SELECTION AND BEHAVIOR OF INFLATIONARY CD8 T-CELL POPULATIONS DURING MURINE CYTOMEGALOVIRUS INFECTION

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

AIM2	Absent in melanoma-2
APC	Antigen presenting cell
APL	Altered peptide ligand
BAC	Bacterial artificial chromosome
Blimp1	B-lymphocyte induced maturation protein
CD62L	L-selectin
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
E	Early
EBV	Epstein Barr virus
EC	Endothelial cells
Eomes	Eomesodermin
ER	Endoplasmic reticulum
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HCMV	Human cytomegalovirus
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IE	Immediate early
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KLRG1	Killer cell lectin receptor G1
KSHV	Kaposis sarcoma herpesvirus
L	Late
LCMV	Lymphocytic choriomeningitis virus
LSECs	Liver sinusoidal endothelial cells
MACs	Macrophages
MHC II	Major histocompatibility complex class II
MCMV	Murine cytomegalovirus
MCMV-GFP-MSL-8	Major intermediate Early Promoter
MHC I	Major histocompatibility complex class I
MIEP	Major immediate promoter
mZP3	Murine zona pellucida 3
NK	Natural killer
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
RhCMV	Rhesus CMV
RhCMV-SIV	Rhesus CMV-simian immunodeficiency virus
RT-PCR	Reverse-transcription PCR
Tcm	Central memory T-cells
TCR	T-cell receptor

Tem	Effector memory T-cells
TLR	Toll-like receptor
TNF	Tumor necrosis family
Trm	Tissue resident memory T-cells
VZV	Varicella zoster virus
VIPRs	Viral genes that interfere with antigen presentation
WT	Wild type

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With the support of all of these individuals in mind, I present, here, the work of my dissertation.

Abstract

Cytomegaloviruses establish a lifelong, latent/persistent infection in their hosts, tactfully avoiding the many redundant components of the innate and adaptive immune system. In so doing, they provoke a large, functional CD8 T-cell response directed against a subset of viral epitopes. This reponse is maintained for the life of the host and is termed "memory inflation." Inflationary responses are comprised primarily of cells with an effector phenotype, which retain cytotoxic capacity and the ability to produce cytokines upon antigen stimulation. Because of the immunogenic properties of this virus, immunologists anticipate that CMV-vectored vaccines will have the capacity to generate robust, long-lived and fast-acting cellular immunity against defined antigens from pathogens and tumor cells. How and why certain epitopes within the virus are chosen as targets for memory inflation is not fully understood. This knowledge is essential for the intelligent design of CMV-based vaccines. Additionally, the capacity to modulate inflationary responses to individual CD8 T-cell epitopes could greatly enhance the application of this technology.

Here I have used Murine CMV (MCMV) as a model in which to explore the selection and behavior of inflationary CD8 T-cell responses in Human CMV (HCVM) infection. I have used a recombinant MCMV encoding the ovalbumin-derived epitope SIINFEKL as a model antigen. I show that when SIINFEKL occupied the IE2 locus of MCMV, T-cells specific for the SIINFEKL epitope inflated and profoundly dominated T-cells specific for endogenous, MCMV-derived epitopes. The immunodominance of the SIINFEKL epitope could not be altered by modulating the proportion of SIINFEKL or MCMV-specific T-cells available prior to infection. Instead, coinfection with this virus and a WT MCMV enabled co-inflation of T-cells specific for both SIINFEKL and MCMV-derived antigens. Because coinfection allows presentation of

SIINFEKL and MCMV-derived antigens by different cells within the same animal, these data show that competition for, or availability of, antigen at the level of the antigen presenting cell influences the selection of inflationary response during chronic MCMV infection. These findings emphasize the need to select and place exogenous epitopes within CMV vectors such that they have competitive expression kinetics and biochemical properties.

I also found that the SIINFEKL-specific response during chronic infection could be boosted by the intravenous administration of soluble peptide-eptitope. This was true for inflationary responses generated during infection with WT MCMV as well, and for smaller central-memory responses in the same infection. I found, for all but one epitope, that inflationary and central memory responses proliferated and remained expanded as a result of what is traditionally a tolerizing stimulus. These findings suggest a method for boosting T-cell populations in a vaccine setting. Additionally, they contradict recent evidence suggesting that memory and effector T-cells are susceptible to peptide-induced tolerance.

Chapter 1: Introduction

I. Overview

The immune system serves many roles in the human body, from an arsenal of defenses trained against pathogenic invaders, to an exquisitely tempered peacekeeper, able to distinguish harmless from harmful and to regulate the fine balance between inflamation and tolerance. More recently, it has become a tool that we can instruct to protect us from infection and cancer through vaccine technology.

Cytomegalovirus has known the many facets of our immune system for the entire evolution of our species. This virus ducks and dodges immune attacks in ways that we are continuing to discover, a trait that prevents it from being cleared but does not prevent it from generating robust immunological memory. In fact, CMV's capacity to generate immunological memory is exceptional. This disseration explores the processes by which specific elements of CMV are selected as targets of this memory response, with the goal that this understanding can be applied towards using the virus as a novel vaccine strategy.

II. CMV

Basic Virology

Cytomegaloviruses belong to the herpesvirus family, which includes over 120 known viruses that infect a wide range of vertebrates and several known invertebrates, including oysters, abalone, and coral. Most herpesviruses have a narrow host range and cause lifelong, latent infections punctuated by periodic episodes of reactivation. Humans

are the natural hosts of at least eight herpesviruses: CMV, herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), Varicella-zoster virus (VZV), Epstein Barr virus (EBV), and human herpesviruses 6, 7, and 8 (also called Kaposis sarcoma herpesvirus or KSHV).

These viruses are assigned to their family based upon a common virion architecture: linear, double-stranded DNA packaged in an icosahedral capsid (T=16). A structured protein layer known as the tegument surrounds this capsid, which is further enclosed in a lipid bilayer containing glycoproteins. Virions range in size from 150-200 nm.

The family is further subdivided into alpha, beta and gamma herpesviruses. Initially these subfamilies were based on host range and growth kinetics; they have since been shown to accurately reflect genomic relationships as well (Murphy and Shenk, 2008). The alpha-herpesviruses, which include HSV-1, HSV-2 and VZV, have the smallest genomes, the fastest *in vitro* replication cycles, and the largest host range. Gamma-herpesviruses, including EBV and KSHV, are intermediate in size while beta-herpesviruses have the largest genomes. Both gamma and beta herpesviruses have a narrow host range and long reproductive cycles *in vitro*. CMVs are members of the beta-herpesvirvus subfamily.

Genomic sequencing provides evidence that CMVs have co-evolved with their natural hosts for more than one hundred million years (McGeoch et al., 1995). Additionally, tropism is highly restricted—a given virus will infect only one species—and this property has been linked to blocks of genes specific to each CMV (Murphy and Shenk, 2008). These genes most often function to modulate their host's immune system. Indeed, all herpesviruses employ a multitude of tactics to evade, distract and dampen the immune system, facilitating their persistence despite robust host defenses.

For all herpesviruses, viral gene expression is an ordered sequence of events. Genes are divided into three categories: immediate early (IE), early (E) and late (L). As expected, IE genes are the first to be transcribed; they are expressed between one and six hours post infection during Human CMV (HCMV) infection. Their protein products are responsible for transactivating E gene promoters. The E genes, expressed between four and 18 hours post-infection, encode for non-structural proteins and tend to suppress IE gene transcription. L genes are transcribed after 12 hours of infection and encode the structural proteins of progeny virions.

CMV tropism extends to most major organ systems and cell types. Demonstrating the necessity of specific cellular molecules for viral entry has been challenging and, in fact, entry processes may differ substantially depending on cell type. HCMV was initially shown to enter fibroblasts via direct fusion of the viral envelope with the plasma membrane (Compton et al., 1992), but clinical strains of the virus were later shown to enter epithelial and endothelial cells via receptor mediated endocytosis at low pH (Bodaghi et al., 1999; Ryckman et al., 2006). Once inside permissive host cells, fully formed viral proteins from the tegument layer are released and orchestrate the delivery of the viral genome to the nucleus, where gene expression and replication are initiated. During latency, the viral genome is maintained in the nucleus as an episome (Bolovan-Fritts et al., 1999), and is associated with histones in a closed chromatin structure (Nitzsche et al., 2008).

Because most CMV research, including the work presented here, ultimately aims to improve our understanding of HCMV infection, I will describe the key features of HCMV biology in the next few sections. MCMV infection, the primary model used by my laboratory, will be compared and discussed afterwards.

HCMV Pathogenesis

Human CMV infection is widespread, with rates of seropositivity reaching nearly 100% in undeveloped areas, including parts of Africa, Asia and South America. Certain regions in North America and Northern Europe, on the other hand, see infection in less than 30% of their adult population (Britt, 2008). Primary infection of healthy individuals is rarely associated with disease beyond occasional mononucleosis-like symptoms. HCMV infection poses a grave risk, however, to immunocompromised populations, including those with genetic or virally-acquired immune deficiencies, recipients of organ transplants, and the unborn. In the developed world, in fact, HCMV is the most common congenital viral infection and the most common infectious cause of brain damage and hearing loss in children (Manicklal et al., 2013).

HCMV infects a remarkably wide array of cell types within its host. Analysis of patient and autopsy samples indicates that the virus can spread to the parenchyma and connective tissue of virtually any organ, as well as several hematopoietic cell types (Sinzger et al., 1993; Sinzger et al., 1995). Infection of epithelial cells in glands and mucosal tissues presumably facilitates transmission between hosts, while infection of endothelial cells and hemotopoietic cells enables systemic spread within the host. Infection of fibroblasts and smooth muscle cells provides additional sites for replication and proliferation (Sinzger et al., 2008).

HCMV is thought to enter a new host by infection of mucosal epithelium. For newborns, this can happen via breast milk; more than 95% of seropositive breastfeeding women reactivate HCMV locally and shed virus into their milk (Hamprecht et al., 2001). Epithelial cells in the gastrointestinal tract are, in this case, the most likely sites of primary

replication. Breast milk is not the only vehicle for viral transmission, however; saliva, urine, semen and feces can all be sources of infection. Epithelial cells in the intestines, salivary glands and kidney are responsible for shedding virus into these bodily fluids (Bissinger et al., 2002; Sinzger et al., 1995).

Dendritic cells (DC) are another potential target for acute infection. Immature dendritic cells can be found surveying virtually all mucosal and epidermal surfaces by ingesting small quantities of extracellular fluid. Upon uptake of pathogens, DCs mature: they express costimulatory molecules and migrate via homing receptors to the lymph nodes, where they activate antigen-specific lymphocytes. Uptake of HCMV by DCs can result in viral replication and release of viral progeny, as well as the downregulation of molecules necessary for lymphocyte activation (Riegler et al., 2000; Hertel et al., 2003; Moutaftsi et al., 2002). If an infected DC is interacting with different cells types in the lymph nodes, this could lead to viral dissemination without immune activation.

HCMV is thought to disseminate throughout the body by infecting other types of leukocytes as well. In particular, monocyte-derived macrophages can support full viral replication (Ibanez et al., 1991). Polymorphonuclear cells (basophils, eosiniphils, neutrophils) do not support replication but can take up virus particles and express viral IE proteins, and thus can function as vehicles for passive transport of infectious virus (Gerna et al., 2000).

Due to the virus's extensive tropism, HCMV disease in immunocompromised populations is associated with a broad range of symptoms: hepatitis, encephalitis, esophagitis, colitis, pneumonia, enteritis, retinitis, etc. Within the immunocompetent population, it has been proposed that chronic, subclinical HCMV infection could result in

(or exacerbate) certain inflammatory conditions such as atherosclerosis and neurodegeneration, or be a causative agent in specific types of cancer. While many of these links remain circumstantial, as it has been difficult to prove cause and effect, the possibility remains that CMV may impact its host in subtle ways over a lifetime of interaction (Hill, 2012).

Superinfection

CMV has a remarkable ability to reinfect—or superinfect—a host that already harbors one or more virus strains, despite the host's preexisting CMV immune response. A number of studies indicate that immunocompetent populations can acquire and carry different CMV genomes. Strain-specific neutralizing antibody titers and sequencing of HCMV glycoprotein H were used to show that a majority of pregnant women sampled (Boppana et al., 2001), as well as a significant number of healthy blood donors (Ishibashi et al., 2008; Ross et al., 2010), had evidence of infection with two separate strains. Restriction fragment length polymorphism analysis of serial HCMVs isolated from children in daycare centers also showed evidence of serial superinfection specifically, rather than simultaneous coinfection (Bale et al., 1996).

Moreover, coinfection is associated with worse outcomes for the developing fetus (Arav-Boger et al., 2002), and for transplant recipients (Coaquette et al., 2004; Humar et al., 2003). Evidence in the mouse model suggests that MCMV coinfection may actually increase the collective fitness of multiple virus strains, specifically through *trans*-complementation in the same cells rather than homologous recombination (Cicin-Sain et al., 2005).

Prior immunity may offer some degree of protection from superinfection, however. Immunocompromised populations exhibited greater rates of coinfection in studies similar to

those referenced above (Baldanti et al., 1998; Meyer-Konig et al., 1998a; Meyer-Konig et al., 1998b), and amongst newborns, the risk of congenital infection was three times higher in the offspring of women who were initially HCMV seronegative (Boppana et al., 2001; Fowler et al., 2003). This gives hope to those seeking a vaccine that could be protective against congenital CMV infection.

Latency and Reactivation

Much confusion can be found in sorting out various disciplines' respective definitions of viral latency and persistence. Latency can be described from a clinical perspective as the maintenance of viral genomes without the production of virus capable of spreading to new hosts or causing overt disease. Alternatively, from a molecular perspective, latency could be defined as genome maintenance without the expression of specific lytic gene products. IE genes, under control of the major immediate early promoter (MIEP), are used a proxy for detecting lytic replication of HCMV, as their products are necessary for the trans-activation of E gene promoters. Whereas HCMV can be found replicating in numerous tissues and cell types during acute disease, carriage of latent genomes in healthy individuals appears to be more tightly restricted.

Based on the finding that depleting donor blood of leukocyte populations greatly reduced HCMV transmission to transfusion recipients, it was strongly suspected that one or more peripheral blood mononuclear cells (PBMCs) populations harbored infectious virus (Gilbert et al., 1989; Tolpin et al., 1985). With the advent of highly sensitive DNA detection methods, it could be determined that healthy carriers have a load of one genome-positive cell per 10,000 PBMCs, and that the predominant cell type associated with HCMV DNA is

CD14+ monocytes (Slobedman and Mocarski, 1999). These cells are short-lived and arise from CD34+ haematopoietic cell precursors in the bone marrow, which is now thought to represent a significant latent reservoir of virus in humans (Mendelson et al., 1996; Sindre et al., 1996). The argument that these are a site of true latency is based upon the lack of detectable IE gene expression by reverse-transcription PCR (RT-PCR), despite carriage of HCMV DNA (Taylor-Wiedeman et al., 1994; Mendelson et al., 1996). In spite of the fact that CD34+ stem cells give rise to both lymphocyte and myeloid lineages, HCMV DNA has not been found in the lymphocyte or polymorphonuclear cell fractions of the blood. An as yet unknown mechanism restricts viral carriage to cells of the monocytic and dendritic cell lineages (Sinclair and Sissons, 2006).

Other sites of HCMV latency or low-level persistence may exist in vivo. In particular, endothelial cells (EC), which form an interface between blood and underlying tissues, are another attractive site for HCMV carriage. Evidence both in vivo and in vitro indicates that ECs can be infected with the induction of only minimal cytopathology (Jarvis and Nelson, 2007). Because CD34+ stem cells also give rise to some endothelial cell lineages, it is plausible that viral genomes may selectively segregate in specific endothelial cell subsets, just as they do in monocytes. The difficulty of obtaining organ-specific EC subsets from healthy donors, however, has made it difficult to determine whether ECs are a site of true latency, or instead, a site of low-level persistent infection (Sinclair and Sissons, 2006).

A crucial step in the establishment of latency is thought to be the silencing of the MIEP, which controls IE gene transcription (Sinclair and Sissons, 1996). Cellular transcription factors, histone proteins and chromatin structure have been shown to regulate the MIEP, thus partially dictating permissive and non-permissive cell types

(Ioudinkova et al., 2006; Meier and Stinski, 1996; Nevels et al., 2004; Reeves et al., 2006). HCMV does not yet have a defined latent origin of replication, as many other herpesviruses do, yet there is no evidence that genome replication happens, instead, simply as the result of low-level persistence in latently infected cells either. A number of viral gene products associated with latent infection have been identified, some of which have been shown to aid in the establishment of latency, silencing of the MIEP, and immune evasion (Reeves and Sinclair, 2008).

Cellular environment strongly influences the capacity of HCMV for reactivation. In vivo studies using experimental infection of granulocyte colony stimulating factor (G-CSF) mobilized CD34+ cells, CD34+ cells isolated from bone marrow aspirates, cord blood CD34+ cells, and fetal liver CD34+ cells, have indicated that viral genomes are carried by monocyte precursors (CD34/CD33/CD14) and dendritic cells (CD34/CD33/CD1a), and that reactivation of lytic gene expression requires the terminal differentiation of these cell types into mature macrophages and DCs (Goodrum et al., 2002; Hahn et al., 1998; Kondo et al., 1994; Maciejewski and St Jeor, 1999; Minton et al., 1994; Reeves et al., 2005). Pro-inflammatory factors from activated T-cells (including TNF-α, IFN-γ, IL-2 and GM-CSF) have been shown to induce reactivation in infected monocytes (Soderberg-Naucler et al., 2001). Changes in cellular transcriptional regulation and chromatin remodeling which occur with exposure to inflammatory stimuli and growth factors could be enough to trigger reactivation, but additional, as yet undiscovered viral products may also be required.

MCMV as a Model

The experiments in this dissertation use Murine Cytomegalovirus (MCMV) as a proxy to explore processes that occur in HCMV infection. Mouse models are routinely employed for a variety of practical and ethical reasons. One strength of this system as opposed to other murine models of infection, however, is the appropriate evolutionary relationship between the host's immune system and the actions of the virus; MCMV is a natural mouse pathogen. As discussed above, CMVs are highly host-specific. This is reflected in part by the significant number of private genes not shared by different species of CMVs, suggesting specific adaptations of each CMV to its host species. Importantly, many of these adaptations are involved in the modulation of the host's immune surveillance mechanisms.

The two viruses share a basic virology: virion structure, genome organization and gene expression patterns are similar. Sequencing of the Smith strain of MCMV has revealed that its 230 kB size compares to that of HCMV and that the two viruses are colinear over a central 180 kB region. Of the 170 open reading frames encoded by MCMV, 78 share significant amino acid homology with HCMV (Rawlinson et al., 1996), whereas a number of private genes encoded by MCMV are functionally synonymous to private genes encoded by HCMV, in particular gene products which interfere with the MHC class I pathway of antigen presentation (Lemmermann et al., 2012; Reddehase, 2002).

Importantly, HCMV and MCMV exhibit similar behaviors with regards to pathogenesis, tissue tropism, establishment of latency, and reactivation after immunosuppression or transplantation. MCMV, like HCMV, infects its host via the mucosal epithelium and spreads through the blood to various organs, infecting many different cell

types. Notably, unlike HCMV, MCMV does not cross the fetal placental barrier (Juanjuan et al., 2011), and as such, is not a suitable model for congenitally acquired infection. MCMV does behave like HCMV with respect to transmission and superinfection in the wild; mixed infections are common in feral mouse species and can be acquired by immunocompetent mice through simultaneous or consecutive infections (Farroway et al., 2005; Gorman et al., 2006). Modeling superinfection with laboratory strains of mice has been more difficult, however (Doom, 2009), even with the use of wild viral isolates. The reason for this is unclear.

HCMV and MCMV may or may not share the same sites of latency. There is evidence that haemotopoietic cells of the myeloid lineage do contain MCMV genomes for a time after initial infection (Balthesen et al., 1993; Kurz et al., 1997), but not for the lifespan of the animal (Balthesen et al., 1993). Instead, high genome loads are maintained in numerous organs in models of neonatal infection and bone marrow transplant, especially the lungs (Balthesen et al., 1993; Kurz et al., 1999; Reddehase et al., 1994). EC subsets—which have a wide tissue distribution and a very low rate of proliferation—are thought to be the most likely candidate reservoir for lifelong MCMV persistence, if not latency.

Evidence for MCMV persistence in these cells came first from in situ hybridization experiments, which localized viral RNA to stromal cells of the spleen. These cells stained positive for Factor VIII-related antigen, present on EC populations, and negative for MHC class II and T-cell markers. Electron microscopy indicated that the infected cells were predominantly from the sinusoidal lining (Mercer et al., 1988; Pomeroy et al., 1991). In situ immunofluoresence revealed MCMV DNA in ECs of diverse organs, including spleen and liver, lung alveolar macrophages, and bone marrow cells (Koffron et al., 1998).

More recently, highly sensitive quantitative PCR (qPCR) and rigorous adoptive transfer models have identified liver sinusoidal endothelial cells (LSECs) as a definitive site of latency. By transferring highly purified cell fractions to naïve recipient mice, Seckert et al were able to show that reactivated virus could only be obtained from the LSEC fraction (Seckert et al., 2009). Importantly, RT-PCR revealed IE gene expression in LSECs taken from infected animals, but not E or L gene expression. Twenty-four hours of ex-vivo culturing produced E gene RNA, but still no L gene RNA. Therefore, in this scenario, latency was defined not as the absence of IE gene expression, but as the absence of infectious virion production.

Thus, LSECs and other organ-specific subsets of ECs are the likeliest site for the maintenance of MCMV genomes. Reconciling murine and human models of CMV latency is challenging given the constraints of working with human subjects and discrepancies in the preferred definition of latency itself. Are haematopoeitic stem cells infected for the life of human hosts, or are there, instead or in addition, EC subsets that are sites of lifelong viral carriage? Are ECs responsible for low-level persistence while haematopoetic stem cells exhibit true, non IE-expressing latency? It is uncertain whether these inconsistencies are a matter of biology or a reflection of the difficulty of obtaining human tissue at defined time points after acute CMV infection.

Like HCMV, silencing of MCMV's MIEP is also thought to preserve latency, and is attributed to the maintenance of a closed chromatin structure about this genomic region. Stochastic desilencing of these genes may occur as a rare event in all latently infected tissues, as evidenced by IE1 and IE2 expression from random lung samples in the absence of infectivity (Grzimek et al., 2001; Kurz and Reddehase, 1999). Further, it is argued that at

least two checkpoints, if not more, may exist before replicative latency is fully abrogated: the first is the expression of the IE1 gene, the second is the differential splicing of the same mRNA into what forms the IE3 gene, a crucial transactivator of E gene expression (Reddehase et al., 2008a). A role for the immune system, specifically CD8 T-cells, in sensing these random moments of desilencing and preventing the virus from crossing further transcriptional checkpoints has been proposed and will be discussed in more detail in the coming sections. Supporting a role for the immune system in maintaining replicative latency is the observation that conditions of unchecked immune activation or situations where immune control is interrupted—for instance, allogeneic transplantation, polymicrobial sepsis, immune cell depletion, and inflammatory disease states—all trigger full reactivation of the virus (Hummel and Abecassis, 2002; Reddehase et al., 2002). This is similar to what is observed with HCMV, lending confidence to the use of MCMV as a model infection.

III. CD8 T-Cell Biology

This dissertation is concerned with the CD8 T-cell response to chronic MCMV infection. Accordingly, various aspects of CD8 T-cell biology will be addressed in the following sections, with the aim of preparing the reader for a more specific discussion of the interplay between MCMV and this particular subset of immune effectors. CD8 T-cells are key component in the immune system's defense against viruses; these effectors are responsible for monitoring the contents of cells presented through the MHC class I pathway, thereby identifying infected targets by their display of foreign protein components. CD8 T-cells respond to viral infection through the release of cytokines and cytolytic granules,

which contain molecules capable of inducing apoptosis in infected target cells. Thus, these cells have been given the alternative name of cytotoxic T lymphocytes (CTL). In addition to these immediate effector functions, CD8 T-cells form a significant element of immunologic memory. The persistence of CTL specific for previously encountered pathogens aids in the quick response to secondary infection.

MHC I Processing and Presentation

CD8 T-cells recognize and are stimulated by foreign peptides only in the context of cell-surface glycoproteins called major histocompatibility class I (MHC I) molecules, which are expressed on all nucleated cells of the body. Because viruses are obligate intracellular parasites, dependent upon host machinery to synthesize and assemble their various components, viral proteins are subject to the same degradation pathways as endogenous proteins. Via the proteasome (and immunoproteasome in cells stimulated by inflammatory cytokines) cytosolic proteins are cleaved and degraded into peptides that are then transported into the endoplasmic reticulum (ER) and loaded onto MHC class I molecules via the transporters associated with antigen processing (TAP1 and TAP2). Exogenous proteins, encountered as infected cells or cellular debris, can be taken up by certain cell types and presented via this pathway in a process called cross-presentation, or cross-priming when it results in the priming of naïve T-cells. Both direct and cross presentation play important roles in initiating and maintaining MCMV-specific CD8 T-cell responses. The intricacies of antigen processing impact the specificity of this response, as do viral mechanisms directed towards modulating this process. Thus, the information discussed below will be applied more specifically to MCMV infection in subsequent sections.

Structure of the MHC I Molecule

MHC class I proteins are comprised of two polypeptide chains, the α chain and the β2-microglobulin chain. The α chain is polymorphic and is encoded in the Major Histocompatibility complex, a genetic locus that includes components of both classes of MHC molecules, as well as genes involved in all stages of antigen processing and presentation. This chain spans the cell membrane and has three subunits: α1, α2 and α3. The β2-micoglobulin chain is not polymorphic and is encoded elsewhere in the genome. Together, the folded α1 and α2 domains form a cleft where peptide binds as well as the polymorphic surface that is recognized by T-cells. Importantly, MHC I molecules are highly unstable without loaded peptide. They preferentially bind peptides that are eight to eleven amino acids long, and stabilize these molecules by interactions with invariant sites found at each end of the peptide-binding cleft (Janeway et al., 2008a).

Proteasome Degradation & MHC I Loading

MHC I is assembled and loaded with both self and non-self peptides in the ER. Newly synthesized α chains translocate to the ER, where they associate with the chaperone calnexin. This complex then binds β2-microglobulin, dissociates from calnexin and associates instead with a complex of proteins (calreticulin, tapasin, TAP and ERp57) that facilitate editing and coupling of peptides to the class I molecule. Peptides are brought into the ER from the cytosol via TAP1 and TAP2. Once peptide is bound to MHC-I, the complex associates with transporter proteins and travels through the Golgi compartment to the cell surface (Janeway et al., 2008a).

The proteasome is responsible for creating MHC I-worthy peptides via the degradation of damaged, misfolded and short-lived regulatory proteins. This is a multi-subunit enzymatic

complex consisting of the modular 20S proteasome associated with two 19S regulatory complexes, which manage access to its catalytic cavity. 20S is barrel shaped, consisting of four stacked rings of seven subunits each. The β1, β2 and β5 subunits of the two inner rings contain the six catalytically active sites, which together can cleave a protein at virtually any residue, ensuring the generation of an almost infinite variety of MHC-I bound peptides (Sijts and Kloetzel, 2011; Shastri et al., 2002).

Proteasomes containing β1, β2 and β5 are referred to as S-proteasomes and are expressed constitutively in almost all cell types. An alternative proteasome, called the immunoproteasome, is assembled upon cytokine stimulation by replacing these subunits with β1i/LMP2, β2i/MECL1 and β5i/LMP7. These variants have different cleavage site preferences as well as different cleavage rates, which can change the nature and relative abundance the peptide epitopes presented by an infected cell (Sijts and Kloetzel, 2011). The immunoproteasome is expressed constitutively only in certain relevant immune cells (DCs, macrophages, T-cells and B-cells), but is thought to help non-immune cells rapidly signal their infected state to the adaptive immune system via enhanced MHC I epitope presentation (Ferrington and Gregerson, 2012). Together with its immune functions, the immunoproteasome has been demonstrated to relieve oxidative stress by the more efficient turnover of oxidatively-damaged proteins, averting the formation of harmful protein aggregates that could be brought about by bystander exposure to cytokines (Angeles et al., 2012).

Cross Presentation

Most CD8 T-cells cannot directly eliminate infected cells without first being activated by professional antigen presenting cells (APCs). Uninfected APCs therefore need a way to present

exogenously acquired antigens via the MHC I pathway. This is done using a process known as cross-presentation, referred to as cross-priming when it results in the activation of naïve T-cells.

Intracellular loading of MHC I with exogenous peptides can happen in one of two ways: via the 'cytosolic pathway' or via the 'vacuolar pathway' (Joffre et al., 2012). In the first scenario, internalized proteins enter the cytosol, where they are degraded by the proteasome and fed into the classical MHC I antigen presentation pathway. Peptide loading might occur in the ER, however the presence of TAP and MHC I loading complexes in phagosomes and endosomes suggests that peptide loading could also occur in endocytic compartments (Burgdorf et al., 2008; Houde et al., 2003). By contrast, cross-presentation via the vacuolar pathway is resistant to proteasome inhibitors and independent of TAP, but is sensitive to inhibitors of lysosomal proteolysis (Bertholet et al., 2006; Shen et al., 2004), suggesting that both antigen processing and loading occur in endocytic compartments.

DCs are the principal cell type associated with cross-presentation; however, macrophages, B cells, neutrophils and some EC subsets (including LSECs) can also cross-present (Basta and Alatery, 2007). The low level of proteolysis specific to DC endocytic compartments is thought to facilitate cross-presentation by preserving potential class I binding epitopes. This is the result of reduced numbers of lysosomal proteases as compared to other phagocytic cells, and of higher pH, rendering those proteases less efficient (Delamarre et al., 2005). Also, various ER-resident proteins, including those related to MHC class I loading, are found in the phagosomes of DCs (Ackerman et al., 2003; Guermonprez et al., 2003).

Amongst DCs, only particular subsets are able to present exogenous antigens efficiently, although the respective contribution of these subsets varies depending on the experimental model used. Most studies indicate that two types of conventional DCs have an enhanced ability to

cross-present: lymphoid organ-resident CD8+ DCs and migratory CD103+ DCs (Joffre et al., 2012). Cytokines that promote the development of CD8+ lineage DCs also improve these cells' ability to present exogenous antigen, suggesting that cross-presentation is a function acquired at late stages of DC development (Sathe et al., 2011).

DCs can acquire exogenous antigen through a few mechanisms: phagocytosis, macropinocytosis and cell-surface receptor-mediated engulfment of dead cells. All of these allow a DC to sample its environment (Joffre et al., 2012). Uninfected APCs can also capture and present pathogen-derived antigen through a process known as "cross-dressing," which involves the acquisition of fully-formed MHC I-peptide complexes. This was shown to happen via "trogocytosis," which is the transfer of membrane fragments and their associated proteins from one cell to another (Wakim and Bevan, 2011).

The relative contributions of direct and cross presentation to the CD8 T-cell response during infection vary largely by the type of pathogen studied. For MCMV, cross-presentation is thought to be of considerable importance, as the virus encodes multiple genes capable of interrupting direct presentation. This will be discussed in detail later.

TCR Signaling

Antigen recognition is mediated by a cell surface complex called the T-cell Receptor (TCR). Since the majority of the work in this thesis concerns events stemming from the engangement of TCR with peptide-MHC I complexes (activation and proliferation of T-cells, as well as their antiviral effector functions), a brief description of the signaling events downstream of this interaction follows. While specific signaling events are not directly

explored in this work, their outcomes are. Thus, the following background may provide a helpful context.

The TCR is composed of an α and a β chain linked by a disulphide bond. Each chain contains a constant region and a variable region, which attains its variability from the rearrangement of genomic segments during T-cell development. These variable regions are responsible for the immense specificity of each T-cell for its antigen. Associated with the α and β chains are invariant accessory chains that carry out the signaling function of the receptor. Two ε, one γ and one δ chain make up a complex that is called CD3. The α and β chains of the TCR are also associated with an intracellular component, which is homodimer of ζ chains. These components of CD3 each contain sequences called immunoreceptor tyrosine-based activation motifs (ITAMs) that can signal to the interior of the cell (Janeway et al., 2008b).

CD8—a cell surface molecule that is often used to distinguish cytotoxic T-cells from those bearing CD4 instead—associates with the TCR on the cell surface and binds to invariant sites on the MHC portion of the peptide-MHC I complex. CD8 is referred to as a co-receptor, as its concurrent ligation is required for an effective T-cell response. The strength of CD8/MHC binding can be modulated by altering the number of sialic acid residues on the carbohydrate portion of the receptor. The configuration of these residues changes after activation and during maturation of CD8 T-cells (Daniels et al., 2001).

Contact with peptide-MHC brings together the CD8 co-receptor and the TCR. Tyrosine kinases associated with cytoplasmic regions of each receptor, Fyn and Lck, can then phosphorylate ITAMs, initiating a cascade of events that leads to the activation of

transcription factors in the nucleus, including NFκB, NFAT and AP-1. These initiate gene transcription that results in differentiation, proliferation and maturation of T-cells.

A host of other molecules serve as co-stimulatory or co-inhibitory receptors on CD8 T-cells. In particular, CD28 is present on the surface of naïve T-cells and binds the co-stimulatory ligands B7.1 and B7.2 expressed on professional APC. Naïve lymphocytes must engage both their TCR and a co-stimulatory molecule to be activated. CTLA-4, PD-1 and BTLA serve as co-inhibitory receptors on activated T-cells. The cytoplasmic tails of these receptors contain a motif called an immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIMs bind and activate phosphatases that inhibit signals derived from ITAM-containing receptors. These inhibitory receptors serve to contain and modulate the T-cell response to infection, limiting immune-related pathology and preventing autoimmunity (Janeway et al., 2008b). Additionally, the relative expression levels of costimulatory and coinhibitory molecules are modulated upon antigen encounter, and these characteristics are commonly used to determine the activation status of individual populations of T-cells.

T-Cell Effector Function

Once CD8 T-cells are activated, they become poised to perform two functions in response to further antigen stimulation, both of which help to combat virus infection. The first is the release of cytotoxic granules that contain granzymes and perforin, molecules that can breach lipid bilayers and trigger apoptosis in infected targets. The second is the release of small, soluble proteins called cytokines that can alter the characteristics and behavior of neighboring cells.

Cytotoxic granules are similar to lysosomes and contain at least two types of cytotoxic effector proteins. The first is a class of serine proteases called granzymes, of which granzyme B is the best studied. The second, perforin, is important for the delivery of the other proteins into the cytosol of the target. The third is expressed in humans but not mice and is called granulysin. This molecule has antimicrobial properties and can induce apoptosis at high concentrations. All of these proteins come pre-loaded, but deactivated, in granules awaiting release (Janeway et al., 2008c).

Granzymes trigger apoptosis by activating caspases, which initiate proteolyic cascades resulting in the cleavage of genomic DNA and the disruption of the outer mitochondrial membrane. This, in turn, causes the release of more pro-apoptotic molecules, including cytochrome c. The mechanism by which perforin mediates delivery of granzymes to target cells has yet to be fully described. Initially, perforin was thought to form a pore in the plasma membrane, allowing granzymes to enter. Instead, it appears that perforin and granzymes form a multimeric complex with the proteoglycan serglycin acting as a scaffold. This complex is delivered into the cytoplasm via a mechanism thought to be more similar to that of viral entry (Metkar et al., 2002). Perforin is necessary for the translocation of these complexes across the target's plasma membrane and for the release of bound granzyme into the cytosol, but the exact mechanics of this process are unclear.

In addition to killing infected cells via the targeted release of cytotoxic granules, CD8 T-cells can induce death using membrane receptors in the tumor necrosis family (TNF), particularly Fas and Fas ligand. This pathway is used primarily to regulate lymphocyte numbers, particularly when proliferation in response to infection needs to be curtailed.

Ligation of Fas activates caspases and induces apoptotic pathways similar to those affected by granzyme release. Activated lymphocytes express both molecules (Green et al., 2003).

Cytokines released by cytotoxic T-cells include IFN-γ, TNF-α, LT-α (formerly known as TNF-β) and IL-2. IFNγ directly inhibits viral replication and works to increase the expression and loading of MHC class I molecules. This increases the likelihood that infected cells will be recognized by patrolling CD8 T-cells. IFNγ also works to activate and recruit macrophages, in part by instructing the endothelium to bind these cells and allow their exit from blood vessels to sites of infection. TNF-α and LT-α also help to activate macrophages and can induce apoptosis in some target cells through their interaction with TNFR-I (in the same family as Fas). Finally, IL-2 works to promote T-cell proliferation (Janeway et al., 2008c).

Activation, Contraction and Generation of Stable Memory

The initiation of the T-cell response to pathogen occurs in the lymphoid organs, where captured antigen is presented to CD8 T-cells by professional APCs, primarily by DCs but also by macrophages. Initial activation of a naive T-cell requires three signals: (1) peptide-MHC recognition via the TCR and CD8, (2) costimulation through molecules like CD28, CD40, 41BB, CD27, ICOS, and OX40, and (3) exposure to inflammatory cytokines, including IL-12 and interferon α. Naïve CD8s receiving all of these signals will undergo vigorous proliferation, thereby generating a large effector population. Activation also results in acquisition of the effector functions discussed above and the migration of effector cells to non-lymphoid tissues to patrol for infected cells.

After an infection is contained, an estimated 95% of the newly generated army of effectors will die by apoptosis (Obar and Lefrancois, 2010a). Whether or not a cell dies depends on the balance of pro-apoptotic and pro-survival molecules it generates, and this can be modulated through TNF receptor family signaling. The remaining cells form the memory population, which can persist independent of TCR stimulation (Leignadier et al., 2008; Murali-Krishna et al., 1999) but not without specific cytokines, most importantly Il-7 and IL-15 (Becker et al., 2002; Schluns et al., 2000). Memory T-cells can respond much more efficiently to a repeated antigen exposure for several reasons: they persist in larger numbers than their naïve counterparts, they are stationed in multiple front-line tissues like the lung, intestines and skin, and they can swiftly re-express effector molecules (Obar and Lefrancois, 2010a).

Subsets of Memory T-Cells

The properties of memory T-cells vary by phenotype and location. Three general categories of memory T-cells have been identified based on these characteristics (Kaech and Cui, 2012). Central memory (Tcm) T-cells have the greatest proliferative potential and can be found in secondary lymphoid organs and bone marrow. These cells are identified by their expression of CD62L (L-selectin) and CCR7 (the receptor for chemokines CCL19 and CCL21), which facilitate trafficking of T-cells from the blood to the lymph nodes through high endothelial venules. T-cells lacking expression of these molecules are considered effector memory (Tem) T-cells. These cells localize to the peripheral non-lymphoid organs and constitutively express cytotoxic proteins. Both subsets circulate through the blood and may actually interconvert as they traffic through different tissues (Marzo et al., 2007).

Despite these neat classifications, CD62L and CCR7 are not sufficient to define all of the relevant subsets of T-cell memory. Additional phenotypic heterogeneity is observed within the memory pool and can vary substantially by the type and location of an infectious agent (Hikono et al., 2007; Obar and Lefrancois, 2010a).

A third subset of memory T-cells with the phenotype $CD103^{hi}CD69^{hi}CD62L^{low}$ $CD27^{low}$ has been described in a number of recent studies (Sheridan and Lefrancois, 2011) and has been designated tissue resident memory (Trm). These cells can be found in the skin, lung, gut and brain, and express high levels of granzyme B. They exhibit limited recirculation, presumably facilitated by CD103 expression (also known as b7 integrin) which binds E-cadherin in epithelial tissues, and by CD69 expression, which helps prevent egress from tissues. This diversity of memory subsets likely guarantees optimal protection through division of labor. In the event of repeat infection, Tem and Trm serve as a first-line defense, wheareas Tcm can quickly generate more effectors if a pathogen overwhelms the localized response (Kaech and Cui, 2012).

How and when these different memory subsets are formed from a much larger initial effector population is an area of intense study and debate. Remarkably, single-cell adoptive transfer experiments indicate that one naïve T-cell has the potential to form both effector and memory T-cells, as well as Tcm and Tem (Stemberger et al., 2007). These findings were corroborated by experiments using cellular bar-coding, where retroviral vectors transduced heritable genetic material into the cellular DNA such that clonal progeny could be rigorously tracked (Gerlach et al., 2010). These data indicate that T-cells are not programmed during thymic development to accept one fate or the other, suggesting

instead that the descendants of an activated cell must decide their fate based on a combination of downstream signals.

Three prevailing models attempt to explain this fate decision (Kaech and Cui, 2012). The linear progression, or decreasing potential, model suggests that as a population of T-cells is repeatedly stimulated with antigens and pro-inflammatory cytokines, lineages that receive more signals will experience greater proliferation leading to terminal effector cell differentiation. This cumulative signaling history creates a continuum of effector T-cells at various differentiation states. Alternatively, the signal strength, or early fate determination (Obar and Lefrancois, 2010a), model suggests that a T-cell's fate depends upon the overall strength of signals 1 (antigen), 2 (costimulation) and 3 (proinflammatory cytokines) encountered during or soon after activation. Thus, cell fates are fixed at the onset of the response rather than in a linear stepwise manner.

It is clear that signal strength does significantly influence T-cell fate, as situations of limited antigen availability and high competition amongst responding CD8's for stimulatory signals result in enhanced memory T-cell development and limited effector proliferation (Obar et al., 2008; Obar and Lefrancois, 2010b; Zehn et al., 2009). Additionally, levels of costimulatory molecules like OX40; inflammatory cytokines like IL-12 and IFNγ; CD4 help by way of DC licensing and IL-2 expression; and the temporal coupling of all of these signals can influence the proportion of memory cells versus effectors generated during infection (Cui and Kaech, 2010). Nonetheless, it is difficult to ascertain from these studies whether signal strength exerts its influence early during activation or cumulatively over many divisions. Experiments where antibiotics were given shortly after infection to curtail the duration of antigen exposure and inflammation resulted in more rapid memory

formation (Badovinac et al., 2004), lending support to the idea of a linear progression. Yet, other work has shown that repeated antigenic stimulation is not required to sustain effector expansion—that, in fact, brief exposure could result in both effector and memory formation (Kaech and Ahmed, 2001; van Stipdonk et al., 2001).

The third attempted explanation for CD8 T-cell fate decisions is the asymmetric cell fate model, which proposes that a single activated precursor gives rise to both memory and effector daughter T-cells through asymmetric cell division. Daughter cells in closer proximity to the APC will encounter stronger TCR and co-stimulatory signals, driving them towards a terminal effector state. This idea is supported by experiments visualizing the asymmetric inheritance of cellular proteins during the first division after priming (Chang et al., 2007). Importantly, these three models are not mutually exclusive; they can be taken separately or integrated. All result in a spectrum of T-cells with varying effector and memory potential, proportions of which can be modulated in response to the characteristics of a particular infection.

Memory cell potential does not appear to be inherited equally by all effector cells. Certain CD8 T-cells are better suited than others to persist and populate the memory pool. In the widely used LCMV and *Listeria monocytogenes* infection models, these memory precursor cells are distinguished based on their increased expression of IL-7 receptor α (CD127) and co-stimulatory molecule CD27, as well as their decreased expression of killer cell lectin-like receptor G1 (KLRG1) (Kaech et al., 2003). Cells with the reciprocal expression of these markers are associated with effector or memory CD8 T-cells with Tem characteristics (i.e. cytotoxic potential, IFNγ production, low proliferative capability, shortened telomeres, less IL-2 production). This paradigm is not universal for all infectious

conditions, however, as persistent CD127^{low}KLRG^{hi} cells have been found after secondary challenge and a proportion of CD127^{hi} KLRG1^{low} cells have been shown to undergo apoptisis after infection (Obar and Lefrancois, 2010a).

Profiling of transcription factor expression in subsets of T-cells indicates that T-bet, eomesodermin (Eomes), B-lymphocyte induced maturation protein 1 (Blimp1), Bcl6 and inhibitors of DNA binding 2 (ID2) and 3 (ID3) all have roles in the generation of short-lived effectors or long-lived memory cells. Over-expression of T-bet leads to enhanced generation of short-lived terminal effector cells (Joshi et al., 2007). Blimp1 is highly expressed in cells that also express T-bet (Joshi et al., 2007). Eomes expression is reciprocal to that of T-bet, and Blimp1 activity is opposed by Bcl6 (Cui et al., 2011). ID2 expression supports the survival of effector CD8 T-cells, while ID3 supports the survival of long-lived memory T-cells (Yang et al., 2011). Underlying these transcriptional programs may be the metabolic switch from fatty acid oxidation in resting, naïve T-cells to aerobic glycolysis in activated effectors (Kaech and Cui, 2012). This switch is reversed after pathogen clearance, during the transition from effector to memory. These states are governed by nutrient-sensing molecules, in particular mTOR, which can integrate external signals and initiate T-bet expression to drive a cell towards effector differentiation. mTOR can be inhibited by AMPK, which senses cellular stress and ATP deprivation, leading to the adoption of a more metabolically quiescent, long-lived memory state.

T-Cell Exhaustion

The persistent encounter with antigen in certain chronic viral infections, including HIV, hepatitis B and hepatitis C in humans and LCMV clone 13 in mice, can alter the

function and gene expression of virus-specific CD8 T-cells. These cells experience a hierarchical loss of cytokine expression, proliferative capacity, and cytotoxicity referred to as T-cell "exhaustion." The precise features of exhaustion vary by infection, but particular characteristics are found at various levels throughout (Wherry, 2011).

One of these attributes is the reliance of memory cells on antigen stimulation rather than IL-7 and IL-15 for maintenance and turnover (Shin and Wherry, 2007). Exhausted T-cells respond poorly to these cytokines *ex vivo*, in part because they downregulate their receptors—specifically CD127, which is the α-chain of the IL-7 receptor, and CD122, which is the β-chain of the IL-2 and IL-15 receptors. Furthermore, when transferred into infection free mice, exhausted T-cells do not persist nor do they regain characteristics of functional memory (Wherry et al., 2004).

These functional changes have been associated with increased expression of inhibitory receptors such as PD-1, LAG-3, 2B4, TIM3 and CD160 (Wherry, 2011). These receptors are expressed transiently upon activation, and have important roles in maintaining self-tolerance (discussed below); however, their persistent expression indicates a state of exhaustion. Interestingly, blocking PD-1 ligation during LCMV infection reversed exhaustion and lowered viral loads (Sijts and Kloetzel, 2011), suggesting that exhaustion must be actively maintained. The inhibitory cytokines interleukin-10 (IL-10) and TGF-β also have a role in promoting T-cell exhaustion (Wherry, 2011).

Central and Peripheral Tolerance

T-cells exhibit a staggeringly varied repertoire of specificities. Cross-reactivity of T-cell receptors further expands the number of antigens this cell type is capable of

recognizing. While the diversity of this response is important for identifying harmful pathogens, it carries with it a great danger: that of autoimmune tissue damage.

Much of this danger is reduced in the thymus, where most immature T-cells that recognize self-antigens are eliminated through negative selection. This process results in central tolerance. However, because many self-antigens are not expressed in the thymus, central tolerance is not sufficient to protect against all self-reactive T-cells, nor those that may respond to innocuous antigens acquired through diet or the environment (Redmond and Sherman, 2005; Srinivasan and Frauwirth, 2009). Additionally, because TCRs are so highly cross-reactive, an especially stringent negative selection process would too severely limit the repertoire. Therefore, low-affinity self-reactive clones are allowed to pass this checkpoint.

Luckily the complexities of the immune system provide additional checkpoints to ensure what is called peripheral tolerance; self-reactive T-cells that escape negative selection in the thymus may still be deleted or functionally inactivated (rendered anergic) before they can participate in a damaging attack elsewhere in the body. The first way in which the immune system ensures peripheral tolerance is through ignorance (Srinivasan and Frauwirth, 2009). Naïve CD8 T-cells are activated when they see their cognate antigen presented by self-MHC class I molecules on professional APCs. In immune privileged sites, like those that sit on the other side of the blood/brain barrier or the maternal/fetal barrier, neither naïve T-cells nor APCs have the opportunity to encounter antigens specific to these tissues. Additionally, the amount of self or innocuous antigen may not reach concentrations high enough to be cross-presented by APCs , and finally, the TCR/peptide-MHC affinity

requirement for T-cell activation may be higher than what results in thymic deletion, thus clones that have a low affinity for self may never be activated in the periphery.

If a self-reactive T-cell does encounter its cognate self-antigen, it is likely to happen in the lymph node. Tissue-resident dendritic cells are continuously sampling their environment by phagocytosing apoptotic cells. This process can induce chemokine expression and migration of the DC without promoting the expression of costimulatory molecules (Redmond and Sherman, 2005). Thus, the DC remains "unlicensed." Encountering antigen in this context, by seeing peptide/MHC (known as signal 1) without costimulation (known as signal 2), can result in deletion or inactivation of the offending T-cell.

This tolerizing stimulus is often the result of a combination of activating and inhibitory receptors. Just as B7.1 and B7.2 bind CD28 on the T-cell surface to provide activating signals, molecules of the same family can bind B7 instead and provide opposing signals. The list of inhibitory receptors found on T-cells now includes BTLA, TIM-3, LAG-3, KRLG-1, 2B4, CD160, PD-1 and CTLA-4 (Chen and Flies, 2013). The last two have been studied the most extensively.

CTLA-4 is member of the CD28 receptor family but binds B7 molecules with a 20 to 50 fold higher affinity than CD28. Expression is upregulated in activated CD8s, and engagement prevents cell cycle progression. This engagement is necessary for tolerance induction in CD4s, but not CD8s. PD-1 is also an inducible receptor found on T-cells. Engagement downregulates proliferation, activation and development of effector functions. The ligands for this molecule are PD-L1 and PD-L2, which are structurally similar to B7 molecules. PD-L1 is expressed on all cells of hematopoietic and parenchymal origin, while

PD-L2 is only expressed in MACs and DCs. PD-1 is upregulated by the first cell division after self-antigen stimulation and works to promote tolerance by attenuating TCR signals and the CD28 activation pathway (Srinivasan and Frauwirth, 2009).

CD8 T-cells appear to require additional signals beyond costimulation to reach full effector capacity. IL-12 and type I IFN have been shown to fill this role by enhancing the proliferative and cytotoxic capacity of activated CD8 T-cells (Curtsinger et al., 2003; Curtsinger et al., 2005). Also, CD4 T-cells stimulate DC's through CD40/CD40L interactions, allowing DCs to activate CD8 T-cells. Thus, additional networks of cellular interaction are important for helping the immune system differentiate between activation and tolerance (Srinivasan and Frauwirth, 2009).

Anergy is the state ascribed to T-cells that become nonresponsive after encountering a tolerizing stimulus. While functionally similar to exhaustion, anergy is distinct in that it arises from a single event and is initiated rapidly, whereas exhaustion is gradual and progressive (Wherry, 2011). Additionally, gene-expression profiling has highlighted differences between the two T-cell states (Wherry et al., 2007). Notably, exhausted CD8 T-cells do not upregulate the anergy-associated transcripts Grail, Egr2 and Egr3.

Soluble Peptide-Induced Tolerance

Deletional or anergic tolerance to a given peptide antigen can be induced by injecting large amounts of synthetic peptide intravenously (i.v.), without adjuvant. Here, T-cells are encountering their cognate peptide in the absence of inflammatory stimuli that would normally upregulate costimulatory molecules and/or inflammatory cytokines.

This effect was first described as “immunological paralysis” in the 1960s, when naïve animals were injected with full-length protein antigen and subsequent cellular and humoral immune response were prevented (Dixon and Maurer, 1955; Dresser, 1962a; Dresser, 1962b). Later, i.v. administration of smaller synthetic peptides was shown to specifically delete or inactivate naïve T-cells in an epitope-dependent manner (Dubois et al., 1998; Kyburz et al., 1993; Ria et al., 1990). Effector/memory T-cell subsets are generally assumed to be more resistant to peripheral tolerization as a consequence of their less stringent costimulation requirements *in vitro* (Pihlgren et al., 1996; Sagerstrom et al., 1993). In these studies, primed TCR Tg T-cells activated by plate-bound peptide-MHC did not require anti-CD28 or protein kinase C activator (PMA) stimulation in order to produce IL-2. Additionally, primed T-cells required 50 to 100-fold less peptide than naïve T-cells for activation. There is, however, significant *in vivo* evidence that memory T-cells can undergo peripheral tolerization in response to soluble i.v. peptide as well, and this is discussed below.

This strategy has been explored as an attractive therapy for T-cell mediated autoimmune disease. Experimental allergic encephalomyelitis (EAE) is a CD4 T-cell mediated animal model of multiple sclerosis that can be induced by the transfer of L10C1 TCR transgenic T-cells specific for myelin basic protein epitope p87-99 into healthy mice. In this model, disease onset has been prevented and ongoing disease ameliorated with the intraperatoneal administration of 0.5 mg of p87-99 (Brocke et al., 1996). A reduction of inflammatory infiltrates mediated by IL-4 was responsible for this effect.

CD8 memory/effector T-cells also succumb to peripheral tolerization in a number of systems. Mouse models of diabetes have shown that disease can be disrupted with

administration of agonist peptide, which promotes the downregulation of autoreactive CD8 T-cells through apoptosis-induced cell death. In (CL4-TCR x Ins-HA)F_1 double transgenic mice, spontaneous diabetes develops due to HA expression in the β-islet cells. When these mice were given 30μg of HA peptide intravenously, for three consecutive days starting on day three after birth, disease progression was blocked. Reduced CD8 T-cell numbers were noted in the secondary lymphoid organs, suggesting deletion. Additionally, peptide administration did not induce bystander tissue damage in the pancreas (Bercovici et al., 2000). In nonobese diabetic (NOD) mice, where disease develops by 10 weeks of age, weekly intraperitoneal injections of 100 μg of NRP peptide delayed the development of diabetes, whereas injections with NRP-A7, a superior agonist, completely protected these mice from diabetes (Amrani et al., 2000). This was attributed to the deletion of high-affinity clones specific for NRP-A7.

In a study by Kreuwel et. al., BALB/c mice were infected with influenza virus and given 250 μg of soluble HA peptide i.v. three weeks later (Kreuwel et al., 2002). Two weeks after peptide treatment, splenocytes were cultured with APCs and pulsed with HA peptide. After 6 days, the cytotoxic capacity of these cells was tested in a chromium release assay. Diminished cytotoxicity, indicative of tolerance, was observed from the cells of mice treated with HA peptide.

Finally, soluble i.v. peptide treatment reversed CD8 T-cell mediated disease in an OVA-based mouse model of outer ear-specific autoimmunity (Paek et al., 2012). In this study, K14-sOVA/OT-1 double transgenic mice, which express keratin 14-soluble chicken ovalbumin in the external pinnae, demonstrate tissue damage due to OT-1 T-cell-induced inflammation in the first few days of life. When 200 μg of SIINFEKL peptide was

administered i.v. to pregnant mothers , and to newborn pups at 50 μg i.p., ear pathology was eliminated. Deletion of OT-1 T-cells and downregulation of CD8 on those remaining was shown in response to peptide treatment.

IV. CD8 T-cell response to CMV

The Big Picture

The immune system's reaction to CMV is complex; multiple layers of innate and adaptive immunity—which are, in large part, redundant—work to prevent pathology. Yet CMV is never fully eradicated, a consequence of the numerous immune evasion strategies born out of the co-evolution of virus and host. Many different cell types play a role in this response, and CMV has an answer to all of them.

After initial infection, before a specific adaptive response has been formed, DCs, macrophages and natural killer (NK) cells all work to control infection and to prime humoral and cellular immunity. Macrophages and DCs are the primary sensors of MCMV infection via a number of innate immune receptors – specifically TLR3, which senses double stranded RNA, TLR 9, which senses CpG-rich DNA, and TLR-2, which may sense peptidoglycan components of the envelope (Hoebe et al., 2003; Krug et al., 2004; Szomolanyi-Tsuda et al., 2006). Viral DNA may also be sensed in the cytoplasm by receptors like the absent in melonoma-2 (AIM2) inflammasome (Rathinam et al., 2010). The recognition of these pathogen associated molecular patterns (PAMPs) is inherently

unspecific; as the infection progresses, more precise mechanisms of viral recognition are triggered.

Immature DCs are activated by PAMP recognition and TLR ligation. Once activated, they traffic to the lymphoid organs and upregulate MHC I, MHC II, and costimulatory molecules in order to prime CD4+ and CD8+ T-cells. Additionally, DCs produce Type I IFN, IFN-γ, IL-12, IL-2, IL-15 and IL-18. These last four cytokines are important for NK cell activation. As discussed above, CMV can infect DCs. This allows the virus to modulate their function: antigen uptake and degradation is inhibited, maturation and migration is impaired, and expression of class I, costimulatory molecules and cytokines is altered in infected cells (Andrews et al., 2001; Loewendorf et al., 2011b; Raftery et al., 2001). Macrophages are also able to recognize PAMPS via TLRs, phagocytose and present antigen, and produce TNF-α, IL-1 and IL-12.

NK cells are large granular lymphocytes that are able to secrete cytokines (IFN-γ, TNF-α, GM-CSF, IL-3, M-CSF), and trigger apoptosis in target cells via cell surface receptors and the release of cytotoxic granules. NK cells are prompted to release cytokines and induce killing by integrating combinations of activating and inhibitory signals, which arise from the ligation of their large array of surface receptors. Many of their inhibitory receptors recognize class I and class II-like molecules. NK cells are thus very sensitive to class I down-regulation, a viral strategy for evading CTL that will be discussed below. Many of their activating receptors recognize proteins that are induced as a cellular response to stress or adhesion proteins that are exposed upon the breakdown of normal intercellular communications. There is functional heterogeneity among subsets of NKs, and emerging research shows that NK cells can exhibit properties of education and memory. NK Subsets

recognizing features of CMV-infected cells will proliferate during infection and remain at higher frequencies in chronically infected individuals (Wilkinson et al., 2013).

The importance of NK cells in the control of MCMV infection can be seen when comparing two commonly used strains of laboratory mice, C57BL/6 and BALB/c. The latter lack the NK activating receptor LY49H, which recognizes the m157 gene product of MCMV, and these mice consequently suffer higher viral loads upon infection (Lee et al., 2001). Further evidence for the significance of these cells in the immune response to CMV can be found in the substantial number of genes encoded by both MCMV and HCMV that are able to modulate NK cell function, including MHC-I homologues and other proteins that either inhibit activating receptors or activate inhibitory receptors (Wilkinson et al., 2013).

In both HCMV and MCMV infection, antibodies are produced to the main glycoprotein complexes of each virus. These antibodies can prevent virus attachment and receptor binding, inhibit conformational changes in glycoproteins that are required for virus-cell fusion, prevent the assembly of the viral fusion complex, and block uncoating of the virus and capsid release (Mach et al., 2013). These antibodies are substantially effective; in the mouse model, combined depletion of CD8, CD4 and NK cells does not result in notable virus reactivation. Only when B-cell deficient mice are used did this depletion induce reactivation (Polic et al., 1998). Unsurprisingly, CMV dodges these antibodies in a number of ways. Different strains exhibit substantial antigenic variation, helping new infections to elude existing antibody responses (Mach et al., 2013). Additionally, the virus encodes receptors that bind the Fc portion of immunoglobulin molecules, and these are expressed on infected cells presumably to prevent antibodies from inhibiting capsid release (Antonsson and Johansson, 2001; Atalay et al., 2002).

CD4 T-cells, activated through MHC-II presentation, go on to license DCs through ligation of CD40 by CD40L, induce somatic hypermutation and class switching in B-cells, and produce cytokines that promote proliferation and differentiation of virus-specific CD8 T-cells. Evidence in mice indicates that without CD4 help, there can be extended periods of persistent replication in the salivary gland (Jonjic et al., 1989; Jonjic et al., 1990), while in humans, children with compromised CD4 T-cell function show increased HCMV shedding in the urine and saliva (Tu et al., 2004). As might be expected, HCMV employs a number of mechanisms to inhibit this particular cell type by disrupting MHC-II presentation.

Importance of CD8 T-Cells

CD8 T-cells, activated through direct and cross-presentation in the lymph nodes by professional APCs, and via cytokines secreted by all of the immune cells mentioned above, also play a significant role during CMV infection. Evidence for their protective capacity in HCMV is inferential; an increasing prevalence of HCMV disease is seen in subjects with impaired T-cell immunity (Wills et al., 2013). Additionally, a strong correlation was shown between the recovery of CD8 T-cell activity and recovery from HCMV infection after the reconstitution of the immune system by bone marrow transplantation (Cwynarski et al., 2001; Reusser et al., 1991; Gratama et al., 2010). In contrast, a more recent study indicated that CMV-specific CD4 T-cells, rather than CD8 T-cells, inversely correlated with CMV viral loads in stem cell transplant recipients (Widmann et al., 2008).

Evidence in the mouse model is stronger. In a model of bone marrow transplant, where mice are irradiated and then infected with MCMV, these immunocompromised animals are protected from lethal MCMV challenge by the transfer of CD8 T-cells specific for IE genes

(Reddehase et al., 1987). Yet CD8 T-cells are not essential for viral control in immunocompetent hosts, as CD4 T-cells and NK cells are an adequate substitute for controlling MCMV reactivation in B-cell deficient mice (Polic et al., 1998). Furthermore, mice depleted of CD8 T-cells prior to infection survive and are able to clear virus with similar kinetics to control mice (Jonjic et al., 1990). Thus, CD8 T-cells are clearly very effective at controlling CMV infection; however, other components of the immune system— in particular NK cells and humoral immunity— can serve redundant functions.

MHC I Immune Evasion

The importance of CD8 T-cells to CMV control is further supported by the discovery in all major model systems of multiple viral genes that function to disrupt normal MHC I antigen presentation. In HCMV, these actions are principally mediated by the genes encoded by the US2-11 region. For example, US2 and US11 gene products are responsible for the degradation of newly synthesized class I heavy chains and US3 binds tapasin in the ER, causing retention of MHC I. The US6 gene product binds the cytosolic face of TAP, blocking peptide translocation into the ER (Powers et al., 2008).

MCMV encodes three proteins that disrupt MHC I antigen presentation: m4, m6 and m152. MHC I complexes with m4 in the ER and accompanies it to the cell surface. This molecule's role in limiting CTL detection is unclear, as it may actually function to upregulate MHC I expression (Holtappels et al., 2006; Wagner et al., 2002; Kavanagh et al., 2001), but m4 has more recently been established as a negative regulator of NK cell activation (Kielczewska et al., 2009). m6 functions by binding stably to pMHC complexes and sorting them into lysosomes for degradation (Reusch et al., 1999), whereas m152

retains peptide-MHC complexes in a cis-Golgi, ER-Golgi intermediate compartment (Ziegler et al., 1997). M152 can also modulate NK cell function by reducing the cell surface expression of ligands that bind the activating receptor NKG2D (Krmpotic et al., 2002).

Immune evasion genes may not completely protect infected cells from CD8 T-cell recognition, and evidence for HCMV indicates that this may depend on the antigen specificity of the T-cell. The US2-11 region prevented presentation of IE antigen by HCMV-infected cells; however, pp65 (a tegument protein expressed as a late gene) was still able to provoke T-cell responses in these cells (Besold et al., 2007). TCR avidity may be a factor in this discrepancy. Evidence in the mouse model, using various permissive cell types infected with either WT MCMV or a virus lacking all three MHC-I modulating genes, showed that T-cells could produce IFN-γ and TNF-α in response to cells infected with either virus, but that cytotoxicity was limited to cells infected with the mutant lacking immune evasion genes (Pinto, 2006).

One might expect that a virus lacking MHC-I modulation genes would be better at priming CD8 T-cell responses, but our laboratory has shown that this is not the case. Comparisons of mice infected with wild type (WT) MCMV or a virus lacking m4, m6 and m152 found no difference in the magnitude or phenotype of the CD8 T-cell response to an epitope in the protein encoded by the M45 gene (Gold et al., 2002; Gold et al., 2004). Later analysis of the T-cell responses elicited to 26 different MCMV epitopes concluded that MHC-I downregulation has little impact on the specificity or the overall scale of the MCMV response (Munks et al., 2007).

Instead, predicted functions for these immune evasion genes include delaying the rate of viral clearance, which could enhance the chance of host-to-host transmission, and

increasing the speed and ability of the virus to establish latency (Bohm et al., 2009). An interesting study in the Rhesus CMV model concluded that the RhCMV homologues of HCMV US 2, 3, 6 and 11 are necessary for super-infection but are not required for primary infection nor for the establishment or maintenance of latency (Hansen et al., 2010).

Memory CD8 T-Cell Inflation

CMV provokes a unique T-cell response during chronic infection. Whereas in most viral infections, the T-cell response to *all* viral epitopes contracts and stabilizes at a low level after the initial burst of viral replication, in chronic CMV infection, a subset of T-cells accumulates in large numbers and remains with the host for the duration of its life. This accumulation of virus-specific T-cells has been termed "memory inflation," and is described both in mice and in people (Holtappels et al., 2000; Karrer et al., 2003; Munks et al., 2006a; Northfield et al., 2005).

The majority of inflationary CMV-specific T-cells bear the phenotypic signature of repeated antigen stimulation (reviewed in Snyder, 2011, Immunol Res, 51, 195-204)—that is, they lack expression of the co-receptors CD27 and CD28 (the latter only in humans), they lack CD62L and CCR7, which would allow access to the lymph nodes, and they have reduced expression of the IL-7 receptor α chain (CD127) and the IL15 receptor β chain. These cells also upregulate the NK cell-associated receptors NKG2D, NKG2A and KLRG-1. KLRG-1 in particular is associated with repeated antigen stimulation and reduced proliferative potential (Masopust et al., 2006). Additionally, in humans, CD57 (an inhibitory molecule associated with highly differentiated cells) and CD85j (an inhibitory NK-associated receptor) are upregulated on HCMV-specific T-cells . A subset of HCMV-specific

inflationary T-cells reverts from CD45RO expression to CD45RA expression (Iancu et al., 2009; Wills et al., 1999), and these cells have shortened telomeres (Romero et al., 2007). Yet *in vitro* stimulation can provoke the re-expression of CD45RO (Wills et al., 2002). These isoforms of the tyrosine phosphatase CD45 are used to distinguish naïve (usually CD45RA+) and memory/effector (usually CD45RO+) T-cells in humans.

Despite this highly differentiated phenotype, these inflationary CMV-specific T-cells appear to be functional, both in mice and in humans (reviewed in Snyder, 2011, Immunol Res, 51, 195-204). They express perforin and granzymes, they can kill antigen-bearing targets *ex vivo*, and they can secrete IFN-γ and TNF-α upon antigen stimulation, although they secrete very little IL-2. Notably, neither HCMV nor MCMV-specific T-cells express the inhibitory molecule PD-1, which is associated with terminal differentiation and the phenomenon of T-cell "exhaustion" seen in many other chronic viral infections. However, one might expect thaT-cells with the phenotype described above would have significantly reduced proliferative capacity. This is only partially true. In humans, deuterated glucose-labeling experiments showed that these cells have a low proliferative rate *in vivo* (Wallace et al., 2011), but can be induced to divide by the correct stimuli, which includes TCR engagement and either common gamma chain cytokine receptor signaling or 41BB ligation (Waller et al., 2007; van Leeuwen et al., 2002). In mice, MCMV-specific inflationary cells can divide after challenge in an adoptive transfer model, but are not sustained and instead decay with a half-life of one to two months (Snyder et al., 2008).

A population of MCMV-specific cells with a more central memory-like phenotype is also seen during chronic infection in the mouse model (Snyder et al., 2008). Most do not recognize the same antigens as the inflationary populations, although a subset of

inflationary epitope-specific T-cells also have more central memory-like characteristics. These included reduced KLRG-1, NKG2D and NKG2A expression, and increased CD27, CD127, CD62L and CCR7 expression. These cells expand and persist better than effector phenotype cells after adoptive transfer and viral challenge.

Maintenance of Inflationary Memory

The mechanisms by which these inflationary populations are produced and maintained over the lifetime of a host have yet to be fully elucidated. Our laboratory has shown that naïve T-cells can be recruited during chronic infection to supply some new effector cells, but this recruitment is insufficient to maintain the entire inflationary population (Snyder et al., 2008). Additionally, data from thymectomized mice revealed that priming of recent thymic emigrants is not necessary for memory inflation, nor does it make a large contribution to the size of the response (Loewendorf et al., 2011a). Yet, when cells bearing effector-like characteristics were transferred into naïve or chronically infected recipients, they were unable to sustain themselves after viral challenge. Thus, the inflationary population must be comprised of continuously renewed short-lived effectors, replenished by another population of more long-lived memory cells. This memory population could be readily transferred with a whole splenocyte suspension at day seven, but not later during chronic infection (Snyder et al., 2008). The source of these inflationary cells at later time points has yet to be defined.

Based on the phenotype of these inflationary effectors and their inability to sustain themselves after adoptive transfer, it was concluded that continued antigen presentation must be necessary for their renewal. Yet, our laboratory has also shown that inflationary

memory does not require virus spread. To do this, Snyder et. al. used a recombinant MCMV lacking glycoprotein L (MCMV-ΔgL), which is essential for entry into cells. This virus can be grown on a complementing cell line that provides gL in trans, but once the virus is harvested and used to infect an animal, it can infect only one round of cells. Despite this fact, mice chronically infected with MCMV-ΔgL display memory inflation of the characteristic effector-like phenotype described above (Snyder et al., 2011). In the same study, a virus containing the thymidine kinase (TK) gene from HSV was used to infect mice that were later treated with famcyclovir, an antiviral drug that, once phosphorylated by TK, inhibits viral replication by interfering with the viral polymerase. In these experiment, famcyclovir treatment had little impact on CD8 T-cell response once the infection was established. Thus, full viral replication is not needed to maintain memory inflation, but some antigen presentation must occur even in the case of a replication-deficient virus.

Given this data, we are forced to conclude that a cell type present during the first round of infection is responsible for driving memory inflation. Evidence that this cell type is non-hematopoetic comes from two studies using bone marrow chimeric mice. In the first, presentation of inflationary MCMV epitopes was limited to hematopoetic cells by the reconstitution of irradiated H-2K$^{b-/-}$ mice with bone marrow from WT mice (Torti et al., 2011a). When TCR transgenic T-cells specific for the inflationary, H-2K^{b} restricted epitope M38 were transferred to recipients, accounting for the lack of positive selection of H-2K^{b} restricted CD8 T-cells, these mice did not develop memory inflation. M45-specific T-cells, specific for an H-2D^{b}-restricted epitope, were generated in large numbers during acute infection in these mice, similar to WT mice. The same study also explored the ability of IE3-specific CD8 T-cells to inflate in DC-MHCI mice, which express β2-microglobulin only in

select tissues, confining MHC I presentation to DCs and keratinocytes. When irradiated DC-MHCI mice were reconstituted with bone marrow from WT mice, IE3 and M38-specific cells were unable to inflate. A similar observation was made in the Balb/c system using a bone marrow chimeric model where only donor-derived cells of haematopoetic origin were able to present the IE1 epitope (Seckert et al., 2011). Here, male donors carrying the Y-chromosomal gene *sry* as a genotypic marker and expressing the IE1 epitope-presenting MHC I molecule L^d, were transered to females lacking L^d expression. In this scenario, the IE1 epitope can only be presented by donor-derived professional APCs of hematopoetic origin. Acute responses were normal in these mice, but memory inflation in response to IE1 was impaired. Together, these studies indicate that memory inflation is driven by cells of non-hematopoetic origin, but that these cells are dispensable for the priming of the acute response.

Consistent with this, the B7-CD28 costimulatory pathway was shown to play an important role in the generation of the acute response to MCMV, but its absence had little effect on the maintenance of inflationary memory populations (Arens et al., 2011). Conversely, 41BB/41BBL was shown to promote inflationary responses while antagonizing the acute response (Humphreys et al., 2010). Additionally, the absence of CD4 T-cell help was shown to abrogate memory inflation to the IE3 epitope in one study (Snyder et al., 2009), and to multiple epitopes in another study, where the effects of increased viral replication were controlled for by using MCMV-TK infection treated with famcyclovir (Walton et al., 2011).

This body of data supports a model in which MCMV establishes a latent infection in some type of endothelial cell. Latency is punctuated by rare periods of viral gene

expression that stimulate a population of memory "stem" cells. These cells divide and give rise to the short-lived effectors that constitute the majority of the inflationary response. An interesting question then arises: why do these memory cells not exhaust after enough antigen stimulation, as they do in LCMV infection? An alternative hypothesis states that the short-lived effectors are in fact the subset that sees the majority of antigen, and these cells serve as a "buffer," protecting the memory subset from all but the occasional antigen encounter (Snyder, 2011). This occasional event is enough to promote division and boosting of the effector population, leading the memory inflation.

HCMV and Immunosenescence

It is hypothesized that this large, oligoclonal expansion of CMV-specific T-cells that occurs during chronic infection may have unfavorable effects on the immune system, particularly in the elderly. HCMV-specific CD8 T-cells have been found to occupy more than 5% of total CD8 responses in old age, and in one case greater than 20% (Khan et al., 2002). Several explanations are offered for this HCMV-induced immunosenescence. For one, if these T-cells become dysfunctional, individuals may lose control of HCMV. The implication that these cells are senescent or exhausted, however, is not supported by data which indicate little defect in *ex vivo* antigen-driven proliferation (Waller et al., 2007; Wills et al., 2002), and no difference in phenotype, proliferative capacity, or cytokine function between RhCMV-specific T-cells extracted from young and old animals (Cicin-Sain et al., 2011) or HCMV-specific T-cells extracted from young and old people (Wallace et al., 2011).

Additionally, responses to new antigens could be impaired if a large percentage of the T-cell compartment is "filled-up" by CMV-specific cells. CMV could, in other words, drive T-

cell immunosenescence, which would lead to poor immune control of other pathogens and an overall increase in inflammation (Hill, 2012). A number of studies have looked at the response to influenza vaccination in the elderly, HCMV positive population, but not all of them show an association between seropositivity and poor vaccine responses. Additional studies have looked for a correlation between HCMV and all-cause mortality. Again, results are inconsistent (reviewed in Wills MR et al., 2013, Cytomegaloviruses, 142-172). Thus, the connections between CMV, immunosenescence and increasing inflammation remain unproven.

V. CMV as a Vaccine Vector

Viral vectors are an attractive potential vaccination strategy because they act simultaneously as adjuvant and antigen delivery system. They offer a promising option for pathogens that require robust cellular responses for protective immunity. CMV is particularly appealing as a viral vaccine vector for three reasons. First, as discussed, CMV's high immunogenicity elicits and maintains a large effector-memory dominated T-cell response in chronic infection. This inflationary memory response is generated even in the case of a single cycle virus (Snyder et al., 2011). Second, the virus has a remarkable ability to superinfect. Third, technology allowing CMV to be cloned as a bacterial artificial chromosome (BAC) has greatly enhanced the efficiency of its genetic manipulation (Borst et al., 1999). Molecular tools designed for *Escherichia coli* can now be used to rapidly insert exogenous antigens and alter existing ones.

Interestingly, the first use of CMV as an antigen delivery system was borne out of the need to battle plagues of invasive mouse species in Australia. Recombinant MCMVs

expressing murine zona Pellucida 3 (mZP3) were engineered and tested as immunocontraceptives. mZP3 is one of three glycoproteins that surrounds the oocyte of the growing follicle and later the ovulated egg, and serves as the primary sperm receptor. Responses generated after a single inoculation of mice with this virus were sufficient to cause ovarian pathology and complete sterility, despite mZP3's status as a self antigen (Lloyd et al., 2003; Redwood et al., 2005). This break in peripheral tolerance and the induced pathology were primarily antibody mediated (Redwood et al., 2005).

T-cell epitopes from influenza A and lymphocytic choriomengitis virus have been expressed in MCMV as fusions to the C-terminal end of the IE2 gene. Responses to these antigens were shown to expand during latency and to provide protection to recombinant poxviruses expressing the same antigens (Karrer et al., 2004). Moving towards a more clinical application of these concepts, recombinant CMVs that target simian immunodeficiency virus in rhesus macaques (Hansen et al., 2009; Hansen et al., 2011) and Ebola in mice (Tsuda et al., 2011) have been tested with significant initial success.

With the eventual objective of creating a disseminating vaccine against Ebola virus to be administered to great ape populations in Africa, both to save these endangered animals from lethal disease and to minimize transmission to human populations, Tsuda et. al. created an MCMV-based vector containing a CD8 T-cell epitope from the nucleoprotein complex of Ebola. Mice inoculated with this virus developed long lasting effector CD8 T-cell responses and were protected from challenge with a mouse-adapted Ebola strain (Tsuda et al., 2011).

Several HIV vaccines have been constructed with the goal of generating large CD8 T-cell responses to the virus. These are hypothesized to restrict acute-phase replication and

lower the chronic-phase set point, thus reducing transmission rates. Yet a recent trial of an adenovirus-based vaccine met with little success, perhaps because the memory T-cell response that was elicited was predominately of the central memory phenotype (Walker and Burton, 2008; Watkins et al., 2008). In the wake of these failures, Hansen et. al. hypothesized that by using a CMV vector to deliver and persistently present HIV antigens, the ensuing T-cell response would be swayed toward effector memory, would localize to mucosal sites, and would have more immediate effector potential. This could initiate adaptive immunity at very early time points after mucosal infection (Hansen et al., 2009).

Rhesus CMV-simian immunodeficiency virus (RhCMV-SIV) vectors were constructed to contain SIV Gag, a Rev-Tat-Nef fusion protein, and Env. Rhesus macaques inoculated with these constructs developed and maintained strong CD4 and CD8 responses to each of the SIV antigens, despite competing responses to RhCMV antigens. These responses, especially the CD8 responses, were strongly skewed toward an effector memory phenotype. When RhCMV-SIV-vaccinated animals were then inoculated with repeated doses of SIV, the median number of doses necessary to achieve systemic infection in the control group was significantly lower than in the vaccinated group. Notably, four of 12 animals showed resistance to SIV infection, even after CD8 T-cell depletion, indicating that the vaccine completely protected these animals from systemic infection (Hansen et al., 2009).

A more comprehensive study was done with a larger cohort of animals given either the persistent RhCMV-SIV vaccine, the previously developed non-persistent DNA prime/replication-deficient Adenovirus-based vaccine, or a combination (Hansen et al., 2011). All animals developed plasma viral loads after a similar number of infections; however, slightly more than half of the animals receiving the RhCMV-SIV vaccine were soon

able to control viral titers to undetectable levels, with some small, short-lived blips of viremia. These controllers did not develop SIV pathology, measured as the loss of effector CD4 T-cells, whereas there were no complete SIV controllers in the non-RhCMV-vaccinated groups. Depletion of CD8 T-cells in these controllers did not result in increased viral loads or changes in CD4 T-cell numbers, indicating that the frequency of SIV-infected cells in these individuals was reduced to non-pathological levels over time or that residual CD4 and CD8 T-cells in the tissues were enough to control replication in the infected cells that remained (Hansen et al., 2011).

VI. Immunodominance

Definition

CD8 T-cells responding to a viral infection are specific to only a small portion of the potential peptide epitopes encoded by the pathogen's genome. The size of the T-cell responses specific to each of these epitopes can be arranged into reproducible hierarchies, a phenomenon that is called immunodominance. Thus, "immunodominant" epitopes are those that provoke the most abundant cognate T-cell numbers, while "subdominant" epitopes provoke comparatively fewer cognate T-cells.

This hierarchy is the end result of a complex interplay of positive and negative factors that influence antigen presentation and T-cell activation. When considering the CD8 T-cell response to viral infection, these factors can be broken down into three categories: virus-intrinsic, APC-intrinsic, and T-cell intrinsic (Munks and Hill, 2006). Virus-intrinsic

factors affect the abundance of a particular antigen; how much and when a protein is produced will influence how available its epitopes are for presentation. APC-intrinsic factors affect the ability of an epitope to be processed and presented by relevant APCs, and T-cell-intrinsic factors include the availability of cognate T-cells and their comparative ability to proliferate and respond to specific epitopes. Each of these categories will be further dissected below.

All told, these factors render less than 0.001% of possible peptide epitopes immunogenic— that is, capable of generating a sizeable T-cell response (Yewdell and Bennink, 1999). If we are to design rational vaccines given this severe limitation, it becomes immensely important to understand what the qualities of immunodominant epitopes are. Practically speaking, the ability to create immunodominant epitopes or to manipulate responses to subdominant epitopes within a viral vector could be very powerful.

Factors that Determine Immunodominance Hierarchies

Virus-Intrinsic Factors

In general, three qualities of a virus can influence the availability of viral antigen. These include the lytic cycle gene expression cascade, the nature of viral latency (if relevant), and the existence of viral genes that interfere with antigen presentation (VIPRs). As discussed above, herpesvirus gene expression occurs in a regulated cascade starting with the expression of immediate early genes, followed by early gene and then late gene expression. The immune system may encounter virion structural proteins even before

replication begins. Thus, if most infected cells are lysed by immune effectors early during infection, IE proteins and preformed structural proteins will be the most abundant viral antigens encountered.

If the virus is not cleared and instead enters a state of latency, a different set of viral proteins may be expressed, and these are likely to provoke a different set or proportion of responses during the latent state. Additionally, if directly infected cells are important for priming the CD8 T-cell response, VIPRs should have a profound impact on the specific epitopes that are seen by CD8 T-cells. The influence of these viral characteristics on the specific immunodominance profile of CMV will be discusses in greater detail below.

APC-Intrinsic Factors

By far the most important factor in determining the ability of an epitope to provoke a sizeable T-cell response is its ability to bind with sufficient affinity to a given class I molecule. Only about one out of 200 possible peptide sequences possess the appropriate amino acids for this interaction, accounting for 99.5% of what makes an epitope immunodominant (Yewdell and Bennink, 1999).

Peptides that bind MHC I molecules are almost always between eight and 11 amino acids in length, owing to the fact that the amino and carboxy termini must interact with residues within the peptide binding groove of the class I molecule, and any residues extending beyond these sites interfere with binding. Each variety of class I molecule binds a unique set of peptides. The antigen-binding groove has two to three pockets that each interact with only a limited number of amino acids (one to five), the specificity of which depends upon the MHC molecule in question. One pocket accommodates the carboxy

terminus of the peptide, while the other(s) bind either the second, third or fifth residue from the amino terminus. This knowledge allows for the prediction of all of the peptides within a protein that might bind to a given class I molecule.

It is not simply affinity for MHC I that determines immunogenicity, however. Amongst epitopes that can bind a given class I molecule, the stability of the peptide-MHC I complex formation, i.e. the dissociation rate, most closely correlates with immunodominance (Busch and Pamer, 1998; van der Burg et al., 1996). A role for tapasin in editing the peptide repertoire during direct presentation to favor peptides with low dissociation rates was shown using mice deficient for this particular ER chaperone (Howarth et al., 2004; Thirdborough et al., 2008). Again, immunodominance correlated with peptide-MHC I stability in these experiments.

Before a peptide can bind MHC I, it must be degraded by the proteasome, transported from the cytosol to the ER by TAP, then trimmed and delivered by other chaperones to an empty class I molecule. Thus, a peptide's ability to bind TAP or to bind other chaperones may affect the efficiency with which it is presented. To bind TAP, a peptide must be between eight and 16 amino acids in length, with the appropriate carboxy-terminal residue. Both human and mouse TAP prefer hydrophobic residues in that position (Yewdell and Bennink, 1999).

The regions immediately flanking an epitope within the full-length protein can also influence its immunogenicity by affecting how well it is preserved by the proteasome (Del Val et al., 1991; Niedermann et al., 1995). This was shown in a series of experiments exploring the processing efficiency of two epitopes within the chicken ovalbumin protein OVA. 22-mer synthetic peptides were composed, one with the first (immunodominant)

epitope surrounded by the naturally occurring flanking sequences of the other (subdominant) epitope, and another with the converse arrangement. For the first, processing efficiency was decreased by twofold, and for the second, processing efficiency was unaffected. These rates correlated with those by which purified 20S proteasomes were able to liberate the epitopes from each 22-mer substrate.

Each of the steps in the antigen-processing pathway can be affected by exposure to cytokines and by the actions and attributes of individual viruses. IFN-γ and TNF-α in particular increase the synthesis of TAP, class I molecules and other chaperones. As discussed, the subunit composition of the proteasome is altered by cytokine exposure, which affects the efficiency and specificity of peptide production. Professional APCs constitutively express this immunoproteasome and are also capable of cross-presentation, thus the nature of the APC responsible for CD8 T-cell activation and the relative importance of direct versus cross presentation in any given infection can have an impact on the epitopes that are most abundantly presented. Viruses have evolved around these antigen-processing mechanisms to elude or minimize detection. For example, a protein in EBV, which is abundantly expressed during latency, is hypothesized to be poorly immunogenic due to a region that interferes with proteasome degradation (Yewdell and Bennink, 1999).

Of the 0.5% of peptides that bind to class I molecules, it is estimated that only about half of these can induce a T-cell response, and only one fifth of these provoke an immunodominant response (Yewdell and Bennink, 1999). There is no simple correlation between immunodominance and the abundance of a particular epitope on an APC's surface, thus T-cell-intrinsic factors have an important impact on the nature of the T-cell response.

T-Cell Intrinsic Factors

Precursor Frequency

The available T-cell repertoire is influenced by germline encoded elements, by positive and negative selection in the thymus, and by peripheral selection. Any correlation between endogenous precursor frequency and the size of CD8 T-cell responses to individual epitopes during infection was not directly explored until the combination of magnetic-bead separation techniques and pMHC I tetramers allowed the direct quantification of endogenous naïve antigen-specific CD8 T-cell responses (Obar et al., 2008). These experiments showed that responses to an epitope with 4-fold higher precursor frequency peaked almost 24 hours earlier than responses to lower precursor frequency epitopes, and had higher numbers at the peak of infection. These responses stayed dominant throughout the memory phase, although the total size of the memory population correlated with size of peak response rather than precursor frequency.

Immunodomination/Cross-Competition

Cross-competition is the active suppression of one epitope-specific response by the presence of another. This is also called immunodomination and should be distinguished from affinity maturation, which results from competition between different T-cell clones for the same epitope. Cross-competition can be said to play a role in a T-cell hierarchy if preventing responses to an immunodominant epitope (by removing the epitope or the T-cells capable of responding to it) enhances responses to a non- or subdominant epitope. This process was demonstrated over a decade ago in the conventional C57BL/6 response

to BALB/b minor histocompatibility antigens. The response to subdominant epitopes was restored when the dominant response was removed or, interestingly, if the subdominant epitopes were presented on separate APCs (Grufman et al., 1999; Wolpert et al., 1998).

Cross-competition could be created in two ways. First, immunodominant epitopes could somehow interfere with the generation or presentation of subdominant epitopes within APCs; however, cross-competition is frequently seen between responses to epitopes that bind different MHC I alleles. Additionally, the abundance of immunodominant epitopes in relation to endogenous cellular peptides is so low as to make this possibility unlikely in most circumstance, though not impossible in all (Yewdell and Bennink, 1999).

Second, T-cells specific for dominant peptides could outcompete T-cells specific for other peptides. This could occur by multiple mechanisms operating alone or in concert: rapidly responding T-cells could reduce the antigen load by killing APCs upon activation; T-cells could out-compete others at the level of the APC for binding sites, costimulation or local cytokines; or dominant responses could systemically suppress less dominant ones.

The strongest evidence for the second explanation— that indeed relevant APCs are the limiting factor— comes from Marrack's group, who demonstrated cross-competition with peptide-loaded DCs and transferred TCR transgenic T-cells (Kedl et al., 2002). They later found that immunodominance was determined early during activation, within the first five hours after immunization of mice with peptide-loaded DCs and T-cells (Willis et al., 2006), and that killing of peptide-loaded DCs was a rare event in their system.

Some evidence for the systemic suppression of subdominant responses comes from a much older study, where mice were co-immunized with two peptides differing 30-fold in their affinity for K^d. Oddly, the weaker binding peptide dominated the response (Eberl et al.,

1996). This immunodomination could be eliminated, however, if mice were treated with IL-12, indicating that some level of cytokine-mediated regulation was possible in this system. Thus, cross-competition is a functionally defined process that can result from multiple mechanisms operating on different levels of T-cell activation.

Immunodominance in CMV Infection

HCMV

While the acute CD8 T-cell response to HCMV infection is difficult to study given that infection is largely asymptomatic, responses in chronic infection have been extensively mapped. A number of somewhat incomplete studies have implicated the responses to pp65 and IE1 gene products as being largely immunodominant in most individuals (Moss and Khan, 2004; Elkington et al., 2003; Manley et al., 2004). More comprehensive examination has involved overlapping 15-mer peptides from all HCMV proteins tested across a large number of HLA types (Sylwester et al., 2005). This work revealed either CD8 or CD4 responses against 151 of the 213 open reading frames tested. The majority of the CD8 responses were directed against UL48, pp65, or IE1, but a significant number were directed towards a much more diverse range of epitopes. Importantly, a strong bias toward IE antigens was detected (Sylwester et al., 2005).

MCMV

In acute infection of BALB/c mice, there is a codominant CD8 T-cell response to IE1 and to the E gene m164, with subdominant responses to m18 and M45 also detectable.

These codominant responses, which account for approximately 30% of the total response early on, later occupy close to 80% of the response in chronic infection (Holtappels et al., 2002). In C57BL/6 mice, the response is larger and more diverse. Our laboratory was the first to use an expression library of genomic MCMV DNA fragments to identify the E antigen M45 as immunodominant during acute infection in these mice (Gold et al., 2002). Munks et. al. later cloned and expressed each open reading frame from MCMV, identifying 27 H2b restricted antigens that are recognized during acute infection (Munks et al., 2006a; Munks et al., 2006b). No IE antigen was recognized during the acute response in these mice. Instead, the responses to M45, M57, m141, M38 and m139 formed the largest percentage of of the MCMV-specific T-cells pool.

In C57BL/6 mice, immunodominance during the chronic phase of infection does not depend on the hierarchy observed during acute infection (Munks et al., 2006a). The CD8 T-cell response to most epitopes, including those that form the largest proportion of the total response, M45 and M57, contract between days seven and 14 and stabilize a low, constant level for the life of the animal. Responses to m139 and M38 epitopes, which rank second and fifth respectively at day seven post infection, become prominent during chronic infection. Reponses to an IE3 epitope, which are barely detectable during acute infection, slowly but dramatically increase over several months post infection. Thus, memory inflation is dominated by responses to IE3, M38 and m139.

If MCMV inflation is independent of the acute immunodominance hierarchy, how then are inflationary responses selected? This question forms the basis for the work presented in this thesis.

Selection of Inflationary Responses in MCMV

One might hypothesize that, due to the wide variety of MCMV genes known to interfere with the MHC I antigen presentation pathway, the T-cell response should focus on those epitopes least likely to be affected by this interference. However, our laboratory has shown that mice infected with a virus lacking these immune evasion genes exhibit the same immunodominance pattern during both acute and chronic infection as a mouse infected with WT MCMV (Munks et al., 2007). The best explanation for this is that CD8 T-cells must be primed by cross-presentation *in vivo*.

Evidence for this idea was also provided by our laboratory. Fibroblasts lacking the H-2b MHC were infected with our spread-deficient deltagL virus and used to immunize C57BL/6 mice (Snyder et al., 2010). This effectively limited priming to cross-presentation since the infected cells could not present any epitopes recognized by the T-cells in these mice, which had of course been positively selected on the H-2b background. Despite this, an immune response almost identical to that of standard intraperotoneal infection with deltagL was observed during acute infection (Busche et al., 2013; Nopora et al., 2012). Additional evidence was provided by experiments done in Batf3-/- mice, which lack CDa+CD103+ DCs and are, as a result, deficient in their ability to cross-present antigen (Busche et al., 2013). The acute response to MCMV was significantly impaired in these mice.

In these same Batf3-/- mice, memory inflation was fairly unaffected, both in magnitude and hierarchy, suggesting that responses in chronic infection are driven largely by direct presentation. This idea is supported by the work discussed previously, which suggests that the cells responsible for driving memory inflation are not of haematopoetic origin (Torti et al., 2011a; Seckert et al., 2011). Given this model, it seems likely that

epitopes dependent on the immunoproteasome for presentation—which should be expressed predominantly in DCs— would dominate during the acute response, whereas those that can be processed by normal proteasomes would have an advantage during chronic viral carriage. Consistent with this idea, a recent study using immunoproteasome-deficient LMP7-/- mice showed significant impairment of the M45-specific response in mutant mice, and somewhat less impairment of the M38 and m139 responses, suggested that antigenic peptides driving memory inflation are less dependent upon the immunoproteasome (Torti et al., 2011a).

The vast majority of reactivation events in CMV infection are thought to be abortive based on evidence in the bone marrow transplant model discussed above. IE1 and IE2 transcripts were found in the lungs during latency; however, neither the alternatively spliced IE3 transcript nor the essential gene gB/M55 were expressed (Grzimek et al., 2001). Rare, stochastic events could lead to IE1 and IE2 gene transcription, which is hypothesized to activate effector-memory CD8 T-cells and promote memory inflation of IE1-specific responses in BALB/c mice. These T-cells would then terminate further viral gene transcription, focusing the inflationary response on those few genes that are desilenced during latency. This theory is called the "silencing/desilencing and immune sensing hypothsis" and has been used to explain the size, phenotype, and epitope specificity of CMV-specific memory (Reddehase et al., 2008b). It was formally tested by a study using BALB/c mice and a virus in which the L^d restricted IE1 epitope was effectively deleted through mutation of the C-terminal MHC I anchor residue (Simon et al., 2006). Spliced IE3 transcripts were found in the lungs of mice latently infected with the IE1 mutant, but not its

revertant, indicating that without T-cell sensing of IE1 transcription, MCMV could proceed to the next checkpoint in reactivation.

This immune sensing model cannot explain the entirety of the inflationary response, however, as a number of epitopes that provoke a response in chronic infection are expressed as early genes (M38, m139, m164), well after IE gene transcription is initiated. Additionally, both M38 and m164 contain multiple CD8 T-cell epitopes (Munks and Hill, 2006). In the case of M38, one epitope is inflationary in the C57BL/6 model, and one is not. In the case of m164, CD8 inflationary T-cells in BALB/c recognize the D^d-restricted epitope, but neither of the H-2b restricted epitopes provoke an inflationary response in C57BL/6 mice.

VII. Conclusions

CMV viruses share an ancient evolutionary relationship with their hosts' immune systems. In circumventing the multipe lines of attack directed against them, they manage to maintain latency while provoking a large, functional and long-lasting effector-dominated CD8 T-cell response. Immunologists looking to exploit these viruses' ability to generate robust cellular immunity have begun to design vaccines using CMV as a vector. Many questions remain to be answered about this unique, "inflationary," T-cell response, however. Specifically, how are these responses primed and maintained? Also, what processes are responsible for the selection of only a few viral epitopes against which to mount inflationary responses? And lastly, how will these T-cells react to further antigen encounter *in vivo*? Answers to these questions are essential for the rational design of CMV-

vectored vaccines. The experiments in this disseration explore these unknowns with an eye toward future vaccine design.

Chapter 2: Competition for antigen at the level of the antigen presenting cell is a major determinant of immunodominance during memory inflation in murine cytomegalovirus infection.

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I. Abstract

Cytomegalovirus's (CMV's) unique ability to drive the expansion of virus-specific T-cell populations over the course of a lifelong, persistent infection has generated interest in the virus as a potential vaccine strategy. When designing CMV-based vaccine vectors to direct immune responses against HIV or tumor antigens, it becomes important to understand how and why certain CMV-specific populations are chosen to inflate over time. To investigate this, we designed recombinant murine cytomegaloviruses (MCMV) encoding a SIINFEKL-eGFP fusion protein under the control of endogenous immediate early promoters. When mice were infected with these viruses, T-cells specific for the SIINFEKL epitope inflated and profoundly dominated T-cells specific for non-recombinant (i.e. MCMV-derived) antigens. Moreover, when the virus encoded SIINFEKL, T-cells specific for non-recombinant antigens displayed a phenotype indicative of less frequent exposure to antigen. The immunodominance of SIINFEKL-specific T-cells could not be altered by decreasing the number of SIINFEKL-specific cells available to respond, or by increasing the number of cells specific for endogenous MCMV antigens. In contrast, coinfection with viruses expressing and lacking SIINFEKL enabled co-inflation of T-cells specific for both SIINFEKL and non-recombinant antigens. Because coinfection allows presentation of SIINFEKL and MCMV-derived antigens by different cells within the same animal, these data reveal that competition for, or availability of, antigen at the level of the antigen presenting cell determines the composition of the inflationary response to MCMV. SIINFEKL's strong affinity for H2-K^b, and its early and abundant expression, may provide this epitope's competitive advantage.

II. Introduction

Cytomegalovirus (CMV) establishes an asymptomatic latent or persistent infection, which is characterized by the lifelong accumulation of a large number of virus-specific T-cells. This process is termed "memory inflation," and has led to the exploration of CMV as a vaccine vector for HIV and for tumor antigens, with significant initial success in the SIV model (Hansen et al., 2009; Hansen et al., 2011). The fact that memory inflation occurs after infection with a single-cycle CMV (Snyder et al., 2011) indicates that CMV-based vaccines may be safely used even in immunosuppressed cancer patients, further increasing the appeal of this approach. The vaccine potential of this virus has elevated the importance of understanding how inflationary CMV-specific responses are selected and maintained during infection.

C57BL/6 mice mount a response to at least 20 viral antigens during acute infection with murine CMV (MCMV) (Munks et al., 2006b). Most of these responses, including those to the immunodominant M45 antigen, then decline precipitously and leave small central memory (T_{CM}) populations. In contrast, memory inflation is dominated by only three responses: those to M38, m139 and IE3, all of which are subdominant to M45 during acute infection (Munks et al., 2006a). These same three epitopes display memory inflation after infection with the single cycle ΔgL-MCMV (Snyder et al., 2011), which implies that non-productively infected cells harboring the viral genome can drive memory inflation.

We presume that ongoing presentation of viral epitopes must be involved in memory inflation. We have shown that memory inflation is sustained by repeated production of short-lived effectors derived from a pool of memory cells established early in infection (Snyder et al., 2008). However, the reason that inflationary responses focus on just a few antigens is not well understood.

MCMV has a highly ordered sequence of lytic cycle gene expression, which starts with the transcription of Immediate Early (IE) genes and is followed by the synthesis of Early (E) and then Late (L) gene products. However, latent MCMV infection in the lungs and liver is characterized by sporadic expression of IE genes without evidence of E or L gene expression (Kurz et al., 1999; Grzimek et al., 2001). This is thought to be abortive reactivation, in which the virus initiates the standard lytic gene cascade, but gene expression is aborted at the IE stage (Simon et al., 2006). This scenario predicts that IE gene products would be the most abundant during latent infection and thus immunodominant, which is at least partly the case: IE3 becomes progressively more immunodominant over time in B6 mice, and pp89 (IE1)-specific responses inflate somewhat more than those specific for the E antigen m164 in BALB/c mice. Furthermore, recombinant epitopes expressed behind IE promoters provoke inflationary responses (Karrer et al., 2004). However, M38 and m139, both E antigens, also provoke immunodominant inflationary responses in B6 mice, as does m164 in BALB/c mice (Munks et al., 2006a). Likewise in humans, T-cells target epitopes expressed with IE, E and L kinetics (Sylwester et al., 2005) and cells specific for the L gene product pp65 are frequently immunodominant (Boppana and Britt, 1996; McLaughlin-Taylor et al., 1994; Wills et al., 1996). The viral gene expression program that drives these diverse responses is not yet clear.

Our data suggest that viral gene expression, and not productive replication, is sufficient to promote inflation of T-cells specific for E gene products. This is evidenced by the ability of a single cycle ΔgL-MCMV to stimulate inflation of T-cells specific for the E genes M38, m139 and m164 (Snyder et al., 2011). Abortive reactivation may sometimes proceed to expression of E genes, as suggested by Simon et. al. (Simon et al., 2006). An alternate possibility is that a completely different gene expression program occurs in some infected cells. Indeed, in the rat

CMV heart transplant model, expression of a subset of E genes without production of infectious virus has been described (Streblow et al., 2007). It is interesting that this "persistent" pattern of gene expression involved very little IE gene expression. Similarly, expression of some viral genes in the absence of IE gene expression is reported in monocytes latently infected with human CMV (Goodrum et al., 2002). Hence, inflationary responses to E epitopes may be driven by different cells harboring a different program of gene expression than those that drive the IE responses.

There is also some evidence that T-cells can influence the pattern of immunodominance during memory inflation. Indeed, Holtappels et. al. (Holtappels et al., 2008) described a "conditional" immunodominant response specific for the viral m145 gene product in Balb/c mice, which appeared when the immunodominant m164- and IE1-derived epitopes were deleted. In line with this, Simon et. al. have suggested that T-cells directly limit the cascade of viral gene expression (Simon et al., 2006). Thus, immunodominant T-cell responses may restrict other epitopes from being produced. Inflationary T-cell responses of particularly high avidity, either due to expression of high affinity T-cell receptors (TCRs), or to abundant antigen expression, might enforce a selective advantage by suppressing expression of additional epitopes.

Here, we describe memory inflation in response to recombinant MCMVs that encode a SIINFEKL-GFP fusion protein under immediate early control. Not only did SIINFEKL promote memory inflation, it became the sole inflationary epitope during chronic infection. We used this model to explore the determinants of immunodominance in the inflationary T-cell response to MCMV.

III. Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. B6.SJL-CD45.1 congenic (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) mice were also purchased from The Jackson Laboratory and bred to C57BL/6 mice in house to generate CD45.1/CD45.2 F1 mice as recipients for adoptive transfer experiments. OVA-Tg mice were bred from the B6.FVB-Tg(MMTV-neu/OT-I/OT-II)CBnel Tg(Trp53R172H)8512Jmr/J strain to express the Erbb2/HER-2/neu oncogene tagged with ovalbumin epitopes recognized by the OT-I and OT-II , but not the Trp53 gene (Wall et al., 2007). Breeders of this strain were obtained from The Jackson Laboratory. Mice were between the ages of 6 and 16 weeks upon infection. All studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at Oregon Health and Sciences University.

Virus Strains and Infections

Mice were infected i.p. with 2 x 10^5 PFU of virus, except in coinfection experiments, where mice were infected with 1 x 10^5 PFU of each virus used. Virus labeled MCMV-WT BAC was of the strain MW97.01, which is derived from a bacterial artificial chromosome of the Smith strain (Borst et al., 1999). MCMV-GFP-SL8 and MCMV-GFP-MSL8 were generated on the MW97.01 backbone. In both recombinant viruses, the SIINFEKL peptide plus 7 N-terminal amino acids from ovalbumin (SGLEQLESIINFEKL, to facilitate normal peptide excision, (Cascio et al., 2001)) were fused to the C-terminal end of eGFP. In the case of MCMV-GFP-SL8, this fusion construct was targeted to replace the m128 (IE2) gene, under the control of the IE2 promoter, using established techniques (Borst et al., 2007). In the case of MCMV-GFP-MSL8, the eGFP-SL8 fusion construct was encoded with the Major Immediate Early promoter (MIEP) of HCMV

and targeted to replace exon 3 of the m128 gene in MCMV. Stocks of these viruses were produced from murine embryonic fibroblasts and titered by plaque assay on Balb3T3s without centrifugal enhancement.

To produce the ΔgL viruses, an ampicillin gene fragment was inserted into the M115 (gL) gene of the MCMV-WT BAC (strain MW97.01, (Messerle et al., 1997)) using homologous recombination. Stocks of this virus were produced on gL-3T3 cells, which provide gL in *trans* (Snyder et al., 2011), and titered by plaque assay on gL-3T3s without centrifugal enhancement. The individual virus stock used in Figure 2.3 was checked for reversion by infecting murine embryonic fibroblasts, a non complementing cell line, then passaging and monitoring these infected cells for 30 days. The growth of cells not infected by the initial inoculum confirmed the inability of this gL-deficient virus to spread from cell to cell.

Intracellular Cytokine Staining and FACS Analysis

For measurement of intracellular IFN-γ, peripheral blood was collected at the indicated time points. Red blood cells were lysed with 3 ml of lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$) and the remaining cells were incubated for 5-6 hrs at 37^oC in the presence of 10 μM of the indicated peptide and brefeldin A (GolgiPlug; BD Pharmingen). Surface staining was done overnight at 4°C, and cells were fixed and permeabilized for intracellular cytokine staining with Cytofix/Cytoperm (BD Pharmingen). The following fluorescently conjugated antibodies were used (CD8α [clone 53-6.7], CD27 [clone LG.7F9], CD3 [clone 145-2C11], CD127[clone A7R34], KLRG1 [clone 2F1], IFN-γ [clone XMG1.2]), and all purchased from either BD

Biosciences, eBioscience, or BioLegend. Samples were acquired on an LSR II or a FACSCalibur (both BD) and analyzed with FLowJo software (Tree Star).

Adoptive Transfers

Splenocytes from congenic mice infected for 7 days with MCMV-WT BAC were harvested, passed through a 70 μm cell strainer, washed twice with T-cell media (RPMI 1640 with L-glutamine + 10% FBS + 1% penicillin/streptomycin + 5 x 10^{-5} M β-mercaptoethanol) and resuspended in PBS at 5 x 10^{8} cells/ml. 100 μl of this unfractionated splenocyte suspension was injected into each congenic recipient via the retro-orbital route. These mice were infected with either MCMV-GFP-SL8 or MCMV WT-BAC the following day.

RMA-S Peptide Binding and Stabilization Assays

For binding assays, TAP-deficient RMA-S cells were plated at 1x10^{5} cells/well in 96-well plates and cultured for 16 h at 25°C in T-cell media buffered with 25 mM HEPES. The cells were then washed with T-cell media, incubated with different concentrations of the indicated peptides at 25°C for 2 hours, and then incubated for an additional 2 hours at 37°C. After this incubation, cells were washed once and stained on ice for 1 hour with PE-conjugated Y3 mAb, which binds to the class I MHC H-2K^{b}. The cells were then washed twice with PBS, fixed with BD Fix/Perm solution, and analyzed on a BD FACSCalibur.

Quantitative Real-time PCR

1 x 10^{6} murine embryonic fibroblasts were infected with WT MCMV or MCMV-GFP-SL8 at a multiplicity of infection of 10. Cells were harvested at 0, 1, 2, 3, 4, 8, 18, and 24 hours post

infection, and RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen). On-column DNAse treatment was performed as described in the Qiagen protocol. cDNA was generated using the Invitrogen SuperScript III First-Strand Synthesis SuperMix. A portion of each sample was treated similarly, but without the addition of reverse transcriptase to ensure that there was no DNA contamination. cDNA was then stored at -20°C. Quantitative PCR was performed using Platinum SYBR green qPCR SuperMix UGD with ROX, using the primers at a concentration of 250 nM. The samples were run on an ABI PRISM 7700 Sequence Detection System. Relative gene expression was determined by normalizing each gene to β-actin, and comparing the gene expression relative to cells at 0 h. The calculations were made following the method described in the User Bulletin Number 2: ABI Prism 7700 sequence detection system; subject, relative quanititation of gene expression (Applied Biosystems). Primer sequences follow:
SL8 F: ACGTAAACGGCCACAAGTTC, SL8 R: TGAACTTCAGGGTCAGCTTG,
IE3 F: GATTCAACCCGCCTGTTATG, IE3 R: GATAATTCAGGCAGCCAACC,
M38 F:TCGATATTGAGCTGCTTGA, M38 R: CCCAGCCTGCAAGACTTC,
m139 F: GCGCTCTGTGACAGAGTTT, m139 R: ACGAGCAACAACATGGAA.

IV. Results

SIINFEKL-specific CD8+ T-cells dominate memory inflation after infection with MCMV-GFP-SL8.

We generated a recombinant strain of MCMV expressing a GFP-SIINFEKL fusion construct under the control of the endogenous MCMV IE2 promoter (Turula et. al., manuscript submitted). After infection with this virus (MCMV-GFP-SL8), the SIINFEKL-specific CD8 T-cell responses in B6 mice steadily inflated over time, becoming the dominant inflationary T-cell population in these animals at chronic time points (Figures 2.1A and B). We also generated a virus in which the GFP-SIINFEKL fusion construct is under control of the HCMV Major Immediate Early promoter (MCMV-GFP-MSL8), resulting in approximately 10-20 fold greater GFP fluorescence after *in vitro* infection (not shown). SIINFEKL-specific T-cells dominated the inflationary response in mice infected with this virus as well (data not shown). Responses to IE3- and M38-derived peptides were barely detectable in these animals, whereas T-cells specific for these epitopes each comprised approximately 5% of the CD8 T-cell compartment in mice infected with WT MCMV (Figure 1A and (Munks et al., 2006a)). Notably, the size of M38- and IE3-specific T-cell populations were similarly reduced when measured as a frequency of all cells in the blood (not shown). Moreover, the proportion of CD8+ T-cells specific for M45, which are resting memory cells that do not inflate during MCMV infection, were comparable in mice infected with either virus (Figure 2.1 and not shown). These data indicate that proportional changes in T-cell numbers cannot explain the disappearance of IE3 and M38 inflation. Thus, the presence of the SIINFEKL epitope and the resulting T-cell response suppressed inflation of IE3- and M38-specific T-cells, despite evidence that M38-specific T-cells were successfully primed during acute infection.

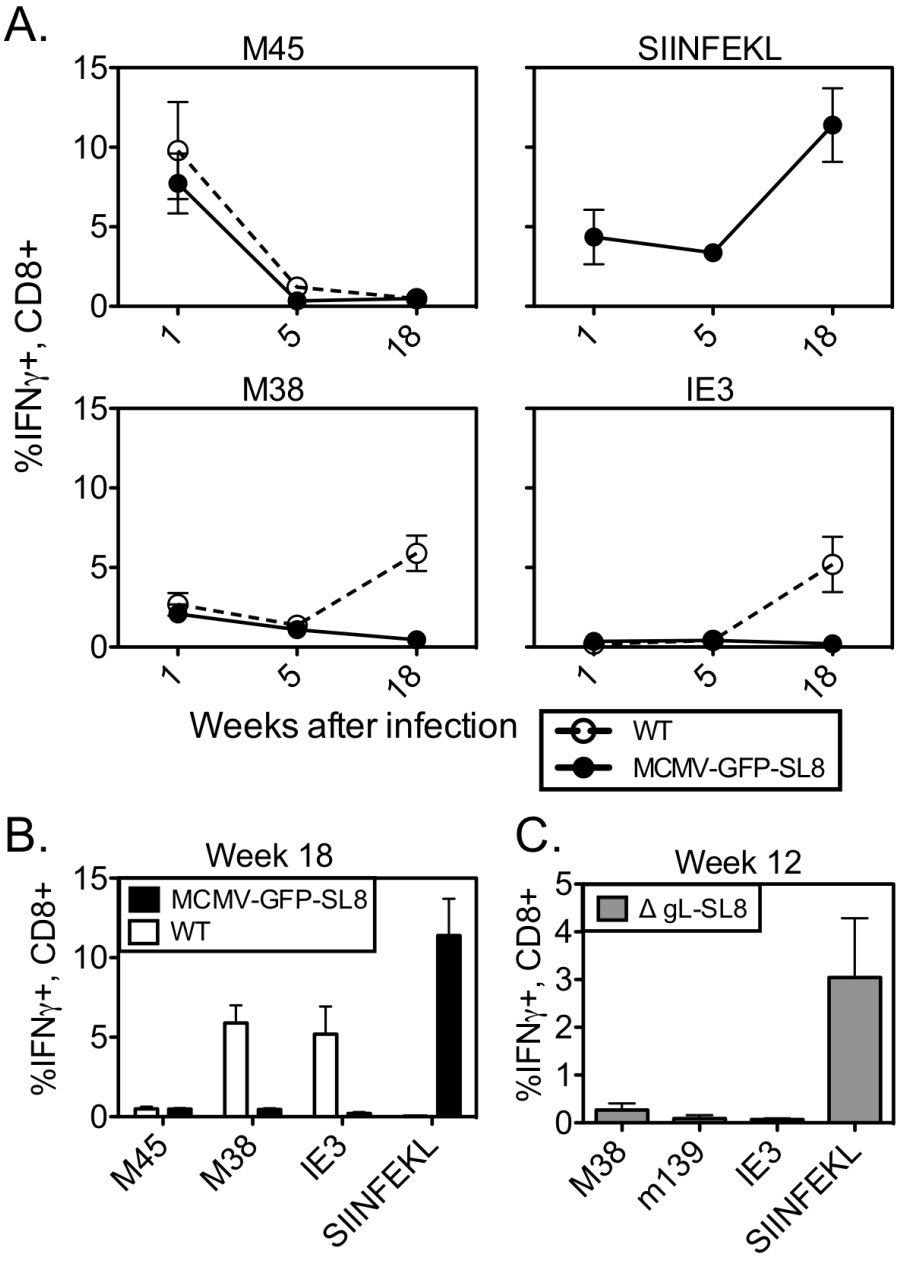

Figure 2.1: SL8 is profoundly immunodominant over normal responses to MCMV. (A) C57BL/6 mice were infected i.p with the indicated MCMV viruses. Virus-specific T-cells were measured in the blood at the indicated times post infection using intracellular cytokine staining. (B) Individual responses from the two infections in part A are contrasted at week 18 post infection. (C) Mice were infected with ΔgL-MCMV and CD8 T-cell responses to the indicated epitopes were measured at Week 12 post infection using intracellular cytokine staining. Individual plot points and bars represent 4-5 mice per group. Experiments were done twice.

SIINFEKL responses dominate memory inflation when SIINFEKL is expressed in a single cycle MCMV.

To determine whether the profound immunodominance of SIINFEKL would also occur in a single cycle MCMV, we produced a version of MCMV-SL8-GFP lacking gL, a glycoprotein necessary for cell entry and spread. The ΔgL-SL8 virus was confirmed to be spread-deficient as described in the methods, but still induced SIINFEKL-specific T-cells to inflate and become dominant (Figure 2.1C), indicating that productive infection is not needed for the immunodominance of this response.

Phenotype of cells specific for inflationary epitopes after infection with MCMV-GFP-SL8.

Inflationary CD8+ T-cells express KLRG1, and have low levels of the IL-7 receptor (CD127) and the costimulatory molecule CD27 (Snyder et al., 2008). This terminally differentiated effector phenotype is consistent with recent or repeated antigen exposure. Conversely, T-cells comprising the memory response to non-inflationary epitopes M45 and M57 exhibit a memory phenotype (KLRG1-, CD27+ and CD127+), which suggests that they are rarely exposed to antigen after the acute phase of infection. Because responses to M38 and IE3 contract sharply after acute MCMV-GFP-SL8 infection, we wondered whether they would also develop a memory phenotype.

Figure 2.2 shows that SIINFEKL-specific CD8s at week 18 post infection exhibit the classic phenotype of inflationary MCMV T-cells, with upregulated expression of KLRG1 and downregulation of CD27 and CD127. In contrast, T-cells specific for M45 mostly lacked KLRG1 and retained expression of CD127 and CD27, although some cells were KLRG1+. This is similar to their phenotype in WT infection. Strikingly, the small M38-specific population

found in MCMV-GFP-SL8 infected mice had a similar phenotype to the M45-specific cells: mosT-cells lacked KLRG1, and retained CD27 and high levels of CD127. IE3-specific cells were so infrequent that an accurate assessment of their phenotype was impossible. These results suggest that SIINFEKL-specific cells have seen antigen recently or repeatedly and that M45- and M38-specific cells encounter antigen rarely.

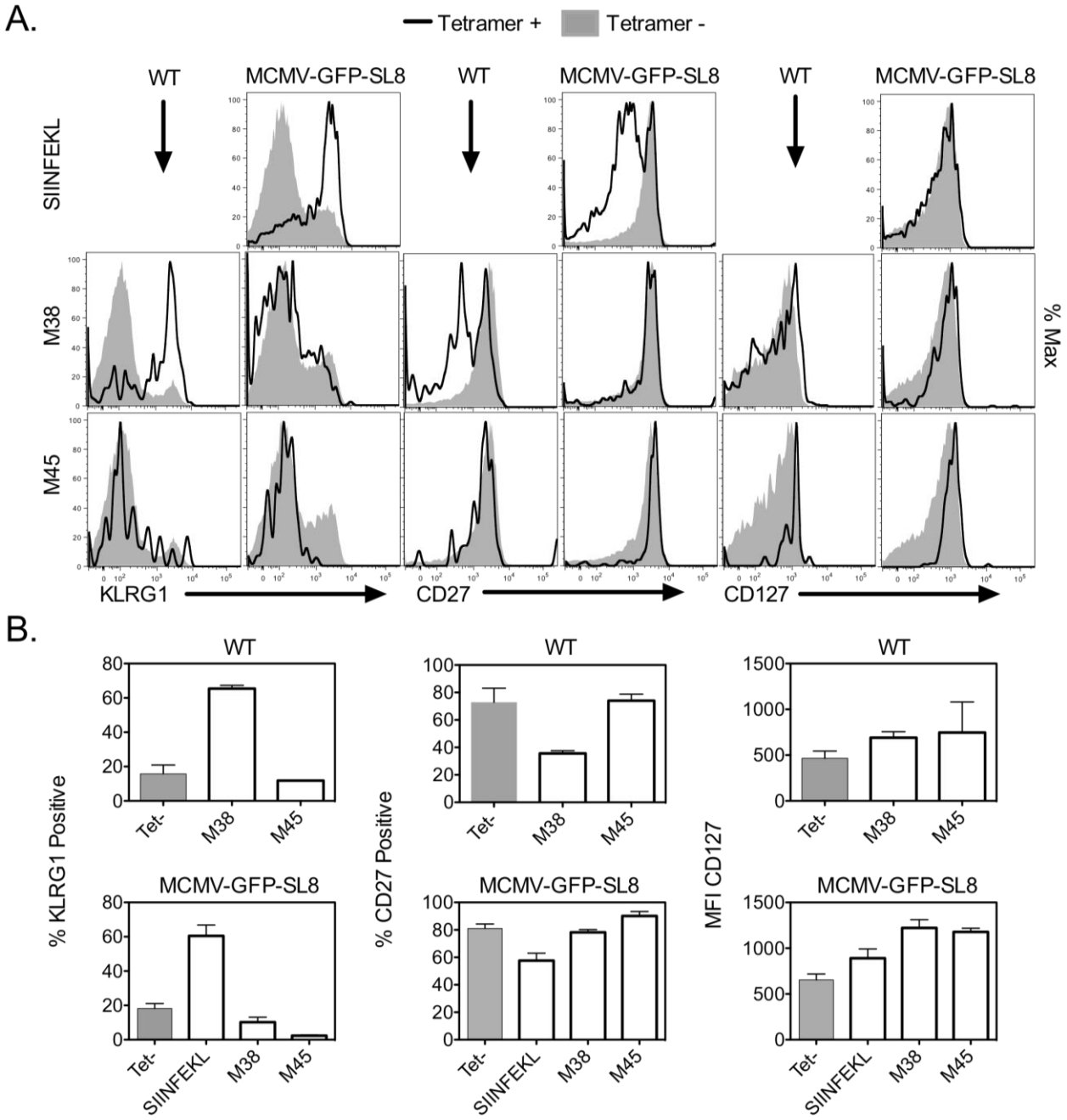

Figure 2.2: Phenotype of SIINFEKL-specific and MCMV epitope-specific responses in chronic infection. (A) Splenocytes from mice infected for greater than 18 weeks with the indicated viruses were stained with SL8 or MCMV-specific tetramers and for the indicated surface markers. The plots shown are gated on Tetramer+ CD8+ cells (black line) or Tetramer- CD8+ cells (shaded histogram). Plots represent one mouse, which is representative of two experiments with 3-4 mice per group. (B) Averages of the percent KLRG1 positive, percent CD27 positive, or CD127 mean fluorescence intensity of tetramer positive and tetramer negative populations from the splenocytes collected in part A. Individual bars represent 3-4 mice per group. Experiment was done twice.

Altering the ratios of functional, epitope-specific cells available to respond to infection does not influence the immunodominance of SIINFEKL-specific T-cells.

The precursor frequency of antigen-specific T-cells—either naïve or memory—is a major determinant of immunodominance during acute infections, and also affects proliferation and memory CD8 T-cell lineage decisions (Obar et al., 2008). We wondered whether we could modify the immunodominance of the SIINFEKL response during chronic infection by altering the ratios of functional, epitope-specific CD8 T-cells prior to infection. We explored this possibility in three ways.

First, we used mice that express OVA as a self-antigen behind the Mouse Mammary Tumor Virus promoter. When these mice were infected with MCMV-GFP-MSL8, the acute response to SIINFEKL was approximately one third of that in WT mice (Figure 2.3A), consistent with a lower number of SIINFEKL-specific precursors. Nevertheless, during chronic infection with either MCMV-GFP-MSL8 (not shown) or MCMV-GFP-SL8, the SIINFEKL response inflated at the expense of the M38 and m139 responses (Figure 2.3A).

Next, we reduced the number of naïve CD8+ T-cells capable of responding to SIINFEKL during acute infection by intravenous injection of SIINFEKL peptide prior to infection. Intravenous peptide provides a large amount of antigen (signal 1) in the absence of costimulation (signal 2), resulting in anergy or deletion of cognate T-cells (Redmond and Sherman, 2005; Srinivasan and Frauwirth, 2009; Walker and Abbas, 2002). Mice were injected i.v. with 10 μg of SIINFEKL peptide on each of the three days prior to infection. SIINFEKL-specific T-cells were not detected by ICS or tetramer staining seven days post-infection, indicating profound suppression and probable deletion of SIINFEKL-specific cells, whereas T-cells specific for MCMV epitopes were primed normally (Figure 2.3B). However, by week 12, SIINFEKL

responses had risen to the same percentage of total CD8s as those of mice left untreated, and responses to IE3 and M38 were barely detectable (Figure 2.3B).

In a third experiment, we asked whether increasing the number of T-cells available to respond to IE3 and M38 would enable those responses to inflate after infection with the SIINFEKL-expressing virus. Splenocytes from CD45.2+ donor mice that had been infected with WT MCMV seven days previously were adoptively transferred into CD45.1+CD45.2+ F1 naïve recipients. These mice were then infected with MCMV-GFP-SL8. A control group received splenocytes from the same donors, but was infected with WT MCMV instead. Figure 3C shows that the SIINFEKL-specific response still dominated memory inflation at the expense of the IE3 and M38 responses. This was not because the transferred cells were unable to proliferate, as the donor cells expanded and contributed to inflation in WT-infected mice (Figure 2.3C). Thus, pre-expanding T-cells specific for MCMV epitopes were not able to override the profound immunodominance of SIINFEKL-specific CD8 T-cells in chronic infection.

Together these results suggest that the frequency of epitope-specific cells available prior to infection is not the most significant factor in determining the size of the SIINFEKL response relative to other MCMV responses during chronic infection with MCMV-GFP-SL8.

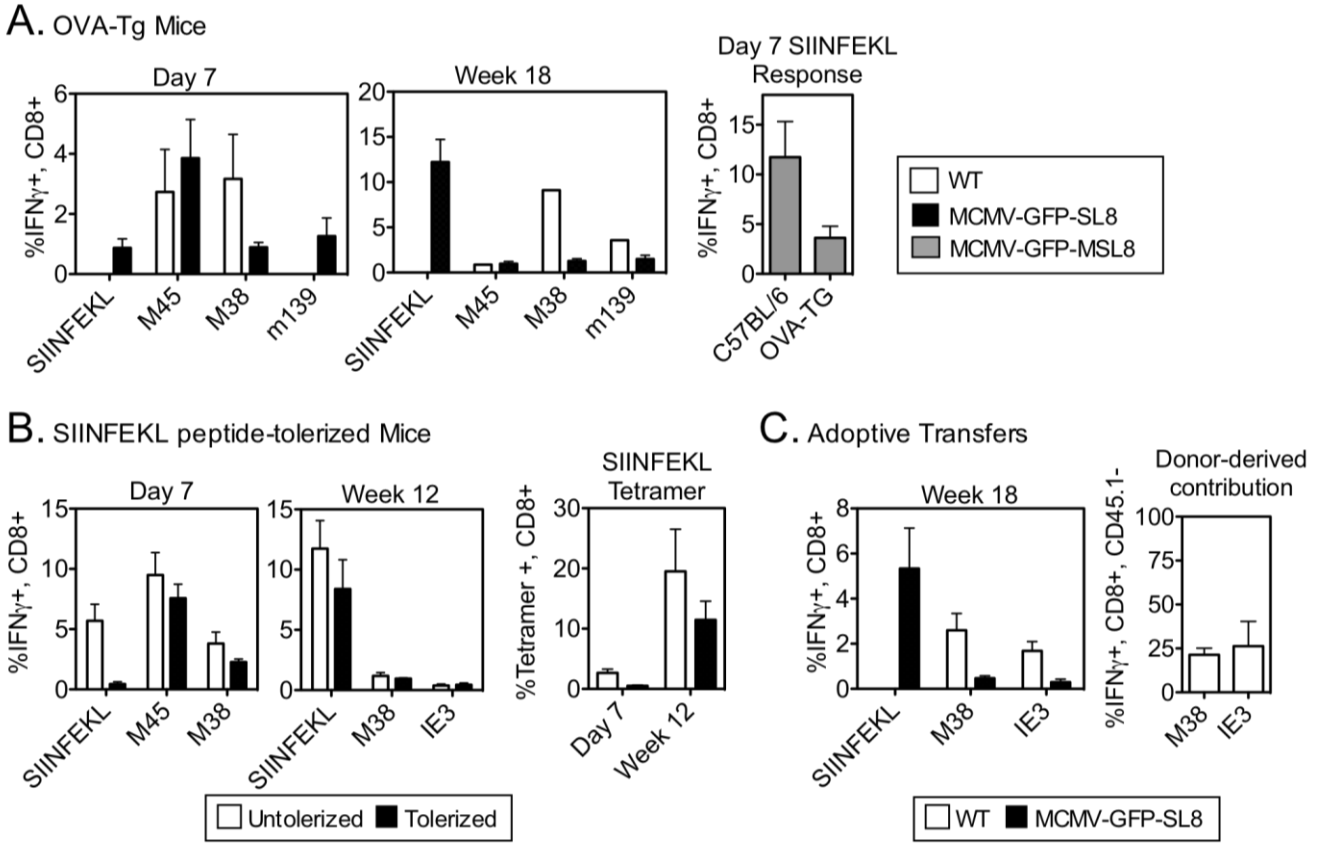

Figure 2.3: Precursor frequency does not contribute significantly to the immunodominance of SIINFEKL-specific CD8 T-cell responses in chronic infection. (A) OVA Tg mice were infected i.p. with the indicated viruses. Virus-specific CD8 T-cells were measured in the blood on day 7 and at week 18 post infection using intracellular cytokine staining. (B) C57BL/6 mice were injected with 10μg SIINFEKL peptide i.v. on days -3,-2 and -1 prior to infection with MCMV-GFP-SL8. Responses were measured in the blood on day 7 and at week 18 post infection (C) CD45.2+, CD45.1+ naive recipients received 2-5x10^7 unfractionated splenocytes from mice infected for 7 days with WT MCMV. Recipients were infected with WT MCMV or with MCMV-GFP-SL8 and virus-specific responses were measured in the blood at week 18 post infection. Total CD8 T-cell responses are shown on the left and percentages of CD45.2-negative donor cells contributing to either IE3 or M38 responses are shown on the right. Bars represent 4-5 mice per group. Experiments were done twice.

Competition for antigen shapes immunodominance during chronic MCMV infection.

Because precursor frequency did not explain SIINFEKL's dominance during chronic infection, we asked whether the phenomenon was the result of competition between T-cells at the level of the APC. This phenomenon has been termed immunodomination (Kedl et al., 2000). To test this, we co-infected mice with both WT MCMV and MCMV-GFP-SL8. Previous work has shown that coinfection with two viruses yields distinct foci of infection with each individual virus (Holtappels et al., 2004). Thus, in our experiments, WT MCMV and MCMV-GFP-SL8 should largely infect different cells within the same host and their epitopes should be presented to T-cells by different APCs. This eliminates competition between T-cells of different specificities at the level of the APC.

In mice receiving both viruses, responses to SIINFEKL and to the MCMV epitopes IE3 and M38 were co-dominant during chronic infection (Figure 2.4A). We interpreted this to mean that T-cells specific for endogenous MCMV gene products were able to inflate when these epitopes were not presented by APCs also presenting SIINFEKL. However, a trivial explanation for this would be that a much faster replicating WT virus would result in a greater abundance of MCMV epitopes in co-infected mice. Indeed, MCMV-SL8-GFP does grow with slightly delayed kinetics in vitro (Turula et. al., manuscript submitted).

To ensure that this was not the case, we repeated these coinfection experiments with a single-cycle virus, ΔgL-MCMV, in place of WT MCMV. Despite lacking gL, this virus can still promote memory inflation during chronic infection (Figure 2.4B and (Snyder et al., 2011)). Nevertheless, in mice co-infected with MCMV-GFP-SL8 and ΔgL-MCMV, antigens from MCMV-GFP-SL8 would clearly be more abundant. Figure 2.4B shows that at 18 weeks post infection, responses to IE3, M38 and m139 were similar in co-infected mice and mice infected

with ΔgL-MCMV alone. These data indicate that the results in Figure 2.4A are not due to differing rates of viral replication. We therefore conclude that competition at the level of the antigen presenting cell influences inflation and immunodominance during MCMV infection.

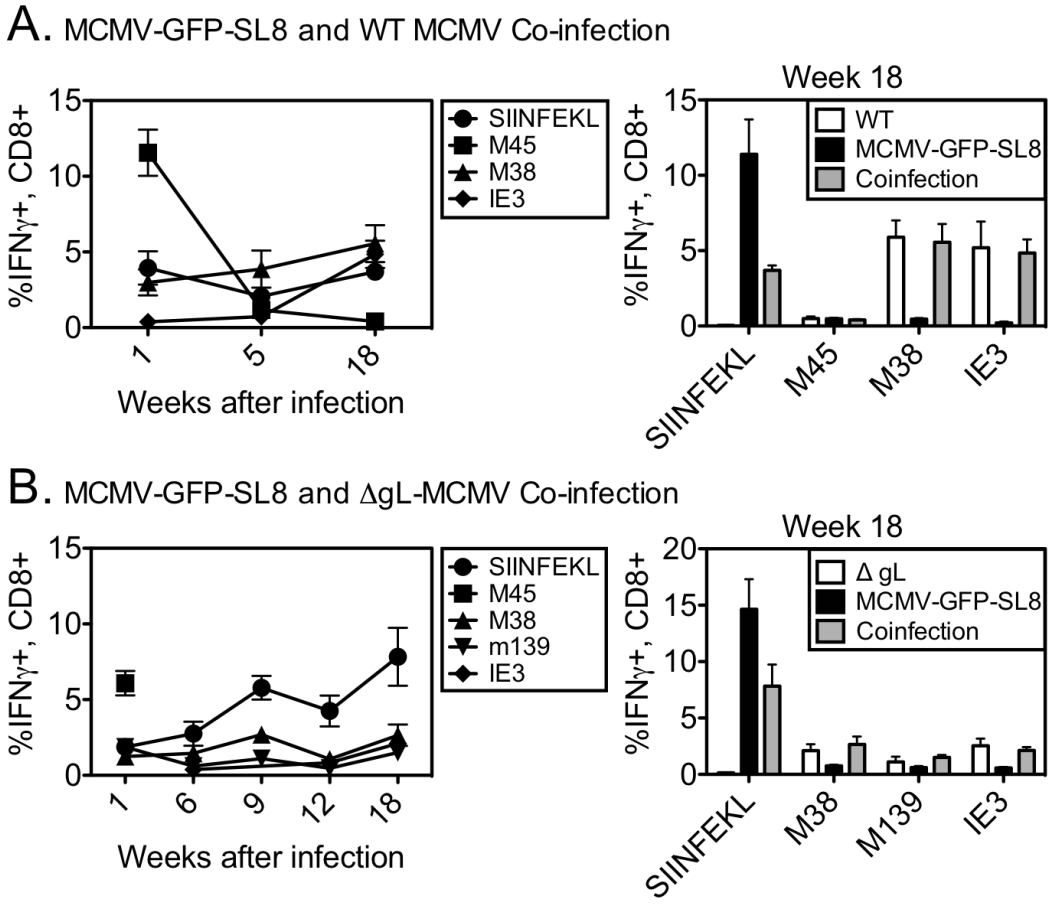

Figure 2.4: Competition for antigen shapes immunodominance during chronic MCMV infection. (A) C57BL/6 mice were infected i.p with WT MCMV and MCMV-GFP-SIINFEKL at the same time. Virus-specific T-cells were measured in the blood at the indicated times post infection using intracellular cytokine staining (B) Mice were infected i.p. with ΔgL MCMV or both ΔgL MCMV and MCMV-GFP-SIINFEKL. Virus-specific CD8 T-cell responses were measured in the blood at the indicated times post infection. The graph on the left shows the T-cell responses at the indicated weeks after coinfection. The graph on the right shows the data from all groups at week 18. Individual bars represent 4-5 mice per group. Experiments were done twice.

SIINFEKL is expressed earlier and has a higher MHC binding affinity than endogenous MCMV epitopes.

The above data established that SIINFEKL is able to out-compete endogenous MCMV epitopes to promote T-cell inflation when presented on the same APC. The mechanisms that cause the immune system to narrowly focus T-cell responses on a few immunodominant epitopes are not completely understood. That being said, some factors are obviously important: peptides that are more abundantly presented, either due to expression, processing, or binding affinity, are more likely to be the focus of these responses (Yewdell and Bennink, 1999; Yewdell, 2006).

To compare the MHC binding affinity of SIINFEKL and the MCMV-derived inflationary epitopes, all of which are presented by H-2K^b, we evaluated the ability of these peptides to stabilize K^b on the surface of the TAP-deficienT-cell line RMA-S. Figure 2.5A shows that SIINFEKL bound K^b most strongly, followed by M38 and m139, with IE3 binding with the weakest affinity. Thus, a better ability to bind K^b would favor SIINFEKL presentation.

Epitope presentation is also affected by the amount of parent protein available for degradation and presentation. Because SIINFEKL dominated memory inflation after infection with the single cycle ΔgL-SL8, we presume that T-cells harboring the latent viral genome, or their progeny, are responsible for the antigen presentation that drives memory inflation. Since the identity of these cells is unknown, it is not possible to definitively describe antigen synthesis and presentation at this site. However, as described above, sporadic expression of IE genes in the absence of detectable E or L genes has been described in latently infected lungs (Kurz et al., 1999; Simon et al., 2006). Preferential expression of IE genes is the likely explanation for the immunodominance of IE-encoded antigens during memory inflation. In MCMV-GFP-SL8, SIINFEKL is encoded behind the IE2 promoter and IE3 is driven by the Major Immediate Early

promoter. To explore the timing of expression of SIINFEKL, IE3, M38 and m139 during lytic cycle infection in vitro, we infected murine embryonic fibroblasts with WT MCMV or MCMV-GFP-SL8, harvested RNA at various time points after infection, and performed quantitative real time PCR. SIINFEKL was expressed immediately and abundantly; IE3 was also transcribed with immediate early kinetics, but probably less abundantly, and, as expected, the E genes were expressed later (Figure 2.5B). These results suggest that SIINFEKL may have a quantitative and kinetic advantage over IE3 in expression during latency.

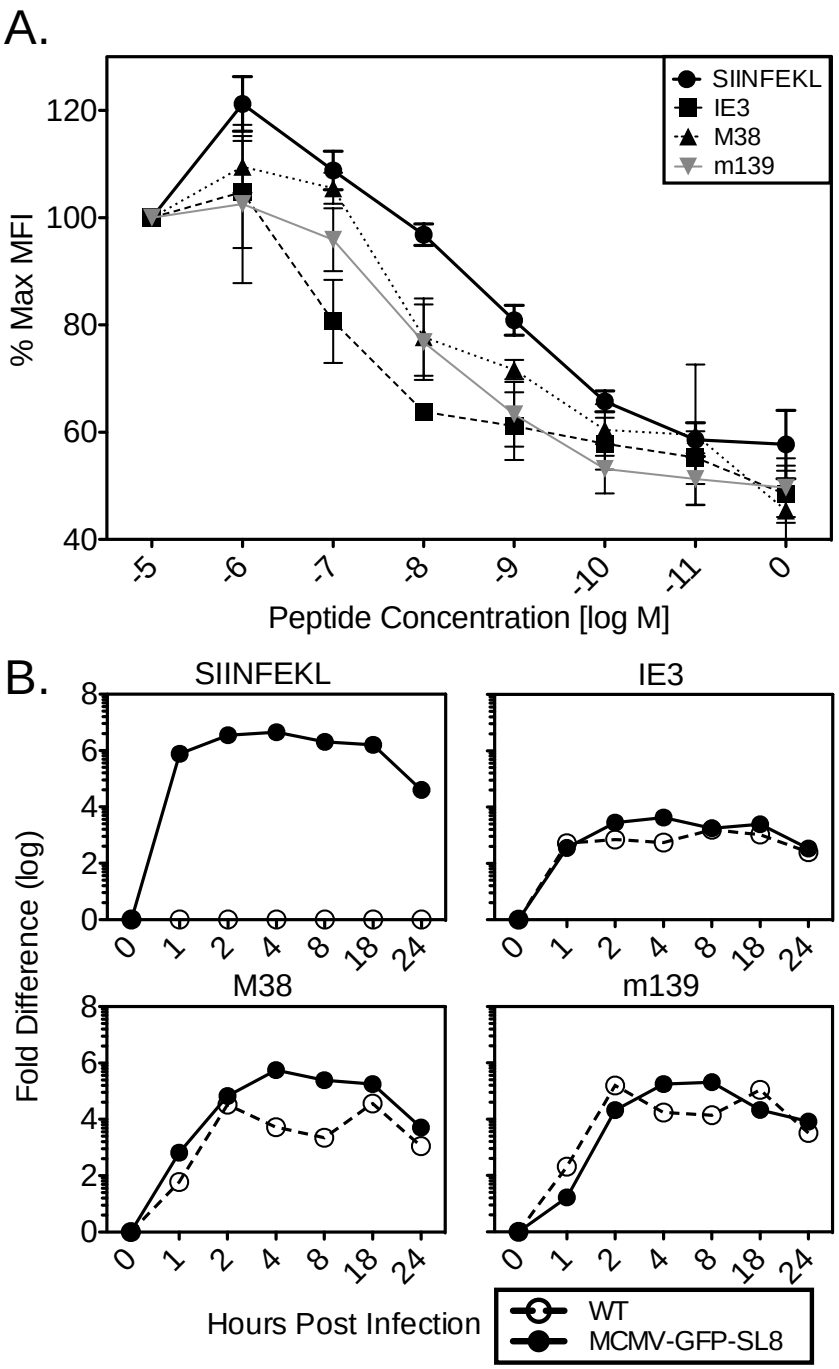

Figure 2.5: MHC binding affinity of MCMV epitopes and kinetics of expression. (A) RMA-S cells were incubated with the indicated concentrations of peptide for 2hrs at 25°C and an additional 2hrs at 37°C, then washed and stained for H2-K^b expression. Experiment was done twice. Shown is the normalized mean fluorescence intensity of class I MHC on the surface of cells. (B) Murine embryonic fibroblasts were infected with the indicated viruses and RNA was harvested at the time points listed on the y-axis. cDNA was made in parallel with no reverse-transcriptase controls for each sample, and qRT-PCR was done for the indicated gene products. No signal was obtained from the no reverse transcriptase controls. Experiment was done twice.

V. Discussion

We have shown that a GFP-SIINFEKL fusion construct, when inserted into MCMV under immediate early control, completely dominates the inflationary memory response during chronic infection with this virus. The number of SIINFEKL-specific T-cells available prior to infection was not the main determinant of immunodominance since the SIINFEKL response was still dominant in mice expressing SIINFEKL as a self-antigen or after specific peptide tolerization. Conversely, adoptive transfer to increase the number of T-cells specific for endogenous MCMV-derived peptides did not enable them to inflate in response to the SIINFEKL-expressing virus. However, when mice were co-infected with WT MCMV and our recombinant MCMV expressing SIINFEKL, inflationary responses developed to both SIINFEKL and endogenous MCMV epitopes. This indicated that when different cells in the same animal were infected with each of the individual viruses, and thus WT-infected APCs were able to present MCMV epitopes without the competing influence of SIINFEKL, T-cells recognizing these epitopes were able to inflate alongside the SIINFEKL response. Yet, when both sets of epitopes were encoded by the same virus and presumably expressed on the same APC, T-cells responding to SIINFEKL outcompeted the MCMV-specific responses. This happened either because these cells had more antigen available to them or because they were better able to access antigen. Thus, competition for—or availability of—antigen at the level of the APC plays a significant role in the selection of inflationary responses during chronic MCMV infection.

This competition may be won by the SIINFEKL response, at least in part, because patrolling SIINFEKL-specific CD8+ T-cells see antigen first and go on to terminate further gene transcription. The silencing/desilencing and immune sensing hypothesis proposed by Simon et.

al. suggests that T-cells specific for the IE1-derived epitope in Balb/c mice prevent further MCMV gene transcription. Consistent with this, only IE1 and IE2 transcripts have been found in latently-infected lung tissue from Balb/c mice (Grzimek et al., 2001). IE3 and gB were found at low levels only when the IE1 epitope was mutated such that it could no longer be presented to T-cells (Simon et al., 2006).

Indeed, work from the Cicin-Sain group shows that the context of MCMV gene expression influences whether or not an epitope generates an inflationary response (Dekhtiarenko et al.). Dekhtiarenko et. al. infected mice with one of two recombinant viruses expressing the gB epitope from HSV-1, linked to the carboxy terminus of either IE2 or M45. Inflating gB responses were seen only when expression was controlled by the IE2 promoter. When gB was linked to M45, an E gene, gB T-cell responses dominated only during acute infection. This study lends support to the idea that ordered, temporal viral gene transcription results in immune recognition of the first viral gene products and immune silencing of downstream transcription, resulting in a bias of the T-cell response toward IE antigens.

A similar scenario is likely at play in our system, where the IE2 promoter controls SIINFEKL expression. In addition, SIINFEKL may be more abundant than other MCMV epitopes as a result of higher MHC affinity and greater transcription levels. However, in both the BALB/c model and the C57BL/6 model, inflationary memory consists of responses to E-encoded antigens as well as IE-encoded antigens. This could be explained by the idea that these responses are programmed to inflate from the time of acute infection, or by the idea that E epitopes are presented by a different cell type during latency, one that is undergoing a different program of viral gene expression. However, our data argue against both of these ideas. Inflationary responses are not programmed early during infection, as MCMV-specific T-cells transferred

seven days after infection did not inflate in a host later infected with MCMV-GFP-SL8. Thus, repeated antigen exposure after priming is a necessary driver of inflationary memory. In addition, different and simultaneous gene expression programs are likely not the cause of E-gene-specific inflationary memory, as IE and E responses were equally silenced by the expression of SIINFEKL under the IE2 promoter. Thus, we favor the hypothesis that competition between T-cell clones for antigen at the level of the infected APC dictates the selection of epitopes that drive memory inflation. This hypothesis implies that, after WT MCMV infection, IE1-specific T-cells (in Balb/c mice) and IE3-specific T-cells (in B6 mice) fail to completely silence MCMV E-gene expression.

When considering the use of MCMV and eventually HCMV as a vaccine vector, these results emphasize the importance of gene expression kinetics and epitope availability in determining the size of inflationary memory responses to individual antigens.

Acknowledgements:

The authors thank Dr. Stephen C. Jameson for supplying SIINFEKL-specific tetramer.

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Chapter 3: The effect of exogenous soluble peptide epitopes on the CD8 T-cell response to murine cytomegalovirus infection

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I. Introduction

Most autoreactive CD8 T-cells are deleted in the thymus via negative selection. A subset of these cells escape to the periphery, where they are most often deleted or rendered anergic by seeing their cognate antigen without costimulation or in the context of inhibitory signals. This process results in peripheral tolerance. Once naïve cells are activated to become effector or memory T-cells, they are thought to be resistant to tolerizing stimuli and thus more easily activated. This idea is based on *in vitro* work demonstrating less stringent costimulatory requirements for effector and memory T-cells to undergo activation (Sagerstrom et al., 1993; Pihlgren et al., 1996).

More recent *in vivo* work has challenged this assumption. Using TCR Tg T-cells specific for the HA antigen from influenza, Kreuwel et. al. show that memory T-cells can be tolerized by intravenous (i.v.) administration of soluble HA peptide, as well as self-antigen expression (Kreuwel et al., 2002). In a mouse model of diabetes, high dose soluble autoantigens were shown to selectively induce apoptosis in autoreactive CD8 effector and memory T-cells (Amrani et al., 2000; Bercovici et al., 2000). Other work, however, shows that shock, hypothermia and death can result from T-cells activated by soluble cognate peptide injection, in a dose-dependent manner. Specifically, this occurred in mice chronically infected with LCMV and Vaccinia (Liu et al., 2006). Thus, the effect of soluble antigen administered without costimulation on effector and memory CD8 T-cell subsets has yet to be fully characterized *in vivo,* and may, in fact, vary by system.

Given these inconsistencies, we were interested in how the effector and memory T-cell subsets present in chronic MCMV infection would react to soluble peptide epitope encounter. Cytomegaloviruses are unique in that they provoke an accumulation of virus-

specific T-cells during chronic infection that remain with the host for the duration of its life (Holtappels et al., 2000; Karrer et al., 2003; Munks et al., 2006a; Northfield et al., 2005). This phenomenon has been termed memory inflation, and the resulting population of T-cells is referred to as "inflationary." Because of CMV's profound immunogenicity extending into chronic infection, the virus has become an attractive potential vaccine vector for preventing infectious diseases (Hansen et al., 2009; Hansen et al., 2011; Tsuda et al., 2011) and for tumor immunotherapy (Klyushnenkova et al., 2012)(Xu et al, in press). This has led to a heightened interest in understanding the development and behavior of inflationary memory.

Inflationary T-cells in chronic MCMV infection are thought to be primarily short-lived-effectors, with a phenotype that suggests recent antigen exposure and low proliferative potential, but a robust ability to degranulate and secrete effector cytokines upon stimulation (Snyder et al., 2008; Snyder, 2011). Inflationary responses develop to only a subset of MCMV epitopes in C57BL/6 mice, however; the viral gene products of IE3, M38 and m139 all contain epitopes that provoke this response. Smaller populations of CD8 T-cells specific for these epitopes exhibit more classic central memory characteristics and are maintained alongside inflationary responses. These cells may be responsible for sustaining the short-lived-effectors. Additionally, small central memory populations specific to other, non-inflationary MCMV epitopes, including components of M45 and M57, can be found during chronic infection.

Given the interest in CMV as a vaccine vector for tumor immunotherapy—a treatment that would, by definition, induce autoimmunity—developing a means by which to modulate inflationary responses to individual CD8 T-cell epitopes could greatly increase

the benefits of this approach. With this in mind, we asked how soluble peptide-epitope exposure would affect inflationary responses, as well as smaller central-memory responses, during chronic MCMV infection. We found, for all but one epitope, that inflationary and central memory responses proliferated and remained expanded as a result of this stimulus; they were not tolerized. This was also true for an exogenous epitope inserted into a recombinant MCMV, mimicking a vaccine vector. This finding suggests a method for boosting anti-tumor T-cell populations in a vaccine setting. Additionally, it is a cautionary tale for those looking to use soluble peptide as a means to combat autoimmunity.

II. Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. Mice were between the ages of 6 and 16 weeks upon infection. All studies were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee at Oregon Health and Sciences University.

Virus Strains and Infections

Mice were infected i.p. with 2 x 10^5 PFU of virus. Virus labeled MCMV-WT BAC was of the strain MW97.01, which is derived from a bacterial artificial chromosome of the Smith strain (Borst et al., 1999). MCMV-GFP-SL8 and MCMV-GFP-MSL8 were generated on the MW97.01 backbone. In both recombinant viruses, the SIINFEKL peptide plus 7 N-terminal amino acids from ovalbumin (SGLEQLESIINFEKL, to facilitate normal peptide excision, (Cascio et al., 2001)) were fused to the C-terminal end of eGFP. In the case of MCMV-GFP-SL8, this fusion construct was targeted to replace the m128 (IE2) gene, under the control of the IE2 promoter, using established techniques (Borst et al., 2007). Stocks of these viruses were produced from murine embryonic fibroblasts and titered by plaque assay on Balb3T3s without centrifugal enhancement.

Peptide Injections

All peptides were synthesized as crude peptides (65–95% pure by HPLC) by Genemed Synthesis and confirmed by mass spectrometry. Peptides were diluted in DMSO and stored as stocks at a concentration of 2 mg/ml. Stocks were diluted to concentration of 0.1 mg/ml

in PBS and 100 μl of this solution injected retro-orbitally into anesthetized mice at the indicated time points. Peptide sequences and epitope characteristics are listed in the table below.

ORF	MHC	Residues	Sequence	Inflationary?
M45	D^b	985–993	HGIRNASFI	No
M57	K^b	816–824	SCLEFWQRV	No
M38	K^b	316–323	SSPPMFRV	Yes
IE3	K^b	416–423	RALEYKNL	Yes
SL8	K^b	n/a	SIINFEKL	Yes

Organ Processing

Lungs were perfused with 50 mL of PBS. Spleens, perfused lungs, inguinal, mesenchymal and cervical lymph nodes were collected, diced, and incubated with Collagenase D at a concentration of 1 mg/ml in T-cell media (RPMI 1640 with L-glutamine + 10% FBS + 1% penicillin/streptomycin + 5 x 10^{-5} M β-mercaptoethanol + 25 μm HEPES) for 20 minutes at 37°C, then for 20 minutes rocking at room temperature. Organ parts in solution were then passed through a 70 μm cell strainer, washed twice with T-cell media (RPMI 1640 with L-glutamine + 10% FBS + 1% penicillin/streptomycin + 5 x 10^{-5} M β-mercaptoethanol) and resuspended at 1 x 10^6 cells/ml for intracellular and surface staining.

Intracellular Cytokine Staining and FACS Analysis

For measurement of intracellular IFN-γ, peripheral blood and/or organs were collected at the indicated time points. Red blood cells were lysed with 3 ml of lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$) and the remaining cells were washed and incubated for 5-6 hrs at 37°C in the presence of 10 μM of the indicated peptide and brefeldin A (GolgiPlug; BD Pharmingen). Surface staining was done overnight at 4°C, and cells were fixed and permeabilized for intracellular cytokine staining with Cytofix/Cytoperm (BD Pharmingen). The following fluorescently conjugated antibodies were used: CD8α [clone 53-6.7], CD3 [clone 145-2C11], and IFN-γ [clone XMG1.2]. All were purchased from BD Biosciences, eBioscience, or BioLegend. Samples were acquired on an LSR II or a FACSCalibur (both BD) and analyzed with FLowJo software (Tree Star).

Tetramer Staining and Co-staining

Tetramers were synthesized by the NIH tetramer core facility (http://www.niaid.nih.gov/reposit/tetramer/overview.html), except in the case of the SIINFEKL-K^b tetramer, which was a gift from the laboratory of Dr. Steve Jameson. Tetramer staining was performed on 50 μl of whole blood or 1×10^6 splenocytes, lymphocytes or lung cells. Red blood cells were lysed with BD lysis buffer after staining (Becton Dickenson). Cells were fixed and permeabalized with FoxP3 Fix/Perm (BD Pharmingen). The following fluorescently conjugated antibodies were used: *CD8α [clone 53-6.7], CD27 [clone LG.7F9], CD127 [clone A7R34], KLRG-1 [clone 2F1], PD-1 [clone RMP1-30], Ki67[cloneB56], 2B4[eBio44F3], 41BB[1AH2], 41BBL[cloneTKS-1], CD160 [clone CNX46-3], TIM3 [8B.2C12], Lag3 [C9B7W].*

III. Results

Peptide injection during acute infection does not induce tolerance.

We asked how the traditionally tolerizing stimulus of soluble-peptide antigen without adjuvant would affect newly generated effector CD8 T-cells during the acute phase of MCMV infection. We used a recombinant MCMV expressing a GFP-SIINFEKL fusion construct under the control of the endogenous MCMV IE2 promoter, MCMV-GFP-SL8 (Turula et al., 2013). After infection with this virus, mice were injected i.v. with 10 μg of SIINFEKL peptide on three consecutive days, either on days one through three post infection (p.i.) or days four through six p.i. Our laboratory has successfully used injections of this dose and duration to delete naïve CD8 T-cells prior to infection (Farrington et al., 2013), This protocol—10ug of peptide for three days prior to infection—was performed alongside the experiments described above. Additionally, this procedure has been used by another group to achieve significant, but not complete, clonal deletion of transferred naïve clone 4 TCR Tg T-cells with injections of the HA peptide from influenza virus (Redmond et al., 2005).

Responses measured in the blood via intracellular cytokine staining (ICS) at day seven, the peak of acute infection, revealed almost no CD8 T-cells responsive to SIINFEKL peptide in any of the experimental groups. Tetramer staining confirmed the lack of SIINFEKL-specific CD8 T-cells in the blood. This effect was epitope-specific, as responses to the endogenous MCMV epitope M45 were unaffected by peptide injections (Figure 3.1B). We used tetramer staining to look for SIINFEKL-specific CD8 T-cells at day 14 p.i. and found that mice injected with peptide on days one through three p.i. now showed a SIINFEKL-specific response that had returned to levels at or above those of the untreated mice. This was also true for mice that were infected with WT-MCMV and injected with M38 peptide, indicating that this outcome of peptide

injection was not specific to our recombinant virus (Figure 3.1C). At four months p.i., the CD8 T-cell response to SIINFEKL in all groups of mice treated with peptide had stabilized near the levels of untreated mice (Figure 3.1C), indicating no long term effect on the magnitude of the epitope-specific response.

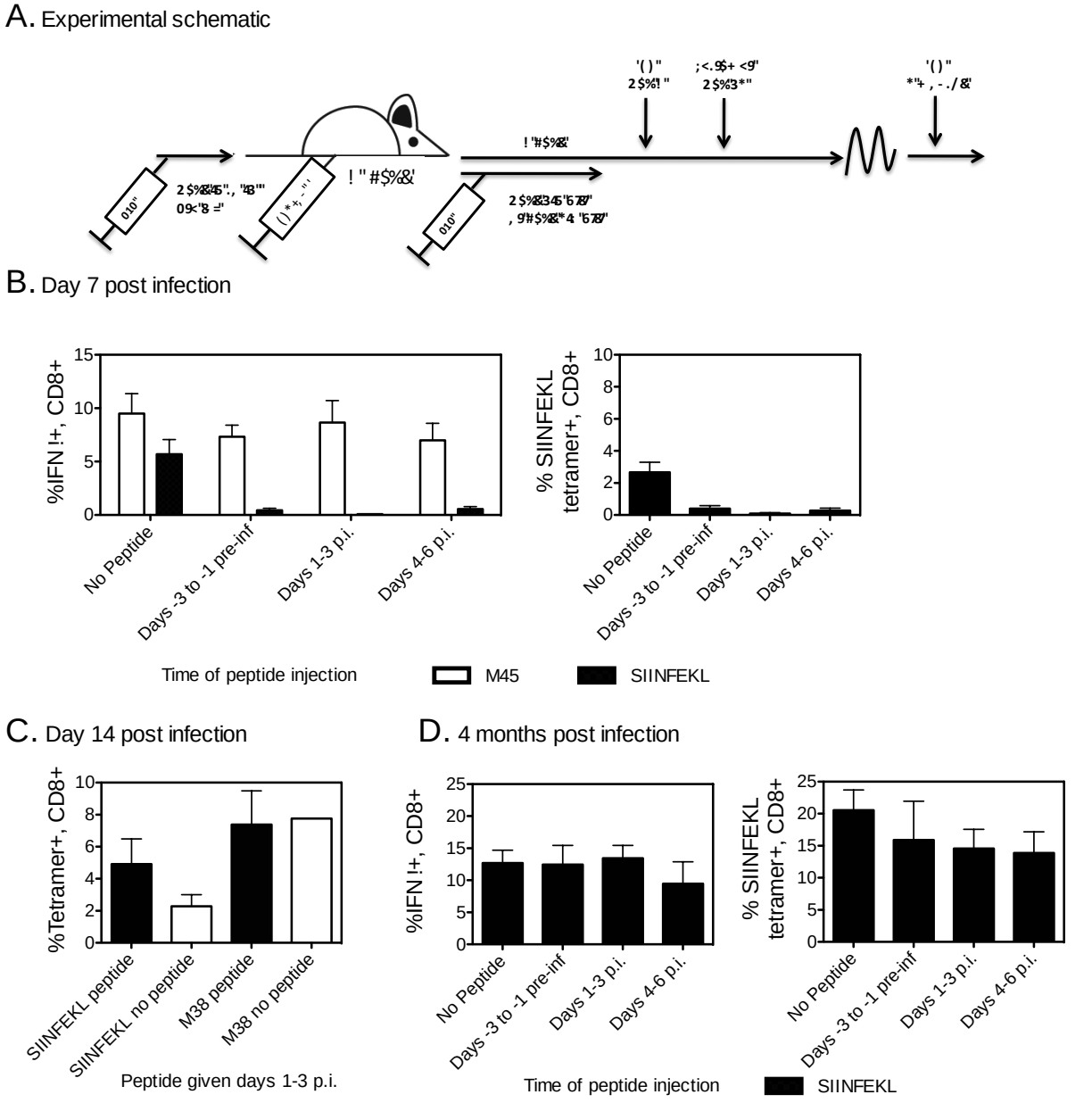


Figure 3.1: Peptide injection during acute infection does not induce tolerance. (A) Experimental schematic: mice were infected with MCMV-GFP-SL8 and injected with 10 µg of SIINFEKL peptide on the indicated days p.i. (B) ICS for CD8+ T-cells secreting IFN-γ in response to the indicated peptides and tetramer staining for SIINFEKL-specific CD8 T-cells was preformed at day 7 p.i. (C) Tetramer staining for SIINFEKL and M38-specific CD8 T-cells was preformed at day 14 post infection on mice injected with the corresponding peptides on days 1-3 p.i. (D) ICS for CD8+ T-cells secreting IFN-γ in response to the indicated peptides was preformed more than 4 months p.i. Experiments in B and D were performed twice with 4-5 mice per group. Experiment in C was performed once with 3 mice per group.

Peptide injection during chronic infection induces expansion of epitope-specific cells.

We next asked how the administration of soluble peptide antigen without adjuvant would affect the inflationary CD8 T-cell response during chronic MCMV infection. Mice were infected with MCMV-GFP-SL8 or WT MCMV. After 12-20 weeks, MCMV-GFP-SL8-infected mice were injected with 10 μg of SIINFEKL on three consecutive days. SIINFEKL-specific CD8 T-cell responses in the blood were measured by tetramer staining and ICS one day after the last day of peptide injections, and roughly every two weeks thereafter. At day one following the third peptide injection, SIINFEKL-specific responses were not detectable in the blood by either ICS or tetramer staining, thus neither functional nor anergic T-cells remained in circulation (Figure 3.2B). Two weeks after peptide injections, the response in the blood, as measured by ICS and tetramer staining, had returned five-fold, and remained at this high level 22 weeks from the initial peptide treatment.

We repeated this protocol in mice infected with WT-MCMV, using soluble versions of the endogenous viral peptide epitopes M38 and IE3. In the case of M38, we saw an effect that was very similar to that of SIINFEKL; epitope specific CD8 T-cell responses were undetectable in the blood via ICS and tetramer staining at day one following peptide injections, but they returned close to three-fold by week two after treatment (Figure 3.2B). Thus, expansion in response to i.v.-administered peptide-epitopes is not an artifact of our recombinant virus. When we injected chronically infected mice with IE3 peptide, however, we saw a different response. We were unable to detect IE3-specific CD8 T-cells at day one as before, but rather than observing a large expansion at week two, we saw a gradual repopulation of the response over the following ten weeks. Looking at other MCMV epitope-specific responses in those mice injected with peptide showed that both the expansion seen with SIINFEKL and M38 peptides, as well as

the disappearance and gradual return seen with IE3 pepetide, were epitope specific phenomena (Figure 3.2C).

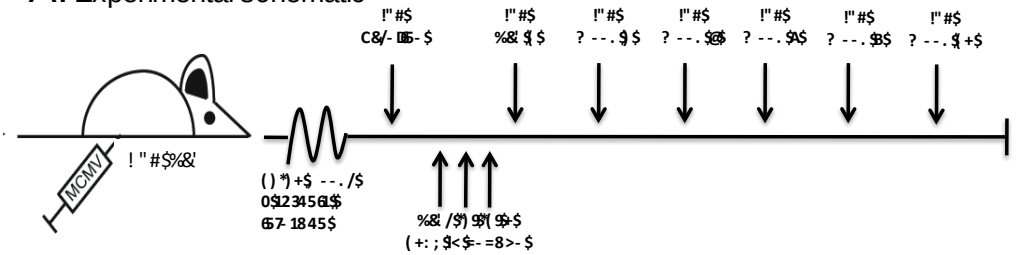
SIINFEKL, M38 and IE3 are all inflationary epitopes in that they provoke an effector-dominated response that grows after acute infection and stabilizes at a high level for the life of the host. We were interested to see whether non-inflationary, central-memory dominated responses would behave the same way in response to i.v. peptide injection. Thus, we repeated the protocol described above using soluble M45 and M57 peptide-epitopes. In mice chronically infected with WT-MCMV, we saw a small, ten-fold increase of M45-specific CD8 T-cells in the peripheral blood at two weeks after peptide injection, whereas M57 peptide treatment did little to affect the M57-specific response (Figure 3.2D). In mice infected with MCMV-GFP-SL8, we saw a very large, approximately eighty-fold increase at week two in response to M45 peptide, and a smaller but still substantial sixteen-fold increase of epitope specific T-cells two weeks after M57 injection (Figure 3.2E).

The reason for this difference in magnitude between mice infected with WT-MCMV and MCMV-GFP-SL8 is unclear. The presence of the SIINFEKL epitope has a profoundly dampening effect on the inflationary responses of IE3, M38 and m139-specific CD8 T-cells in chronic infection (Farrington et al., 2013), thus it is tempting to speculate that M45 and M57-specific cells primed in this enviroment may have an altered capacity to respond to antigen stimulation. We caution against any quick interpretation of this data, however, as the experiment was only performed once and with small experimental groups.

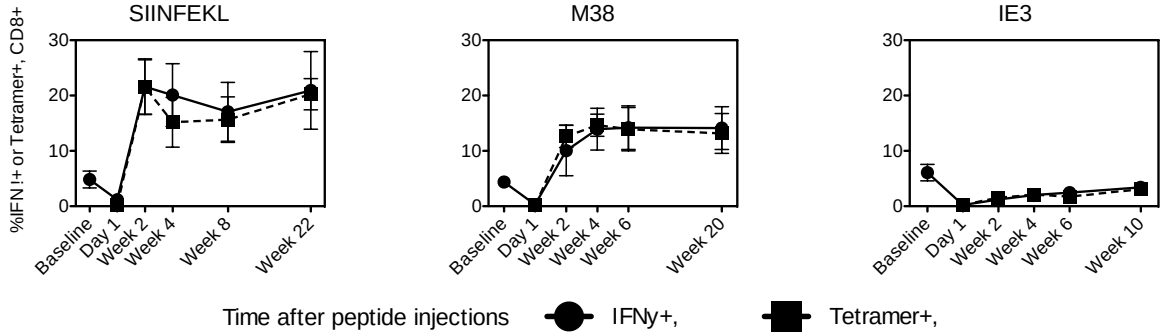
We next wondered if the expansion seen for most peptide epitopes at week two was influenced by low-levels of viral replication in chronic infection, or sustained by reactivation events caused by cytokine release from stimulated T-cells. We asked how peptide injection

might influence the inflationary responses generated by a single-cycle virus, which persist despite viral spread. In the case of ΔgL-SL8-infected mice injected with SIINFEKL peptide, a disappearance from the blood at day 1 after three days of peptide treatment was observed, followed by a return of SIINFEKL-specific cells by week two after treatment. No expansion relative to baseline levels was noted. In the case of ΔgL-infected mice injected with M38 peptide, expansion at week 2 was observed, similar to that seen with a replication competent WT virus. Given these conflicting results obtained with two different single-cycle viruses and two different peptide epitopes, we were unable to draw any conclusions about the effect of low-level virus replication on the response of inflationary CD8 T-cells to peptide encounter during chronic infection.

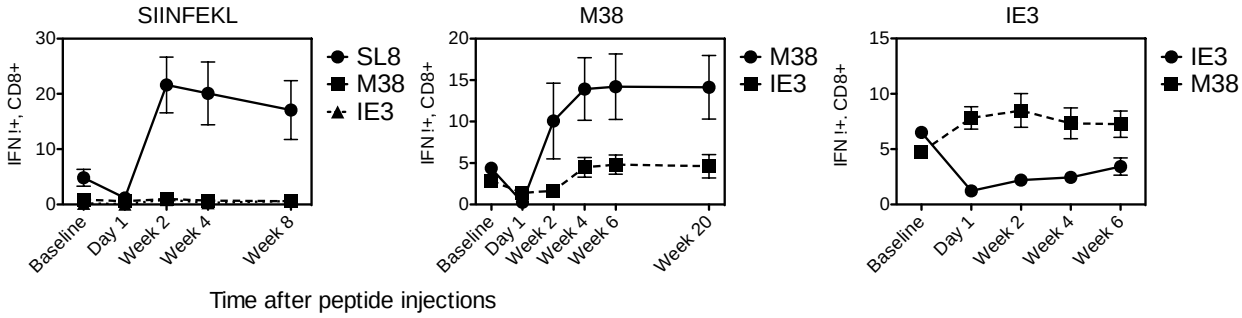
A. Experimental schematic



B. Inflationary responses

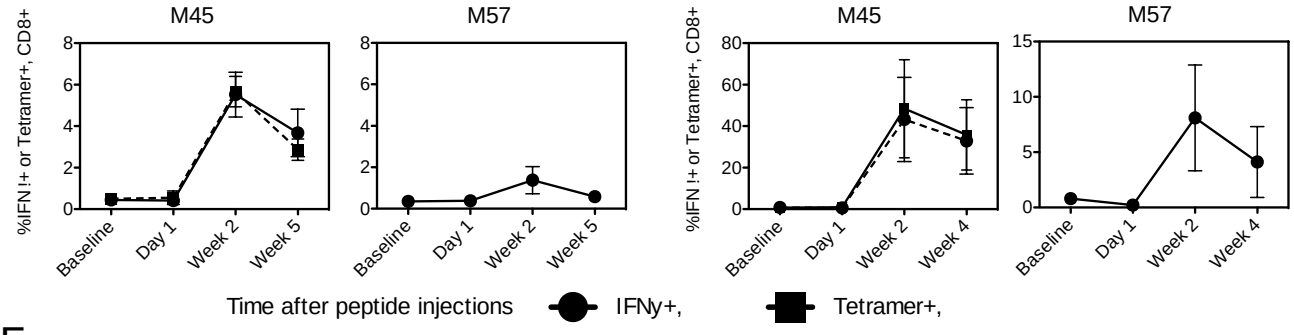


C. Epitope-specifcicity



D. Central memory in mice infected with WT-MCMV

E. Central memory in mice infected with MCMV-GFP-SL8



F. Single-Cycle Virus

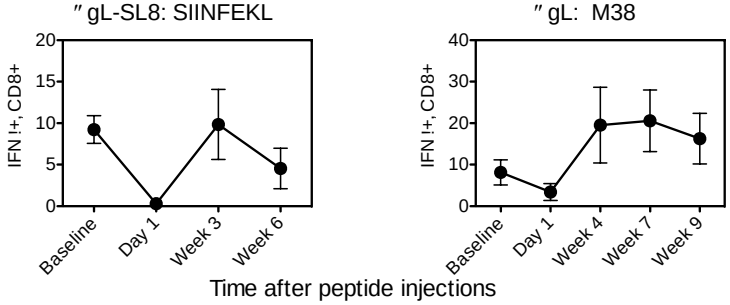


Figure 3.2: Peptide injection during chronic infection induces proliferation. (A) Experimental schematic: mice were infected with WT-MCMV, MCMV-GFP-SL8, ΔgL, or ΔgL-SL8. During chronic infection, these mice were injected with 10 μg of peptide for three consecutive days and bled for ICS and tetramer staining at the indicated times. Mice injected with SIINFEKL peptide were always infected with a SIINFEKL-expressing virus. (B-F) ICS for CD8+ T-cells secreting IFN-γ in response to the indicated peptides and tetramer staining for epitope-specific CD8 T-cells was preformed at the indicated times post infection, on mice infected with the indicated viruses. Experiments in B and C were performed twice with 4-5 mice per group. Experiments in D-F were performed once with 3 mice per group.

Peptide injection during chronic infection causes epitope-specific CD8 T-cells to migrate from the blood to the lymphoid organs.

Given the stark disappearance of epitope-specific CD8 T-cells from the blood at day one following three days of peptide injections, and the subsequent large epitope-specific expansion for all but the IE3 epitope, we presumed that these cells were sequestered in the tissues following peptide injection. Further, we hypothesized that they had traveled to the lymphoid organs to interact with antigen presenting cells. To test this, we removed the spleens, perfused lung tissue and inguinal, mesenteric and cervical lymph nodes at day one following three days of injection with either SIINFEKL peptide (in MCMV-GFP-SL8 infected mice), or IE3 (in WT-MCMV infected mice). These tissues were collagenase-digested to release T-cells from stromal interactions and stained with epitope-specific tetramers.

In the case of SIINFEKL-peptide injection, these T-cells did not completely disappear from the blood as before, but their percentages were substantially decreased from that of untreated mice. Percentages of tetramer-positive cells in the lungs were roughly equivalent between treated and untreated groups. We did, however, see an increase in tetramer-positive CD8 T-cells in the spleen and lymph nodes of treated animals, confirming our initial hypothesis of travel to the lymphoid organs (Figure 3.3B). This increase of SIINFEKL-specific cells in the lymph nodes was interesting given the almost undetectable percentage in untreated animals. After IE3-peptide injection, we saw a substantial decrease in the percentage of epitope-specific T-cells in the blood of treated animals, similar to results from Figure 3.2B. We also found very few IE3-specific cells in the lymph nodes and spleens of treated animals (Figure 3.3B), which was consistent with the lack of proliferation in response to this peptide (Figure 3.2B).

SIINFEKL-specific T-cells in the spleen have an altered phenotype after i.v. peptide injection.

Given that activation followed by deletional or anergic tolerance is the expected outcome of i.v. peptide injection (Redmond et al., 2005), yet SIINFEKL-specific T-cells continued to proliferate after a course of treatment, we wondered whether tetramer-positive cells in the spleen would express common activation or inhibitory markers after peptide encounter. Additionally, we wondered whether all SIINFEKL-specific cells were responding to peptide equivalently, or whether certain populations were disproportionately responsible for the increased response.

To get a sense of the activation state and proliferation status of these T-cells, we stained them for a series of activating and inhibitory cell-surface molecules, as well as Ki67. We found that they downregulated the inhibitory molecule CD160 and the costimulatory molecule 41BB while upregulating the costimulatory molecule CD27 and the inhibitory molecule PD-1, as compared to tetramer-positive cells from untreated animals. Additionally, they stained uniformly positive for Ki67, indicating that they were in a state of proliferation (Figure 3.3C). Thus, we were able to conclude that SIINFEKL-specific T-cells in the blood traveled to the lymphoid organs after peptide injection, where they expressed a unique combination of inhibitory and activating receptors, as well as markers of proliferation. Additionally, all cells at this time point appeared to be reacting uniformly to peptide stimulus.

Differential expression of PD-1 in response to IE3 or SIINFEKL peptide.

In order to address the difference between CD8 T-cell responses to IE3 and SIINFEKL peptides, we looked at the phenotype of tetramer positive, epitope-specific CD8 T-cells in the spleen following peptide injection. We were unable to find enough IE3-specific cells after three days of peptide injections, so we compared the populations after one day of peptide injections

and stained for the three markers that showed the most significant change in the previous experiment: CD27, PD-1 and Ki67. We found similar levels of CD27 and Ki67 expression, but IE3-specific cells did not upregulate PD-1 as uniformly as SIINFEKL-specific cells, suggesting that they had not all seen antigen at the administered dosage of peptide. This suggested to us that the IE3 epitope might be less effective than SIINFEKL at stimulating T-cells per μg of peptide injected.

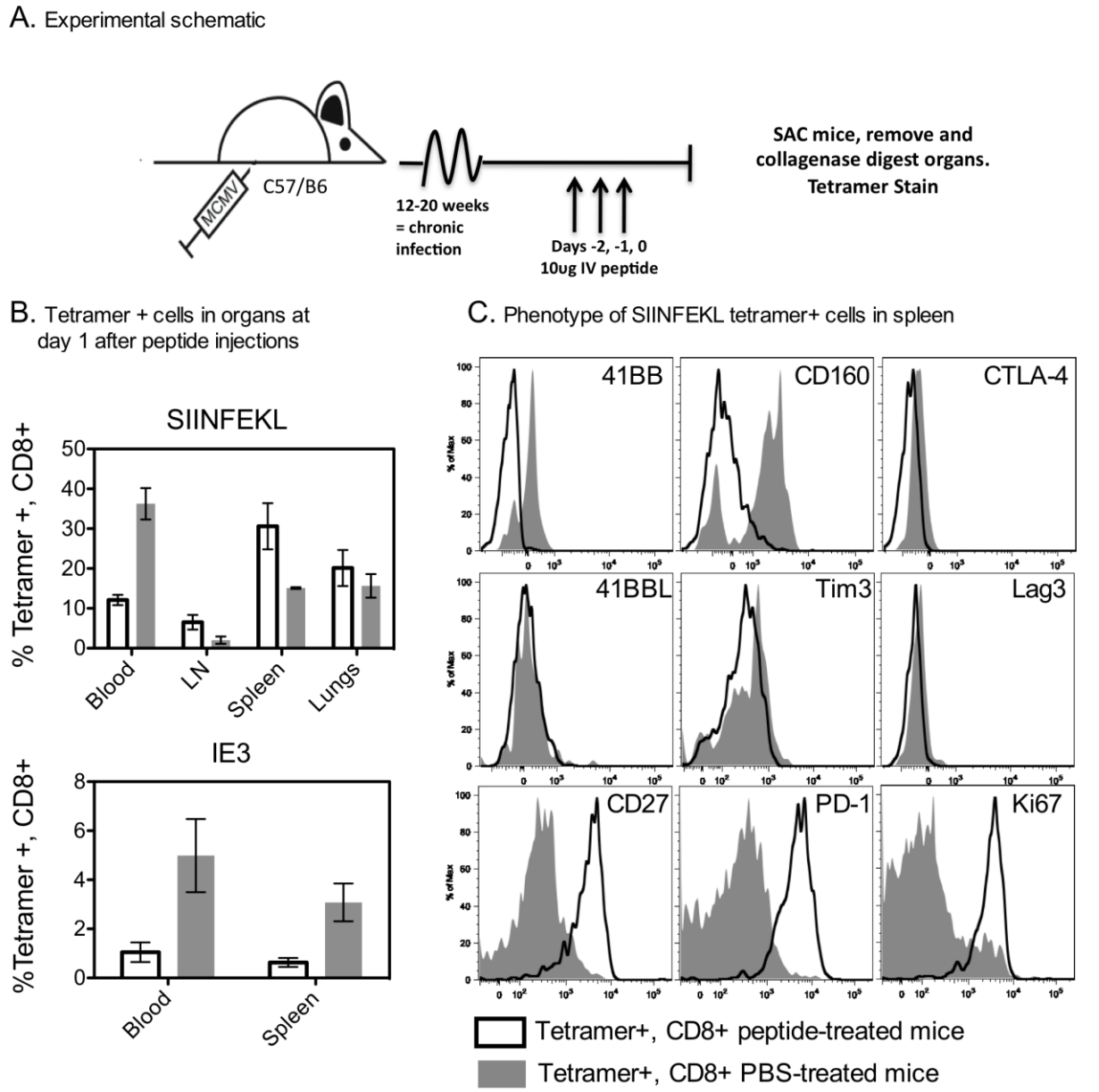


Figure 3.3: Peptide injection during chronic infection causes migration of epitope-specific CD8 T-cells from the blood to the lymphoid organs. (A) Experimental schematic: mice were infected with WT-MCMV or MCMV-GFP-SL8. During chronic infection, they were injected with 10 μg of peptide for three consecutive days. On the following day, blood, perfused lungs, spleen and mesenteric, cervical and inguinal lymph nodes were collected, collagenase digested, and stained with tetramers for epitope-specific CD8 T-cells. Mice injected with SIINFEKL peptide were always infected with MCMV-GFP-SL8. (B) Tetramer staining for epitope-specific CD8 T-cells preformed 1 day after peptide injections. (C) Phenotype of tetramer + cells 1 day after peptide injections. Experiments B and C were performed twice with 3 mice per group.

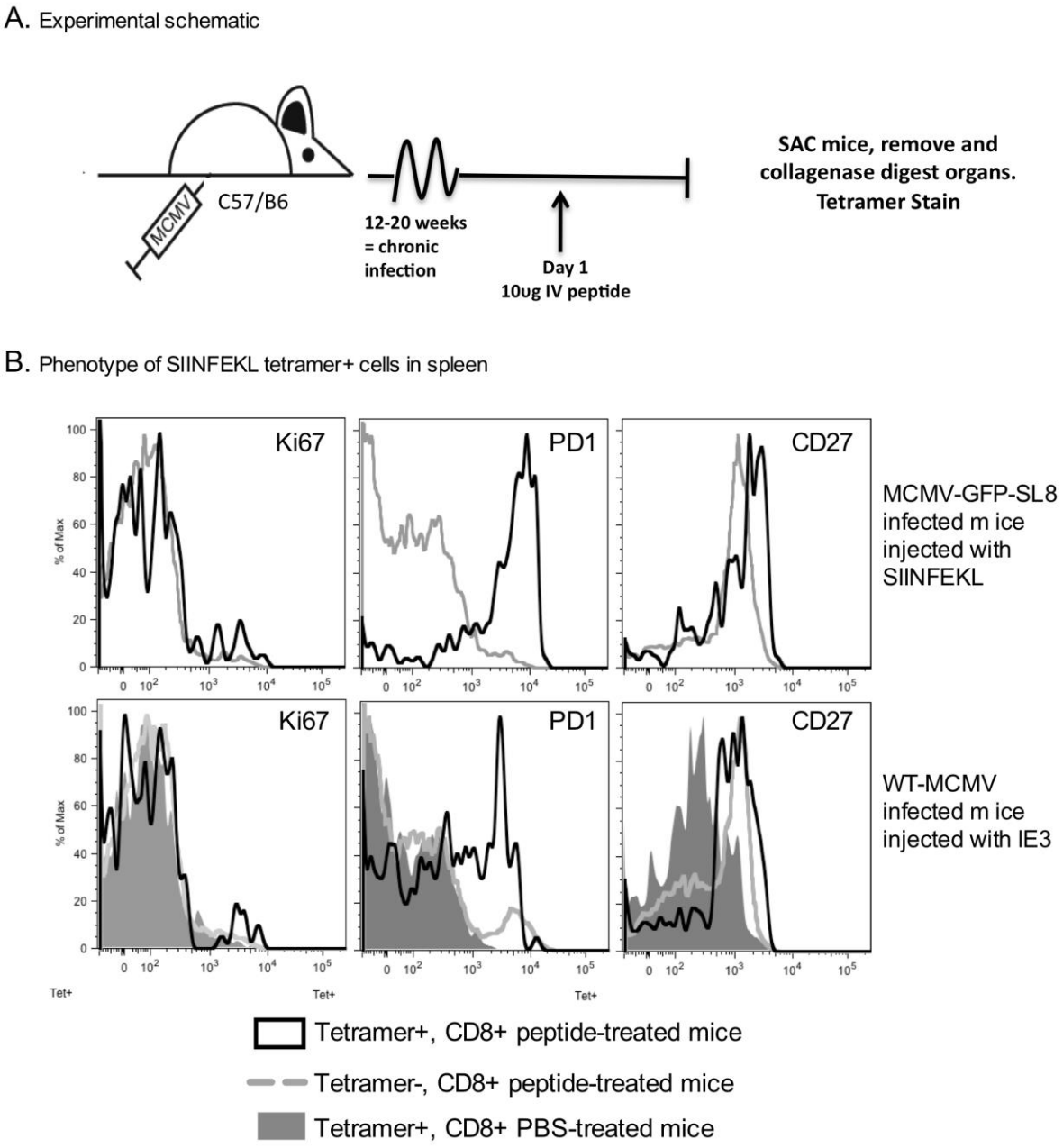


Figure 3.4: IE3 and SIINFEKL-specific CD8 T-cells in the spleen show differential expression of PD-1 after 1 day of peptide injection. Mice were infected with MCMV-GFP-SL8 or WT-MCMV. During chronic infection, they were injected with 10 μg of peptide, SIINFEKL and IE3 respectively. On the following day, spleens were harvested, collagenase digested, and stained for the indicated phenotypic markers. Experiment was performed once with 3 mice per group.

Dose and duration of peptide exposure may affect the magnitude epitope-specific T-cell proliferation.

Given that fewer IE3-specific T-cells upregulated PD-1 after peptide injection, we hypothesized that fewer of these T-cells were seeing peptide as compared to the SIINFEKL-specific T-cells, and that this difference resulted from some quality of the peptides' interaction with the animal's immune system. We know from previous work that the MHC affinity of IE3 is lower than that of SIINFEKL (Farrington et al., 2013). This, and/or differences in the TCR affinity or the half-life of the peptides in the animal may influence their respective abilities to interact with cognate T-cells. Additionally, *in vivo* work with naïve CD8 T-cells has shown that repeated low doses of antigen are better for inducing deletion than single high doses, and that high doses can in fact make naïve CD8 T-cells refractory to further tolerizing stimuli (Redmond et al., 2005).

Thus, we thought that by broadly adjusting the amount or the duration of peptide exposure, we might affect the magnitude of the proliferative response to our peptide epitopes. To test this, we injected mice with higher doses of SIINFEKL peptide (100 μg) for three days (Figure 3.5A), or higher doses of IE3 peptide (100 μg) for three days (Figure 3.5B). In both cases, the overall effect was not significantly different from the original protocol of 10 μg for three days; SIINFEKL-specific cells still proliferated while IE3-specific cells were virtually absent from the blood at week two after treatment. A slight increase in the SIINFEKL-specific proliferation at day one was observed after treatment with the higher dose of peptide, consistent with the idea that more antigen could render these T-cells refractory to deletion.

We also adjusted the duration of peptide exposure to the SIINFEKL and M38 epitopes from three days of 10 μg to six days of 10 μg. Again, no significant difference was seen from the

original protocol, although we did observe a slight decrease in the proliferation of both SIINFEKL-specific and M38-specific T-cells with six days of peptide. We concluded that greater differences might need to be applied to our protocol or that greater mouse numbers might need to be used to achieve a significant result. Ultimately, the differences that led to deletion with IE3 peptide injection and proliferation with SIINFEKL or M38 peptide injection were robust enough so as not to be influenced by a ten-fold increase in peptide amount or a two-fold increase in peptide duration.

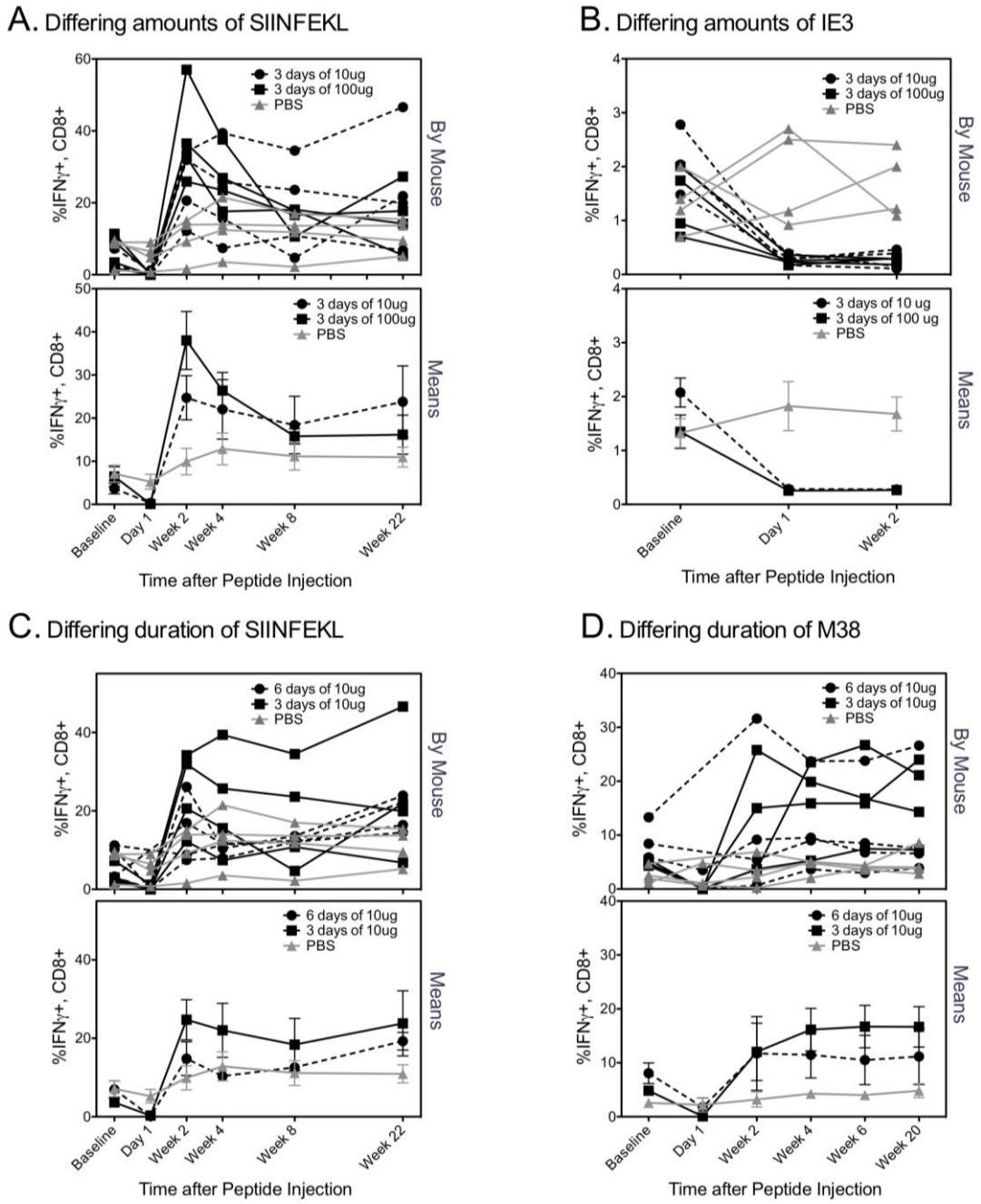


Figure 3.5: Dose and duration of peptide exposure may affect the size of the subsequent epitope-specific T-cell proliferation. Mice were infected with MCMV-GFP-SL8 or WT-MCMV. During chronic infection, they were injected with the indicated amount of peptide, for the indicated number of days. Mice injected with SIINFEKL peptide were always infected with MCMV-GFP-SL8. ICS for CD8+ T-cells secreting IFN-γ in response to the indicated peptides was preformed at the indicated times post infection. Graphs of individual mice appear above graphs showing the mean of each group. Experiments were performed once with 3-4 mice per group.

Altered peptide ligands of SIINFEKL provide inadequate data about the influence of TCR affinity on the epitope-specific response to i.v. administration of soluble peptide epitopes.

We hypothesized that the affinity of IE3 for endogenous C57BL/6 TCRs may be weaker than that of SIINFEKL, leading to less activation and proliferation of IE3-specific CD8 T-cells. In order to more specifically address the role of TCR affinity in the response of epitope-specific CD8 T-cells to soluble i.v. peptide injection, we used a pair of altered peptide ligands (APL) of SIINFEKL: SIIGFEKL (G4) and SAINFEKL (A2). Both are agonists of the OT-1 TCR, which recognizes SIINFEKL with high affinity. G4 is significantly weaker than SIINFEKL, while A2 is only slightly weaker (Carreno et al., 2007). All three peptides bind MHC I with indistinguishable affinity (Zehn et al., 2009).

We followed the protocol used in Figure 2, in which chronically infected mice were injected with 10 μg of peptide for three consecutive days, then bled for ICS on day one after peptide treatment and every two weeks thereafter. G4 treatment did not alter the course of the SIINFEKL-specific CD8 T-cell response in any significant way from that of untreated mice (Figure 3.6A). SIINFEKL-specific cells did not disappear from the blood at day one post treatment, nor did they proliferate from baseline values at two or four weeks post treatment. This indicated to us that G4's affinity for SIINFEKL-reactive TCRs was insufficient to induce either proliferation or deletion of this response. We did not, however, investigate whether G4-reactive T-cells were stimulated or deleted in these mice using a G4-specific ICS.

In mice that were injected with A2, SIINFEKL-specific responses in the blood mimicked the pattern induced by SIINFEKL injections: a disappearance of epitope-specific CD8 T-cells at day one and a huge proliferation by week two post treatment (Figure 3.6B). ICS using A2 instead

of SIINFEKL on blood from these mice indicated that most CD8 T-cells responded equally well to both peptides (Figure 3.6C).

Thus, the APLs used in these experiments were insufficient to draw any conclusions about TCR avidity, as one was of such low avidity as to be ignored by our SIINFEKL-specific T-cells, and the other of such high avidity as to be recognized by all the cells that recognized SIINFEKL.

Proliferated populations are somewhat refractory to further proliferation in response to a second round of i.v. peptide injection.

We were interested in whether the epitope-specific CD8 T-cell populations that had proliferated in response to i.v. peptide injection would react in the same or a different way to another round of peptide injection. Would they proliferate a second time or were they somehow refractory to further peptide stimulation? Mice that had been infected with MCMV-GFP-SL8 and injected during the chronic stage of infection were *re*-injected more than 22 weeks after the initial peptide treatment. At day one after this secondary peptide treatment, SIINFEKL-specific CD8 T-cells did not disappear from the blood as they had after the first peptide treatment, indicating that these cells were, indeed, resistant to further peptide stimulation (Figure 3.7B). Thus, the inflationary SIINFEKL-specific CD8 T-cell population that proliferated in response to peptide encounter was in some way permanently altered by this experience. Defining these alterations will require further investigation.

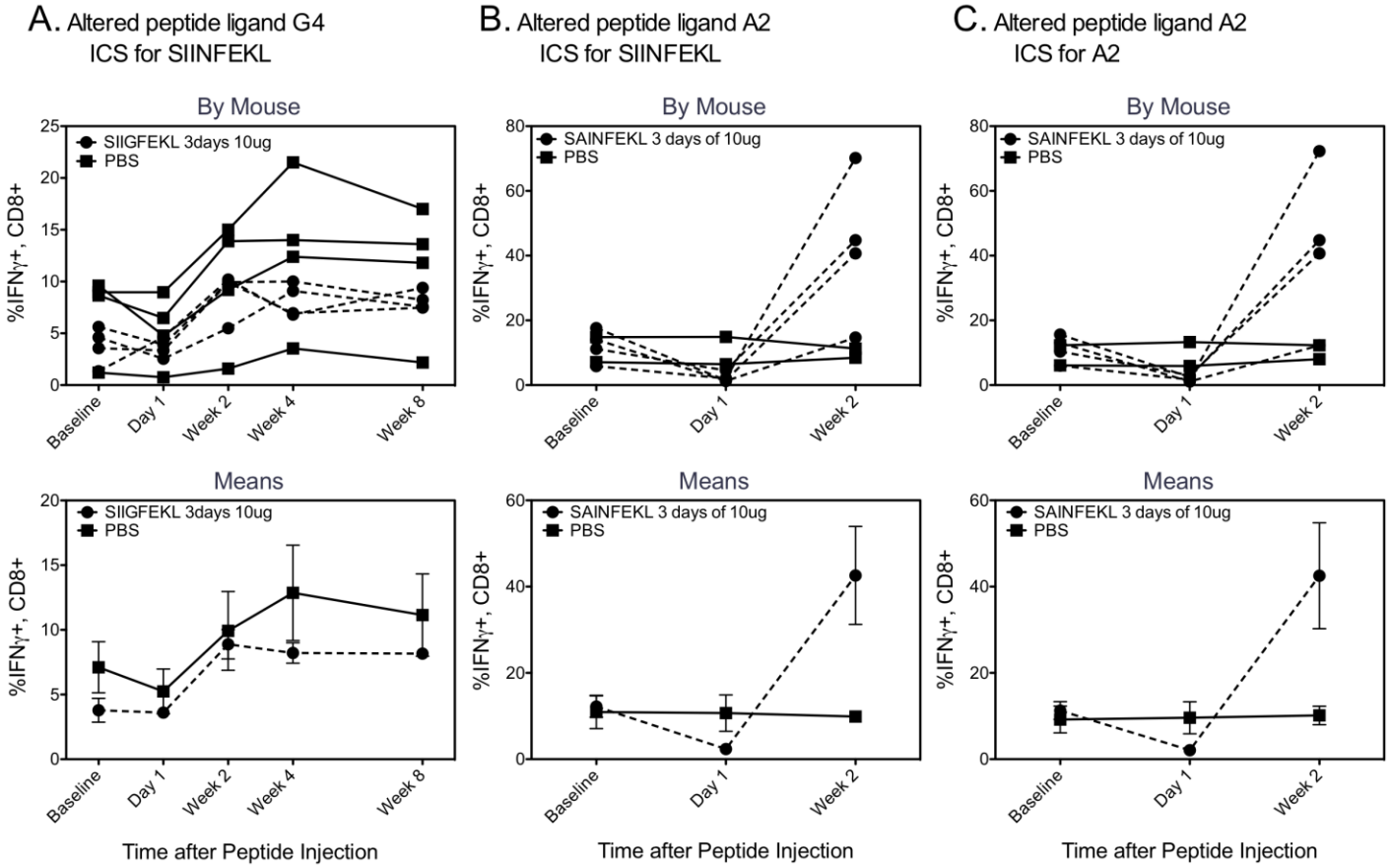


Figure 3.6: Altered peptide ligands of SIINFEKL do not tell us about the influence of TCR avidity on i.v. peptide-induced T-cell proliferation. Mice were infected with MCMV-GFP-SL8. During chronic infection, they were injected with 10 μg of the indicated altered peptide ligands of SIINFEKL for three consecutive days. ICS for CD8+ T-cells secreting IFN-γ in response to the SIINFEKL peptide was preformed at the indicated times p.i. Experiments were performed once with 2-4 mice per group.

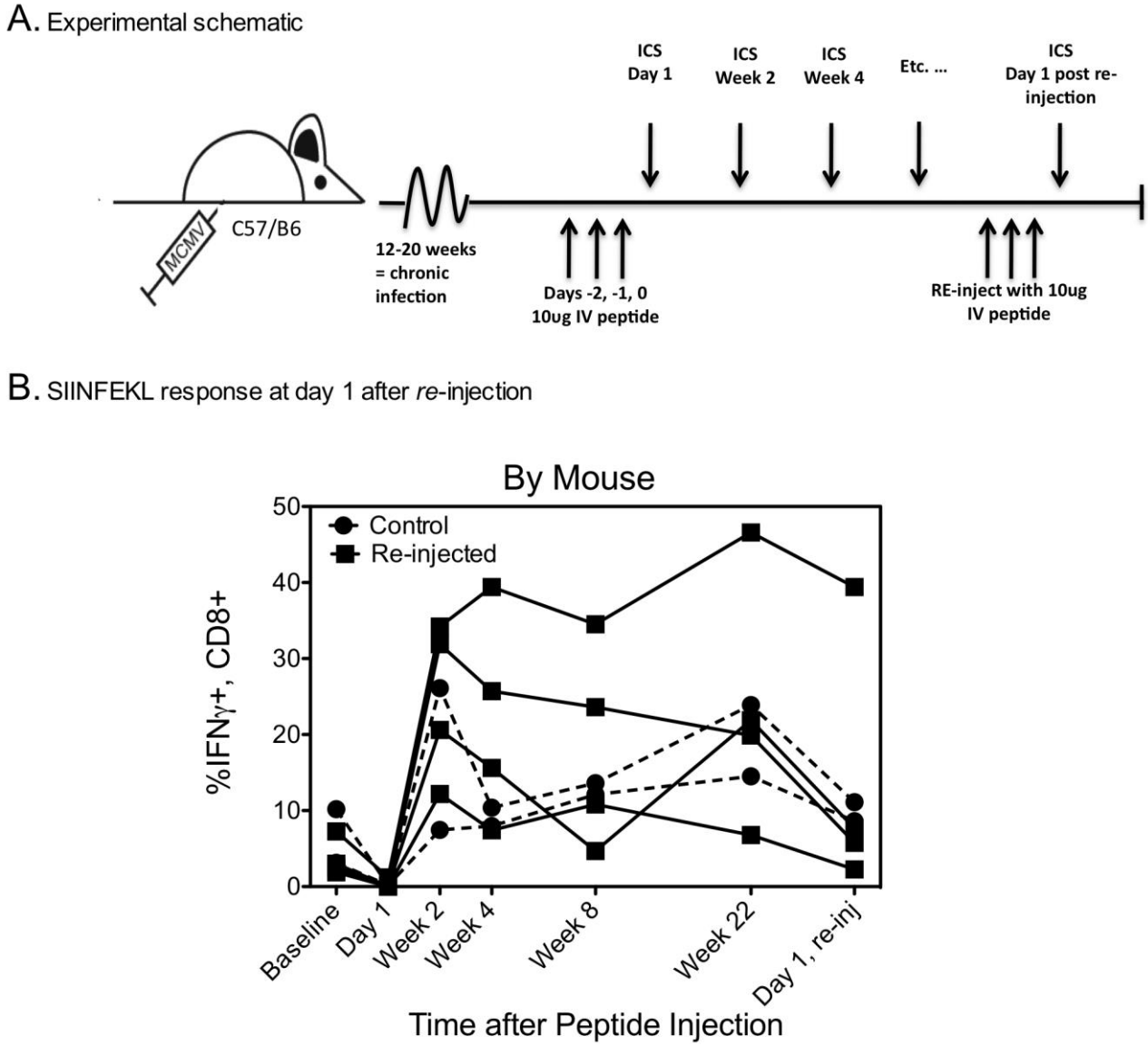


Figure 3.7: Proliferated populations are somewhat refractory to further proliferation in response to peptide-injection. Mice were infected with MCMV-GFP-SL8. During chronic infection, they were injected with 10 μg of the SIINFEKL peptide for three consecutive days and the percentage of epitope-specific CD8 T-cells was followed in the blood via ICS for 22 weeks. After this, 4 mice were *re-injected* with 10 μg of SIINFEKL for three consecutive days and the SIINFEKL-specific CD8 T-cell response in the blood was measured via ICS at day 1 following peptide re-injection. Experiment was performed once with the indicated numbers of mice per group.

IV. Discussion

A number of studies have suggested that memory and effector CD8 T-cells may be just as susceptible to tolerizing stimuli as their naïve counterparts. In particular, Kreuwel et. al. show that HA-specific TCR Tg T-cells undergo tolerance in a variety of situations, including when HA is crosspresented in the pancreatic lymph nodes and when soluble peptide is administered intravenously (Kreuwel et al., 2002). T-cells sorted for phenotypic markers of central-memory were tolerized with the same kinetics as naïve T-cells in this study. Two separate groups show soluble peptide-induced tolerance of effector CD8 T-cells, one in a diabetes model (Bercovici et al., 2000) and the other in TCR Tg mice carrying T-cells specific for an LCMV epitope (Kyburz et al., 1993). (Additional ear model paper?)

Here we have shown that the majority of effector/memory CD8 T-cell responses in chronic MCMV infection do not undergo tolerance in response to exogenous peptide epitopes. Instead, they migrate to the lymphoid organs and undergo a sizeable proliferation. These elevated responses were maintained well after the initial peptide stimulus, and this pattern was true for all epitopes tested except IE3. The majority of IE3-specific T-cells appeared to be deleted from the blood and lymphoid organs after three days of peptide stimulus. Yet, even this response eventually returned, albeit gradually. Whether this slow return was the result of new thymic emigrants or a small subset of IE3-specific memory that did not initially see peptide was not determined.

It is unclear why IE3-specific T-cells behaved differently than other populations. This variation could be attributed to the IE3 peptide's relatively reduced ability to bind MHC (Farrington et al., 2013); however, a ten-fold increase in the amount of administered peptide indicated that this was likely not the case. Our experiments with APLs of SIINFEKL were

insufficient to determine the affect of a peptide's TCR affinity on our system. This was likely due to the heterogeneous nature of the endogenous response, thus adoptive transfer of TCR Tg T-cells specific for SIINFEKL (OT-1 T-cells) could provide more conclusive information about this variable. The response to IE3 is somewhat unique among inflationary responses due to its late onset (Munks et al., 2006a), its dependence upon cross-presentation (Torti et al., 2011b), and its dependence upon CD4 help (Snyder et al., 2009). Together these observations indicate that the expansion of IE3-specific T-cells during chronic infection may be more reliant on elements of antigen presentation outside the strictly MHC I-driven direct presentation that occurs during soluble peptide injection.

It is also unclear whether the entirety of the inflationary population is proliferating in our system or whether those T-cells with central memory characteristics, which are expected to have greater proliferative potential (Snyder et al., 2008), contribute disproportionately to the response. Phenotypic analysis of SIINFEKL-specific populations in the spleen just one day after peptide injection showed a fairly homogenous population of activated, dividing cells. Adoptive transfer experiments of sorted cell populations would be necessary to determine whether central-memory and effector subsets respond similarly to peptide stimulus.

These experiments emphasize that tolerance is not the universal response of all antigen-experienced CD8 T-cell populations to exogenous peptide. Why MCMV-specific T-cells should behave differently than those of other systems is uncertain. Given MCMV's remarkable capacity to elicit large T-cell responses in chronic infection without inducing exhaustion, it is reasonable to hypothesize that some subset of the inflationary response has a very high antigen sensitivity and is programmed to expand immediately upon encounter. Experiments done in mice chronically infected with LCMV or Vaccinia, however, indicate that peptide injection is not

tolerizing in these situations either (Liu et al., 2006). Small differences in the protocol of peptide administration are unlikely to be responsible for these discrepancies, as our own experience with different amounts or duration of peptide did not significantly affect the outcome of treatment. Thus, further study is necessary to determine why the same stimulus is tolerizing in some situations and activating in others.

While these results confound attempts to use soluble peptides to eliminate autoimmune responses, either those of a dysregulated immune system or responses purposefully generated to eliminate tumors, they suggest a way in which to boost anti-tumor responses generated by a CMV-vectored vaccine. Further investigation into the qualities of these boosted responses is necessary, as our experiments indicate that inflationary T-cells that have proliferated in response to one round of peptide injection may be refractory to further peptide stimulus. How this result translates to the potential effectiveness of these T-cells in a tumor environment requires further study. In conclusion, for those interested in treating autoimmunity and in general mechanisms of tolerance, it will be important to determine why antigen-experienced populations of CD8 T-cells respond differently to the same stimulus in different models of infection and autoimmune pathology.

Chapter 4: DISCUSSION

I. Overview

The common theme throughout this work is the desire to understand the development, selection and behavior of the unique inflationary CD8 T-cell response generated during chronic CMV infection. Of immediate relevance is the application of this understanding to the design of CMV-based vaccines. This technology would harness the profound immunogenicity of the virus to direct lasting cellular immune responses against hard-to-target pathogens and tumors. Given that this inflationary response develops to a minority of CMV epitopes, discerning how and why certain responses are selected is necessary for the design of rational vaccines. Additionally, developing ways to manipulate—either by boosting or deleting—the resulting T-cell populations could further enhance the value of this technology.

The significance of this work extends beyond vaccine design, however, to add to our ever-evolving picture of CMV's interaction with the immune system during latency. As discussed in the introduction, CMVs have co-evolved with mammalian species for one hundred million years. One could view this 230 kb passenger as a bit of extra-chromosomal inheritance. As such, insight into the inflationary CD8 T-cell response is of deep relevance not only to CMV biology, but also to the understanding of our own immune systems. Although memory inflation is most often hailed as a distinctive feature of CMV infection, emerging evidence (discussed below) suggests that it may be a more common reaction to repetitive antigen stimulation than previously thought. Thus, universal tenants of immunological memory lay waiting to be uncovered in this model of chronic infection.

II. Summary of Results

- In a recombinant MCMV virus, where the highly immunogenic epitope SIINFEKL is placed in the IE2 locus behind the endogenous IE2 promoter, the CD8 T-cell response to SIINFEKL dominated inflationary memory during chronic infection with MCMV-GFP-SL8. This was also true for MCMV-GFP-MSL8, where SIINFEKL is placed behind the major immediate early promoter, and for the spread-deficient MCMV-ΔgL–SL8. (Chapter 2, Figure 2.1)

- During chronic infection with MCMV-GFP-SL8, SIINFEKL-specific CD8 T-cells exhibited an effector phenotype whereas the small M38 and m139 responses, which normally have an effector phenotype as inflationary responses in WT-MCMV infection, exhibited a central-memory phenotype. (Chapter 2, Figure 2.2)

- Diminishing functional SIINFEKL-specific T-cells by using OVA-Tg mice or deleting naïve cells prior to infection with soluble peptide injection did not alter the immunodominance of the SIINFEKL response in chronic infection. (Chapter 2, Figure 2.3)

- Increasing antigen-primed MCMV-specific T-cells prior to infection by adoptive transfer did not alter the immunodominance of the SIINFEKL response. (Chapter 2, Figure 2.3)

- Coinfection with WT and MCMV-GFP-SL8 allowed MCMV responses to inflate alongside those to SIINFEKL. This was also true for coinfection with ΔgL and MCMV-GFP-SL8. (Chapter 2, Figure 2.4)

- Coinfection with WT and MCMV-ΔgL-SL8 eliminated SIINFEKL as an inflationary epitope, responses to M38, m139 and IE3 inflated instead. (Appendix A)

- SIINFEKL may be expressed earlier and bind to MHC I better than MCMV epitopes. (Chapter 2, Figure 2.5)

- Most effector/memory MCMV-specific CD8 T-cells, including those specific for SIINFEKL, M38, M45 and M57, proliferated in response to i.v. peptide injection. Conversely, IE3-specific T-cells disappeared in response to peptide and returned very gradually. (Chapter 3, Figures 3.1&3.2)

- The proliferative response of SIINFEKL and M38-specific T-cells was not very sensitive to differing amounts or duration of peptide injection, nor was the disappearance of IE3-specific T-cells sensitive to a ten-fold higher dose of peptide. (Chapter 3, Figure 3.3)

- The boosted SIINFEKL-specific CD8 T-cell response was somewhat refractory to further proliferation in response to i.v. injected peptide. (Chapter 3, Figure 3.5)

- The memory CD8 T-cell response to a recombinant Vaccinia expressing SIINFEKL also proliferates in response to i.v. peptide. Additionally, an anergic population of epitope-specific cells was generated by this treatment. (Appendix C)

III. Implications

For understanding the selection of inflationary responses during chronic MCMV infection

The coinfection experiments from chapter two show that competition at the level of the APC—either amongst T-cells for antigen or between epitopes for antigen binding sites—dictates which responses inflate during CMV infection. When separate cells were infected with the individual viruses, the cross-competition that allowed responses to SIINFEKL to dominate responses to M38, m139 and IE3 could no longer occur. This indicated either that these MCMV epitopes were not being presented by MCMV-GFP-SL8 infected cells, or that they could not activate T-cells when SIINFEKL was also being presented.

As discussed in the introduction, the abundance of immunodominant epitopes in relation to endogenous cellular peptides is generally so low as to make competition for MHC I binding between viral epitopes within the ER unlikely in most circumstances. Additionally, cross-competition readily occurs between epitopes presented by different MHC isoforms on the same cell (Willis et al., 2006). Nevertheless, it is difficult to rule out this idea entirely, as all of the inflationary epitopes in the C57BL/6 model bind K^b. It would be interesting to monitor T-cell responses in a mouse expressing both K^b and L^d such that the same cells could present SIINFEKL and the inflationary IE1 epitope. We suspect, instead, that T-cells are not seeing M38, m139 and IE3 as often as they are seeing SIINFEKL in the single MCMV-GFP-SL8 infection. This could happen for a number of reasons. For one, there

is likely more SIINFEKL being presented due to its higher MHC affinity, although studies indicate that half-life is a greater predictor of immunodominance than strict affinity (Galea et al., 2012; Busch and Pamer, 1998; van der Burg et al., 1996). Thus, it would be interesting to compare the stability of each epitope on MHC I using an RMA-S fall-off assay. Increased TCR avidity could also play a role in activating SIINFEKL-specific T-cells faster than MCMV-specific T-cells. Any one of these more biochemical attributes could allow SIINFEKL-specific T-cells to gain greater access to antigen, costimulation and/or local stimulatory cytokines.

Additionally, viral expression kinetics influence which epitopes are seen by T-cells. We show that in an immortalized fibroblast line, SIINFEKL is expressed slightly earlier than endogenous MCMV epitopes. While it is unclear from these *in vitro* experiments whether this pattern translates to *in vivo* latent gene expression, the concept is supported by work from the Cicin-Sain group, published concomitantly with ours (Dekhtiarenko et al., 2013). The authors of this study designed two recombinant MCMVs, each containing an epitope from the structural glycoprotein B (gB) of HSV-1. In the first, gB was placed downstream and in frame with IE2. In the second, gB was placed such that it was expressed with M45. The CD8 T-cell response to gB differed depending upon which virus was used, and importantly, it matched the pattern of the response generated to the endogenous epitope. That is, when gB was expressed with IE2, gB-specific T-cells inflated during chronic infection, whereas when gB was expressed with M45, gB-specific T-cells mounted a strong acute response, but contracted and stabilized at low levels during chronic infection.

As discussed at the end of Chapter 2, the "silencing/desilencing and immune sensing hypothesis" tested by Simon et. al. provides a mechanism for the influence of viral

expression kinetics on immunodominance during chronic infection (Simon et al., 2006) . In this model, IE1 and IE2 are expressed before IE3 because IE3 results from a separate splicing event, which is controlled by a downstream transcriptional checkpoint. T-cells would see IE1 or IE2 epitopes on infected cells in BALB/C mice and shut off any ensuing viral gene expression. As we are unaware of any K^b-binding epitopes in IE1 or IE2, IE3 is presumed to be the first gene product detected by patrolling MCMV-specific T-cells in C57BL/6 mice. Alternatively, the ability of C57BL/6 mice to present an IE3 epitope may allow these T-cells to outcompete others specific for IE1 or IE2 epitopes. Whatever the case, this model cannot explain the inflation of non-IE epitopes (like m164 in BALB/C mice or M38 and m139 in C57BL/6 mice), unless a separate latent gene expression pattern exists, or these genes are de-silenced independently and out-of-order as suggested by Reddhase and colleagues (Seckert et al., 2012). Determining whether either of these latter two possibilities is occurring will necessitate identifying the cell type responsible for harboring latent virus and driving inflationary memory.

Our results obtained from coinfections with MCMV-ΔgL-SL8 and WT MCMV add yet another layer of complexity to the system. Here, when SIINFEKL was expressed on separate APCs but with presumably very low abundance due to the spread-deficient nature of the virus, responses to SIINFEKL were unable to inflate. Responses to SIINFEKL did inflate and dominate during the single infection with the MCMV-ΔgL-SL8 virus, however. Given that the replicating virus in this scenario should generate T-cells specific for MCMV epitopes, which would be able to recognize those epitopes if they are presented by cells infected with MCMV-ΔgL-SL8, SL8-specific T-cells may not be able to compete with this elevated number of primed MCMV-specific T-cells. As in the previously described co-infections, this

competition could happen on an APC level (more MCMV-specific T-cells could "crowd out" the SL8 ones), or on a systemic level via competition for cytokines. Given the results in chapter two, competition at the APC level is more likely. The important difference between this scenario and that tested in the adoptive transfer experiment, where mice were given T-cells primed by acute WT-MCMV before infection with MCMV-GFP-SL8, is that the elevated numbers of MCMV-specific cells may be continuously stimulated throughout chronic infection by cells infected with WT-MCMV. This assumes that cells infected with the SL8 virus are presenting MCMV-specific epitopes, i.e., the presence of the SL8 epitope does not prevent M38, M139 and IE3 from binding MHC I in the ER. It also assumes that ongoing antigen presentation events influence immunodominance rather than just those events that occur during acute infection. Alternatively, this elevated number of MCMV-specific T-cells generated over the course of chronic infection by the replication-competent virus might be better able to irradicate cells infected with MCMV-ΔgL-SL8 than the inflationary populations generated by the spread-deficient virus alone. This would, of course, lead to significantly less, or perhaps no, SIINFEKL presentation after acute infection.

Importantly, it is almost impossible to separate all variables influencing immunodominace; more than one is likely at play in our system. A second virus designed by my laboratory contains a D^b-binding epitope from the human melanoma antigen gp100, also in the IE2 locus of MCMV. In response to this virus, gp100-specific CD8 T-cells inflated, but they did not out-compete the MCMV-specific responses (unpublished observations). This might be due to gp100's utilization of an MHC molecule other than K^b, or because the epitope has comparatively less affinity for MHC I or TCR. Our work, as well as that of others (Dekhtiarenko et al., 2013; Karrer et al., 2004), suggests that any peptide placed in the IE2

locus will inflate, provided that it can be processed by the conventional proteasome (Hutchinson et al., 2011) and bind MHC I. The properties of the peptide itself (i.e. which MHC molecule it binds, its relative binding affinity, etc.) will determine how competitive it is with respect to endogenous MCMV epitopes.

For understanding the immune response to CMV

The data discussed in the proceeding section provide support for the idea that continued antigen presentation is a driving force of memory inflation. This has previously been suggested based on the phenotype and activation status of inflationary cells, as well as their inability to sustain themselves after adoptive transfer into naïve hosts (Snyder et al., 2008). We show that ongoing events at the level of the APC influence the inflationary response, and that these events likely include the order in which viral genes are expressed. What is unclear is whether cross-competition during the acute phase of infection is enough to determine which responses inflate or whether cross-competition needs to be sustained during chronic infection.

Our investigations into the influence of precursor frequency provide some support for the latter. Regardless of whether fewer SIINFEKL-specific T-cells were available to respond to acute infection, the response still dominated memory inflation, suggesting that a small number of SIINFEKL-specific cells were able to "catch up" during chronic infection. The adoptive transfer experiment showed, not only that a supplement of antigen-primed MCMV-specific T-cells given prior to infection had no impact on SIINFEKL's immunodominance, but also that these MCMV-specific cells likely were not "programmed" to inflate during the first seven days of infection. Given that events in the recipient animal could potentially shut off this programming, a more comprehensive test of this idea would

be to transfer cells seven days after infection into an infection-matched animal such that this hypothetical acute "programming" phase would have passed in both donor and recipient.

The conclusion that continued antigen presentation is, indeed, a necessary determinant of memory inflation, implies that our single-cycle viruses continue to present antigen well into chronic infection. The first round of cells infected by these viruses may be long-lived and very resistant to CTL-mediated lysis. Alternatively, some type of episomal replication may allow viral genomes to be maintained in daughters of the originally-infected cells. The possibility that secreted virions lacking gL are taken up by phagocytic cells also exists, though cross-presentation is an unlikely driver of memory inflation (Snyder et al., 2010; Torti et al., 2011b).

Results from Chapter 3, where peptide-epitope injection (in the absence of adjuvant) induced proliferation of MCMV-specific effector/memory CD8 T-cell populations, imply that inflationary responses—or some subset thereof—are still very responsive to at least one round of antigen stimulation. Their proliferative capacity is not stunted in this circumstance, as might be expected from their terminally-differentiated phenotype and from adoptive transfer experiments. It is unclear, however, whether short-lived-effectors are proliferating or whether just those T-cells with a central-memory phenotype are participating. Alternatively, one population of T-cells could be stimulated to make cytokines that influence the response of another population of T-cells. These possibilities could be tested by transferring CD8 T-cells sorted for their differential expression of CD27 into infection-matched recipients and following these individual populations after a series of peptide injections.

It is also unclear whether it is the unique environment of chronic MCMV infection that encourages expansion rather than deletion in reponse to i.v. peptide antigen. Chronic, low level inflamation could keep APCs in an activated state rather than a tolerogenic one. Results from the experiment in figure C.3, where mice were infected with Vaccinia, would suggest otherwise, but a more thorough exploration of this question is necessary. This could be done by transferring primed TCR transgenic T-cells into both naïve and chronically-infected mice and following their behaviour subsequent to peptide injections. Alternatively, T-cells primed in an MCMV infection could be transferred to naïve recipients and followed subsequent to peptide injections. In either scenario, if peptide injected into naïve reciepients resulted in deletion rather than expansion of T-cells, some element of the the chronic MCMV environment would be suspected as the cause of these T-cells' expansion in response to peptide.

Inflammatory cytokines produced by T-cells encountering peptide antigen could reactivate MCMV, as these factors have been shown to induce reactivation from infected monocytes in HCMV infection (Soderberg-Naucler et al., 2001) and in MCMV infection (Hummel and Abecassis, 2002). The emigration of immature monocytes from bone marrow to blood after M45 and M57 peptide injection in figure C.1, as well as after SL8 injection when OT-1 TCR Tg T-cells are present in figure C.2, indicates that these cytokines are being produced in response to peptide in some circumstances. Further analysis of the serum cytokine levels in mice injected with peptide would be useful in determing exactly what these cells are doing and how their behavior compares to previous reports showing cytokine release in response to peptide (Liu et al., 2006). Additionally, virus titers could be

calculated via plaque assay or PCR after peptide injection to deduce whether reactivation events might be further stimulating T-cell populations.

This question could also be explored through the use of spread and replication-deficient MCMVs, as reactivation events during infection with these viruses should be unable or at least less effective at stimulating the immune response. Thus, if T-cell responses did not expand after peptide injection in these infections, reactivation events would be suspected as the cause for the expansion seen with replication-competent virus infections. This could happen either through direct stimulation of the immune response, or by the establishment of a larger latent reservoir from which to continuously stimulate T-cells over the life of the animal. Results using ΔgL in figure 3.2F were somewhat contradictory in that SIINFEKL responses did not expand beyond baseline after peptide injection, but M38 responses did. These experiments should be repeated not only with spread-deficient viruses, which may still be able to stimulate the immune system through antigen presentation or the release of virions, but with a replication-deficient virus similar to the thymidine kinase-expressing virus used by Snyder et al. (Snyder et al., 2011).

Importantly, the peptide-injection experiments indicated that the size of the inflationary response can be increased by antigen exposure during chronic infection, rather than permanently constrained by a ceiling determined during acute infection. This suggests that at the point that the inflationary response stabilizes during chronic infection, antigen is limiting, perhaps because an equilibrium has been reached between the number of latently-infected cells presenting antigen and the number of T-cells responding to that antigen.

These peptide-injection experiments also provide more evidence for the unique nature of the inflationary IE3-specific response. This was the only response tested that did not proliferate after soluble peptide injection. IE3-specific T-cells, in contrast to other inflationary MCMV responses, may depend upon cross-presentation (Torti et al., 2011b) and/or CD4 help (Snyder et al., 2009), suggesting that all inflationary responses are not maintained by the same mechanisms. This is an intriguing issue that deserves more exploration. As CD4 help is primarily mediated through CD40 ligation, anti-CD40 antibody could be added along with IE3 peptide injection to trigger CD40 and simulate CD4 help. This technique was used by Diehl et al. to convert the tolerizing effects of subcutaneous injection of human adenovirus-derived epitopes E1A and E1B to enhanced T-cell responses (Diehl et al., 1999).

For vaccine design

Given that some epitopes even within the same protein inflate while others do not, the selection of inflationary responses will likely prove to be a complex phenomenon, influenced by the kinetics of viral gene expression, antigen processing pathways, and MHC affinity relationships. Thus, great care will need to be taken when selecting epitopes from other viruses or tumor cells to place in CMV-based vectors, and equal consideration will need to be given to deciding where in the viral genome they should be placed.

From this work and that of others, we can conclude that epitopes placed in the IE1 or IE2 loci of MCMV have a reasonable chance of becoming inflationary in the mouse model, provided that they are not dependent on the immunoproteasome for processing. Translating this finding to HCMV, the IE1 locus should be a good candidate for the placement of exogenous antigens, given that most human subjects mount inflationary

responses against at least one IE1 epitope (Sylwester et al., 2005). Additionally, because we have shown that cross-competition plays a role in the selection of inflationary responses, exogenous epitopes should be chosen that have competitive affinities for any HLA types that also present inflationary HCMV epitopes. This will be especially true for cancer vaccines, where we are asking our vaccine to overpower the immune system's propensity towards self tolerance. On this front, our results in the OVA-Tg mice, where SIINFEKL is a self-antigen and yet MCMV-GFP-SL8 still provokes an inflationary response, are quite promising.

Recent work with RhCMV-based SIV vaccine vectors raises the possibility that CMVs can actively manipulate the specificity of T-cell responses directed against them (Hansen et al., 2013). Here, proteins from SIV were inserted into non-coding regions of a laboratory-passaged strain of RhCMV under the control of the exogenous EF1α promoter. Responses to this vaccine were extraordinarily broad and included unconventional, MHC II-restricted CD8 T-cells. Rh189 (the homologue of HCMV's US11) and Rh157.4-.6 (the homologues of HCMV's UL128-131) were absent in this particular strain of RhCMV. The absence of these genes was shown to be responsible for the highly unusual T-cell response. These results raise the possibility that additional, virus-intrinsic forces are responsible for the selection of inflationary epitopes during CMV infection. Further investigation is needed to determine the mechanisms through which these proteins influence CD8 T-cell priming, and whether this effect is unique to the rhesus system. If similar proteins are at work in HCMV infection, we will need to understand their influence in order to design HCMV-based vaccines to predetermined epitopes. This understanding offers the possibility of more precisely programming T-cell responses.

For immunological memory

As alluded to above, emerging work suggests that what is thought of as a unique feature of CMV infection may actually be a more stereotypical pathway of immunological memory. T-cell exhaustion, therefore, is not the only outcome of repetitive antigen stimulation. For example, memory inflation occurs during systemic HSV-1 infection in mice, in response to an epitope from gB. These gB-specific T-cells accumulate months after acute infection and are maintained for the life the infected animal. Analogous to CMV-specific memory, they retain their ability to produce IFN-γ and to divide *in vivo* (Lang et al., 2009; Lang and Nikolich-Zugich, 2011). Two members of the small DNA virus family *Parvoviridae*, parvovirus B19 and PARV4, provoke an expansion of effector CD8 T-cells many months after exposure in humans. These T-cells have a phenotype that is very similar to CMV-specific memory (Isa et al., 2005; Norbeck et al., 2005; Simmons et al., 2011), despite the fact that these viruses do not establish a classic latent infection.

Bolinger et. al. have very recently established an adenovirus-based model of memory inflation. In this system, a replication-deficient adenovirus containing β-galactosidase (βgal) behind the HCMV promoter provoked an inflationary response to one epitope from βgal, but not to another (Bolinger et al., 2013). This inflationary response exhibited all of the characteristics of CMV-inflation, including an effector phenotype and polyfunctionality, and was shown to be dependent on CD4 help but independent of immunoproteasome function. Importantly, low-level antigen persistence was found at very late time points, suggesting that continued antigen presentation was responsible for

inflationary memory. This model will provide an opportunity to dissect the requirements for memory inflation with a smaller, less complex virus.

It will be important to definitively determine whether repeated antigen encounter is *always* necessary for the maintenance of inflationary memory. If indeed it is, then the key question becomes: what determines whether repetitive antigen encounter leads to memory inflation or exhaustion? This may be a matter of antigen amount—that is, T-cells may only see a small amount of intermittent antigen during chronic MCMV infection, whereas more antigen exposure might drive them towards exhaustion. It will be valuable to determine whether our i.v. peptide-expanded T-cells are, indeed, refractory to further stimulation and/or show signs of exhaustion given their large amount of antigen exposure.

IV. Summary and Future Directions

In summary, we show that competition at the level of the APC determines immunodominance during MCMV infection, and we provide some evidence that ongoing antigen presentation events maintain this hierarchy beyond what is determined during acute infection. These ongoing antigen presentation events may drive memory inflation, but they result in an eventual ceiling—that is, T-cell populations do not expand forever (Munks et al., 2006a). Presumably some sort of equilibrium is reached between the number of infected cells presenting antigen and the number of memory cells that produce short-lived effectors. This apparent ceiling is broken, however, in the case of i.v. peptide epitope injection. Why this happens is unclear, but it could result from an expansion in the memory pool prompted by antigen encounter, leading to the generation of more short-lived effectors. Alternatively, inflammatory cytokines from stimulated T-cells could provoke a

fleeting reactivation of the virus, resulting in a larger population of latently infected cells capable of stimulating and maintaining a larger memory pool. Our limited understanding of the maintainence of inflationary memory makes this distinction difficult to explore, but adoptive-transfer experiments with sorted memory and effector T-cell populations, as well as infections with replication-deficient MCMV viruses would help to do this.

Some important questions remain with regard to inflationary memory; answers to these will aid the efficient and sensible design of CMV-based vaccine vectors. First, is continued antigen presentation necessary for memory inflation? What circumstances prevent CMV-specific T-cell from undergoing exhaustion? What cell type is responsible for harboring latent virus and maintaining inflationary responses? Are all inflationary responses sustained in the same way? How does CMV manipulate the priming of CD8 T-cells, beyond MHC I downregulation? This information will undoubtedly provide us with ways to improve CMV-vectored vaccines. Moreover, these investigations will broaden our understanding of immunologic memory in ways that can help us treat chronic infections.

Appendix A: Functional avidity of SIINFEKL-specific response during chronic infection unchanged by partial tolerance in acute infection

In Chapter 2 I explored the effects of tolerance toward the SIINFEKL epitope on the immunodominance of SIINFEKL-specific CD8 T-cell responses generated during chronic infection with MCMV-GFP-SL8. In both the case of self-antigen expression, where OVA-Tg mice were used, and when soluble peptide was injected into naïve C57BL/6 mice to induce deletional tolerance, a sizeable, immunodominant SIINFEKL-specific response was seen at 18 weeks p.i. The mice in both experiments were expected to have significantly fewer SIINFEKL-specific CD8 T-cells at the outset of infection due to mechanisms of either central or peripheral tolerance, yet this had little effect on the size and hierarchy of T-cell responses during chronic infection.

We hypothesized that, in both cases, low-avidity T-cell clones may have escaped tolerance-induction and expanded when SIINFEKL was presented by infected cells. To test the functional avidity of the resulting inflationary responses, we preformed an ICS with ten-fold dilutions of SIINFEKL peptide on blood from WT C57BL/6 mice, OVA-Tg mice, and peptide-injected mice. All had been infected with MCMV-GFP-SL8 for more than 18 weeks. We expected T-cells from the untolerized C57BL/6 mice to be more sensitive to smaller amounts of peptide than the other groups; however, in all cases, the minimal amount of peptide necessary to generate an IFNy-producing cells was $1x10^{-10}$ M. Thus, the sensitivity of this peptide-titration ICS was insufficient to distinguish any differences in the TCR avidity of SIINFEKL-specific responses generated in OVA-Tg, peptide-injected, and untreated WT mice.

Considering these data, it is unlikely that the incomplete tolerance we observed in OVA-Tg and peptide-treated mice resulted from the escape of low-avidity clones. We do not know if OVA-Tg mice express SIINFEKL in the thymus, thereby deleting SIINFEKL-reactive clones, or whether, instead, these T-cells are deleted in the periphery after maturation. If the latter is true, small numbers of naïve, SIINFEKL-specific cells must escape regulatory mechanisms during chronic viral infection. For mice injected with soluble SIINFEKL peptide in the absence of adjuvant, it is likely that new thymic emigrants repopulated the response in chronic infection. This is an interesting idea, as it implicates continued antigen presentation as a driving force behind memory inflation, rather than programming during acute infection. However, the possibility that small numbers of naïve SIINFEKL-specific T-cells remained in some location other than the blood after peptide injection cannot be ruled out.

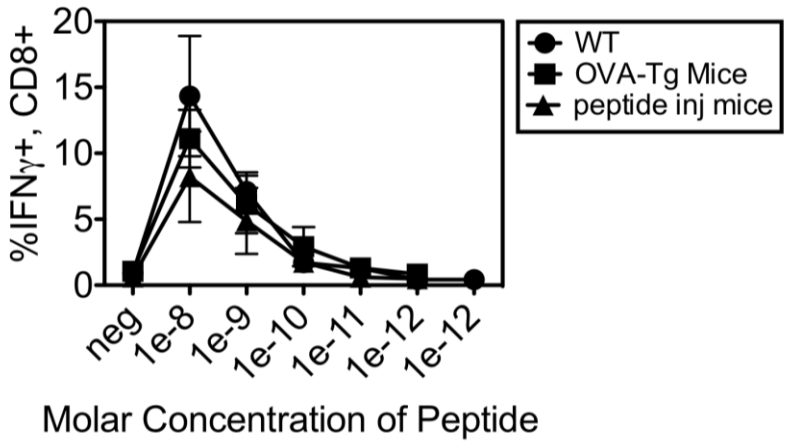


Figure A.1: No difference in TCR avidity of SIINFEKL-specific T-cells from chronically infected WT mice, OVA-Tg mice, or SIINFEKL-peptide injected mice. C57BL/6 mice or OVA-Tg mice were infected with MCMV-GFP-SL8. Half of the C57BL/6 mice were injected with 10 μg SIINFEKL peptide i.v. on days 1, 2, and 3 following infection. At week 20 post infection, an ICS was done with the indicated concentrations of SIINFEKL peptide to measure TCR avidity of the SIINFEKL-specific T-cells from each group of mice.

Appendix B: SIINFEKL is not immunodominant in chronic coinfection when expressed as a single cycle virus

In Chapter 2, Figure 2.4B, I compared the immunodominance hierarchy generated by coinfection with fully replicating versions of WT MCMV and MCMV-GFP-SL8 to coinfection with replication-deficient ΔgL and replication-competent MCMV-GFP-SL8. In both cases, CD8 T-cell responses to endogenous MCMV epitopes inflated alongside those to SIINFEKL. This indicated that even a modest amount of M38, m139 or IE3 presented by separate APCs was enough to generate an inflationary response, despite a simultaneous inflating SIINFEKL-specific response.

As a separate experiment, I also co-infected mice with a replication-deficient MCMV-ΔgL-SL8 and the replication-competent WT MCMV. Interestingly, I did not observe co-dominance of SIINFEKL and MCMV-specific responses in this situation. When the SIINFEKL-expressing virus was significantly less abundant, only a modest SIINFEKL response was generated during acute infection and this became almost undetectable at week eight p.i.. MCMV-specific responses, while dominant in chronic infection, did not exhibit the same magnitude that they did in single WT-MCMV infection. This is most likely a reflection of the fact that only half the amount of WT virus ($1x10^5$ PFU of each virus) was used in the coinfection as compared to the single infection ($2x10^5$ PFU).

These results suggest that the amount of SIINFEKL peptide expressed during acute and chronic infection contributes substantially to the immunodominance of the response. They also indicate that M38-, m139- and IE3-specific responses are participating in some type of competition beyond the level of the APC. They appear to “win out” over small

numbers of SIINFEKL-specific cells, despite the fact that SIINFEKL should be expressed by different APCs during coinfection. Whether this competition is won early on during infection or later, continuously, during chronic infection, is unclear. Our experiments in Chapter 2 showed that altering the ratios of functional, epitope-specific cells available to respond to early infection did not influence the immunodominance of SIINFEKL-specific T-cells. Thus, it is more likely that the SIINFEKL response is continuously repressed during chronic coinfection with WT-MCMV and MCMV-GFP-SL8.

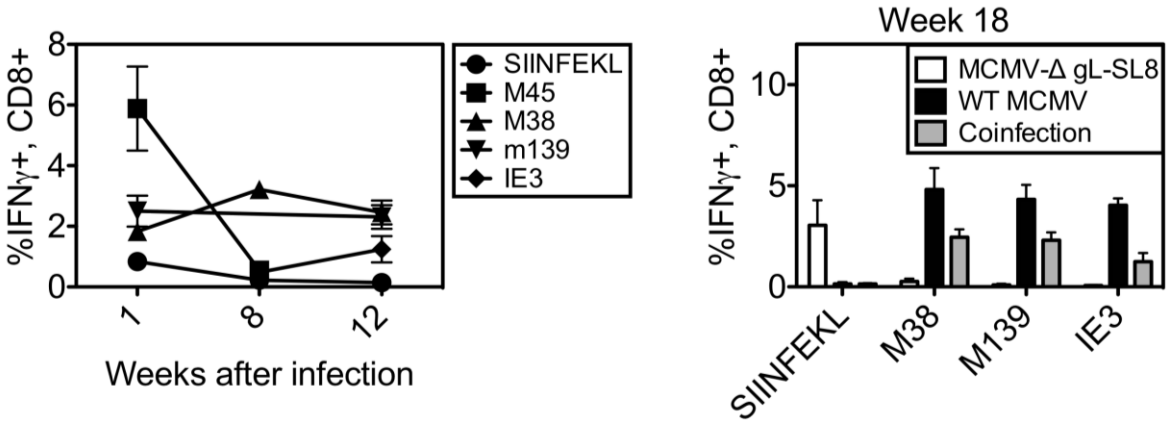


Figure B.1: SIINFEKL is not immunodominant in chronic coinfection when expressed by a single-cycle virus. C57BL/6 mice were infected i.p with the indicated MCMV viruses. Virus-specific T-cells were measured in the blood at the indicated times post infection using intracellular cytokine staining. Experiment was done once with 3-4 mice per group.

Appendix C: Soluble peptide injection induces epitope-specific proliferation in vaccinia-infected mice

In Chapter 3, Figure 3.2, I looked at how soluble peptide injection during infection affected the epitope-specific CD8 T-cell response over time. In the process of quantifying epitope-specific T-cells after these injections, I noticed an increase in cells registering high on the SSC axis (Figure C.1A) in the blood of mice taken one day after a series of injections with either M45 or M57. Expecting that T-cells with high granularity should be of the myeloid lineage, I repeated this experiment and found that this population was comprised primarily of GR-1+, CD11b+, Ly6C hi cells (Figure C.1B). By week two after peptide treatment, the proportion of these cells in the blood was no longer greater than that of untreated mice. Cells of this phenotype are known to be immature myeloid cells (IMCs) of monocytic morphology, which emigrate from the bone marrow in response to monocyte chemoattractant proteins 1 and 3 (MCP-1 and MCP-3), migrate through the blood to inflamed tissues, and then differentiate into macrophages and CD11c+ dendritic cells (Gabrilovich and Nagaraj, 2009).

This increase in IMCs at day one after peptide treatment did not occur with injections of the SL8 or the IE3 peptide (Figure C.1B). As these are inflationary epitopes, which generate a large number of short-lived-effector CD8 T-cells, and M45 and M57 provoke a smaller, central-memory dominated response, I thought that the phenotype of the responding T-cells might be responsible for the increase in IMCs. Central-memory T-cells might secerete unique cytokines or chemokines that draw IMCs from the bone marrow into the blood after antigen encounter. To test this idea more specifically, I infected

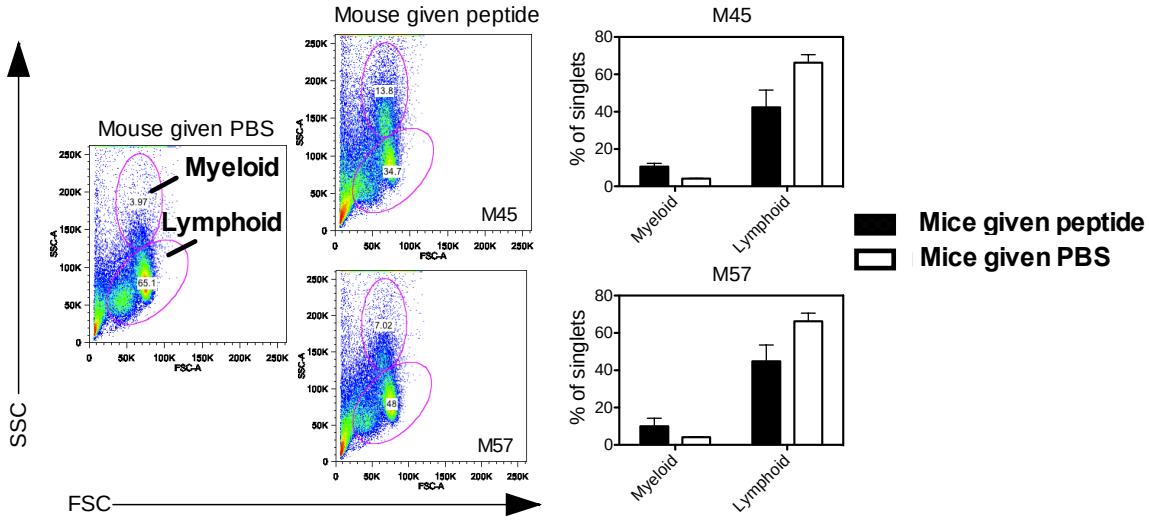
mice with a recombinant Vaccinia expressing SIINFEKL. As Vaccinia is not anticipated to generate any inflationary responses, I assumed SIINFEKL-specific T-cells in this infection would be biased toward a central-memory phenotype. Thus, I could compare the outcome of injecting the same peptide into two different infections, each of which generates a different type of T-cell memory.

Because the recombinant vaccinia used in this experiment provokes a very poor SIINFEKL-specific response (unpublished observations), I boosted the response by transferring whole blood from OT-1 TCR Tg mice into C57BL/6 recipients. These mice were infected the next day with either Vaccinia-SL8 or MCMV-GFP-SL8. Both groups of mice received OT-1 blood prior to infection. Five weeks later, they were injected with 10 μg of SIINFEKL peptide i.v. on three consecutive days. Blood was taken at day one after three days of peptide treatment and stained for IMC markers. In both infected populations, I noted a very significant (greater than ten-fold) increase in GR-1+, CD11c+ cells in comparison to mice that received no peptide (Figure C.2C). Thus, this experiment did not validate the correlation between central-memory and IMC mobilization. Something about the nature of transferred OT-1 Tg T-cells and their response to i.v. peptide may be responsible for this profound increase in GR-1, CD11b+ cells in the blood. I did not, however, confirm that SIINFEKL-specific T-cells in Vaccinia-infected mice were truly central memory. More careful phenotyping of the transferred OT-1 T-cells would be helpful in confirming or negating the idea that T-cell phenotype has a differential affect on IMC mobilization. Additionally, I did not see a disappearance of SIINFEKL-specific CD8 T-cells from the blood at day 1 after peptide injections in either virus infection, as I had in similar experiments described in figure 3.2B. This is also, most likely, the result of the OT-1 TCR Tg

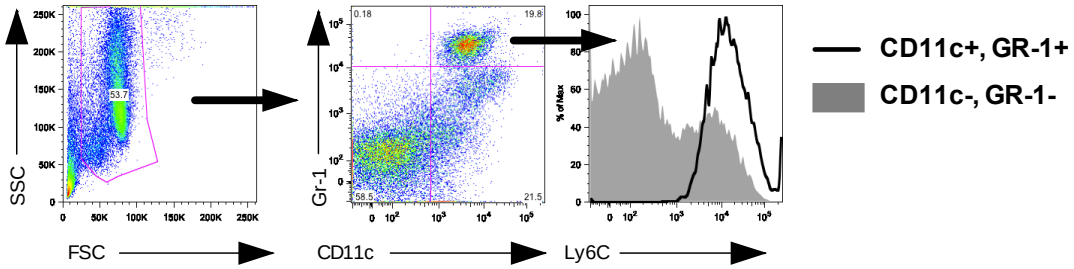
T-cells. The amount of peptide administered may not have been enough to affect all SIINFEKL-specific cells in recipients of the transfer.

I was also interested in whether the proliferation that we saw in response to peptide injection was specific to MCMV infection. In mice receiving OT-1 blood, infected with Vaccinia-SL8, and injected with SIINFEKL peptide, I followed the SIINFEKL-specific CD8 T-cell response in the blood with ICS and tetramer staining. As with MCMV infection, the SIINFEKL-specific response increased over the five weeks in which it was monitored (Figure C.3B). In contrast to MCMV infection, tetramer staining consistently showed more epitope-specific cells than did the ICS, indicating a significant proportion of unresponsive cells. Thus, proliferation in response to peptide-epitope injection is not unique to MCMV infection and can, in fact, happen with other memory populations generated by acute, cleared infections. Peptide injection has the capacity to induce deletion (as seen with the IE3 epitope in Chapter 3), proliferation (as seen with SIIINFEKL and M38 in Chapter 3), and anergy, as seen here with a subset of SIINFEKL-specific T-cells in Vaccinia infection. Why different situations elicit different outcomes is unclear and demands further study.

A. FSC and SSC profiles of mice chronically infected with WT, injected with M45 or M57



B. Gating strategy for quantifying CD11c+, Gr-1+ cells in the peripheral blood



C. CD11c+, Gr-1+ cells in the blood of mice chronically infected with MCMV-GFP-SL8 and injected with the indicated peptide

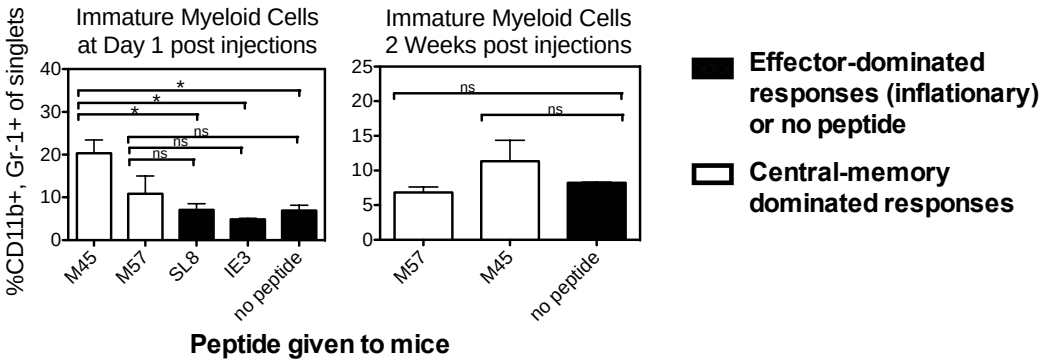
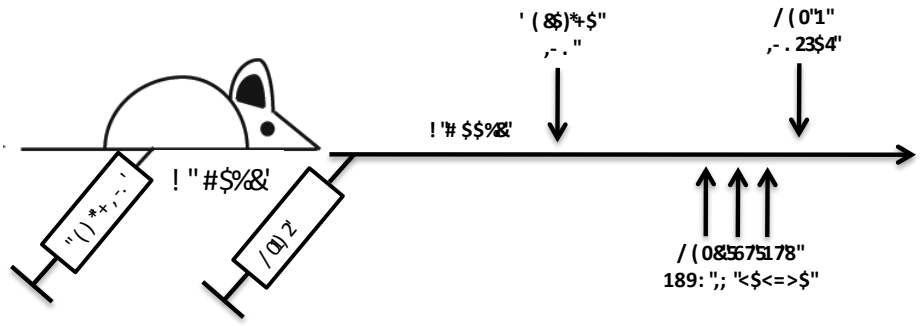
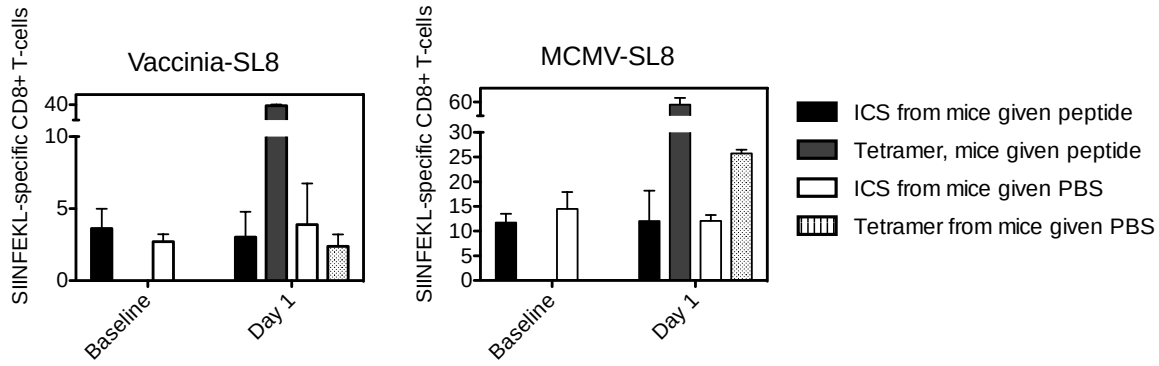


Figure C.1: Injection with M45 or M57 leads to a transient increase in immature myeloid cells in the blood. C57BL/6 mice were infected i.p with WT MCMV (A) or MCMV-GFP-SL8 (B). 12-20 weeks p.i., mice were injected with 10 µg of the indicated peptide for 3 consecutive days. Blood was taken at day 1 after peptide injections and analyzed by flow cytometry for the indicated markers. Results in A and B are from the experiments described in figures 3.2D and 3.2E, respectively. Each was done once with 3-4 mice per group. * $p<0.05$

A. Experimental Schematic



B. Epitope-specific T-cells in mice infected with Vaccinia-SL8 and MCMV-GFP-SL8 after SL8 peptide injections



C. CD11c+, GR-1+ cells at day 1 after SIINFEKL injections in infected OT-1 recipients

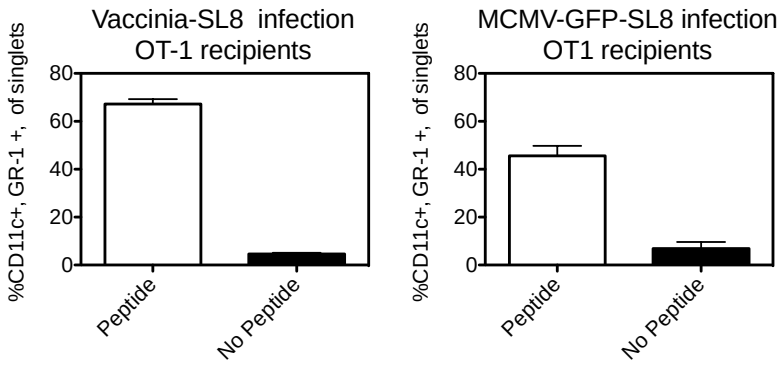
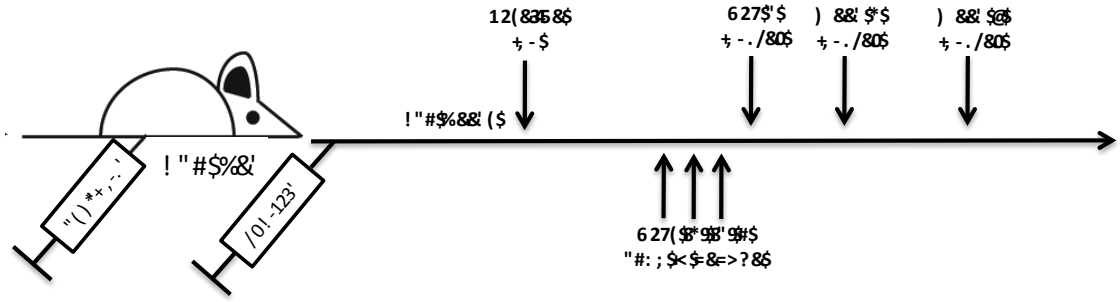


Figure C.2: SIINFEKL peptide injection of mice receiving OT-1 TCR Tg blood leads to an increase in immature myeloid cells in the blood of both MCMV-SL8 and Vaccinia-SL8 infection mice. C57BL/6 mice received 50 µl of whole blood from OT-1 Tg mice. Recipients were infected i.p with Vaccinia-SL8 or MCMV-GFP-SL8 the next day. 6 weeks p.i., mice were injected with 10 µg of SIINFEKL peptide for 3 consecutive days. Blood was taken at day 1 after peptide injections and analyzed with flow cytometry for the indicated markers. This experiment was done once with 3-4 mice per group.

A. Experimental Schematic



B. Vaccinia-SL8 infected mice injected with SIINFEKL peptide

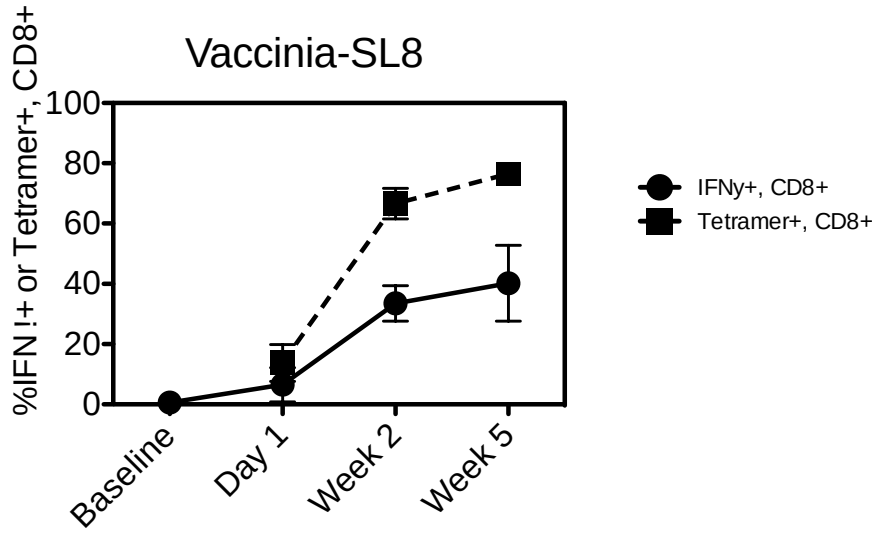


Figure C.3: SIINFEKL peptide injection of mice chronically infected with Vaccinia-SL8 also induces epitope-specific T-cell proliferation. C57BL/6 mice received 50 µl of whole blood from OT-1 Tg mice. Recipients were infected i.p with Vaccinia-SL8 the next day. 10 weeks p.i., mice were injected with 10 µg of SIINFEKL peptide for 3 consecutive days. ICS for CD8+ T-cells secreting IFN-γ in response to SIINFEKL and tetramer staining for SIINFEKL-specific CD8 T-cells was preformed at the indicated times post peptide-injection. Experiment was done once with 3 mice per group.

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