD8 T cells from chronically infected mice, and transfer them into naïve mice before infection with MCMV.

Wt-BAC and MCMV-TK co-infection

It is not completely clear why MCMV-TK co-infection with wt-BAC (with Famcyclovir treatment) results in an elevated M45- and M57-specific CD8 T cell responses, while co-infection with dm157-MCMV ablates these responses (Figure 2-4A and C). The difference between wt-BAC and dm157-MCMV is the expression of the m157 ORF. Wt-BAC infected cells are susceptible to NK cell-mediated control via Ly49H, which results in lower titers and a weaker inflammatory response (Figure 2-4D, IFN-α ELISA). Lower titer and IFN-α levels are the main factors in the premise of my hypothesis of Famcyclovir treatment enhancing CD8 T cell responses in MCMV-TK infection. Because wt-BAC is in line with my MCMV-TK hypothesis I believe this is the reason why wt-BAC/TK co-infection forms elevated M45- and M57- specific CD8 T cells responses, in comparison to a dm157/TK co-infection–despite that both wt-BAC and dm157 are replication-competent viruses.

Wt-BAC does not form elevated CD8 T cell responses (Figure 2-4C). Yet, mice

wt-BAC infected mice under Famcyclovir treatment form elevated CD8 T cell responses, although the elevated responses are less than 2-fold. Snyder et al. also found that Famcyclovir treatment decreased titers of wt-BAC by 1 log, indicating that endogenous kinases are activating Famcyclovir to limit viral replication. Therefore, this data suggests that there is a suppressive threshold of viral replication/ IFN-α that affects the CD8 T cell response–meaning that wt-BAC limits viral replication and type I IFN levels but not to the point where it may affect cross-presenting DC populations. However, if viral replication is completely blocked or IFN-α is neutralized, lower than the levels set during a wt-BAC infection, the M45- and M57- specific CD8 T cell response results in a higher frequency and number.

Contraction of M45- and M57-specific responses

In this study, we find the contraction of M45- and M57-specific CD8 T cells to be rapid and faster in Famcyclovir-treated mice verses untreated mice. There are two hypotheses that may explain this phenomenon. First, it is possible that blocking viral replication inhibits survival cytokine production. The IL-2 ELISA to measure production between Famcyclovir-treated and -untreated mice was not sensitive enough to identifying a difference between these groups, and IL-7 and IL-15 CD8 T cell survival cytokines were not measured in this assay. However, the general cytokine response to blocking viral-replication during acute infection is one of inhibition, as our data show that in acute infection, cytokines are decreased or unchanged in comparison to replication-competent infection. Based on these data, it is plausible that survival cytokines are also inhibited in mice treated with Famcyclovir. Even if IL-2, IL-7, and IL-15 were not decreased from

the inhibition of viral-replication, the total number of CD8 T cells, specifically effector M45 and M57 T cells, is significantly increased during acute infection, which would deplete cytokine resources. Therefore, I hypothesize that the lack of survival cytokines is driving a steep contraction rate in Famcyclovir treated mice in comparison to untreated mice.

Second, McNally et al. show that type I IFN induces bystander elimination of CD8 T cells during acute viral infection (McNally et al., 2001). It is proposed that this elimination of non-specific CD8 T cells in the acute stage of infection helps create space, which Ag-specific CD8 T cells can fill during clonal expansion. Inhibiting the virus with Famcyclovir and reducing the type I IFN response eliminates any vacancy in which proliferating CD8 T cells can settle. Therefore, peak numbers are reached by the highly replicating M45- and M57-specific CD8 T cells in Famcyclovir-treated mice, but with limited space and cytokine-mediated resources, these CD8 T cells contract at a greater pace in comparison to their counterparts in Famcyclovir-untreated mice.

Implication of this work in immune therapy

I do not believe the research described in this thesis would be useful for the development of immune therapy to prevent CMV infection. However, this research could have implications for the development of vaccine therapy. One theme that is reinforced throughout this thesis is the importance of timing. As discussed earlier, type I IFNs have functions to help activate and suppress the immune system. Manipulating the type I IFN

response during the administration of a vaccine can be useful in generating a robust CD8 T cell response, perhaps by giving a recipient of a vaccine a dose of neutralizing IFNα/β receptor mAb after vaccination. It is doubtful this strategy would work in vaccines that contain large inert proteins (e.g., influenza vaccines). Many of these vaccines include adjuvants, including the activation of type I IFNs, to help with the priming of the immune system. A neutralizing mAb would counter the adjuvant function. Administration of neutralizing IFNα/β receptor mAb in a live-attenuated vaccine may be effective, as the infection would induce a systemic inflammatory response. In particular, a CMV-based vaccine would be an ideal parallel model to test such type I IFN-based therapy. However, using a neutralizing IFNα/β receptor would block the beneficial antiviral functions of the type I IFNs, thereby making the host susceptible to increased viral loads and CMV-related disease.

CHAPTER 4: MATERIALS AND METHODS

Mice

C57BL/6, CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), BALB/c, and CD11-c DTR (B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J) were purchased from Jackson Laboratory. C57BL/6 mice were bred to CD45.1 mice, in-house, to generate CD45.2 x CD45.1 F1 mice used for adoptive transfer experiments. Mice used were between the ages of 6 and 36 weeks.

Virus strains

Preparation of MCMV-TK and MCMV-dm157 mice has previously been described by Snyder et al. (Snyder et al., 2011). Briefly, HSV-1 TK gene was inserted in the m157 locus of pSm3fr, the Smith-based BAC from which MW97.01 virus is derived. Virus was passaged in mouse embryonic fibroblasts more than 8 times before being injected into BALB/c mice. All mice were inoculated with $2*10^5$ PFU i.p..

Tissue processing

Spleens were collected and placed in cold RPMI media. Splenocytes were collected after spleens were crushed in a 70 μm cell strainer (BD pharmingen) with a syringe plunger. Cells were spun down and red blood cells were lysed by the addition of ACK buffer (Gibco) for 10 minutes at room temperature. The splenocytes were then washed with FACS buffer and counted. Cells were then plated for surface staining.

Antibodies

The following antibodies were used: KLRG1 FITC (Biolegend, clone:2F1/KLRG1), CD127 PerCp-Cy5.5 (Biolegend, clone: A7R34), PD-1 PE-Cy7 (Biolegend, clone: RMP1-30),CD8 Brilliant Violet 405 (Biolegend, clone: 53-6.7), PDL-1 Brilliant Violet 421 (Biolegend, clone: 10F.9G2), CD3 PerCP-Cy5.5 (eBiosciences, clone: 17A2), Ki67

FITC (BD, clone: B56), CD11b APC-efluor 780 (eBiosciences, clone M1/70), CD11c PE-Cy 7 (eBiosciences, clone: N418), Live/Dead Fixable Aqua Dead Cell Stain Kit 405 (Invitrogen, cat#L34957), tetramers were made from the NIH tetramer core (M45 APC, m139 APC, M57 PE, and M38 PE).

Antibody Staining

Cells were plated at approximately $5*10^5$ per well in a 96-well round bottom plate in 1% FACS buffer (PBS + 1% BSA + 2mM EDTA). The cells were spun down at 4^oC and vortexed in on a plate shaker to disrupt the cell pellets. An antibody cocktail mix was made with surface-staining antibodies, including the live/dead cell marker Amine Aqua (Invitrogen). Antibody cocktail was added at 50 µl per well and mixed with a pipette 3–5 times. Cells were then incubated in the dark at 4^oC for 1 hour. After incubation the cells were washed with FACS buffer 3 times. If cells were from an ICS assay and subjected to intracellular staining, then follow the steps below in the ICS assay.

Intracellular Cytokine Staining (ICS)

Cells were cultured for 6 hours at 37^oC with peptides and brefeldin A [10 µg/ml]. DMSO was cultured with cells as an experimental negative control. Cells where then stained for cell surface markers (detailed above). After surface staining the cells were incubated with 100 µl of Cytofix/Cytoperm reagent (BD pharmingen) for 10 min at room temperature, or at 4^oC overnight. After fixation cells were washed with 1x Permwash (BD pharmingen) 2 times. An intracellular antibody cocktail was made with the 1x Permwash as the solution buffer. The intracellular antibody cocktail was added to the wells at 50 µl per well and incubated in the dark at 4^oC for 2 hours.

FACS Analysis

Cells were transferred into FACS tubes (Corning) with a 1% paraformaldehyde (PFA) solution. Cells were then analyzed, uncompensated, on a BD LSRII flow cytometer located in the OHSU Flow Cytometry Flow Core. Data was analyzed using Flowjo software (Tree Star, Inc.).

Adoptive Transfer

CD45.1 x CD45.2 F1 mice were bred in house. Negative selection of CD8 T cells was performed by magnetic bead isolation (StemCell Mouse Negative Selection Kit). $3*10^6$ pure CD8 T cells were resuspended in 100 µl of cold PBS and adoptively transferred by retro-orbital i.v. injections.

Cytokine array

Quantification of circulating cytokines and chemokines were measured in the plasma of infected mice. Approximately 400 µl of blood was collected and mixed with 10 µl of heparin via retro-orbital bleeding. Collection tubes were spun down and plasma was collected and placed into a 96-well plate. Plasma was collected on multiple days and frozen at -80^{o}C until all specimens were collected. Plasma was thawed and analyzed by a multiplex ELISA assay according to manufacturer's instructions. (Quansys Biosciences: Mouse Cytokine Screen cat#110951MS or Life Technologies: Cytokine Mouse Magnetic 10-PLEX Kit cat# LMC001M).

TLR agonist and Diphtheria Toxin administration

Poly(I:C) (Invivogen) was administered i.p. at 100ug per injection. Diphtheria toxin (Sigma) was administered i.p. into CD11c-DTR positive and littermate controls animals at 3ng/mg of body weight.

Tissue sectioning and microscopy

Spleens from mice were collected and embedded in 1.5% agarose TAE. 500um sections were made and pretreated with CD16-pure antibody before being stained with CD8-Cy and B220-alexa488 overnight at 4^{o}C. Cells were washed and mounted on glass slides with Fluoromount G. Sections were analyzed on a Zeiss Apotome wide-field microscope with a 10x magnification.

Statistics

Student's t-test was performed where appropriate. All statistics were calculated using Prism software (GraphPad, Inc.)

Contributions

The Obhrai and Stenzel-Poore laboratories at OHSU contributed TLR agonist reagents. The O'Rourke laboratory (special thanks to Garen Gaston) at OHSU contributed the initial diphtheria toxin reagent.

CHAPTER 5: APPENDIX

Data not included in Chapters, Pilot Experiments, Future Studies, etc.

5.1 FoxP3 expression on CD4 and CD8 T cells is greater in replication-competent MCMV infection

Introduction

Regulatory T cells (Tregs) are subsets of T lymphocytes that modulate the immune system by suppressing the proliferation and activation of conventional T lymphocytes. They function to prevent excessive or aberrant responses against pathogen recognition or self antigens. Tregs were originally identified as "Suppressor T cells" with a CD8 T cell lineage (Gershon and Kondo, 1970). However, CD4 T cells expressing the master regulator FoxP3 and CD25 are widely known to be the prominent regulatory T cell and are extensively investigated as such (Sakaguchi et al., 2007). The potential uses of Tregs are vast, as they help maintain the tolerance to self-antigens, help with the maternal-fetal interface during pregnancy, and mediate the inflammatory response to an infection. Deletion of the Tregs can also help unmask effector responses to viral infections (Legrand et al., 2006). Therefore, understanding the function of Tregs is an important area of research, as modulation of Tregs can help with treatment of autoimmune disease, or in cancer or infection therapy.

Because my thesis is focused on understanding the mechanism of CD8 T cell responses in a replication-deficient MCMV infection, I questioned whether Tregs play a role in acute CD8 T cell responses. Specifically, I was interested in whether Tregs exist in greater frequency during a replicating viral infection (MCMV-TK without

Famcyclovir treatment) for the purposes of lowering acute CD8 T cell responses. I therefore explored the frequency of FoxP3 expressing CD4 and CD8 T cells in MCMV-TK infection as described below.

Results:

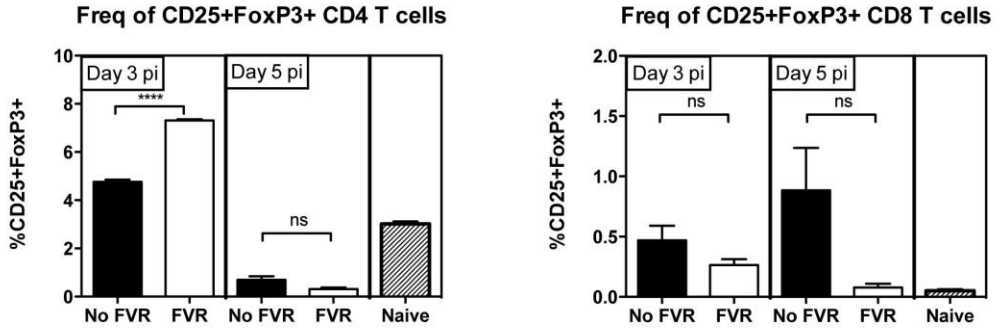
Appendix Figure 5-1A and B show the frequency of CD4+CD25+FoxP3+ and CD8+CD25+FoxP3+ splenocytes at days 3 and day 5 pi. The CD4 Treg population at day 3 pi was significantly higher in Famcyclovir treated mice versus untreated mice (4.763 ± 0.08838 vs. 7.313 ± 0.04667%). However, at day 5 pi, the population of CD4 Tregs in both groups of mice dropped below 1% with no significant difference between them. The CD8 Treg population at both day 3 and day 5 pi was not significantly different between Famcyclovir treated versus untreated mice.

Conclusions:

The greatest difference in the Treg frequency between Famcyclovir treated and untreated mice was within the CD4 Treg subset at day 3 pi. This result was not expected because Tregs are activated by antigen recognition via the MHC recognition, and Famcyclovir prevents the early inflammatory response and the antigen-load of MCMV. At day 5 pi, at the start of the expansion phase of Ag-specific CD8 T cells, the CD4 Treg population was below 1% in both Famcyclovir treated groups. This decrease of CD4 Tregs supports the expansion phase of effector CD8 T cells since a primary function of Tregs is to limit T lymphocyte proliferation. However, the role of CD4 Tregs is unclear, since both groups had similar frequencies at day 5 pi. Based on my data in Chapter 2, Famcyclovir treated mice had significantly higher expression of proliferation marker Ki67 at day 6 pi. If the frequency of CD4 Tregs is marginal at day 5 pi, yet Ag-specific CD8 T cells are

proliferating with Famcyclovir treatment, then it is likely that Tregs are not playing a role in the difference between Famcyclovir treated and untreated mice.

To fully understand whether CD4 Tregs are playing a role in masking CD8 T cell responses to MCMV-TK infection, one possibility is to use the FoxP3^{DTR} mice, where conditional depletion of FoxP3 is achieved through diphtheria toxin administration (Kim et al., 2007). Infection of the FoxP3^{DTR} mice is more elegant than using a broad CD4 depleting antibody (GK1.5 mAb) since it would allow dissection of the Treg function versus FoxP3^{DTR} negative CD4 T cells that could provide CD4 help. The CD8 Treg populations were not significantly different at either day 3 or day 5 pi, which indicates that they have little impact on the acute CD8 T cell responses. Furthermore, a recent report has stated that CD8+FoxP3+ Tregs share the same lineage as CD4 Tregs, however, the suppressive function of CD8 Tregs is weak in comparison to their CD4 counterpart (Mayer et al., 2011). Overall, the data collected here do not support the theory that Tregs are driving the difference between Famcyclovir treated and untreated Ag- specific T cell responses.



5-1: Treg frequency is dynamic with viral replication.

Spleens from MCMV-TK infected C57BL/6 mice were harvested on days 3 and 5 pi. FoxP3 expression was measured in CD25+ (A) CD4 T cells and (B) CD8 T cells. Graphs shows statistics from a student's t-test. ****p<0.0001

5.2 The CD8 T cell response to MCMV-TK in IFNα/β receptor-/- and IL-12p35-/- mice

To identify whether stimulatory factors are contributing to the enhanced CD8 T cell response, we investigated signal 3 cytokines IFNα/β and IL-12. Previous work has suggested that these cytokines drive primed CD8 T cells to form better memory and effector responses (Curtsinger et al., 2005; Curtsinger and Mescher, 2010). We performed multiple experiments addressing the Ag-specific CD8 T cells by tetramer binding and production of IFN-γ. In experiment 1, blood was collected from both IFNα/βR KO and wild-type mice. Blocking viral replication with Famcyclovir did not affect the CD8 T cell response in IFNα/βR KO-infected mice, as the frequency of IFN-γ+ CD8 T cells was similar between Famcyclovir-treated and -untreated mice. However, the magnitude of the M45 and m139 responses was surprisingly high. These responses were similar or higher than the CD8 T cell response from C57BL/6 mice under Famcyclovir treatment. In experiment 2, the frequency of IFN-γ+ CD8 T cells from IFNα/βR KO was measured from splenocytes at day 7 pi. The M45, m139, and M57 specific response was significantly elevated with Famcyclovir treatment. In experiment 3, we measured splenocytes for tetramer-specific CD8 T cells at day 7 pi. With Famcyclovir treatment, only the M45 tetramer+ CD8 T cells were significantly higher versus the response from untreated mice. Together, these results show that blocking MCMV-TK viral replication in IFNα/βR KO is not completely understood; the CD8 T cell responses between blood and spleen are not consistent, and the correlation between IFN-γ+ cells and tetramer+ cells in the spleen varies among the epitopes.

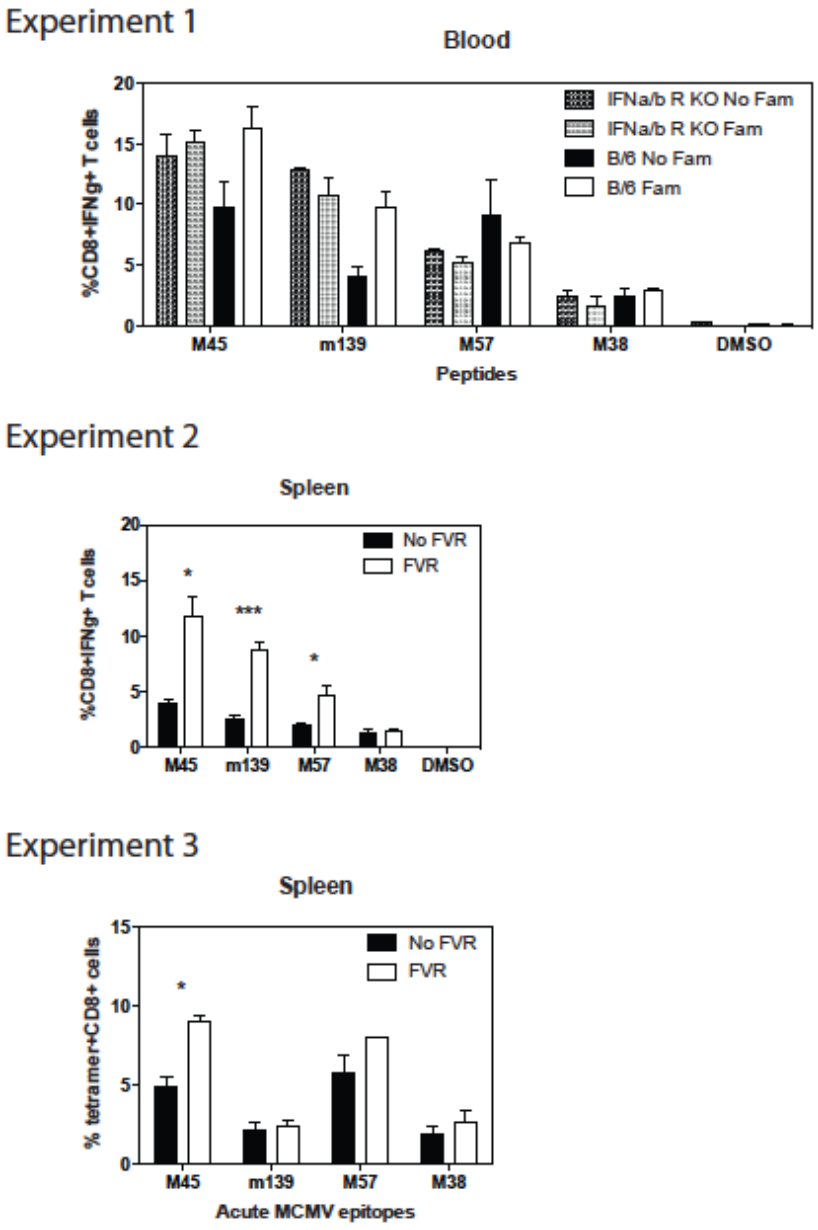


Figure 5-2.1: The CD8 T cell response in IFNα/β receptor KO mice to MCMV-TK infection.

Graphs represent 3 different experiments in MCMV-TK infected IFNα/β receptor KO mice. Experiments 1 and 2 represent an ICS assay on day 7 pi to measure IFN-γ production from peptide-stimulated splenocytes. Experiment 3 shows the frequency of splenic tetramer+ CD8 T cells in immunized mice at day 7 pi. Graphs represent the average with the SEM and significance as determined by a student's t-test (*$p<0.05$, ***$p<0.0005$).

We next pursued the CD8 T cell response in IL-12p35-/- mice (Figure 5-2.2). IL-12p35 is a subunit in the IL-12 receptor complex, and the lack of p35 prevents the binding and signaling of soluble IL-12. Since IL-12 is a signal 3 cytokine, we hypothesized that a robust CD8 T cell response to MCMV-TK would necessitate IL-12 signaling. The CD8 T response, measured by IFN-γ secretion, is elevated with M45, m139, and M57 peptide stimulation. The frequency of M45- and m139- specific responses was significantly elevated in Famcyclovir-treated mice verses -untreated mice,

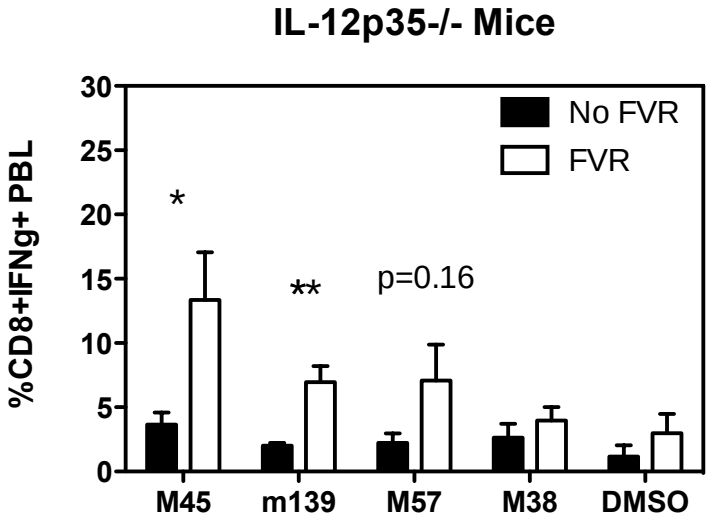


Figure 5-2.2: The CD8 T cell response in IL-12p35 KO mice infected with MCMV-TK
IL-12p35 KO mice were either treated or untreated with Famcyclovir and injected with $2*10^6$ PFU of MCMV-TK 3 days later. Peripheral blood leukocytes were collected at day 7 pi and an ICS assay was performed. Graph represents the average with the SEM and significance as determined by a student's t-test (*p<0.05, **p<0.005).

while the difference between Famcyclovir-treated mice stimulated with M57 peptide was

not. These results show that the enhanced CD8 T cell response in Famcyclovir-treated mice is not dependent on the signal 3 cytokine IL-12.

5.3 CD8 T cell death in MCMV-TK infected mice

In Figures 2-1F and 2-2A, the total number of CD8 T cells was higher in comparison to untreated mice, which suggests two possibilities: the CD8 T cells are expanding faster when viral replication is blocked, or CD8 T cells are dying in untreated mice, which prevents maximal expansion. To address the latter hypothesis, we measured the expression of cell death and apoptosis markers 7AAD and Annexin V. 7AAD (7-Aminoactinomycin D) is a DNA intercalating agent that identifies dead cells, while apoptosis is measured by staining for inner cell surface protein Annexin V (Figure 5-3A). A comparison between Famcyclovir-untreated mice versus -treated mice showed significantly more dead cells in mice without Famcyclovir treatment (Figure 5-3B), which indicates that viral replication is causing more CD8 T cells to die at day 7 pi. Because this data is only at one time point, it is not clear how early or rapidly cell death may be occurring at earlier time points. Cells that are going through apoptosis (7AAD-AnnexinV+) are not significantly different between Famcyclovir-treated and -untreated groups. However, Annexin V staining was significantly higher in both groups compared to naïve mice.

We also performed an additional apoptosis experiment and stained CD8 T cells for intracellular apoptosis protein Bim (BH3-only protein). Figure 5-3C shows an overlay of histograms of Bim expression in CD8 T cells from infected and naïve mice. Blocking viral replication with Famcyclovir decreased the frequency of Bim+ CD8 T cells, but not

to the level of expression in naïve mice. Collection of Bim data was technically difficult as the Bim expression varied on the method of permeabolization buffer used. This experiment was performed with BD Perm Buffer 2. Together, these data show that blocking viral replication preserves CD8 T cells from death and possible apoptosis, although the AnnexinV and Bim data is not consistent. Unfortunately, tetramer staining within these experiments was poor, and distinct Ag-specific populations expressing these death/apoptosis markers could not be identified.

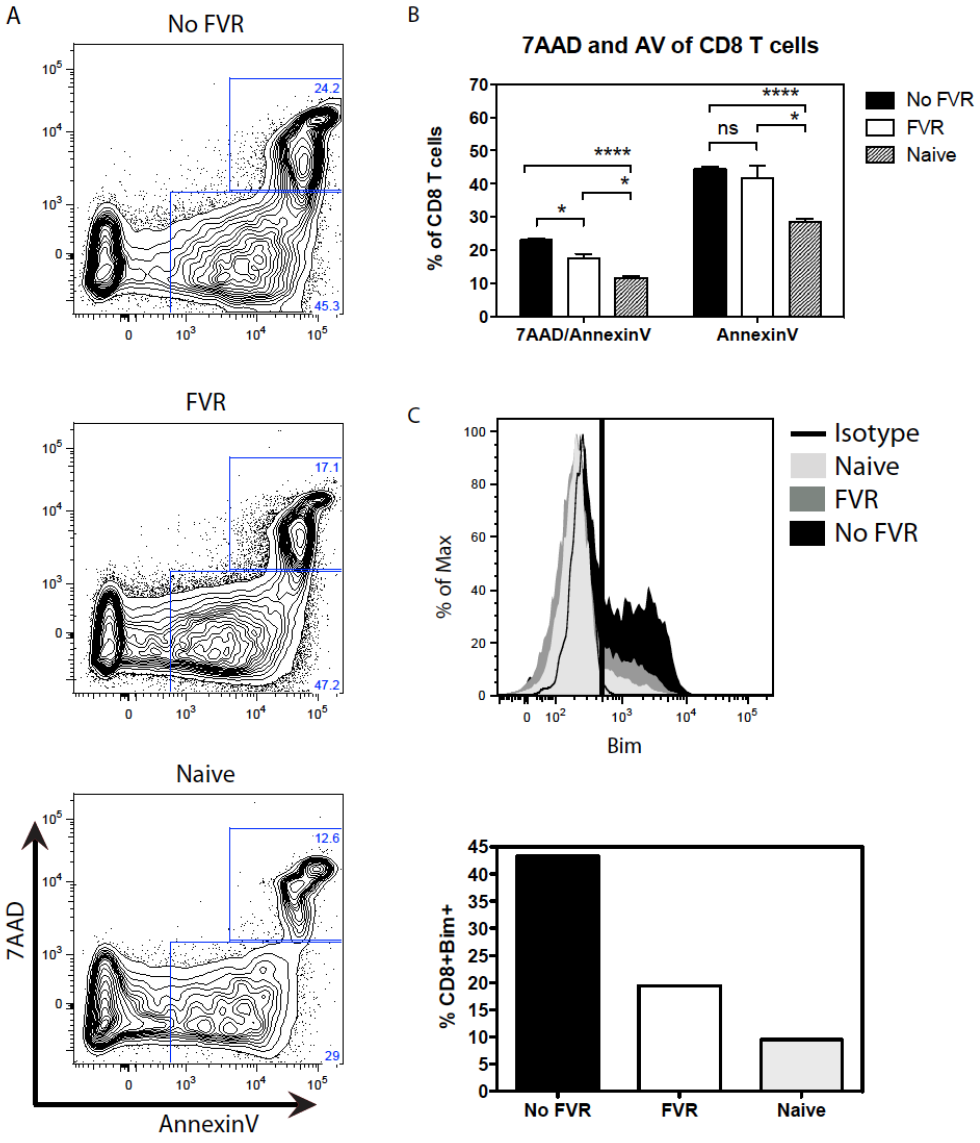


Figure 5-3: Blocking viral replication reduces CD8 T cell death and apoptosis during acute MCMV-TK infection

Spleens were collected from C57BL/6 mice at day 7 pi. (A) Contour plots show the expression of 7AAD and AnnexinV in CD8 T cells from mice without Famcyclovir treatment (No FVR), with Famcyclovir treatment (FVR), and uninfected mice (Naïve). (B) Bar graph shows the frequency of dead (7AAD+AnnexinV+) and apoptosis CD8 T cells. (C) Histogram identifies the expression of Bim staining of CD8 T cells between No FVR vs. FVR-treated mice at day 7 pi. Bar graph represents the CD8 expression of Bim. Each group represents a pool of three mice. Graphs represent the average with the SEM and significance as determined by a student's t-test (*p<0.05, ****p<0.0001).

5.4 Dose-dependent CD8 T cell response to MCMV

In an effort to compare antigen dose to the Ag-specific CD8 T cell response, we infected mice with a stratified dose of virus ($2*10^2$– $2*10^5$ PFU/mouse) (Figure 5-4A). We found that increased dose of virus resulted in an enhanced Ag-specific CD8 T cell response in both Famcyclovir-treated and untreated mice –meaning that increasing the viral load in conjunction with the inflammatory response forms a better CD8 T cell response. The only exception is the M57-specific population in Famcyclovir-untreated mice where both the frequency and absolute numbers at $2*10^4$ PFU was slightly higher than those measured at $2*10^5$ PFU. Like the data in Figure 2-1, the M45- and M57-specific responses were preferentially increased with Famcyclovir treatment. Famcyclovir treatment causes clearance of the virus and limits the viral load to what is produced in the first round of infection, yet CD8 T cell responses from treated mice out performs responses than non-treated mice. Therefore, antigen load cannot be the only or strongest predictor of CD8 T cell responses.

In Figure 5-4B, we show the M45- and M57-specific CD8 T cell populations from mice infected with dm157, MCMV-TK, and wt-BAC. We did not measure the titers of virus from each group but we ordered the columns in this graph from left to right based on the predicted viral loads, respective of Famcyclovir treatment. For instance, dm157 and MCMV-TK should have higher viral titers than wt-BAC because they are m157-null virus and are not susceptible to acute NK cell responses. dm157 would have the highest titer in Famcyclovir-treated groups since it does not initiate the m157 NK cell response and it does not contain TK to activate Famcyclovir, followed by wt-BAC, and MCMV-TK. If the order of these groups of mice is correct, then this result shows that decreasing

the viral load in C57BL/6 mice correlates to an increase in the M45- and M57-specific CD8 T cell response. This result now suggests the opposite from what we described in Figure 5-4A. Our cytokine data in Figure 2-5 was measured in mice infected with $2*10^5$ PFU of MCMV-TK (the highest dose measured in this thesis). By day 2 pi, many of the inflammatory cytokines were negated in Famcyclovir-treated mice, in particular IFN-α. Even though we do not have data to show the inflammatory response in this experiment, we can assume a lower dose of infection produces both a lower viral load and inflammatory response. This suggest that the inflammatory and antigen together are influencing the enhanced M45- and M57-specific CD8 T cell responses in this system.

5.5 Construction of Delta-gL-TK virus

Introduction

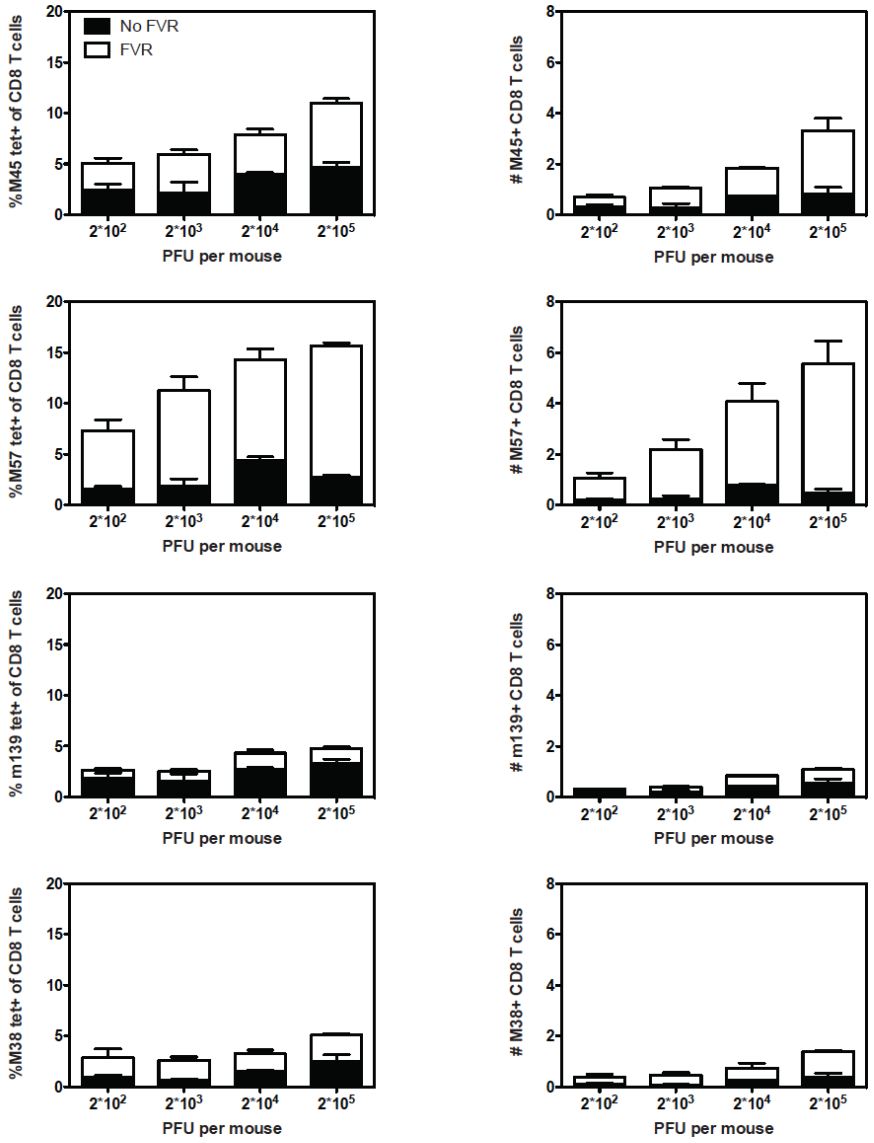
The MCMV-TK virus is replication- and spread-deficient when under Famcyclovir treatment (Snyder et al., 2011). In our laboratory, we also study delta-glycoprotein L (dgL) MCMV virus, which is spread-deficient and produces undetectable viral titers at 3 days pi. Glycoprotein L (gL) is a surface molecule that is necessary for the fusion events of the virion to interact and enter the plasma membrane of a target cell. gL is not a necessary protein for viral replication, but it is essential for the infectivity of the virion. In culture, dgL-MCMV is grown in complementing gL-fibroblasts that express the gL protein on the membranes of the Golgi transport vacuoles. Therefore, as a dgL virion buds from the cytosol into a vacuole, it acquires gL as part of the envelope membrane structure and enables one round of infection.

Despite MCMV-TK and dgL-MCMV being spread-deficient, the acute CD8 T cell response infection is not comparable. While MCMV-TK infection produces an enhanced CD8 T cell response when viral-replication is inhibited early after infection, dgL-MCMV infection forms an attenuated response (Snyder et al., 2011). It is unclear why both of these attenuated viruses form disparate CD8 T cell responses during acute infection; however, I hypothesize that production of non-infectious virions from dgL-MCMV infection may modulate the immune response and suppress CD8 T cell responses. To address this hypothesis I constructed an MCMV-TK virus that lacks gL (dgL-TK virus).

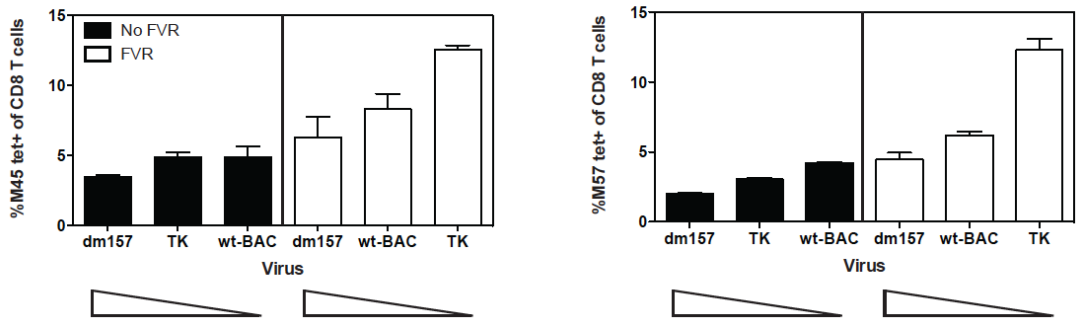
Figure 5-4: Increasing the infectious dose of MCMV leads to better M45- and M57-specific CD8 T cell responses

C57BL/6 mice were treated or untreated with Famcyclovir and infected (i.p.) with $2*10^2$, $2*10^3$, $2*10^4$, or $2*10^5$ PFU of virus. Spleens were collected on day 7 pi. (A) Graphs show the frequency and absolute numbers of Ag-specific CD8 T cells. (B) Graph shows the M45- and M57-specific response from dm157, wt-BAC, and MCMV-TK. The gradient at the bottom of (B) represents the level of viral load from each infection group.

A


No FVR
FVR
%M45 tet+ of CD8 T cells
M45+ CD8 T cells
%M57 tet+ of CD8 T cells
M57+ CD8 T cells
% m139 tet+ of CD8 T cells
# m139+ CD8 T cells
%M38 tet+ of CD8 T cells
M38+ CD8 T cells
2*10^2
2*10^3
2*10^4
2*10^5
PFU per mouse

B


No FVR
FVR
%M45 tet+ of CD8 T cells
%M57 tet+ of CD8 T cells
dm157
TK
wt-BAC
dm157
wt-BAC
TK
Virus

Construction of the virus:

Our plan was to delete gL from the MCMV-TK virus (as opposed to replacing m157 with TK in a dgL virus). The process of generating MCMV-TK is described in the paper by Snyder et al., Sustained CD8 T cell memory inflation after infection with a single cycle cytomegalovirus, PLoS Pathogens, 2011:

To produce the MCMV-TK virus, the Thymidine Kinase gene from HSV-1 was inserted into the m157 locus of MCMV using lRED mediated recombination. Briefly, TK from HSV-1 was amplified from a plasmid (kindly provided by David Johnson) and sub-cloned into a second plasmid containing kanamycin flanked by FRT recombination sites (kindly provided by Jay Nelson). PCR was performed to generate the TK-Kan construct flanked with MCMV sequences in the m157 region and this PCR product was recombined with wild-type MCMV cloned into a bacterial artificial chromosome (BAC, strain MW97.01 [57]). The final product replaced the entire m157 coding region with HSV-1 TK. Kanamycin was removed by Flp-mediated recombination of the FRT sites and the final product was verified by PCR and sequencing.

Using the TK-Kan construct, we deleted the gL gene by homologous recombination with an ampicillin gene sequence with gL overhangs. Table 6.4.1 shows the PCR reaction of the gL–ampicillin–gL 1.5 kb fragment. We used the pGEM-T Easy plasmid (Promega, cat#A1360), which contains ampicillin, and designed “primer 28”—homologous sequence to gL (fragment= 1.5kb=2 minute extension).

Table 4: PCR reaction for gL-Ampicillin-gL fragment

PCR Master mix		PCR reaction		
10x dilution of template	1 µl	Denature	2min	94°C
primer 28 F+R	1 µl	**Denature**	**30sec**	**94°C**
10x Buffer dNTPs	5 µl	**Annealing**	**30sec**	**58°C**
Taq	0.2 µl	**Extension**	**2min**	**72°C**
Water	42.8 µl	Extension	7min	72°C
		Hold	24hours	10°C
		Bold: 30 cycles		

Primer 28F:
ACATATGGCCTAAGATGGTACGGAGGAGGTTATATGTGCCGTCGAGCAGGCATTCA
AATATGTATCCGCTCATG

Primer 28R:
CCCATCCGTCCGGACCCGTATAGGTTCTCGACGATGATCAGGTTCAAGAGAACTTG
GTCT

(Invitrogen AccuPrime Taq DNA pol HIFI #12346-086)

BAC cloning protocol by George Xu MD, PhD, and Tameka Smith

Preparation of Competent Bacteria

1. Grow 3 ml O/N culture of EL250 with 25 mg/ml chloramphenicol.
2. Start 50 ml culture with 1 ml of the O/N culture.
3. Grow at 30°C until the spec reaches 0.4 wavelength 600 (about 3-4hours).
4. Induce at 42°C for 17 min; swirl every 3 minutes.
5. Place on ice for 10 min. A slushy water ice mix works best.
6. Spin at 3.5K for 10 min at 4°C in 50 ml tubes.
7. Pour off LB supernatant, resuspend pellet with 10 ml cold water and fill the 50 ml tube with cold water. Spin at 3.5K for 10 min at 4°C.
8. Pour off water supernatant carefully (pellet is not sticky). Resuspend pellet with 10 ml cold water and fill the 50 ml tube with cold water. Spin at 3.5K for 10 min at 4oC. Use Vacuum to remove water.

9. Resuspend pellet in 3-8 ml cold water. The volume of water depends on the number of transformations; 1 transformation= 1 ml. Aliquot to 1.5 ml tubes (1ml/tube), spin at 5K for 4 min at 4oC and resuspend pellets with 60ul cold water.

Electroporation of Bacteria

1. Add 2-4ul (0.4 μg) DNA to the 60 μl of competent cells.
2. Add mixture to one chilled 0.2 cm cuvette.
3. Use Bio-Rad pre-set program "bacteria 2".
4. Put cuvette into electroporator, press pulse.
5. Immediately add 1 ml LB broth to cuvette using a barrier tip.
6. Slowly transfer all liquid from cuvette in to 15 ml snap cap tube.
7. Place in 30^{o}C shaker for 2 hours to recover.
8. Spin at 5K for 4 min in tabletop centrifuge.
9. Discard supernatant.
10. Resuspend with 100ul, plate onto kan+chlora LB plates, and put in 30^{o}C incubator for 2 days.

Miniprep of BAC cultures

1. Pick colonies and seed each of the into 10 ml LB broth with kan and chlora, grow O/N
2. Spin down cultures for 10 min at 3.5K
3. Pour off supernatant. Resuspend in 300 μl cold P1 Buffer
4. Transfer to a 2 ml Eppendorf tube
5. Add 300 μl P2 buffer (Qiagen) and mix gently by inverting
6. Add 300 μl N3 buffer (Qiagen) and mix gently by inverting
7. Add 1 ml of phenol/chloroform. Invert the tube repetitively by hand for 60 sec.
8. Spin for 10 min at 13K at 4oC.
9. Move the top phase to a 1.5 ml Eppendorf tube, spin for 5 min at 13K.
10. Move the top phase to a 2 ml Eppendorf tube.
11. Add 1/10th volume of 3M NaAce and 1 ml isopropanol then put at -80^{o}C for 30 min.
12. Spin at 13K for 10 min at 4^{o}C.
13. Remove supernatant. Add 1 ml of 70% ethanol to wash the pellet.
14. Spin at 13K for 5 min at 4oC.
15. Remove supernatant carefully by pipetting. Air-dry the pellet for 15 min at room temperature.
16. Add 100 μl of 55^{o}C heated water or heater EB buffer to pellet. Sit in heat block for 30 min to allow to the pellet to dissolve.
17. Run 20 μl of the extraction on a 1% agarose gel to check for plasmid contamination.

FLP recombination

1. Grow 2 ml culture with kan and chlora O/N at 30^{o}C.

2. Add 200 µl to 9.8 ml LB with chlora only.
3. Grow at 30°C for 3 hours.
4. Add 100 µl of 10% L-arabinose solution* to the culture and return to 30°C for 1 hour.
5. Dilute 1 ml of the culture in 9 mls LB broth and grow for another hour at 30 °C.
6. Dilute the culture and spread 50 µl of 10^{-3}, 10^{-4}, and 10^{-5} on chlora+ LB plates O/N at 30 °C.
7. Pick up colonies and dot each colony on both a kan+ LB and a chlora+ LB plate. Grow O/N at 30 °C.
8. Those colonies only growing on the chlora+ LB plate are correct colonies that do not have the kan resistance gene anymore.
9. Pick up colonies to seed into cultures for BAC miniprep. Perform PCR to confirm the Kan cassette is gone.

* 10% L-arabinose solution: 1 g in 10ml LB-sterilized with 0.2 µm filter.

Electroporation of BAC DNA into cells

1. Isolate BAC DNA form a 10 ml O/N culture and resuspend BAC DNA in 100 µl water.
2. Passage 3T3 cells. When cells are 80-90% confluent, prepare the cells for electroporation.
3. Trypsinize cells and resuspend cells in DMEM or RPMI at 10^7 cells/ml.
4. Add 10 µl of BAC DNA to 1.5 ml tube and add 100ul cells.
5. Load the cells to a 0.2 cm cuvette. Electroporate using Bio-Rad pre-set protocol for 3T3 cells.
6. Add 1 ml medium to the cuvette and seed cells to a 25 cm flask. Change medium every 3-4 days.
7. Around day 10, there will be cytopathic effect (CPE).

Results:

The construction of dgL-TK virus has been completed, but at this time further processing and experimentation is still needed. In particular, the virus is concentrated only in cell culture supernatant. From here, a single clone should be made, which can be performed by a limited dilution assay. Once a few single clones have been isolated, they must be made into a stock and passaged until the BAC is removed, then confirmed by size of the virion genome and PCR sequencing.

Discussion:

It is not completely clear why dgL-MCMV and MCMV-TK form disparate responses during acute infection. An interesting difference between these two viruses is that dgL-MCMV is a spread-deficient virus but not replication-deficient, while MCMV-TK is both spread- and replication-deficient. In theory, dgL-MCMV still produces virions and releases virions from infected cells (personal communication and unpublished data from Angela Tatum, Ph.D., OHSU). I hypothesize that the constant release of non-infectious virions is acting as a PAMP that can initiate an inflammatory immune response. At this point, no one in our laboratory has measured the inflammatory cytokine profile of acute dgL-MCMV infected mice. In particular, what is the type I IFN response to infection, and are there any changes to the DC subsets during acute infection?

Experimentally, to identify whether the viral replication of non-infectious dgL-MCMV leads to a suppressive mechanism, I suggest co-infecting mice with MCMV-TK and dgL-MCMV and measuring the CD8 T cell responses after 1 week of infection. This process would be very similar to the setup for my experiment with the co-infection of MCMV-TK and dm157-MCMV in Chapter 2. I would predict that a co-infection in mice with Famcyclovir treatment would not form elevated CD8 T cell responses, and that they would be similar to solo infection of MCMV-TK without Famcyclovir treatment.

5.6 Reactivating latent MCMV infection

My initial project in the laboratory was to create a reactivation model during chronic MCMV infection. Reactivation of human CMV is commonly reported from the

inflammatory response to transplantation, irradiation, and sepsis. Reactivation also occurs when there is a release in adaptive suppression of the virus, such as the depletion of CD8 T cells.

Reactivation is typically measured in the laboratory by a plaque assay (culturing infected samples on a bed of mouse embryonic fibroblast cells and measuring the patches of cytopathic effect from infected cells) or viral transcripts (e.g., IE transcripts). Figure 5-5A is the model that describes a latently infected cell receiving a stimulus. From there, the virus reactivates and differentiates the cell to produce both virus and antigen that is presented on the cell surface via MHC molecules. The cell then proceeds to die from the adaptive immune response or quiesce to escape the immune response and settle back into a latent state of infection.

Measuring reactivation by viral transcripts and a plaque assay is requires the killing of the mice to harvest tissue samples, which prevents long-term research studies. These assays require a particular amount of sample to produce a valid result. However, it would be advantageous to form a faster and more sensitive assay to measure reactivation.

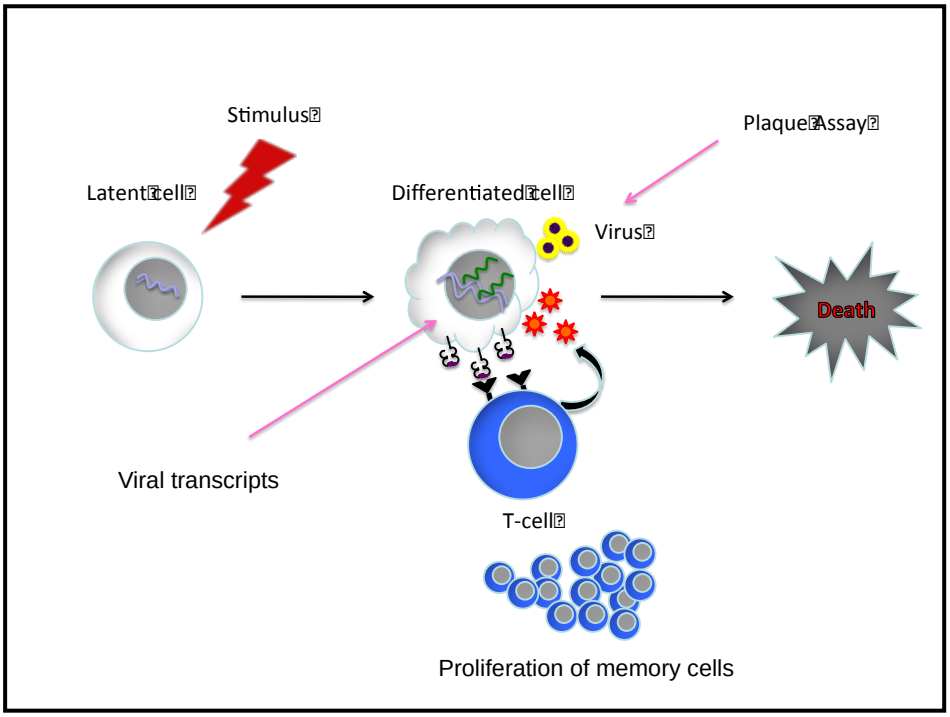
Results

Since a latently-infected cell can produce antigen when stimulated, we hypothesized that the CD8 T cell response would be a good marker of reactivation. Figure 5-5B represents a model of how this concept would work. Using an MCMV expressing the ovalbumin gene under the major immediate promoter (MCMV-OVA), we infected mice and adoptively transferred naïve OT-1 splenocytes. These OT-1 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) prior to transfer. A day after OT-1 cell transfer (letting the cells rest in the host and find their niche), we administered

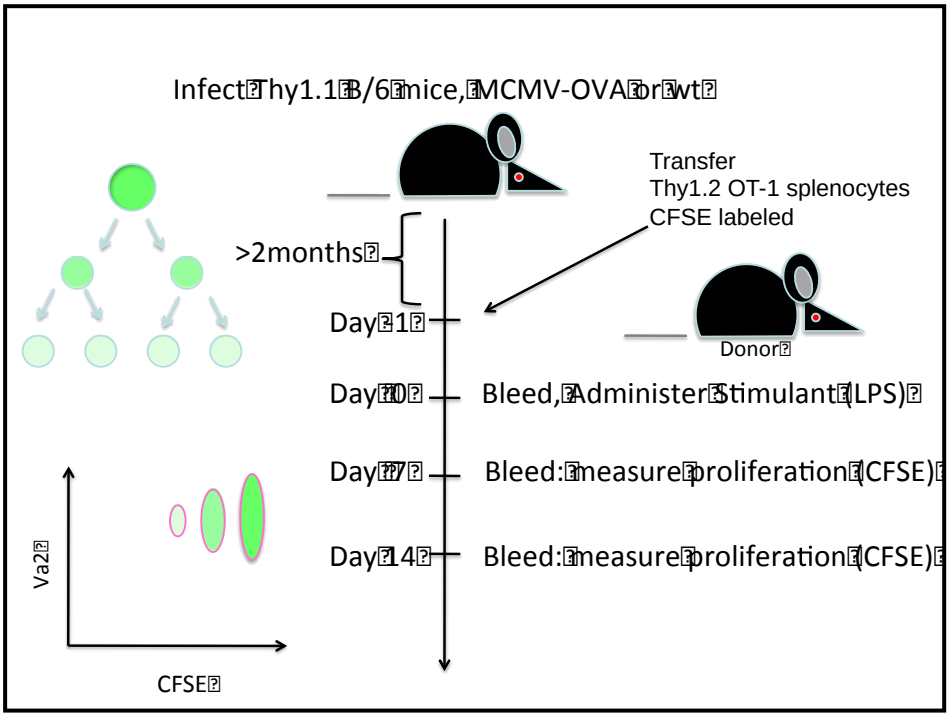
LPS to induce a strong inflammatory response and reactivate the virus. After 7 and 14 days we bled the mouse to measure for the dilution of CFSE in OT-1 cells.

Our results show that OT-1 CD8 T cells disappear in mice infected with MCMV-OVA after 7 days of LPS administration (Figure 5-5C). In contrast, wt-MCMV-infected mice do not show a disappearance of OT-1 cells after 7 or 14 days LPS administration. While this project did not continue after this point, we hypothesize that the OT-1 cells in MCMV-OVA-infected mice are leaving the blood and homing to sites of reactivation. The identification of OT-1 cells in wt-MCMV-infected mice after LPS administration suggests that the cells are capable of existing 14 days after transfer. The OT-1 T cells used in this experiment were naïve T cells. It would be interesting to know if using memory OT-1 T cells would allow for better identification of these cells in the blood after LPS administration, as memory CD8 T cells are able to traffic in and out of tissues better than naïve T cells.

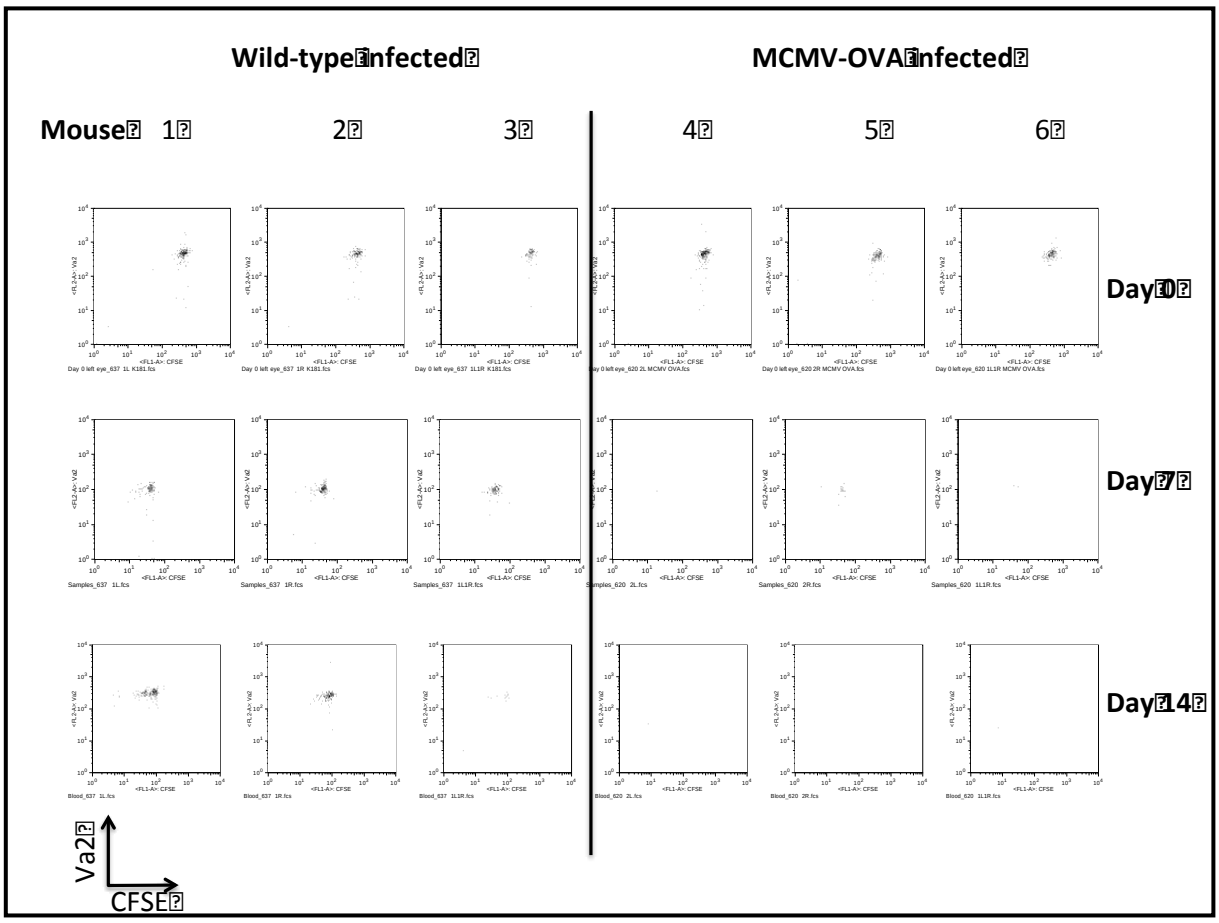
A


Stimulus
Plaque Assay
Latent cell
Differentiated cell
Virus
Death
Viral transcripts
T-cell
Proliferation of memory cells

B


Infect Thy1.1 B/6 mice, MCMV-OVA or wt
Transfer
Thy1.2 OT-1 splenocytes
CFSE labeled
>2months
Donor
Day 1
Day 0
Bleed, Administer Stimulant (LPS)
Day 7
Bleed: measure proliferation (CFSE)
Day 14
Bleed: measure proliferation (CFSE)
Va2
CFSE

C


Wild-type infected
MCMV-OVA infected
Mouse 1
2
3
4
5
6
Day 0
Day 7
Day 14
Va2
CFSE

5.7 Rejection of adoptively transferred CD8 T cells between Thy1.1 and CD45.1 mice

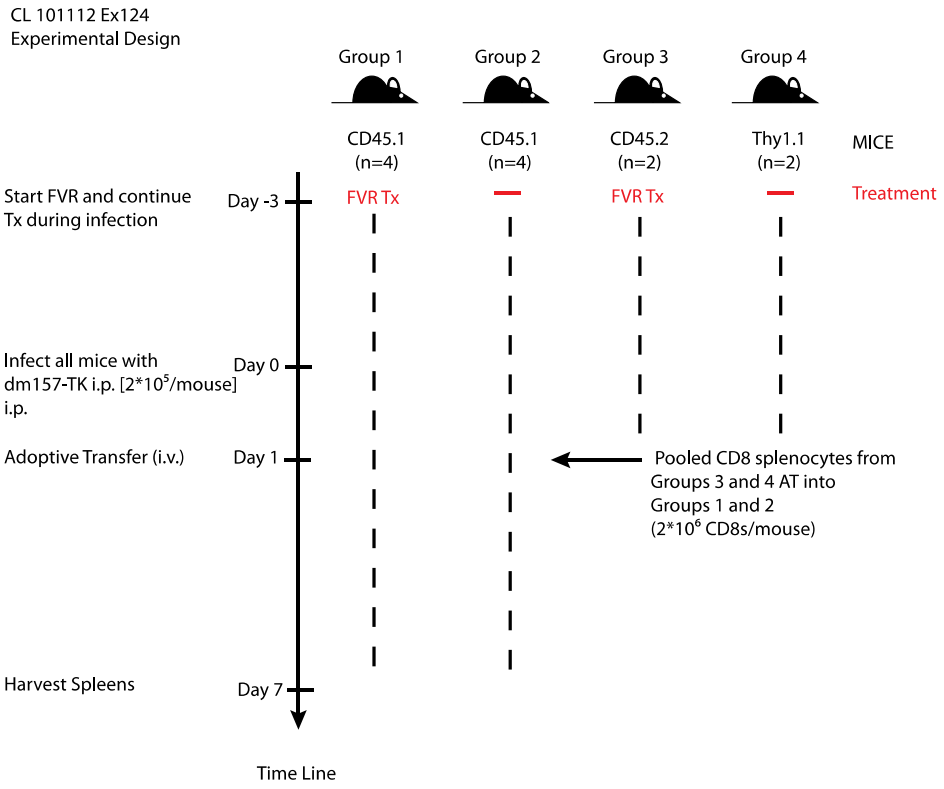
In an effort to determine whether priming events in Famcyclovir treated mice is the cause of enhanced M45- and M57-specific responses in MCMV-TK infection or if the events after priming (days 2–5 pi) are driving these responses, I performed an cross adoptive transfer experiment. This experiment was to take CD8 T cells from Famcyclovir treated mice (at day 2 pi) and put them into infected mice without treatment. Initially, I hypothesized that early priming events in an infection with Famcyclovir treatment would cause Ag-specific CD8 T cells to set on a program to proliferate regardless of the environment after day 2 pi. What I would now hypothesize is that the events after day 2 pi is more important to the robust M45- and M57-specific CD8 T cell response.

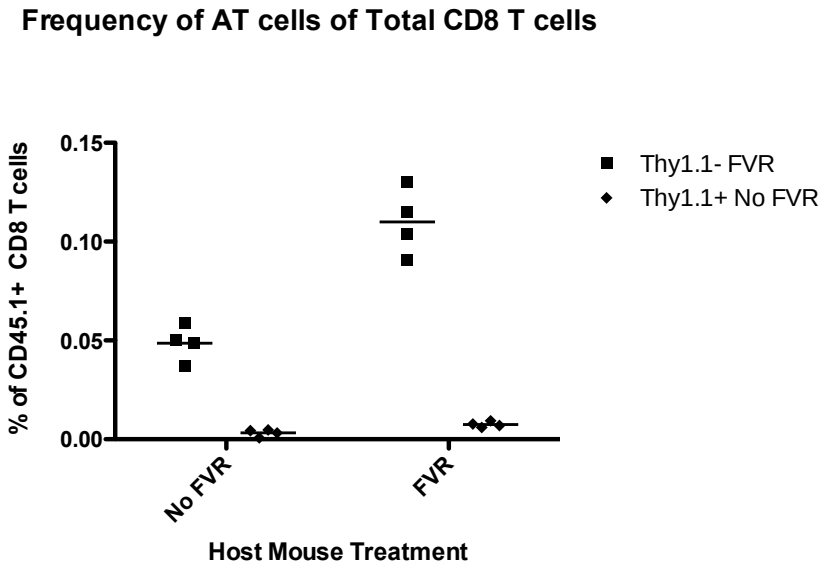
To decipher host and recipient CD8 T cell from each other I used male CD45.1, CD45.2, and Thy1.1 mice and stained for their respective congenic markers. This experiment involved infecting 4 groups of mice with MCMV-TK (2 with Famcyclovir treatment and 2 groups untreated). At day 2 pi, I harvested spleens from 1 Famcyclovir treated group and 1 from an untreated group. CD8 T cells were magnetically sorted by negative selection, mixed in a 1:1 ratio and adoptively transferred into the remaining groups of mice, and the splenocytes from the recipient mice were harvested at day 7 pi.

Below are the results of 3 experiments. The first two experiments (Ex124 and Ex125) I used CD45.1 as recipients, and Thy1.1 and CD45.2 mice are the donors. In the third experiment (Ex131) I used Thy1.1 as the recipients, and CD45.1 and CD45.2 are the donors. These results show, regardless of Famcyclovir treatment, the transfer of Thy1.1

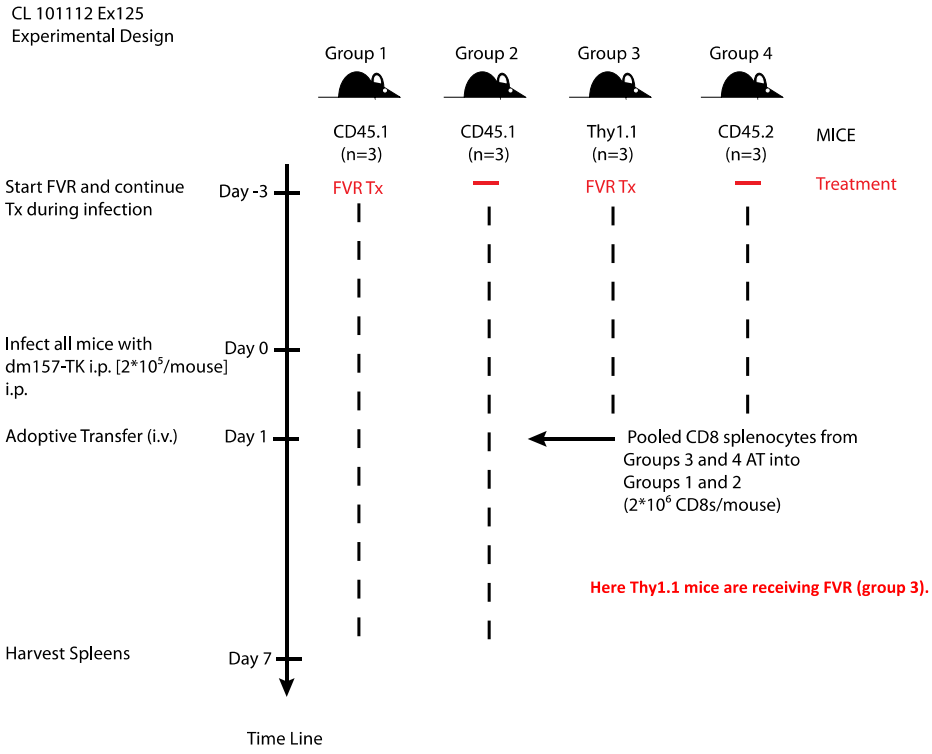
into CD45.1 or vice versa lead to donor rejection. I did not pursue this phenomenon any further, but it is worth noting that in Ex125, recipient CD45.1 naïve mice still rejected donor Thy1.1 cells.

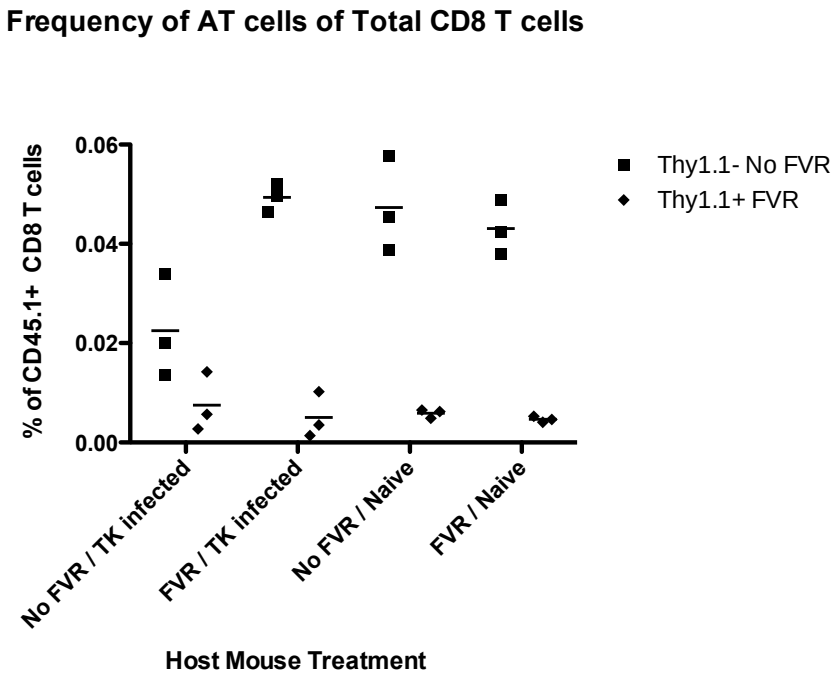
Ex124


CL 101112 Ex124
Experimental Design
Group 1
Group 2
Group 3
Group 4
CD45.1
(n=4)
CD45.1
(n=4)
CD45.2
(n=2)
Thy1.1
(n=2)
MICE
Start FVR and continue
Tx during infection
Day -3
FVR Tx
FVR Tx
Treatment
Infect all mice with
dm157-TK i.p. [2*10^5/mouse]
i.p.
Day 0
Adoptive Transfer (i.v.)
Day 1
Pooled CD8 splenocytes from
Groups 3 and 4 AT into
Groups 1 and 2
(2*10^6 CD8s/mouse)
Harvest Spleens
Day 7
Time Line

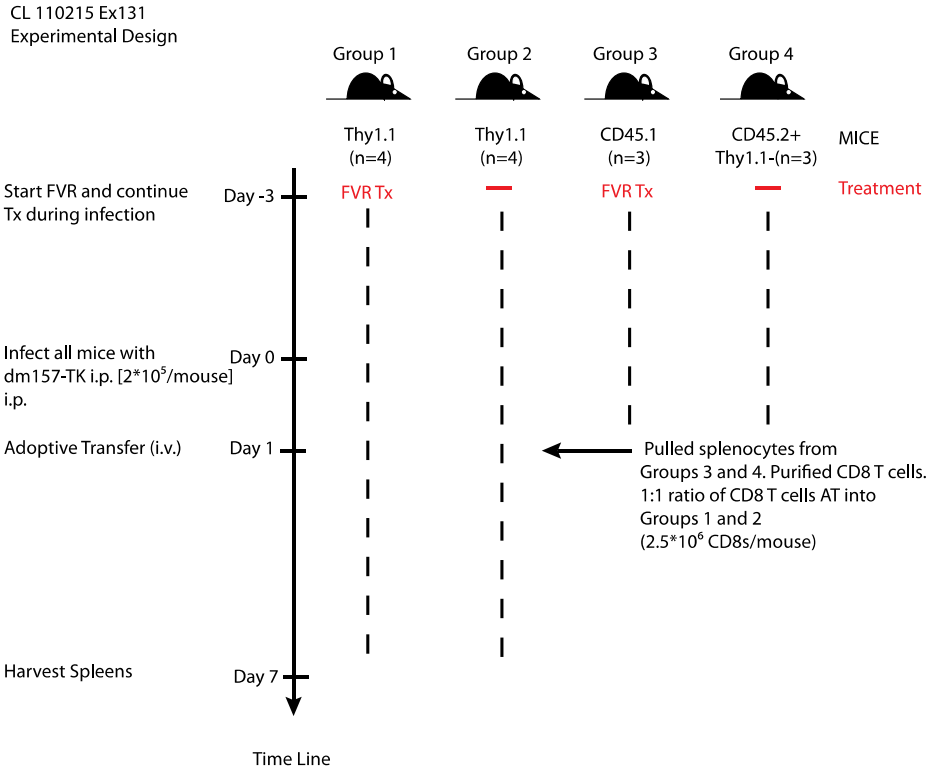

Frequency of AT cells of Total CD8 T cells
% of CD45.1+ CD8 T cells
0.15
0.10
0.05
0.00
No FVR
FVR
Host Mouse Treatment
Thy1.1- FVR
Thy1.1+ No FVR

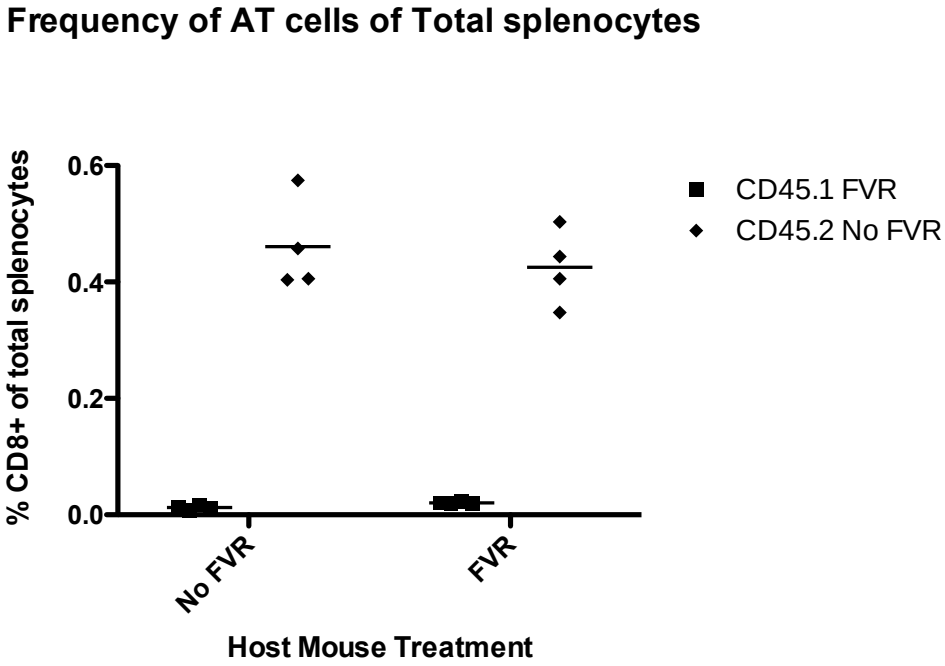
Ex125


CL 101112 Ex125
Experimental Design
Group 1
Group 2
Group 3
Group 4
CD45.1
(n=3)
CD45.1
(n=3)
Thy1.1
(n=3)
CD45.2
(n=3)
MICE
Start FVR and continue
Tx during infection
Day -3
FVR Tx
FVR Tx
Treatment
Infect all mice with
dm157-TK i.p. [2*10^5/mouse]
i.p.
Day 0
Adoptive Transfer (i.v.)
Day 1
Pooled CD8 splenocytes from
Groups 3 and 4 AT into
Groups 1 and 2
(2*10^6 CD8s/mouse)
Here Thy1.1 mice are receiving FVR (group 3).
Harvest Spleens
Day 7
Time Line


Frequency of AT cells of Total CD8 T cells
% of CD45.1+ CD8 T cells
0.06
0.04
0.02
0.00
Thy1.1- No FVR
Thy1.1+ FVR
No FVR / TK infected
FVR / TK infected
No FVR / Naive
FVR / Naive
Host Mouse Treatment

EX131


CL 110215 Ex131
Experimental Design
Group 1
Group 2
Group 3
Group 4
Thy1.1 (n=4)
Thy1.1 (n=4)
CD45.1 (n=3)
CD45.2+ Thy1.1-(n=3)
MICE
Start FVR and continue Tx during infection
Day -3
FVR Tx
FVR Tx
Treatment
Infect all mice with dm157-TK i.p. [2*10^5/mouse] i.p.
Day 0
Adoptive Transfer (i.v.)
Day 1
Pulled splenocytes from Groups 3 and 4. Purified CD8 T cells. 1:1 ratio of CD8 T cells AT into Groups 1 and 2 (2.5*10^6 CD8s/mouse)
Harvest Spleens
Day 7
Time Line


Frequency of AT cells of Total splenocytes
% CD8+ of total splenocytes
0.6
0.4
0.2
0.0
No FVR
FVR
Host Mouse Treatment
CD45.1 FVR
CD45.2 No FVR

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