Generation and Function of CD8 T Cell Memory to \( \gamma \)-Herpesviral Infection

Dissertation

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By

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ABSTRACT

γ-herpesviruses are a worldwide health concern, and result in a lifelong persistent infection that is associated with some degree of immune control preventing the virus from reactivating and causing disease. However, if this immune control is compromised, γ-herpesviruses can reactivate and cause a variety of tumors in the host. Thus, there is a great need to increase our understanding of the immune response to this family of oncogenic viruses.

CD8 T cells have been shown to be critical for viral control. The generation, maintenance, and function of CD8 T cell memory during persistent viral infections is poorly understood. The overall objective of my dissertation is to gain an in depth understanding of CD8 T cell memory generation, maintenance, and function during low load persistent viral infections. We are using a well-established murine model of γ-herpes viral infection, murine gamma herpesvirus 68, or γHV68, to study the in vivo effects of a low load persistent infection on the generation and function of memory CD8 T cells. We determined that virus specific memory CD8 T cells are generated and maintained long term during γHV68 persistence through a variety of phenotypic and functional assays. We found that true bona fide memory CD8 T cells are fully functional during γHV68 latency, which is contrary to other chronic viral infections characterized by high antigenic...
load. We discovered that the γHV68 specific memory CD8 T cells are capable of surviving in a naïve mouse after adoptive transfer and subsequently proliferating homeostatically in response to cytokines. Additionally, the adoptively transferred memory CD8 T cells have the ability to protect against secondary infection with γHV68. We found heterogeneous virus-specific CD8 T cell memory populations during γHV68 latency, and demonstrated that the terminally differentiated CD8 T cell memory is also equally as functional as the central memory CD8 T cell memory.

The current two dominating principles in the field of CD8 T cell memory are: 1) acute infection followed by viral clearance and stable memory formation and 2) persistent infection where there is no viral clearance which leads to T cell dysfunction. My data has contributed to the field of immunology by demonstrating that there is an additional option for how CD8 T cells function during persistent viral infections, where viral persistence does not always lead to memory CD8 T cell dysfunction.

Our objective is to gain critical knowledge about CD8 T cell memory to elucidate the possible mechanisms of CD8 T cell function and dysfunction during γHV68 infection. The in vivo model provides relevant information for how memory is generated and maintained in the host, and hopes to offer insights to new therapies and prevention strategies, which is important for many diseases afflicting the human population.
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CHAPTER 1

INTRODUCTION

1.1 Herpesviruses

Herpesviridae are a large family of viruses that can cause diseases in a wide range of hosts, including humans. Herpesviruses have a considerable worldwide prevalence, and are associated with a variety of human malignancies. Herpesviruses are composed of double stranded DNA genomes that replicate in the cell’s nucleus and possess both lytic and latent viral life cycles. They establish latency in specific groups of cells depending on the type of virus, and are categorized into three distinct subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae. Alpha-herpesvirus, include herpes simplex 1, herpes simplex 2, and varicella zoster virus, infect the mucosal epithelium in their primary infections and establish latency primarily in neurons [1]. One clinical complication of the alpha-herpesviruses is the reactivation of varicella zoster virus, which can cause shingles, manifesting as a painful rash on the skin. A primary example of a beta-herpesvirus is cytomegalovirus, or CMV, which can establish latency in monocytes as well as the vascular endothelium [2]. Although CMV infection rarely results in significant disease in healthy populations, it can become a considerable pathogen for immunocompromised individuals and neonate populations. In addition, CMV is
associated with a severe immunosenescent phenotype in the aged population due to “memory inflation”, where there is a large increase of CMV virus specific cells [3]. γ - herpesviruses can establish latency in macrophages, dendritic cells, and B lymphocytes [4]. However, the major reservoir of viral latency is in splenic B cells propagated by germinal center reactions [5].

Herpesviruses have co-evolved with their hosts for more than 100 million years [6]. Consequently, the family of herpesviruses uses a variety of immune evasion strategies in order to establish persistent infections despite an intact immune system. Herpesviruses have evolved numerous strategies to block both the innate and adaptive branches of the immune response, contributing to the considerable success of these viruses. The main immune evasion technique herpesviruses use is to essentially hide from the immune system by establishing latency, where there is a large decline in virus specific gene products during this period. Another common strategy for herpesviruses is to encode homologues for an assortment of host cellular genes such as anti-apoptotic molecules that prevent infected cells from undergoing apoptosis. Herpesviruses also encode complement control regulators, chemokine scavengers, IL-10 orthologues, antigen presentation inhibitors, and cell cycle regulators to name a few[7-9]. These viral gene products help herpesviruses avoid detection by the immune system and establish successful infections within their hosts. However, these immune evasion tactics can contribute to the malignancies caused by herpesviruses. In particular, the cell cycle regulatory proteins have been associated with oncogenesis [10]. Herpesviruses establish an equilibrium in their hosts between viral persistence and immune control, and when this balance is perturbed herpesviruses can cause disease. By studying the host immune
response to herpesviruses, we can target specific tactics to combat this family of viruses, whether it is by prophylactic vaccinations, antiviral therapies or by enhancing the immune system.

1.2 γ-herpesviruses and γHV68

γ-herpesviruses are a worldwide health concern and are widely distributed, infecting approximately 95% of the world’s population (Center for Disease Control). γ-herpesviruses result in a lifelong persistent infection that is associated with some degree of immune control preventing the virus from reactivating and causing disease. Most individuals are able to control the virus due to immune surveillance. However, if immune control is compromised, γ-herpesviruses can reactivate and are associated with a variety of lymphomas [9, 11], as well as lymphoproliferative disorders after organ transplantation[12]. The γ-herpesvirus family includes Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein Barr virus (EBV) in humans, and murine gamma-herpesvirus 68 (γHV68) in mice. KSHV was initially discovered in 1993 when researchers were looking for the infectious etiology of Kaposi’s sarcoma (KS), a cancer of the lymphatic endothelium, in acquired immunodeficiency syndrome (AIDS) patients [13]. KSHV is considered the leading cause of malignancies in AIDS patients. EBV is ubiquitous in the human population and is frequently associated with infectious mononucleosis, as well as a variety of malignancies [14]. With a growing population of immunocompromised patients due to organ transplantation, and AIDS due to HIV infection, this family of viruses is becoming an increasing health concern. Thus, there is a
great need to increase our understanding of the immune response to this family of oncogenic viruses.

1.2.1 γHV68: a murine model

γHV68 is a natural pathogen of rodents and it follows similar biological functions to the human infection and disease [15]. We are utilizing a murine model of γ-herpesvirus to better understand the in vivo immune response to this virus in its natural host. γHV68 is a convenient small animal model that allows the genetic manipulation of both the virus and the mouse for experimental studies. The infection kinetics of γHV68 is an acute lytic infection of epithelial cells in the lung, which peaks at approximately day 7-post infection and is cleared by day 12-post infection, corresponding to the increase of virus specific T cells in the lung. Viral latency is established primarily in splenic B cells appearing as early as day 6-post infection, and peaking at day 14-post infection followed by a low level latency throughout the life of the host [16]. The γHV68 genome consists of 118 kilo-base pairs, containing 73 protein-encoding open reading frames [7]. The proteins encoded by γHV68 include a variety of immune system modulators, as well as latency associated proteins and structural proteins.

1.2.2 γHV68 Immune Evasion Strategies

γHV68 has evolved a multitude of techniques to evade and exploit the immune system in order to accomplish such successful infections. A few examples of the many immune system modulators specific to γHV68 are included. γHV68 encodes proteins that are specific to the virus as well as host cellular homologues that are advantageous for
infection. γHV68 encodes a novel viral mitochondrial anti-apoptotic protein (vMAP) that can inhibit the release of cytochrome C from the mitochondria, which inhibits apoptosis of the infected cell [17]. A BCL-2 homologue, derived from B-Cell lymphoma-2, is another example of immune evasion genes that γ-herpesvirus encode [7]. BCL-2 can inhibit apoptosis of cells that express it, which can be advantageous for the survival and propagation of virally infected cells [18]. γHV68 can infect immature dendritic cells (DCs) without activating them and may actually prevent their maturation, and thus the induction of the adaptive immune response [19, 20]. γHV68 gene product M3 functions as a broad-spectrum chemokine scavenger, by binding to chemokines with high affinity and inhibiting their function [21, 22]. There is also evidence for inhibition of MHC (major histocompatibility complex) class I antigen presentation in lytically infected DCs by the viral gene product K3 [20, 23]. γHV68 has been shown to inhibit type I interferon secretion by DCs [24]. In summary, γHV68 employs a large repertoire of mechanisms to increase infection efficiency by exploiting and evading the immune system. However, these immune evasion strategies can be detrimental for the host, in particular proteins that inhibit apoptosis and increase cell survival, because they can also play a role in the progression of malignancies and disease. This provides a delicate balance of effective infection and latency, and host immune control.

1.2.3 Immune Response to γHV68

Studies using knockout mice have provided insight on which immune cells and immune components are important for each portion of γHV68 control. In previous studies, it has been shown that the route of administration and dose of γHV68 infection
does not alter the virus’ ability to establish latency [25]. The knockout of granzymes in mice have shown the importance of these enzymes contributing to the control of viral reactivation [26], while mice that lack perforin did not seem to have an effect on the kinetics of virus infection [27]. Additionally, Natural Killer cells (NK cells) seem to have little impact on the acute or latent infection with γHV68 [28]. Antibodies, CD8 T cells, and CD4 T cells all contribute to the ability of the immune system to control γHV68 infection. However, there are two life cycles of γ-herpesviruses, an acute lytic infection of epithelial cells in the lung, and a long-term latent infection in splenic B cells that the immune system must combat. The majority of previous studies have implicated CD8 T cells in the control of the acute lytic infection, while CD4 T cells are more important for the control of latent infection, and vaccination studies have provided a significant role for antibodies in the protection against secondary infection [29].

CD4 T cells have been shown to be necessary to control γHV68 latency, where mice that are deficient in MHC class II, succumb to an eventual death from γHV68 infection [30], while the CD8 T cell population appears to be functionally normal [31]. IFNγ has been shown to prevent γHV68 reactivation from latency [32], and CD4 T cells as well as CD8 T cells produce low-level IFNγ during γHV68 latency [33-35]. More recently CD4 T cells have been shown to have a direct cytotoxic effect during γHV68 latency [34, 36]. Although CD4 T cells are important for the control of viral infection, CD8 T cells also play a vital role in infection control. CD8 T cells or cytotoxic T cells are the cells that are responsible for detecting and killing virally infected cells. CD8 T cells are critical for viral control of both the lytic and latent γHV68 infection [37]. In addition, CD8 specific for latent epitopes of γHV68 have been shown to control the reactivation of
latency [38]. Because CD8 T cell function and memory formation are poorly understood during persistent viral infections, we focus on the CD8 T cell response to γHV68 in this project. In addition, we utilize γHV68 persistence to study the memory CD8 T cell population in hopes of finding new approaches to increase immunity and target new therapeutic treatments to persistent viral infections.

1.3 The CD8 T Cell Response

The T cell receptor (TCR) on CD8 T cells recognizes peptides of 8-9 amino acids in length within the context of self-MHC class I molecules on target cells. Under normal conditions, MHC class I presents portions of cellular proteins on the cell surface, however, when a cell is infected with a virus, viral peptides can also be presented on the cell surface. Nearly every nucleated cell expresses MHC class I on its cell surface, thus allowing CD8 T cells to monitor the cells’ intracellular state. Naïve CD8 T cells require 3 signals to differentiate and proliferate into a population of activated effector CD8 T cells [39]. First, the naïve CD8 T cell needs to encounter its specific antigen through the TCR within the context of self-MHC class I on the antigen-presenting cell (APC). The second signal required is costimulation, where CD28 on the CD8 T cell binds CD80 or CD86 on the antigen-presenting cell sending a signal to the CD8 T cell to become activated [40]. Professional antigen presenting cells express costimulatory molecules upon activation from a pathogen. Thus, the requirement for costimulation prevents the CD8 T cell to become activated without a pathogen or inflammation. Lastly, cytokines provide the third signal to the CD8 T cell to become a fully functional effector population. IL-12 and type I interferons have been shown to provide the third signal to CD8 T cells [41-43].
After initial activation of the CD8 T cells by their specific antigen within the context of self-MHC class I molecules on antigen-presenting cells and the corresponding co-stimulatory and cytokine signals, CD8 T cells proliferate and differentiate into cells with effector functions [44]. These “effector CD8 T cells” function to control the viral infection, by killing virally infected cells by releasing their cytotoxic granules, which contain perforin, granzymes, and granulysins, in an antigen specific manner. The release of perforin causes holes in the plasma membrane of the infected cell and allows granzymes, types of serine proteases, to enter and initiate a cascade of capases leading to the apoptosis of the target cell. The effector CD8 T cells also produce the cytokines IFNγ and TNFα that contribute to clearing viruses from infected cells [32, 45]. Once the infection is controlled, the majority of these antigen specific effector cells undergo a contraction phase via programmed cell death [46]. However, a small percentage of cells remain, and these are known as the long-lived memory cells.

1.4 CD8 T Cell Memory

Immunological memory is the hallmark of the adaptive immune response, and allows the immune system to “remember” a previously encountered antigen and to protect against secondary infection. Memory is characterized by a rapid, more robust response to a secondary challenge. Memory CD8 T cells proliferate in response to homeostatic cytokines IL-7 and IL-15, proliferate in response to secondary antigenic challenge, have the ability to survive long-term without their cognate antigen, have ability to give rise to new effector CD8 T cells that can kill virally infected cells (cytotoxicity), and provide a more vigorous response against secondary challenge with
the same pathogen, or protection [47-49]. In addition, CD8 T cell memory cells have a higher antigen specific precursor frequency than in naïve mice, and have reduced costimulatory requirements [50, 51]. The conditions of CD8 T cell memory commitment is still under debate, where some studies suggest memory follows a path of linear differentiation from a naïve T cell to an effector T cell to an effector memory T cell to a central memory T cell [52, 53]. Other studies show the commitment of CD8 T cell memory occurs during the initial cell division [54]. CD8 T cell memory to viral infections is heterogeneous in nature, composed of diverse populations of CD8 T cells. CD8 T cell memory is categorized into two main groups based on homing characteristics and effector functions: effector memory (TEM) and central memory (TCM) [55]. Effector memory cells are located in the periphery, due to their lack of lymphoid homing molecules, L-selectin and CCR7. Effector memory cells have an activated phenotype and function to produce cytokines and lyse virally infected cells in a more rapid fashion than their central memory counterparts. Central memory cells are known to be a quiescent long-lived T cell that is located in the lymphoid organs, and express L-selectin and CCR7. The generation and maintenance of CD8 T cell memory is critical for long-term protection against intracellular pathogens and is an important component for vaccination strategies. The contributions of TEM and TCM differs depending on the route and type of viral infection [49]. One goal of this project is to provide a better understanding of how CD8 T cell memory is generated, maintained, and functions during a persistent viral infection.
1.5 CD8 T Cell Memory to Persistent Viral Infections

Viral infections are divided into two categories based on the time it takes to clear the infection: acute and persistent viral infections. Acute infections occur when a virus first infects a susceptible host. Persistent infection is the continuation of infection beyond the time when the immune system might reasonably be expected to clear the acute infection. Persistent infections can be further divided into categories based on the general mechanism the virus uses to maintain a long-term infection. The two strategies to establish a persistent viral infection are continuous replication and the establishment of latency. In order to succeed by continuous replication, the virus must produce enough new infectious particles that despite an effective immune response, the viral infection can be productive by essentially overwhelming the immune system. Viruses that utilize this strategy can be categorized as high titer infections, where there are high amounts of virus and viral antigens in the host. Latency is another strategy viruses use to persist in a host, where viral gene expression is restricted, and the virus can survive without any active lytic replication, and the virus is maintained in the host by a very low level of viral reactivation into lytic virus (Fields Virology).

These two strategies of persisting in the host have two different consequences on the amount of viral antigens in the host and consequently have an effect on the immune response to these two different types of persistent infections. Persistent viral infections where there is active viral replication and high antigen load have been well studied, and during these types of viral infections the CD8 T cells become dysfunctional with increasing amounts of antigen. Low antigen levels and low amounts of lytic virus are characteristics of latent viral infections. The CD8 T cell memory response to latent viral
infections is not as well studied as to high titer viral infections. CD8 T cell memory responses to acute infections and high titer infections are relatively well defined [56-59]. However, the events that lead to the generation and maintenance of memory are still unresolved during latent viral infections, where there is relatively low amounts of viral antigens [60]. It is well established that CD8 T cells are critical for control of γ-herpesviral infection [37], however, the CD8 T cell memory response to γHV68 latency has not been well characterized.

During chronic high titer viral infections, there is evidence that CD8 T cells become increasingly dysfunctional during viral persistence, where the presence of constant antigen is thought to exhaust the CD8 T cell functional capabilities. The functional disabilities of the CD8 T cells depend on the antigen load of the virus with high antigen load corresponding with increased CD8 T cell dysfunction and decreased CD4 T cell help [61]. In addition, CD8 T cell memory to chronic high titer viral infections has been shown to be maintained through constant antigen stimulation and recruitment of newly activated CD8 T cells, and characterized by a dependence on viral antigen for survival [62, 63]. Chronic high titer viral infections are associated with a level of immune exhaustion, however, during latent infection there is some degree of immune function due to the fact that the viral latency and active infection are controlled. γHV68 is a latent viral infection with low-amounts of antigen. γHV68 infection will allow us to decipher the effect of low-level antigen and latency on the in vivo immune system.

1.6 Aims and Structure of Dissertation
The generation, maintenance, and function of CD8 T cell memory during latent persistent viral infections is poorly understood. In this study, we will use a well-established murine model of \(\gamma\) -herpes viral infection, \(\gamma\)HV68, to study the \textit{in vivo} effects of a low load persistent infection on the generation and function of memory CD8 T cells. The overall objective of this study is to gain an in depth understanding of CD8 T cell memory generation, maintenance, and function during low load persistent viral infections that establish latency. Additionally we want to gain critical knowledge about CD8 T cell memory to elucidate the possible mechanisms of CD8 T cell dysfunction using the murine \(\gamma\) -herpesvirus infection.

\textit{Aim 1: Are memory CD8 T cells generated and maintained during \(\gamma\)HV68 persistence?}

Chapter 2 will focus on the different functions of CD8 T cells during the early phase of infection and during the late phase of infection (latency) to determine whether bona fide memory is generated and maintained within our model, and what, if any, functions are compromised due to viral persistence.

\textit{Aim 2: Are functional antigen-independent memory CD8 T cells generated during \(\gamma\)HV68 persistence?}

In Chapter 3 we will evaluate the ability of the CD8 T cells to survive and proliferate without viral antigens. We will also test the ability of the transferred CD8 T cells to mount a recall response to secondary antigenic challenge.
Aim 3: What is the role of specific γHV68 memory CD8 T cell subsets on the in vivo immune response? Chapter 4 will test the contribution of terminally differentiated effector memory CD8 T cell subsets and central memory CD8 T cells on the in vivo immune response. We will test the CD8 T cell memory subsets functional abilities to: produce cytokines, kill virally infected cells, survive and proliferate without antigen, and mount an protective recall response to secondary γHV68 challenge.

Our in vivo mouse model will allow us to gain a better understanding of the role of CD8 T cell memory to latent and persistent viral infections, which is essential for understanding many diseases afflicting the human population. The in vivo model will provide relevant information for how CD8 T cell memory is generated and maintained in the host, and offer insights to new therapies and prevention strategies.
CHAPTER 2

MEMORY GENERATION AND MAINTENANCE OF CD8 T CELL
FUNCTION DURING VIRAL PERSISTENCE

2.1 INTRODUCTION

Immunological memory is one of the hallmarks of the adaptive immune system and it can be functionally defined as the stronger protective response of the host to secondary antigen challenge [50, 64]. It, thus, allows the immune system to respond more vigorously to infectious pathogens that have been encountered previously. Upon primary activation by antigen, CD8⁺ T cells follow a program of proliferation and differentiation into effectors that control the infection [44]. After this expansion phase, the majority of antigen-specific CD8⁺ T cells undergo programmed cell death, leaving a population of memory CD8⁺ T cells [46]. A rapid and efficient recall response is partially due to the ubiquitous presence of increased frequencies of memory T cells in peripheral tissues and secondary lymphoid organs [65]. In addition, several characteristics of memory T cell populations contribute to enhanced secondary responses including the ability to self-
renew in the absence of antigen, higher activation status and reduced costimulatory requirements, expression of lymphoid homing molecules and homeostatic cytokine receptors, higher frequency than naïve precursors, rapid proliferation upon secondary stimulation, and the ability to maintain the integrity of the T cell repertoire [50, 51, 66]. Nevertheless, some of these characteristics may be arbitrary (i.e. markers [67]) or not universal (i.e. long lived [68]. The heterogeneity of memory T cells in phenotype, function and location has led to the idea that they can be divided in two major subsets, effector- (CD62L^{low}CCR7^{low}) and central-memory (CD62L^{high}CCR7^{high}) cells based on their expression of lymphoid homing receptors [55].

Memory CD8^+ T cells during viral infections play a major role in protection by rapid recognition and lysis of virus-infected cells. The contribution of memory CD8^+ T cells to recall responses is well defined during acute infections where antigen is cleared [53, 56]. There is, however, little and contradictory information regarding memory generation during persistent infections. This is of special relevance because persistent infectious diseases are a major health concern worldwide [69, 70]. The current paradigm, mostly derived from studies with LCMV and HIV, is that persistent infection leads to some degree of CD8^+ T cell effector dysfunction or deletion and lack of T cell memory formation [60, 71-74]. CD8^+ T cell dysfunction during chronic infection has been raised as one reason for pathogen persistence [61]. This loss of function in the presence of persistent antigen follows a progression in the sequence of cytotoxicity, IL-2, TNF-α and IFNγ production, exhaustion and deletion [61, 75]. However, these findings appear to be at odds with situations where viruses persist and CD8^+ T cell functions are not apparently compromised [76-79], with reports of residual antigen presentation [80] or with a
possible role for antigen in the maintenance of immunological memory [81]. g-herpesviruses are characterized by the establishment of life-long asymptomatic infection, the persistence in more than 90% of the human population and the association with a large list of life-threatening conditions [9, 14]. CD8\(^+\) T cells are thought to be the main effectors of long-term virus control [14, 82, 83]. How do CD8\(^+\) T cell populations maintain control of infection without pathogen recrudescence during persistent infections? Humans encounter different pathogens through life of which a considerable number reach a persistent, parasitic, commensal or a latency state and the human system has to provide life-long protection. Herpesvirus infections are a clear example: Initial infection with CMV or EBV may occur early in the childhood, which demonstrates that the immune system is capable of controlling infection for 70 years or more. Although persistent infections induce a continual evolution of the function and numbers of T cells that are specific for the infecting pathogen, there seems to be a substantial difference between infections with impaired CD4\(^+\) T cell help and/or a high antigenic load such as HIV or LCMV-clone 13 and infections with intact CD4\(^+\) T cell help and low-load antigen persistence, such as herpesvirus infections [84, 85].

To understand more clearly how CD8\(^+\) memory T cells are generated, maintained and function during low-load persistent viral infections, we studied their fate and function during the acute and long-term response to a \(\gamma\)-herpesvirus infection. Using a murine model of infection with \(\gamma\)HV68, we show that antigen-specific CD8\(^+\) T cell function is maintained during long-term, low-level viral and antigen persistence.
2.2 MATERIALS AND METHODS

*Mice and Viral Infection.*

C57BL/6J and Balb/cJ mice were obtained from Jackson Laboratory, Harlan Farms or breed at CCRI. γHV68, clone WUMS, was propagated and titered on monolayers of NIH-3T3 fibroblasts. Mice were housed in BL2 containment under pathogen-free conditions. The Institutional Animal Care and Use Committee at the Columbus Children’s Research Institute approved all the animal studies described here. Mice were anesthetized with 2,2,2,-tribromoethanol and intranasally inoculated with 1000 PFU γHV68 in 30 ul of HBSS.

*Virus analysis.*

To determine the number of cell containing γHV68 genomes long-term after infection, a combination of limiting dilution analysis and nested PCR (LDA-PCR) was used [4]. Splenocytes were isolated, red blood cells were lysed, and the number of cells per spleen was determined. Cells were serially diluted with NIH-3T3 cells in 96 well plates, lysed, and viral DNA was amplified by PCR using primers specific for γHV68 Orf50. A 2 mL aliquot of the product was re-amplified using nested primers. The final product was analyzed by ethidium bromide staining of DNA after electrophoresis in 3% agarose gel. This procedure was able to consistently detect a single copy of target sequence. Twelve replicates were assessed for each cell dilution and linear regression analysis was performed to determine the reciprocal frequency (95% degree of confidence) of cells positive for γHV68 DNA. At least 3 independent experiments were
used to determine the mean reciprocal frequency and standard deviation of γHV68 DNA in splenocytes. Infectious center assays were performed as described [30].

**Antigen presentation assays.**

Splenocytes were isolated and processed into single cell suspensions using collagenase D (5 mg/ml) treatment for 45 min. Cells were next incubated with 2 mM PBS/EDTA for 10 min. at room temperature to disrupt multicellular complexes. Cells were stained with an anti CD11c antibody conjugated to magnetic beads (Mylteni Biotechnology) and separation columns were used to purify cells into CD11c⁺ and CD11c⁻ fractions. 3x10⁴ antigen presenting cells from each fraction were plated with 1x10⁵ γHV68-specific lacZ-inducible T cell hybridomas (49100.2 and 4943.4) [86] in 96-well plates in triplicate and incubated overnight. β-galactosidase activity was assessed in individual wells as described [86]. Background stimulation was determined by using antigen-presenting cells from naïve spleens as stimulators.

**Flow cytometry analysis.**

Splenocytes were processed into single cell suspensions as described above. Cells were stained with Fc-block (CD16/32) and then washed. The cells were then stained with a combination of the following γHV68-specific MHC-tetramers: ORF61487-495/Dᵇ, ORF61524.531/Kᵇ, ORF65131-140/Dᵈ and M291-99/Kᵈ, and antibodies against CD62L (MEL-14), CD8 (53-6.7), CD43 (1B11), CD122 (TM-b1), CD127 (A7R34), CD44 (IM7) and CD4 (GK1.5). All antibodies were purchased from eBiosciences with the exception of CD43 (BD Pharmingen). MHC-tetramers were generated as described [87] or obtained from the NIH Tetramer Core Facility. The surface expression of CCR7 was analyzed
using the recombinant ligand CCL19-Ig followed by anti-human Fc-biotin and streptavidin-PE. This process can be slightly detrimental for the intensity of the tetramer staining. Flow cytometry data were acquired on FACSCalibur or BD LSR and analyzed using FlowJo software. Gates were set using negative controls, isotype controls and following the staining pattern of each marker on the bulk lymphocyte and CD8 population. For any given activation marker and tetramer combination all the analysis gates are identical in size and position.

*Tetramer Dilution.*

Single cell suspensions were obtained as indicated above. Cells were plated in flasks coated with anti-mouse IgG+IgM antibodies (Jackson Immunochemicals) for 1 h to enrich for T cells. The non-adherent cells were incubated with Fc block (CD16/32) and washed. 2x10^6 cells were plated per well in 96-well plates and stained with ORF6_{487-495}/D^b at 2-fold dilutions for 1 hour at RT. Cells were washed and subsequently incubated with anti-CD8 antibody, washed and then fixed with 1% paraformaldehyde. The results were expressed as the percentage of tetramer/CD8 double-positive cells over the log concentration of tetramer. In order to compare groups within each experiment, the data were normalized to the response at the highest concentration of tetramer. The affinity, \( K_D \), is derived from the slope by \( K_D = 1/\text{slope} \).

*Tetramer Decay.*

Cells were processed, enriched by panning and Fc-blocked as described above. After staining with ORF6_{487-495}/D^b and anti-CD8 antibody for 1 h at RT, cells were washed twice and incubated in an excess of anti-D^b 28-14-8s purified antibody and
incubated at 37°C to allow tetramer dissociation. The anti-D\textsuperscript{b} antibody was used to block rebinding of the tetramer to the TCR. Decay was followed at intervals from 0 to 50 min. at which times the cells were fixed with 1% paraformaldehyde. The results were expressed as the percentage of tetramer/CD8 double-positive cells over time. In order to compare groups within each experiment, the data were normalized to the percentage of maximum tetramer binding over time. The half-life was derived from the slope by \( t_{1/2} = \ln 2/\text{slope.} \)

_Intracellular cytokine staining._

Single cell suspensions were obtained as indicated above. 2x10\(^6\) cells/sample were incubated in complete media in the presence of IL-2 (10U/mL), Brefeldin A (10ug/mL) and 10-fold dilutions of purified ORF6\textsubscript{487-495} peptide for 5 h at 37\(^\circ\) C. As positive control, cells were stimulated with PMA/ionomycin. After Fc-blocking, the cells were stained with antibodies anti-CD44 and anti-CD8, fixed, permeabilized, and stained with anti-IFN\(\gamma\) or isotype control antibodies. The data were expressed as the percentage of IFN\(\gamma\)/CD8 double-positive cells over the log peptide concentration after subtracting the background obtained from the negative controls. The data were normalized to the response at the highest concentration of peptide. The EC\textsubscript{50} was determined as the peptide concentration provoking a response halfway between baseline and maximum.

_In vivo cytotoxicity assay._

Single cell suspensions obtained from naive spleens were pulsed with ORF6\textsubscript{487-495} peptide or irrelevant influenza NP\textsubscript{366-374} peptide (1 mg per 10\(^6\) cells) for 2 h at 37\(^\circ\)C. The NP-pulsed control cells were stained with 50nM carboxyfluorescein succinimidyl ester
(CFSE) and the relevant targets with 0.5 mM CFSE for 15 min. at 37°C. The cells were thoroughly washed, combined in a 1:1 ratio and a total of 0.5-1 x 10⁷ cells were injected intravenously into mice. The presence of CFSE positive cells was analyzed in spleen cell suspensions from the recipient mice 6 h or 40 h later. Percent specific killing was calculated according to the formula: % specific killing = (1 – (ratio of naïve recipients / ratio of infected recipients) x 100), where ratio = (number of CFSE<sup>high</sup> / number of CFSE<sup>low</sup>).

*Adoptive cell transfers.*

Splenocytes from Balb/cJ mice at 8 months after γHV68 inoculation were processed into single cell suspensions as described above and stained with anti-CD4 and CD8 antibodies. Cells were purified using a FACS Vantage with Diva option and 4 x10⁵ purified cells (purity 99%) were transferred intravenously into naïve Balb/cJ mice. The recipient and control mice were infected with γHV68 24 hours later. Viral latency was determined in the spleen on day 16.

*Statistical analysis.*

The data were fitted to linear and non-linear regression lines. The form with the highest R² was used in further comparisons between groups. Early and late groups were compared using two-way analysis of variance with group and replicate as main effects while controlling for concentration or time. The extra sum-of-squares F test was used to compare the models and reject or not the simpler null hypothesis model. Statistical analyses were run several times if there were outliers in the data. One analysis included all observations. A second analysis was run excluding outliers that were outside a range.
of 3 standard deviations. There was one observation dropped from the tetramer dilution studies, none from the tetramer decay studies and one from the IFNγ studies. The statistical analysis was performed using GraphPad Prism and SigmaStat software.
2.3 RESULTS

\textbf{\(\gamma\)-herpesvirus persistence: low antigenic load}

\(\gamma\)-herpesviruses are characterized by the establishment of life-long asymptomatic infection by, among other strategies, persisting in a latent state in memory B cells. There is, however, a low level of viral reactivation from latency that is thought to contribute to an active process of infection of new cell reservoirs and to the release of infectious viral particles [4, 14, 88]. Thus, \(\gamma\)-herpesviruses represent a good model to study immune responses during conditions of low-level antigen persistence. To study the low levels of viral latency during \(\gamma\)HV68 persistent infection we determined the viral load in the spleen during long-term \(\gamma\)HV68 infection of mice. We used a sensitive cell limiting dilution assay combined with nested PCR for \(\gamma\)HV68 orf50 (LDA-PCR) to detect the number of cells carrying viral DNA. The data show that there were consistently less than 500 latently infected cells per spleen in C57BL/6 and Balb/c mice at 5 m.p.i. (Figure 2.1A). In addition, we sought to determine the number of infectious viral particles at the same times after infection using a plaque assay. The data show that no infectious virus was detected (Figure 2.1A), indicating that there was lack of lytic virus or that the number of lytic viral particles at 5 m.p.i. is very low and thus, below the detection level of this assay. Altogether, these data indicate that the viral load during long-term \(\gamma\)HV68 carrier state is very low and suggest that the levels of persistent antigen in the host must also be low. To determine the levels of persistent antigen during long-term infection we used two \textit{lacZ}\textsuperscript{+}-inducible T cell hybridomas specific for two class-I-restricted lytic-phase epitopes from the ssDNA-binding protein (ORF6\textsubscript{487-495}/D\textsuperscript{b}) and from the large ribonuclease reductase subunit (ORF61\textsubscript{524-531}/K\textsuperscript{b}) of \(\gamma\)HV68. As shown in Figure 2.1B, hybridoma
stimulation with bulk spleen cells did not detected the presence of viral antigens on 4 m.p.i. splenocytes. However, enrichment of CD11c⁺ cell fraction using magnetic beads induced the stimulation of γHV68-specific hybridomas. No stimulatory capacity could be detected in the CD11c⁻ population. Together, these data demonstrate the during persistent γ-herpesvirus infection a small population of antigen presenting cells, likely dendritic cells, is capable presenting lytic cycle-associated viral antigens on the cell surface in the context of MHC molecules.

**Generation of central-memory CD8⁺ T cells during virus persistence**

Previous studies have shown that there is a shift in the composition of the memory T cell pool from an effector-memory to a central-memory phenotype over time [53, 56, 89]. However, during chronic LCMV infection the presence of persistent antigen is thought to prevent T cell memory generation [90]. We sought to gain a clearer understanding of the generation and heterogeneity of different subsets of memory CD8⁺ T cells in lymphoid organs and the respiratory tract during low-level antigen persistence using γHV68 infection as a model. We performed a temporal kinetic analysis of the evolution of γHV68-specific CD8⁺ T cells in two different mouse strains: C57BL/6 and Balb/c. In order to track antigen-specific T cells we used tetramers against three different CD8⁺ T cell epitopes (lytic cycle: ORF61524-531/Kᵇ and ORF6487-495/Dᵇ, latent cycle: M2⁹1.99/Kᵈ). We analyzed the level of cell surface expression of the lymphoid homing molecule L-selectin (CD62L) and the activation-associated glycoform of CD43 (1B11) to distinguish subpopulations of effector and memory CD8⁺ T cells. Up-regulation of L-selectin expression on antigen-experienced cells is a common characteristic of memory T cells and their precursors [53, 65]. The activation-associated glycoform of CD43 (1B11)
has been extensively used to distinguish memory from effector T cells in mice [91, 92]. Our data show that antigen-specific CD8$^+$ T cells with a central-memory phenotype (CD62L$^{\text{high}}$CD43$^{\text{low}}$) could be readily detected at 14 d.p.i. in spleen and their frequency increased over time during the course of persistent infection (Figure 2.2A, B). Central-memory CD8$^+$ T cells constituted between 15 and 50% of the γHV68-specific CD8$^+$ T cells independent of the tissue or epitope analyzed during long-term infection (9 m.p.i.) (Figure 2.2C). The kinetic analysis also shows that the effector CD8$^+$ T cell population (CD62L$^{\text{low}}$CD43$^{\text{high}}$) contracts from day 14 after infection until day 90 (Figure 2.2B). This process is accompanied by a progressive increase in the frequency of effector-memory T cells (CD62L$^{\text{low}}$CD43$^{\text{low}}$). This shift in the composition of the T cells from effectors (CD43$^{\text{high}}$) to memory cells (CD43$^{\text{low}}$) stabilizes 3 months after infection and the frequency of total memory CD8$^+$ T cells plateaus and remains remarkably stable until 18 months after infection, constituting 70-90% of the CD8$^+$ T cells for any epitope analyzed. There is, however, a shift in the composition of the CD8$^+$ memory T cell pool over time that starts 3 months after infection with central-memory T cells (CD62L$^{\text{high}}$CD43$^{\text{low}}$) gradually increasing their frequency while the effector-memory subset (CD62L$^{\text{low}}$CD43$^{\text{low}}$) contracts (Figure 2.2B). Similar kinetics were found for ORF6487-495/D$^b$-specific CD8$^+$ T cells (data not shown). Altogether, the data show that virus-specific CD8$^+$ T cells primarily had an effector-memory phenotype and were heterogeneous long-term after virus inoculation in both lymphoid organs and the lung. In addition, a population of virus-specific CD8$^+$ T cells resembling a central-memory phenotype could be detected at early stages after infection and progressively increased its frequency over time.
Our previous data suggest the possibility that memory CD8\(^+\) T cell precursors are being generated during the initial stages of the adaptive immune response to a \(\gamma\)-herpesvirus infection. To investigate this possibility we analyzed the expression of two of the hallmarks of bona fide memory T cells on virus-specific CD8\(^+\) T cells: homeostatic-cytokine receptors (IL-7 and IL-15) \([89, 90]\) and lymphoid homing molecules (L-selectin and CCR7) \([53, 65]\). The strategy for the gating of these populations is described in Figure 2.3. The analysis was performed 14 d.p.i., which marks the peak of \(\gamma\)HV68 latency and of viral antigen expression \([86, 93]\). As shown in Figure 2.4A, B, subsets of \(\gamma\)HV68-specific CD8\(^+\) T cells expressing L-selectin, CCR7, and receptors for IL-7 and IL-2/IL-15\(\beta\) on their cell surface could be detected in bone marrow and spleen two weeks after virus inoculation. These data indicate that phenotypically “normal” memory CD8\(^+\) T cell precursors are being generated during the peak of the T cell response to a persistent \(\gamma\)-herpesvirus infection. It should be noted that the analysis of C57/BL6 mice at 14 d.p.i. shows two populations of tetramer positive cells, one that is tetramer low and positive for L-selectin, IL-2/IL-15\(\beta\), IL-7\(\alpha\) and CCR7, and one that is tetramer high and negative for the markers tested. Transient loss of tetramer binding and TCR/CD8 down-regulation has been observed after T cell activation \([94-96]\) and could be explained by the asymmetric T cell lymphocyte division after antigen stimulation \([54]\).

To determine whether memory T cell precursors generated during primary infection had a central- or effector-memory phenotype and their anatomical distribution we analyzed simultaneously the presence of L-selectin and IL-7 receptor on ORF6\(_{487-495}\)/D\(\beta\)-specific CD8\(^+\) T cells in different lymphoid tissues (Figure 2.4C). Antigen-specific CD8\(^+\) T cells consisted of cells with an effector phenotype (CD62L\(^{low}\)CD127\(^{low}\)), central-memory
phenotype (CD62L\textsuperscript{high}CD127\textsuperscript{high}), and effector-memory phenotype (CD62L\textsuperscript{low}CD127\textsuperscript{high}) in MLN, spleen and bone marrow. The analysis shows that memory populations constitute a significant fraction of the cells in the lymphoid tissues analyzed. Although most of the memory cells are found in the spleen, Figure 2.4D shows that their frequencies are higher in bone marrow and MLN than in spleen (CD62L\textsuperscript{high}CD127\textsuperscript{high}: bone marrow 8.7%, MLN 10.7% and spleen 3.3%; CD62L\textsuperscript{low}CD127\textsuperscript{high}: bone marrow 9.8%, MLN 12.1% and spleen 7.3%). Similar numbers and frequencies were obtained using CD43 and CD62L to divide the subpopulations of antigen-specific cells (data not shown). Taken together, these results provide strong evidence that during primary γHV68 infection CD8\textsuperscript{+} memory T cell precursors with a central-memory and effector-memory phenotype are being generated within lymphoid tissues.

**Memory CD8\textsuperscript{+} T cells express homeostatic cytokine receptors and lymphoid homing molecules during virus persistence**

Continuous antigen stimulation prevents the expression of homeostatic cytokine receptors and lymphoid homing molecules on CD8\textsuperscript{+} memory T cells during LCMV persistence, which partially contributes to explain their dysfunction [90]. Thus, we analyzed the expression of L-selectin, CCR7, IL-2/IL-15b receptor and IL-7 receptor on γHV68-specific CD8\textsuperscript{+} T cells during long-term infection. The analysis of CD8\textsuperscript{+} T cells specific for two lytic cycle epitopes (ORF61\textsubscript{524-531}/K\textsuperscript{b}, ORF6\textsubscript{487-495}/D\textsuperscript{b}) during long-term infection of C57BL/6 mice shows that virus-specific CD8\textsuperscript{+} T cells have down-regulated the expression of IL-2/IL-15b receptor, with only a small fraction of the tetramer-positive cells expressing CD122 on their cell surface (1-4%) (Figure 2.5). However, the majority of the virus-specific CD8\textsuperscript{+} T cells show surface expression of the IL-7 receptor (70-80%).
In addition, a subpopulation of the antigen-specific CD8\(^+\) T cells express the lymphoid homing molecules L-selectin and CCR7 on their cell surface (20-30\%). These data are in contrast with results from other chronic infections [90], and indicate that CD8\(^+\) T cells expressing molecular determinants of central-memory cells are maintained during persistent \(\gamma\)HV68 infection. To determine whether the down-regulation of the IL-2/IL-15b receptor is specific for lytic cycle antigens or it also occurs on CD8\(^+\) T cells specific for latent cycle epitopes we did a temporal kinetic analysis of \(\gamma\)HV68-specific CD8 T cells in Balb/c mice comparing the response of a lytic-epitope (ORF65\(_{131-140}\)/D\(^d\)) and a latent epitope (M2\(_{91-99}\)/K\(^d\)). As shown in Figure 2.6, CD8\(^+\) T cells specific for the latent epitope M2\(_{91-99}\)/K\(^d\) maintain the expression of the IL-2/IL-15b receptor overtime. Lytic-epitope specific CD8\(^+\) T cells, however, progressively down-regulate the surface expression of IL-2/IL-15b receptor. Both populations of CD8\(^+\) T cells maintain the expression of IL-7 receptor and L-selectin. Together, these data indicate that during long-term persistent \(\gamma\)-herpesvirus infection memory CD8\(^+\) T cells retain the expression of homeostatic cytokine receptors (IL-7) and lymphoid homing molecules (L-selectin, CCR7) at their cell surface. However, CD8\(^+\) T cells specific for lytic cycle antigens (ORF65\(_{131-140}\)/D\(^d\), ORF61\(_{524-531}\)/K\(^b\) and ORF6487-495/D\(^b\)) specifically down-regulate CD122 surface expression.

**Antigen-specific CD8\(^+\) T cells maintain TCR affinity during virus persistence**

High avidity CD8\(^+\) T cells are thought to play a major role in terminating viral infections [97, 98]. The kinetics and affinity of TCR-peptide/MHC interactions drive the selection of TCR clones during the course of an immune response resulting in an overall
increased affinity for antigen [99]. This mechanistic model suggests that continuous antigen stimulation influences the CD8\(^+\) T cell pool by inducing changes in TCR usage or affinity that translate in the selection of high-affinity immunodominant clones [100]. Other studies show that high avidity CD8\(^+\) T cells are more susceptible to apoptosis or clonal senescence under conditions of excessive antigen load [101, 102], which may explain a decrease in immunodominance and clonal dominance during memory to herpesvirus persistence compared with primary responses [103]. In addition, the down-regulation of the IL-2/IL-15B receptor expression on lytic antigen-specific CD8\(^+\) T cells during persistent \(\gamma\)HV68 infection may be related to a decrease in clonal affinity. IL-15/IL-15R signaling mimics TCR cross-linking [104], activates CD8\(^+\) T cells [105] and mediates avidity maturation of CD8\(^+\) T cells at the single cellular level by increasing TCR functional affinity [106]. Thus, it is possible that in the presence of low levels of persistent antigen high avidity memory CD8\(^+\) T cells are being continuously stimulated by both antigen and IL-15 and driven to clonal exhaustion.

High avidity CD8\(^+\) T cells bind preferentially under conditions of limited peptide/MHC complex availability [107, 108]. To compare the relative TCR affinity of ORF6\(_{487-495}^{\text{b}}\)/D\(_b\)-specific CD8\(^+\) T cells during early (14-35 days) and long-term infection (at least 3 m.p.i.) we performed a dose-response titration using tetramer dilution under conditions ranging from saturation to limiting availability (Figure 2.7A). The data show that the normalized titration curves for early and late time-points after infection were super imposable (Figure 2.7B). The analysis of the data indicated the fit was the same for linear and non-linear approaches. Thus, in this situation the simpler linear model was chosen \((R^2\text{-early}: 0.9887, R^2\text{-late}: 0.9674)\). The slopes, elevations and Y intercepts of
both curves were not significantly different and a common slope (9.2409) and common Y intercept for all the data (95.6591) could be determined. There was no effect of the time after infection on the affinity of tetramer binding ($K_D$-early: 0.1017, $K_D$-late: 0.1157). These results indicate that there was no significant change in the average TCR peptide/MHC affinity over time between primary infection and γHV68 persistence.

The half-life of the TCR-peptide/MHC complex is related to the strength of signaling [109, 110] and the rate of dissociation of peptide-MHC tetrameric reagents from the T cell surface is related to the off-rate of the TCR-peptide/MHC interaction [99]. Thus, we used tetramer decay analysis to compare the binding of ORF6487-495/D$^b$ to γHV68-specific CD8$^+$ T cells during early and long-term infection by measuring the dissociation rate using flow cytometry (Figure 2.7C). The normalized results show that there were no major differences in the rate of tetramer dissociation time between CD8$^+$ T cells analyzed at early and late time-points during infection (Figure 2.7D). The mathematical analysis indicated a better fit for the non-linear one phase exponential decay function ($R^2$-early: 0.9481, $R^2$-late: 0.9510), so that functional form was used in comparing groups. The comparison of fit indicated that the preferred model was one common curve fitting all data sets independently of group and a common half-life value was determined as 17.19 min. ($K$: 0.0403). Altogether, the tetramer dilution and tetramer decay analyses demonstrate that antigen-specific CD8$^+$ T cells exhibit similar TCR-peptide/MHC interaction affinities during primary and persistent γHV68 infection. These data suggest that persistent antigen expression during γHV68 infection does not substantially modify the TCR affinity of CD8$^+$ T cells.
Antigen-specific CD8$^+$ T cells maintain functional avidity during virus persistence

The functional avidity of CD8$^+$ T cells is mediated by the interaction between TCR/CD8b and peptide/MHC complexes but also by several other mechanisms such as the contribution of accessory molecules in lipid rafts recruitment of signaling proteins and regulatory pathways [111-113]. Persistent viral infections are thought to lead to different degrees of CD8$^+$ T cell dysfunction [71, 85, 90]. This progressive decrease in T cell function during viral persistence has been partially attributed to triggering of inhibitory pathways [72, 114]. To compare the functional capabilities of γHV68 primary and long-term CD8$^+$ T cell responses we sought to determine their effectiveness in IFN$\gamma$ secretion or in cytotoxic killing. These two functions were chosen because they characterize the early (cytotoxicity) and final (IFN$\gamma$) stages of CD8$^+$ T cell functional impairment [61].

We measured the capacity of γHV68-specific CD8$^+$ T cells to secrete IFN$\gamma$ in response to saturating and limiting availability of peptide at early and late times after infection. The data show intracellular IFN$\gamma$ staining of splenic ORF6$^{487-495}/D^b$-specific CD8$^+$ T cells (Figure 2.8A). The data points show that the normalized titration curves for early and late time-points after infection overlapped (Figure 2.8B). A better fit of IFN$\gamma$ production data over peptide concentration was obtained with a non-linear sigmoidal dose-response curve analysis ($R^2$-early: 0.8096, $R^2$-late: 0.9082). The comparison of fits indicated that the preferred model was one common curve fitting all data sets independently of group and a common peptide concentration provoking a response halfway between baseline and maximum was determined ($EC_{50}$: 2.229 x 10^{-4}).
were no significant differences in IFNγ-secreting capacity of each CD8+ T cell population defined as the negative log of the peptide concentration that resulted in 50% maximal IFNγ production. These data indicate that antigen persistence during γ-herpesvirus infection has no detrimental effect on the functional capacity of virus-specific CD8+ T cells to produce IFNγ in response to antigenic stimulus.

We next compared the cytotoxic activity of ORF6487-495/Db-specific CD8+ T cells directly in vivo in mice 3 weeks, 3 months and 8 months after infection. The elimination of ORF6487-495-loaded targets was assessed 6 h and 40 h after transfer. Specific target elimination occurred with faster and more efficient kinetics in mice at 3 weeks after infection, which correlated with a higher frequency of ORF6487-495/Db-specific CD8+ T cells (Figure 2.9). Mice at 3 and 8 months after infection had low specific killing rates 6 h after transfer (5-14% specific killing) but were capable of killing specific targets after a 40 h interval (65-88% specific killing). In addition, no major differences in specific killing were found between mice at 3 and 8 months after infection. It should be noted that, (i) during long-term infection the frequency of splenic ORF6487-495/Db-specific CD8+ T cells is 5 to 20-fold lower than during acute infection (Figure 2.9), and (ii) at 3 and 8 m.p.i. the γHV68-specific CD8+ T cell pool predominantly consists of effector-memory cells (as shown in Figure 2.2). Altogether, these data indicate that CD8+ T cells during persistent γHV68 infection are capable of mounting a specific-CTL response in vivo although with slower kinetics that during acute infection. The analysis of IFNγ production and cytotoxicity provides evidence of the functional capacity of virus-specific CD8+ T cells during persistent infection.
CD8+ T cells generated during long-term viral persistence can mediate protection to γHV68 latency during a recall response

Memory T cells mediate faster and more effective responses to secondary pathogen challenge than naive T cells [50, 64]. To determine whether T lymphocytes from γHV68-immune mice were capable of preventing the establishment of viral latency we tested the capacity of CD8+ T cells from γHV68-infected mice to protect during a recall response. CD8+ T cells were FACS-purified from Balb/c mice that were intranasally inoculated with 1000 P.F.U. γHV68 8-months before. During the sorting procedure B cells were excluded by CD19 staining to avoid carrying latently infected B cells in the purified populations. A total of 4x10^5 CD8+ T cells or a 1:1 mixture of CD4+ and CD8+ T cells were intravenously inoculated in 300 ml PBS into naïve Balb/c recipients. The recipient mice were intranasally inoculated with γHV68 the following day and viral splenic latency was analyzed at the peak of latency on day 16. The results show that adoptive transfer of CD8+ T cells from persistently infected mice reduces the number of γHV68-infected cells during the establishment of splenic latency (Figure 2.10). In addition, the data revealed that the addition of CD4+ T cells did not improve the recall response of CD8+ T cells as measured by protection. These data show that memory CD8+ T cells from γHV68-infected mice can respond to a secondary virus challenge by decreasing the number of latently-infected cells in the spleen during the establishment of latency phase.
2.4 DISCUSSION

Persistent infectious diseases are a major health concern [69, 70]. The general consensus is that chronic viral infections lead to some degree of CD8$^+$ T cell effector dysfunction or deletion and lack of T cell memory formation [71, 72, 90]. γ-herpesviruses are very successful pathogens that persistently infect more than 90% of the human population and initial infection may occur early in the childhood [14]. In this report we demonstrate that herpesvirus-specific central-memory CD8$^+$ T cells develop during infection and that herpesvirus-specific CD8$^+$ T cells maintain functional and protective capacities during long-term persistent infection.

Our results show that during persistent infection with γHV68 there are consistently less than 500 hundred latently infected cells per spleen. In spite of this very low level of viral latency, splenic dendritic cells but not B cells are capable of presenting viral lytic-phase epitopes to γHV68-specific CD8$^+$ T cell hybridomas. This finding is unexpected as germinal center and memory B cells constitute the major viral reservoir during long-term latency [5, 115]. It suggests that dendritic cells cross-presentation lytic-phase viral antigens during γ-herpesvirus persistence and supports the existence of continuous viral reactivation during the latency phase of infection [88, 116, 117]. Reduced levels of latency and continuous presentation of lytic-epitope antigens have relevant implications for the maintenance of CD8$^+$ T cell memory. Low-level latency together with low viral gene expression [118] probably represents a very efficient immune escape strategy and explains the low frequency of latent-phase epitope-specific CD8$^+$ T cells during γHV68 persistence [38]. Continuous presentation of lytic viral epitopes explains the delayed generation of central-memory CD8$^+$ T cells and why effectors and effector-memory cells
still constitute a large fraction of the CD8$^+$ T cell response during long-term latency (50-80%, Figure 2.2, 2.4, 2.5). It is possible that these lytic epitope-specific CD8$^+$ T cells partially represent secondary effectors or de novo generated effectors as result of virus reactivation. In this sense, it has been recently suggested that low-level TCR stimulation that occurs during HSV-latency results in the maintenance of an activated but functional CD8-memory pool [1] and that newly recruited naïve CD8$^+$ T cells influence the heterogeneous CD8$^+$ T cell response during polyoma virus persistence [62].

Our data show that memory CD8$^+$ T cell precursors could be detected at early stages during primary infection and that effector-memory cells constitute the dominant CD8$^+$ T cell subset once primary infection has been resolved. The kinetic analysis of γHV68-specific CD8$^+$ T cell response shows that activated CD8$^+$ T cells (CD62L$^{\text{low}}$CD43$^{\text{high}}$) contract over a period of three months after viral inoculation. This slow process can be in part explained for the prolonged infectious mononucleosis-like syndrome in γHV68-infected mice [119] which results in exacerbated and prolonged CD8$^+$ T cell activation and expansion [120, 121]. In addition, γHV68 persistent replication and reactivation from latency [4, 122, 123] will contribute to keep virus-specific CD8$^+$ T cells stimulated. Although the frequency of memory CD8$^+$ T cells remains stable during long-term viral persistence, there is a shift in the ratio between effector-memory and central-memory subsets, with central-memory cells progressively increasing their frequency over time. Selective expression of IL-7 receptor identifies CD8$^+$ T cells that generate memory cells [124, 125]. In addition to this marker, we have also identified memory precursors using the expression of the IL-15 receptor and the lymphoid homing molecules L-selectin and CCR7 [90]. Our data correlate with results
from acute and persistent infections that demonstrate that memory cell precursors exist at
the peak of the CD8+ T cell response [89, 125, 126]. The generation and stability of CD8+
T cell memory or CD8+ T cell function during antigen persistence is poorly understood. It
has been suggested that long-term T cell memory might not develop in conditions of
chronic antigen stimulation [51]. Infections with high load of persisting antigen result in
the suppression of IL-7 and IL-15 receptor expression and down-regulation of lymphoid
homing molecules on virus-specific CD8+ T cells, and this correlates with progressive T
cell exhaustion [61, 90, 127]. Our results show that IL-7 receptor, L-selectin and CCR7
expression is maintained on 20-70% of antigen-specific CD8+ T cells during viral
persistence 9 m.p.i. This represents a small fraction of the whole CD8+ population
compared with that of acute respiratory infections, where most of the CD8+ T cell
population is constituted by memory cells [128]. It is also possible that small differences
on the level of expression of phenotypic markers between memory cells generated during
acute infections or γHV68 persistence may be biologically relevant. Nevertheless, virus-
specific CD8+ T cells with characteristics of memory cells are maintained during long-
term persistent γHV68 infection, which suggests that T cells may not need to fully rest
from antigen exposure during conditions of low-level antigen persistence to differentiate
into memory T cells expressing lymphoid homing molecules and homeostatic cytokine
receptors. It should be noted that differences in antigen load, T cell precursor frequency
or TCR avidity can also influence the expression of various phenotypic markers (such as
CD62L and IL-7R) and the capacity to generate a diversity of cytokines (such as IFNγ,
TNFα and IL-2), suggesting that the phenotype of memory T cells is ultimately a
consequence of molecular events occurring during the antigen-driven phase of the response [129, 130].

IL-15 is implicated in CD8\(^{+}\) T cell proliferation, survival and avidity [106, 131, 132]. Our data indicate that IL-2/IL-15\(\beta\) receptor expression is down-regulated on lytic epitope-specific CD8\(^{+}\) T cells during long-term \(\gamma\)HV68 persistence, which contrast with its expression on latent epitope-specific CD8\(^{+}\) T cells. Interestingly, EBV infectious mononucleosis induces a permanent deficit in IL-15 receptor expression on CD8\(^{+}\) T cells [133]. It has been shown that \(\gamma\)HV68-specific CD8\(^{+}\) T cell maintenance can be independent of IL-15 [134]. The functional implications of impaired IL-15 signaling during \(\gamma\)-herpesvirus infections are unclear, and we could not find functional deficiencies at the level of IFN\(\gamma\) production and cytotoxic capacity on lytic epitope-specific CD8\(^{+}\) T cells. However, functional and phenotypic differences between latent and lytic antigen-specific CD8\(^{+}\) T cells have been described elsewhere [135-137].

The interaction between herpesviruses and the host presents several specific characteristics that are relevant to the analysis of the CD8\(^{+}\) T cell response: (i) continuous presence of persistent antigen may impact the evolution of the cognate response, (ii) \(\gamma\)-herpesvirus persistence is characterized by low-level viral latency and antigenic load, and T cells should compete for this limited resource, and (iii) antigenic mutation is not likely to affect the T cell response. Our findings indicate that antigen persistence during \(\gamma\)HV68 infection does not modify TCR affinity and it has no detrimental effect on the functional capacity of virus-specific CD8\(^{+}\) T cells to produce IFN\(\gamma\) or to kill in response to antigenic stimulus. These results are in concordance with data from MCMV infection that suggest that CD8\(^{+}\) T cells avidity does not change over time [138] and with studies
that indicate that the TCR memory repertoire to persistent herpesvirus infections, although heterogeneous, can be remarkably stable [100, 139]. Altogether, these findings derived from herpesvirus infections argue against the idea that the continuous presence of antigen will eventually drive T cells into some degree of functional exhaustion [61, 71, 75]. These differences are likely to be explained by the low-level antigen persistence during herpesvirus infections. In these sense, during HSV latency in neurons virus-specific CD8+ T memory cells retain their capacity to produce IFNγ [140]. Although the TCR usage between T cells during primary and memory γ-herpesvirus responses is similar there is a shift in clonotypes during memory formation [141]. γHV68 persistence has also been associated with enhanced effector functions when compared with non-persistently infected mice [142]. These conflicting results may reflect differences in the experimental systems and differences between human and murine studies. Regardless, these studies shed light on the idea that CD8+ T cells during herpesvirus persistence maintain their functional ability to control infection and suggest that low-level antigen persistence may not intrinsically be a detrimental factor for T cell function. It should also be noted that this process might be mediated by the continuous recruitment of naïve T cells during viral persistence [62]. Our analysis of CD8+ T cell protection using adoptive transfers allowed us to determine the specific contribution of long-term T cell populations to the control of viral latency. Although antigen persistence has been linked to poor CD8+ T cell cytotoxic and recall responses [90], this was not the case during γHV68 infection. CD8+ T cells from persistently infected mice were capable of mounting cytotoxic responses in vivo, albeit with slower kinetics than during acute infection. These differences can be explained by the reduced frequency of antigen-specific CD8+ T cells
and the contraction of effector cells after 3 m.p.i. In addition, long-term CD8\(^+\) T cells were capable of eliciting protection against the establishment of viral latency after adoptive transfer, which supports the maintenance of CD8\(^+\) T cell function during \(\gamma\)HV68 persistence. Our findings indicate that protective memory can be generated during herpesvirus infections and suggest that the model of CD8\(^+\) T cell linear differentiation that postulates central-memory T cells as the end of a maturation process [53] also applies with an extended time frame to infections with low levels of persistent antigen. These findings have important implications for our understanding of immune control and protection against pathogens or antigens that persist in the host and for the design of vaccination strategies.
Figure 2.1. Long-term infection is characterized by low viral titers and low antigen persistence.

A. Analysis of γHV68 titers during long-term latency. The number of latently infected cells (dark bars, left Y axis) in the spleen of C57BL/6 and Balb/c mice was determined using LDA-PCR assay at 5 m.p.i. The number of lytically infected cells (ND: not detected, right Y axis) was determined using a modified plaque assay. Three individual mice were independently analyzed at each time point, error bars represent SD.

B. Analysis of γHV68 antigenic load in mice during long-term latency. Spleen cells from mice 4 m.p.i. were used as stimulators in an in vitro assay with two lacZ-inducible γHV68-specific T cell hybridomas to determine the number of antigen presenting cells capable of presenting viral antigens on their cell surface. Left panel: hybridoma 4943 specific for peptide ORF61/Kb, right panel: hybridoma 49100 specific for peptide ORF6/Db. Dendritic cells were purified by anti-CD11c antibody labeling and magnetic bead enrichment (75% purity).
Figure 2.1. Long-term infection is characterized by low viral titers and low antigen persistence.
Figure 2.2. Memory CD8\(^+\) T cells predominate during long-term infection.

Temporal kinetic analysis of the effector and memory CD8\(^+\) T cell response to \(\gamma\)HV68. A. Representative dot-plots showing the CD62L/CD43 distribution of a population of virus-specific CD8 T cells (ORF61\(_{524-531}/K^b\)) at different times after infection (14 days, 3 and 18 months). Note the progressive increase in CD62L\(^+\)CD43\(^-\) \(\gamma\)HV68-specific CD8\(^+\) T cells. Cells have been previously gated as tetramer\(^+\)CD8\(^+\). B. Evolution of the different populations of antigen-specific CD8\(^+\) T cells during \(\gamma\)HV68 infection. CD62L\(^-\)CD43\(^+\): squares, CD62L\(^-\)CD43\(^-\): triangles, CD62L\(^+\)CD43\(^-\): circles. Data are presented for ORF6\(_{487-495}/D^b\)-specific CD8\(^+\) T cells in spleen and lung. Similar kinetics were found for ORF61\(_{524-531}/K^b\)-specific CD8\(^+\) T cells. C. All the antigen-specific CD8\(^+\) T cell populations analyzed in spleen and MLN were highly heterogeneous at 9 m.p.i. No significant differences were found between lytic-cycle epitopes (ORF61\(_{524-531}/K^b\) and ORF6\(_{487-495}/D^b\)) and latent-cycle epitopes (M2\(_{91-99}/K^d\)). All the analyses were performed in C57BL/6 and Balb/c mice. Three individual mice were analyzed at each time point, error bars represent SD.
Figure 2.2. Memory CD8\(^+\) T cells predominate during long-term infection.

Temporal kinetic analysis of the effector and memory CD8\(^+\) T cell response to γHV68.
Figure 2.3. Flow-cytometry analysis of antigen-specific CD8\(^+\) T cells.

A. Representative staining showing tetramer vs. CD8 dot-plots for each one of the tetramers used in this study. B. Distribution of CD122 and CD127 staining on spleen cells, CD8\(^+\) T cells and tetramer\(^+/\)CD8\(^+\) T cells. M2\(_{91.99}/K^d\) staining on day 28 is shown. Gates were set using negative controls, isotype controls and following the staining pattern of each marker on the bulk lymphocyte and CD8\(^+\) population. For any given activation marker the analysis gates are identical in size and X-axis position. Numbers represent percentage of positive cells in each quadrant/gate.
Figure 2.3. Flow-cytometry analysis of antigen-specific CD8\(^+\) T cells.
Figure 2.4. CD8\(^+\) memory T cells are generated during the early phase of infection.

A and B. Memory CD8\(^+\) T cell precursors are detected in bone marrow (A) and spleen (B) of 14 d.p.i. \(\gamma\)HV68-infected C57BL/6 mice. The subset of antigen-specific cells precursors that express L-selectin, CD122, CD127 and CCR7 (X-axis) is shown inside the gate against the binding of ORF61\(_{524-531}/K^b\) and ORF6\(_{487-495}/D^b\) (Y-axis). The numbers indicate the percentage of cells inside the gate. The cells have been previously gated as CD8\(^+\)tetramer\(^+\).

C and D. Central and effector-memory cells are present in lymphoid organs of infected mice 14 days after virus inoculation. The number (C) of CD8\(^-\)ORF6\(_{487-495}/D^b^+\) lymphocytes expressing CD62L\(_{\text{high}}\)CD127\(_{\text{high}}\) (central-memory cells), CD62L\(_{\text{low}}\)CD127\(_{\text{high}}\) (effector-memory cells) and CD62L\(_{\text{low}}\)CD127\(_{\text{low}}\) (effectors) and their frequency (D) among the CD8\(^-\)ORF6\(_{487-495}/D^b^+\) lymphocyte population is shown in bone marrow, mediastinal lymph node and spleen. Data correspond to 6-9 individual mice per group. Error bars represent SD.
Figure 2.4. CD8⁺ memory T cells are generated during the early phase of infection
Figure 2.5. Lytic epitope-specific memory CD8$^+$ T cells express L-selectin, CCR7 and IL-7a receptor but down-regulate IL-2/IL-15b receptor expression during long-term infection.

Analysis of bone marrow and spleen cells from mice at 9 m.p.i. with $\gamma$HV68. Cells were previously gated as CD8$^+$ tetramer$^+$: ORF6487-495/D$^b$ is shown on panel A and ORF61524-531/K$^b$ on panel B. The plots show tetramer staining (Y-axis) vs. memory markers (CD62L, CD122, CD127 and CCR7 on X-axis). Note that the expression of IL-2/IL-15b receptor is down regulated while the rest of memory markers remain expressed at significant levels. The numbers indicate the percentage of cells inside the gate. Representative plots from three independent experiments with similar results are shown.
**Figure 2.5.** Lytic epitope-specific memory CD8+ T cells express L-selectin, CCR7 and IL-7a receptor but down-regulate IL-2/IL-15b receptor expression during long-term infection.
Figure 2.6. Latent epitope-specific CD8\(^+\) T cells maintain the expression of lymphoid homing molecules and homeostatic cytokine receptors during long-term infection.

Contour plots show the temporal evolution of memory CD8\(^+\) T cells in Balb/c mice for a latent epitope (M291-99/K\(^d\), panel A) and for a lytic epitope (ORF65\(_{131-140}\)/D\(^d\), panel B). Spleen cells were previously gated as CD8\(^+\)tetramer\(^+\). Tetramer staining is plotted on the Y-axis and the specific memory marker on the X-axis (CD122, CD127, CD62L). Note that memory CD8\(^+\) T cells specific for the latent epitope maintain expression of the memory markers while cells specific for the lytic epitope progressively lose surface expression of IL-2/IL-15b receptor by day 90 after infection. Representative plots from three independent experiments with similar results are shown. C Bar diagrams show the frequency of IL-2/IL-15b-expressing cells for the two epitopes described above. Error bars represent SD. *: p<0.05.
Figure 2.6. Latent epitope-specific CD8$^+$ T cells maintain the expression of lymphoid homing molecules and homeostatic cytokine receptors during long-term infection.
Figure 2.7. The TCR affinity of virus-specific CD8+ T cells does not change from primary infection to viral persistence.

A and B. Measurement of TCR affinity in CD8+ORF6_{487-495}/D^{b+} cells by peptide/MHC tetramer dilution analysis. Lymphocytes were obtained from spleens of mice infected 14 days before (early group) or 3 months before (late group), pooled, enriched by panning and stained with serial 2-fold dilutions of ORF6_{487-495}/D^{b} tetramer conjugated to APC at concentrations ranging from 1:50 to 1:100,000. All the cells were subsequently stained with anti-CD8 antibody. Data are representative from 3 independent experiments with similar results. A. Representative FACS plots of the CD8-ORF6_{487-495}/D^{b} staining. B. Graphic representation of the normalized frequency of tetramer binding and linear regression analysis for the experiment shown in A. To normalize the groups the data were expressed as the percentage of maximum tetramer binding. The inset depicts raw data.

C and D. Measurement of TCR affinity in CD8+ORF6_{487-495}/D^{b+} cells as determined by peptide/MHC tetramer dissociation. Lymphocytes were sampled and processed as above, stained with ORF6_{487-495}/D^{b} tetramer, washed, saturated with anti-D^{b} antibody and incubated at room temperature to allow for tetramer dissociation. At different time points cells were sampled and stained with anti-CD8 antibody. Data are representative from two independent experiments with similar results. C. Representative FACS plots of the CD8-ORF6_{487-495}/D^{b} staining at different time points during tetramer decay. D. Graphic representation of the normalized frequency of tetramer binding over time and non-linear one phase exponential decay regression analysis of the experiment shown in C. To normalize the groups the data were expressed as the percentage of maximum tetramer binding. The inset depicts raw data.
Figure 2.7. The TCR affinity of virus-specific CD8+ T cells does not change from primary infection to viral persistence.
Figure 2.8. The functional avidity of virus-specific CD8$^+$ T cells does not change from primary infection to viral persistence as measured by their IFN$\gamma$ production capacity.

Lymphocytes were obtained from spleens of 3-4 mice infected 5 weeks before (early group) or 3 months before (late group), pooled, enriched by panning and stimulated with different concentrations of ORF6$_{487-495}$ peptide. All the cells were subsequently processed for IFN$\gamma$ intracellular cytokine staining and surface stained with anti-CD8 antibody. Data are representative from 3 independent experiments. A. Representative FACS plots of the intracellular IFN$\gamma$ staining at two peptide concentrations for the early and late groups. B. Graphic representation of the normalized frequency of tetramer binding over time and non-linear sigmoidal dose response regression analysis of the experiment shown in A. To normalize the groups the data were expressed as the percentage of maximum IFN$\gamma$ staining at 1 mg/ml. The inset depicts raw data minus background (isotype-control intracellular staining).
Figure 2.8. The functional avidity of virus-specific CD8$^+$ T cells does not change from primary infection to viral persistence as measured by their IFN$\gamma$ production capacity.
Figure 2.9. Long-term CD8^+ T cells maintain cytotoxic capacity during persistent viral infection.

Cytotoxicity was assessed by an in vivo CTL assays. Spleen cells from naïve mice were loaded with γHV68 ORF6\textsubscript{487-495} or with influenza NP peptides and stained with different amounts of CFSE before injection into naïve control mice or into mice at different time points after γHV68 inoculation. Killing was calculated 6 h (left column) or 40 h (middle column) after transfer. Histograms show representative killing data from the analysis of individual naïve mice and from mice at 3 weeks, 3 months and 8 m.p.i. Bar diagrams show specific killing data from 4-5 individual mice per time point. The frequency of ORF6\textsubscript{487-495}/D^b+CD8^+ T cells was determined by FACS staining of spleen cells and is shown on the right column as representative dot plots and as a bar diagram. Error bars represent SD.
Figure 2.9. Long-term CD8+ T cells maintain cytotoxic capacity during persistent viral infection.
Figure 2.10. Long-term CD8\(^+\) T cells can mediate protective recall response to \(\gamma\)-herpesvirus latency.

Spleen cells from Balb/c donor mice 8 m.p.i. were sorted by FACS. A total of 4x10\(^5\) immune CD8\(^+\) or a 1:1 mixture of CD4\(^+\)/CD8\(^+\) cells was adoptively transfer by intravenous injection into naïve mice. Control mice received no cells. Mice were challenged with \(\gamma\)HV68 24 h later. The number of latently infected spleen cells was determined by an infectious center assay on day 16 after challenge. Error bars represent SD.
Figure 2.10. Long-term CD8\(^+\) T cells can mediate protective recall response to \(\gamma\)-herpesvirus latency.
CHAPTER 3

PROTECTIVE ANTIGEN-INDEPENDENT CD8 T CELL MEMORY IS MAINTAINED DURING GAMMA-HERPESVIRUS PERSISTENCE

3.1 INTRODUCTION

Immunological memory represents the ability of the adaptive immune system to "remember" a previously encountered antigen and protect the host against secondary infection with that pathogen. The formation of immunological memory allows the immune system to mount a rapid, robust, more effective recall response to a secondary challenge [50, 143]. After the resolution of an infection, the expanded pathogen-specific T cell population contracts via programmed cell death leaving only a small population of long-lived memory T cells [46]. These memory T cells are maintained at increased frequencies than their antigen-specific naïve repertoire, and have reduced costimulatory requirements and a higher activation status than their naïve counterparts [50, 51, 66]. Memory T cells persist independently of antigen and self-renew by homeostatic proliferation in response to the cytokines IL-7 and IL-15 [47, 125, 144].
Although the process of maintenance of memory CD8 T cells during an acute resolving infection is starting to be understood, the fate of CD8 T cell memory during persistent infections is still a matter of debate. Substantial evidence from studies of persistent infection with lymphocytic choriomeningitis virus (LCMV) clone-13 demonstrate that antigen persistence leads to progressive effector CD8 T cell dysfunction and to lack of antigen-independent memory formation [71, 72, 90]. Cognate viral antigen is required for the maintenance of virus-specific CD8 T cells during LCMV persistence [59]. These findings have been partially extended to human infections with HIV and hepatitis C virus (HCV) [73, 74, 145, 146]. Persistent virus infections can appear in two different forms: those that lead to progressive pathogen spread, viremia and clinical disease (high-titer infections such as LCMV or HIV), and those that are immunologically contained after a generally asymptomatic infection (low-titer infections such as \(\gamma\)-herpesviruses). \(\gamma\)-herpesviruses establish a systemic infection with a perpetual dynamic state of latency/reactivation that shapes the ongoing T cell response [35, 127, 137, 142, 147]. Viral antigens are continuously presented during long-term persistence [35] and readily induce naïve and memory CD8 T cell proliferation [148]. In spite of antigen persistence, immune surveillance maintains herpesviruses in check and CD8 T cell responses are not globally compromised [35, 76-78, 142, 149]. Altogether, these results highlight that persistent viral infections can last for the life of the host without apparent T cell dysfunction.

A key unanswered question that these findings elicit is whether memory CD8 T cells generated during a low-titer persistent viral infection can survive independently of cognate antigen and are capable of eliciting long-term protection. This issue is critical for
the maintenance of memory after anti-viral treatment during persistent infections. Some studies suggest that virus persistence may be necessary to maintain a CD8 T cell response [63, 150, 151], while others show stable or elevated T cell responses after anti-viral drug therapy [152, 153]. Whether these sustained or contracted T cell responses are protective to virus re-infection remains unknown and can only be tested in an animal model. We have addressed these issues using a model of γ-herpesvirus infection.

In the present study, we sought to analyze the capacity of antigen experienced CD8 T cells from murine γ-herpesvirus-68 (γHV68) persistently infected mice to survive and proliferate in the absence of viral antigens and to subsequently mount a protective recall response. Our data show that memory CD8 T cells isolated from mice persistently infected with γHV68 have the ability to survive and homeostatically proliferate in vivo in the absence of cognate viral antigens. Furthermore, γHV68-specific memory CD8 T cells elicit a recall response by proliferating during secondary γHV68 infection after being rested in a recipient mouse in the absence of cognate virus. Finally, the data show that recall of antigen-independent γHV68-specific memory CD8 T cells confers enhanced protection against a virus challenge. Altogether, these data demonstrate that protective antigen-independent memory CD8 T cell responses are generated and maintained during herpesvirus persistence.
3.2 MATERIALS AND METHODS

Mice and viral infection.

C57BL/6J and B6.PL-Thy1a/CyJ mice were obtained from The Jackson Laboratory, Harlan Farms, or were bred at The Research Institute at Nationwide Children’s Hospital. γHV68, clone WUMS, was propagated and titered on a monolayer of NIH3T3 fibroblasts. Mice were housed in BL2 containment under pathogen-free conditions. The Institutional Animal Care and Use Committee approved all the animal studies described here. Mice were anesthetized with 2,2,2,-tribromoethanol and intranasally inoculated with 1000 PFU γHV68 in 30 µl of HBSS.

Adoptive Cell transfers and CFSE staining.

Splenocytes from C57B/6 mice at 3-months post γHV68 infection and non-infected age-matched controls were processed into single-cell suspensions as described above. Cells were plated in flasks coated with anti-mouse IgG plus IgM Abs (Jackson ImmunoResearch) for 1 h to enrich for T cells. The nonadherent cells were incubated with Fc-block (CD16/32) and washed and stained with anti-CD44, anti-CD19, and anti-CD8 Antibodies. Cells were purified using a FACS Vantage with Diva option, the CD19+ cells were gated out and CD8+CD44+ cells were purified (purity 98%). The purified cells were labeled with 0.5 µM CFSE for 15 min at 37°C in HBSS. The cells were washed thoroughly, and 1 x 10⁶ purified cells were injected intravenously into the recipient B6.PL mice. The presence of CD8+CD90.2+ CFSE-positive cells was analyzed in spleen, lung, and bone marrow cell suspensions from the recipient mice at different time points after transfer. When indicated, B6.PL recipient mice were exposed to a sublethal dose of
irradiation. Briefly, the recipient B6.PL mice were exposed to a contained cesium source to receive 5.5 Gy (550 rads). The presence of γHV68 was routinely analyzed in the spleen of lymphopenic and non-lymphopenic host mice after adoptive transfer by limiting dilution nested PCR [35] and infective center assays [154].

*Virus titers.*

The number of latently infected cells was determined on splenic cell suspensions on day 14 after infection using a standard infectious center assay [154].

*Flow cytometry analysis.*

Single cell suspensions were obtained from the following organs: bronchial alveolar ravage, lung parenchyma, mediastinal lymph node, peripheral blood, spleen and bone marrow. Cells were stained with Fc-block (CD16/32) and then washed and stained with a combination of the following γHV68-specific MHC tetramers: ORF6487–495/D^b^, ORF61524–531/K^b^, and antibodies against: CD8 (53-6.7) and CD90.2 (53.2-1). MHC tetramers were generated as described [87] or obtained from the National Institutes of Health Tetramer Core Facility. Flow cytometry data were acquired on a BD LSR (BD Biosciences) and analyzed using FlowJo software. Gates were set using negative controls and isotype controls

*BrdU Administration and Staining.*

Recipient B6.PL mice were administered BrdU at 0.8mg/mL in their drinking water for 14 days changing out for fresh BrdU water every other day. Single cell suspensions of the bone marrow and spleen were prepared at day 14 of BrdU administration. The BD Pharmigen BrdU flow kit was used to stain the samples following the manufacturer instructions.
3.3 RESULTS

Memory CD8 T cells undergo homeostatic proliferation and mediate protective recall responses in lymphopenic hosts

The continuous presence of persistent viral antigens is thought to maintain virus-specific CD8 T cells during persistent infections [59, 90]. Using γHV68, it has been shown that memory CD8 T cells can undergo IL-15-independent proliferation and that they fail to proliferate in a naïve secondary host [134]. Thus, to test whether antigen experienced CD8 T cells isolated from long-term γHV68 infected hosts can survive and proliferate in the absence of cognate viral antigens, we used an adoptive transfer system into lymphopenic hosts. The recipient mice were previously sub-lethally irradiated in order to create space for the newly transferred cells and to facilitate their engraftment [155, 156]. We FACS-purified antigen experienced CD8 T cells (CD44^+CD8^+) from the spleens of mice infected with γHV68 for at least 3 months, labeled them with CFSE, and intravenously transferred 1 x 10^6 cells per mouse into naïve CD90.1 congenic recipients. This cell transfer represents an average frequency of 5,000 ORF6487-495/D^b^-specific CD8 T cells and 50,000 ORF61524-531/K^b^-specific CD8 T cells transferred per mouse. To ensure that no γHV68 virus was transferred, B cells and macrophages were depleted by panning and plastic adherence. In addition, CD19^+ cells were gated out during the FACS-sorting procedure. The presence of γHV68 was routinely analyzed in the spleen of adoptively transferred mice by limiting dilution nested PCR [35] and infective center assays [154] and no γHV68 was detected (data not shown). In addition no features of γHV68-associated infectious mononucleosis (splenomegaly, selective expansion of virus-specific or Vb4CD8 T cells) [119] were found in mice recipient of memory CD8 T cells.
during the course of the experiments presented here. The data show that we could detect the presence of donor CD8 T cells in the spleen, bone marrow and lung of the recipient mice at different times after adoptive transfer (Figure 3.1). Donor CD8 T cells constituted between 5 and 30% of the total number of cells in each recipient tissue on day 16, which suggest that they had dramatically expanded in the recipient mouse. The analysis of CFSE dilution shows that the transferred CD8 T cells proliferated in response to homeostatic cytokines in the absence of cognate viral antigens, as typical of antigen-independent memory T cells. High-CFSE fluorescence intensity could still be detected in a small fraction of donor CD8 T cells that had not proliferated on day 16 after adoptive transfer (Figure 3.1), indicating that the decrease in CFSE signal intensity described above could not be due to a global decay in CFSE fluorescence intensity over time. Altogether, these data indicate that antigen-experienced CD8 T cells from mice latently infected with γHV68 have the ability to survive and proliferate in the absence of cognate viral antigen in a lymphopenic host.

Next, we addressed whether the adoptively transferred CD8 T cells could mount a recall response to γHV68 after being rested in the absence of cognate viral antigens. We compared the recall capacity of antigen-experienced CD8 T cells purified from mice latently infected with γHV68 (termed γHV68-memory) with that of antigen-experienced CD8 T cells purified from non-infected control donor mice (termed irrelevant-memory). After FACS-purification and adoptive transfer, the γHV68-memory CD8 T cells or irrelevant-memory cells were rested for 30 days in their respective naïve congenic recipients. The data show that both populations of donor CD8 T cells could be detected in the peripheral blood of their respective recipients at least a month after adoptive transfer.
On day 30 after adoptive transfer, all the mice were intra-nasally infected with γHV68. The percentage of donor CD8 T cells in the peripheral blood was analyzed before infection (day 0) and then on day 7 and day 14 after γHV68 infection. The data show that γHV68-memory CD8 T cells of donor origin expand from 20% of the total CD8 T cell population (day 30 post transfer, day 0 of infection) to 40 and 50% of the total CD8 T cells on day 7 and 14 after infection, respectively (Figure 3.2A). The donor CD8 T cells did not expand in response to a γHV68 challenge in the recipient mice that have received irrelevant-memory CD8 T cells. These data strongly suggest that the recall response was specific to γHV68 and not due to non-specific inflammation or to an artifact of adoptively transferring cells into a lymphopenic environment, because irrelevant-memory donor cells did not proliferate in response to γHV68 infection under similar conditions. Due to the high variability of the response in mice that received irrelevant-memory, we performed an analysis of the recall response in individual mice. The data corroborate that there was a two-fold increase on the frequency of donor CD8 T cells transferred from γHV68-memory mice on day 7 (Figure 3.2B) or on day 14 (Figure 3.2C) after γHV68 recall. The frequency of CD8 T cells of donor origin isolated from irrelevant memory mice either stayed the same or went down in their respective host after γHV68 infection. Taken together, these data indicate that memory CD8 T cells from mice latently infected with γHV68 can mount a recall response to γHV68.

To test whether the recall response was mediated by γHV68-specific CD8 T cells of donor origin, we analyzed the frequency and number of antigen-specific cells of donor (CD90.2) and host (CD90.1) origin on day 14 after γHV68 infection (day 44 after adoptive transfer). The tetramer analysis shows that ORF6487-495/D\(^b\) - and ORF61524-
specific CD8 T cell populations of donor origin were conspicuous and could be detected in every organ analyzed (bronchoalveolar lavage (BAL), lung tissue, peripheral blood (PBL), mediastinal lymph node (MLN), spleen and bone marrow) (Figure 3.3A). The data show that donor ORF6487-495/D\(^b\)-specific CD8 T cells constitute 40-50% of the total epitope-specific pool and that 60-70% of the ORF61524-531/K\(^b\)-specific CD8 T cells are also of donor origin (Figure 3.3B). The analysis of the total numbers of epitope-specific CD8 T cells of donor and host origin in the spleen and MLN of the adoptively transferred \(\gamma\)HV68-infected mice shows that memory CD8 T cells of donor origin make a substantial contribution to the total anti-viral T cell response (Figure 3.3C). Whether this process is due to the faster proliferation of memory cells of donor origin than naïve host cells or to donor T cell quenching of the host response via antigen or cytokine competition remains unclear.

Next, we assessed the ability of these “rested” memory CD8 T cells isolated from mice latently-infected with \(\gamma\)HV68 to protect against secondary infection. After FACS-purification and adoptive transfer, the \(\gamma\)HV68-memory CD8 T cells or irrelevant-memory cells were rested for 30 days in their respective naïve congenic recipients. On day 30 after adoptive transfer, the mice where intra-nasally infected with \(\gamma\)HV68. At the peak of viral infection in the spleen (14 days post infection), we analyzed the frequency of \(\gamma\)HV68-latently infected cells by quantifying the ability of the virus to reactivate and cause plaques. The frequency of infected cells in the spleen was compared between mice that received “\(\gamma\)HV68-memory” or “irrelevant-memory” CD8 T cells. The data in Figure 3.4 illustrate that the mice receiving CD8 T cells from \(\gamma\)HV68-persistently infected donors had significant lower levels of splenic viral latency that the mice that received control
antigen-experienced CD8 T cells (p ≤ 0.0001). These data indicate that γHV68-specific memory CD8 T cells preserve their capacity to mediate protective responses after antigen withdrawal. Altogether, these data demonstrate that after adoptive transfer into a lymphopenic environment free of cognate antigen, herpesvirus-specific memory CD8 T cells have the capacity to survive and to mount proliferative and protective recall responses.

**Survival and proliferation of memory CD8 T cells in γHV68-free mice**

The survival of γHV68-specific CD8 T cells in adoptive hosts was unexpected in light of the previous literature [134] and thus, we considered the possibility that it was an artifact due to the lymphopenic environment. Sub-lethal irradiation creates an environment that enhances adoptive immunity [156]. A potential concern with homeostatic proliferation during lymphopenia is that it can produce memory T cells with protective capacity [157, 158]. We next addressed whether CD8 T cells from γHV68 latently infected mice could survive and proliferate without cognate antigens in a normal environment where they have to compete with “normal” memory T cells from the host. We FACS-purified antigen experienced CD8 T cells (CD44⁺CD8⁺) from the spleens of mice that had been infected with γHV68 for at least 3 months, labeled them with CFSE, and intravenously transferred 1 x 10⁶ cells per mouse into recipient naïve congenic B6.PL mice (CD90.1). CD8 T cells or donor origin could be detected at days 1, 7, and 21 after transfer in the spleen, bone marrow and lung of mice that received γHV68-memory or irrelevant-memory transfers (Figure 3.5A). The analysis of CFSE dilution in the transferred cells suggests that they could proliferate at a slow rate (Figure 3.5A). The
frequency of CD8 T cells of donor origin decreased over time, but their values were
similar between the γHV68-memory and irrelevant-memory transfers in both the bone
marrow and spleen (Figure 3.5B). Next, to determine the ability of the donor CD8 T cells
to proliferate in the naïve recipients we performed BrdU assays. At the time of adoptive
transfer, the recipient mice were administered BrdU in their drinking water for 14 days.
The percentage of cells that incorporated BrdU was analyzed by intracellular staining
(Figure 3.5C). The data show that in the mice that received the γHV68-memory transfer,
the percentage of donor cells that have incorporated BrdU in the spleen was 30% and was
nearly identical to the percentage of host CD8 T cells that incorporate BrdU. In the
irrelevant-memory transfer group, the frequency of cells that incorporated BrdU in the
spleen was 15-20% and also similar between donor and host CD8 T cells. Similar data
were obtained in the bone marrow. On day 14 post transfer, the total number of donor
CD8 T cells in the spleen and bone marrow was assessed in both the irrelevant-memory
transfer and the γHV68-memory transfer mice. The data show that the total number of
donor cells is identical between the irrelevant-memory and the γHV68-memory transfer
mice (Figure 3.5D). Since our data show that adoptively transferred memory CD8 T cells
from latently infected mice incorporated BrdU, these indicates that they can survive and
proliferate in the absence of cognate viral antigens by homeostatic proliferation. Their
ability to survive and proliferate is similar to that of memory CD8 T cells isolated from
non-infected control mice.
γHV68-specific memory CD8 T cells mediate recall responses

To address whether memory CD8 T cells can mount an effective recall response after cognate antigen withdrawal, the adoptively transferred CD8 T cells were rested in naïve recipients for 30 days, and the donor cell frequency among the total CD8 T cell population was determined in the bone marrow, MLN and spleen on day 30 after transfer. The donor cell frequencies in the lymphoid organs of mice that received γHV68-memory cells were between 0.5 and 2% of the total CD8 T cell population, and approximately 0.5% in the mice that received irrelevant-memory (Figure 3.6A). The total donor cell numbers in the recipient mice on day 30 after transfer show no differences between mice that receive γHV68-memory or irrelevant-memory (Figure 3.6B). In order to examine the donor cell response to γHV68 infection, we analyzed the frequency of donor cells before and after infection with γHV68. The data show that 14 days after infection, the CD8 T cells of donor origin from recipient mice that received γHV68-memory expanded approximately 10-fold (Figure 3.6C). The donor CD8 T cells from the mice that received irrelevant-memory cells did not increase their frequency after γHV68 infection, suggesting that bystander activation or inflammation from the infection do not play a role in the cell expansion observed in mice recipient of γHV68-memory cells. Altogether, these data suggest that γHV68-specific memory CD8 T cells can mount a proliferative response during virus re-encounter after being rested in the absence of cognate antigens for up to a month.

Next, to address whether the recall response of the adoptively transfer cells was virus specific, we analyzed the presence of virus specific cells in the donor CD8 T cell populations using γHV68-specific tetrameric reagents. On day 14 after infection, we
determined the frequency of ORF6\textsubscript{487-495}/D\textsuperscript{b} and ORF61\textsubscript{524-531}/K\textsuperscript{b} CD8 T cells of donor origin in mice that had received γHV68- or irrelevant-memory transfers 44 days before (Figure 3.7). The results show that in every tissue analyzed the frequency of γHV68-specific cells was 5 to 25-fold higher in the mice that received the γHV68-memory donor cells than in the mice that received irrelevant-memory donor cells. The analysis of the total cell numbers corroborate these differences and shows that the magnitude of the antigen-specific response is dramatically enhanced in mice that received γHV68-memory cells compared to that of control mice that received irrelevant-memory (Table 3.1).

Altogether, these data demonstrate that γHV68 specific memory CD8 T cells can survive in the absence of cognate antigen, and upon virus recall mount an efficient antigen-specific proliferative response. We considered the possibility that 30 days was not enough time for the γHV68-specific transferred cell to “rest” in the absence of cognate viral antigens. Thus, we performed the transfer experiments waiting 60 days after adoptive transfer of γHV68-memory or irrelevant-memory cells before infecting the recipient mice with γHV68. The data show that CD8 T cells of donor origin donor could be detected 60 days after transfer in both experimental groups (Figure 3.8). Furthermore, the donor CD8 T cells from the mice that received γHV68-memory expanded 10-fold after exposure to γHV68 infection, while the donor cells from the mice that received an irrelevant-memory transfer did not expand. These data corroborate that memory CD8 T cells purified from persistently γHV68-infected mice can survive long-term without cognate antigen and mount a proliferative recall response against γHV68.
Rested memory CD8 T cells mediate anti-viral protective responses

Having established that γHV68-specific memory cells can survive in the absence of cognate antigen and that they can mount a proliferative recall response against γHV68, we wanted to examine the ability of these “rested” γHV68-specific memory CD8 T cells to protect against a secondary infection with γHV68. Therefore, we adoptively transferred memory CD8 T cells from γHV68-persistently infected mice or from naive controls into naïve congenic recipients. 30 days after transfer the mice were intranasaly infected with γHV68 and the number of γHV68-infected cells was determined on day 14 after infection. The data in Figure 3.9 show that the mice that received antigen-experienced CD8 T cells purified from persistently infected mice had significant lower numbers of infected cells than the mice that received control memory CD8 T cells (p ≤ 0.0079). These data demonstrate that γHV68-specific memory CD8 T cells preserve their protective capacity after prolonged cognate antigen withdrawal in a normal host.

3.4 DISCUSSION

The generation and maintenance of memory CD8 T cell responses during persistent viral infections is poorly understood. The current line of thought is that persistent infection leads to progressive CD8 T cell dysfunction and lack of memory formation. Our data show that after adoptive transfer into an antigen free environment, γ-herpesvirus-specific memory CD8 T cells have the capacity to survive and homeostatically proliferate in the absence of cognate antigenic peptide, and to mount a proliferative and protective recall response. Importantly, these responses occur both in lymphopenic mice, where the adoptively transferred memory CD8 T cells encounter an
environment that enhances survival and memory generation, and in normal mice, where the adoptively transferred memory CD8 T cells must compete with the host cells for limited resources and space. These results demonstrate that self-renewing memory CD8 T cells with anti-viral protective capacity can develop during persistent viral infection.

T cells are critical in controlling γ-herpesvirus infections [9, 78, 83, 159]. γ-Herpesviruses establish a life-long infection, which constantly influences the maintenance and function the T cell response during virus persistence both in humans [77, 136] and mice [137, 142]. The capacity of γHV68-specific memory CD8 T cells to survive and proliferate in vivo in the absence of persistent viral antigens represents the maintenance of a crucial attribute of a functional CD8 T cell response. These findings contrast with the maintenance of CD8 T cell memory against γHV68 in absence of IL15 in IL-15-deficient mice [134], when IL-15 is critical for the generation and survival of memory T cells [160]. It is possible that in the absence of IL-15, the survival of γHV68-specific memory CD8 T cells is mediated by viral persistent antigens or other cytokines. However, this finding in a transgenic model does not exclude the possibility that a population of memory CD8 T cells with their capacity to homeostatically proliferate intact is maintained during long-term γ-herpesvirus persistence in immunocompetent hosts. It is possible that the γHV68-specific CD8 T cells that survive and proliferate in cognate antigen-free recipients represent a robust expansion of an otherwise small subpopulation of CD8 T cells or it is also possible that a large pool of γHV68-memory CD8 T cells maintained their homeostatic proliferative capacity intact. Our data showing a global decay in CFSE intensity and the incorporation of BrdU in 20-30% of the transferred CD8 T cells appear to support this second alternative. It should be noted that
previous studies have indicated that a population of central-memory CD8 T cells is maintained during γHV68 infection [35] and the expression of IL-7 and IL-15 receptors on γHV68-specific CD8 T cells does not show major deficiencies [35, 142]. In addition, addition of IL-15 increased the survival of γHV68-specific CD8 T cells [134] and induced proliferation of a subpopulation of memory cells [142]. Although the CD8 T cell responses against γ-herpesvirus infections can be very heterogeneous and epitope-specific [137, 141], anti-viral CD8 T cell functions are not apparently compromised during γ-herpesvirus persistence [35, 76].

Persistent antigen is required for the maintenance of CD8 T cell responses during persistent infection with LCMV-clone 13 [59, 90]. Our data show that γHV68-specific CD8 T cells maintain the central-memory property of long-term antigen independent survival. Can these two independent findings obtained with two different persistent infections be reconciled? First, it is possible that the form of virus persistence in the host can influence the maintenance of T cell memory. γHV68 persists in a latent state in multiple anatomical locations [4, 25] and, although the number of infected cells is low, lytic viral antigens are continuously being presented by professional antigen presenting cells during long-term infection [35]. Importantly, this antigen presentation during the persistent phase of infection is capable of inducing the proliferation of naïve and memory T cells [148]. Thus, γ-herpesvirus lytic antigens are continuously priming CD8 T cells during viral persistence but they do not appear to be sufficient to induce global CD8 T cell dysfunction or lack of memory formation. Second, it is possible that there is a threshold of persistent antigen for maintaining memory CD8 T cells that proliferate in response to homeostatic cytokines. The antigenic load, not merely persistence, will be a
crucial factor in determining the outcome of a memory CD8 T cells response [85]. During γHV68 persistence, the number of infected cells is close to 1,000 per spleen [4, 5, 35] while during LCMV-clone 13 persistence viral titers are $10^4$-$10^6$ pfu/g of tissue [72, 161]. γHV68 represents a persistent systemic infection where the viral and antigenic load is relatively low and, thus, CD8 T cell function and memory are maintained.

We have modeled the elimination of the persistent pathogen using an adoptive transfer strategy because herpesviruses cannot be readily eliminated from the host. Our findings show that γHV68-specific memory CD8 T cells do not require cognate viral antigen for survival. Our experimental approach employs memory CD8 T cells from mice at 3 months after infection and different results may be obtained at earlier or later times after infection. Continuous exposure to low levels of persistent antigen for longer periods of time may alter the properties of γHV68-specific memory cells, although previous analyses at 8-9 moths after infection have shown maintenance of IL-7 receptor expression and of cytotoxic capacity (21). The adoptively transferred memory CD8 T cells are efficiently maintained in lymphoid organs and in the periphery of naïve recipient mice in spite of the lack of cognate viral antigens. Two mutually non-exclusive mechanisms can facilitate this process, cell survival and cell proliferation. Through the use of CFSE and BrdU we observed that cell proliferation is an important component for the maintenance of the transferred memory. These observations were made in the absence of cognate antigen stimulation, suggesting that homeostatic cytokines play a crucial role in this process. Importantly, we observed the existence of long-term memory CD8 T cells with anti-viral protective capacity both in lymphopenic and normal hosts. This finding indicates that lymphopenia is not inducing a non-physiological property on γHV68-
specific memory CD8 T cells but merely amplifying a pre-existent property of a subset of γHV68-specific memory CD8 T cells. Our findings appear to be reminiscent to those of T. cruzi persistence in mice, where stable CD8 T cell function and memory has been reported [162, 163]. Drug-induced elimination of T. cruzi parasites results on the induction of a protective central-memory CD8 T cell response [164]. Along the same lines, anti-viral therapy during chronic HCV or HIV infection can induce enhanced anti-viral T cell responses [152, 153]. Thus, data from several persistent infections support the idea that pathogen persistence is not sufficient to induce lack of memory CD8 T cell maintenance. Other parameters such as antigen load, pathogen tropism, immune evasion mechanisms and quality of the CD4 T cell help are likely to play a fundamental role in shaping the memory CD8 T cell response to a persistent pathogen.

A critical aspect of our findings is that the memory CD8 T cell response generated during a persistent γ-herpesvirus infection has the capacity to mediate anti-viral protection during a recall response. These data support the previous observation that γHV68-specific CD8 T cells maintain antiviral functions and mediate protection during the persistent phase of infection [35]. Our data demonstrate that memory CD8 T cells that have been rested in the absence of cognate γHV68 antigens have the capacity to mount a proliferative response following secondary encounter with γHV68. In addition, these “rested” persistent memory CD8 T cells enhance the protection to a γHV68 challenge by reducing the number of infected cells in both lymphopenic and normal hosts. On day 14 after challenge, mice that received CD8 T cells isolated from γHV68-asymptomatic donors more than a month before had significantly lower viral titers that control mice that received irrelevant CD8 T cells (p ≤ 0.0001 in lymphopenic hosts, p ≤ 0.0079 in normal
hosts). Useful cellular memory must to be long-lived and able to mount an effective recall response, and γHV68-specific CD8 T cell memory shows these two characteristics. These findings suggest a modified model of T cell memory generation during virus persistence where, at least under conditions of controlled virus spread, a population of memory CD8 T cells with capacity for long-term antigen-independent survival and for mediating protection during secondary virus encounter can be maintained in the host. Protective memory T cell responses might be achieved by bringing or maintaining the persistent viral or antigenic load below a certain threshold that prevents triggering of progressive T cell dysfunction.
### Table 3.1. Number of antigen-specific CD8 T cells of donor origin on day 14 after γHV68 infection (day 44 after adoptive cell transfer).

<table>
<thead>
<tr>
<th>Antigen specificity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Transfer&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Tissue analyzed&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Spleen</td>
<td>MLN</td>
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<tr>
<td>ORF6&lt;sub&gt;487-495&lt;/sub&gt;D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>γHV68-memory</td>
<td>35,015</td>
</tr>
<tr>
<td></td>
<td>Irrelevant-memory</td>
<td>893</td>
</tr>
<tr>
<td>ORF6&lt;sub&gt;524-531&lt;/sub&gt;K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>γHV68-memory</td>
<td>220,674</td>
</tr>
<tr>
<td></td>
<td>Irrelevant-memory</td>
<td>3,309</td>
</tr>
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<sup>a</sup> Tissues from 6 individual mice were analyzed per group.

<sup>b</sup> Tetramer-positive lymphocytes were gated as CD8<sup>+</sup>CD90.2<sup>+</sup>.

<sup>c</sup> 1 x 10<sup>6</sup> CD8<sup>+</sup>CD44<sup>+</sup> T lymphocytes were purified from γHV68-persistently infected mice or irrelevant naïve controls, and transferred on day 0 of the experiment, as described in Material and methods.
Figure 3.1. Antigen-experienced CD8 T cells isolated from γHV68-persistently infected mice survive and proliferate in the absence of cognate viral antigens in lymphopenic hosts.

Sub-lethally irradiated B6.PL mice received $1 \times 10^6$ CFSE-labeled CD44$^+$ CD8$^+$ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months. Panel A) Representative FACS plots of the spleen, bone marrow and lung 16 days after adoptive cell transfer. The left column shows CD8 and CD90.2 staining. Numbers show the frequency of CD8$^+$ CD90.2$^+$ cells. The right column shows representative plots of the CFSE and CD90.2 staining of CD8 T cells. Panel B) Analysis of the CFSE fluorescent intensity of donor CD90.2$^+$ CD8$^+$ T cells in the spleen on day 0, 3, 8, 16, and 21 after adoptive transfer. Similar results were obtained in two independent experiments.
Figure 3.1. Antigen-experienced CD8 T cells isolated from γHV68-persistently infected mice survive and proliferate in the absence of cognate viral antigens in lymphopenic hosts.
Figure 3.2. “Rested” memory CD8 T cells from γHV68-persistently infected mice proliferate in response to γHV68 virus recall in lymphopenic hosts.

Sub-lethally irradiated B6.PL mice received 1 x 10^6 CFSE-labeled CD44^+CD8^+ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months or from non-infected age-matched controls. After adoptive cell transfer, the recipient B6.PL mice were rested for 30 days and subsequently infected with γHV68. Panel A) Bar diagrams show the frequency of CD90.2^+ CD8^+ T cells of donor origin in the peripheral blood on day 0, 7, and 14 after γHV68 challenge. The analysis of B6.PL mice that received γHV68-memory is on the left and the analysis of B6.PL mice that received control irrelevant-memory CD8 T cells is on the right. Bars represent the mean value of three experimental animals and error bars indicate SEM. Panel B) The graphs show the frequency of CD90.2^+ CD8^+ T cells in PBLs of the same individual B6.PL recipient mouse before γHV68 infection and at day 7 after infection. Left panel: B6.PL mice that received γHV68-memory, right panel: B6.PL mice that received control irrelevant-memory CD8 T cells. Panel C) The graphs show the frequency of CD90.2^+ CD8^+ T cells in the PBLs of the same individual recipient B6.PL mice before γHV68 infection and at day 14 after infection. Left panel: B6.PL mice that received γHV68-memory, right panel: B6.PL mice that received control irrelevant-memory CD8 T cells. Similar results were obtained in two independent experiments.
Figure 3.2. “Rested” memory CD8 T cells from γHV68-persistently infected mice proliferate in response to γHV68 virus recall in lymphopenic hosts.
Figure 3.3. \( \gamma \)HV68-specific memory CD8 T cells mount a recall response to \( \gamma \)HV68 infection.

Sub-lethally irradiated B6.PL mice received \( 1 \times 10^6 \) CFSE-labeled CD44\(^+\)CD8\(^+\) T cells isolated from the spleens of B6 mice that have been infected with \( \gamma \)HV68 for at least 3 months. The recipient B6.PL mice were rested for 30 days after adoptive cell transfer and subsequently infected with \( \gamma \)HV68. On day 14 after infection we analyzed bronchoalveolar lavage (BAL), peripheral blood leukocytes (PBL), lung, mediastinal lymph node (MLN) and spleen of individual mice. Panel A) Representative FACS plots showing tetramer-positive (ORF61\(_{524-531}/K^b\)) cells of donor origin (CD90.2\(^+\)) after gating on CD8\(^+\) T cells. Numbers indicate the frequency of tetramer-positive cells of donor (upper right quadrant) and host (upper left quadrant) origin. Panel B) Bar diagrams show the frequency of cells of donor origin (CD90.2\(^+\)) among virus-specific ORF6\(_{487-495}/D^{b+}\) CD8\(^+\) T cells (left panel) or ORF61\(_{524-531}/K^{b+}\) CD8\(^+\) T cells (right panel). Panel C) Bar diagrams represent the number of virus-specific CD8 T cells (ORF6\(_{487-495}/D^{b+}\) and ORF61\(_{524-531}/K^{b+}\)) that are of donor origin (CD90.2\(^+\)) or host cells (CD90.2\(^-\)). Left panel: spleen, right panel: MLN. Bars represent the mean value of three experimental animals and error bars indicate SEM. Similar results were obtained in two independent experiments.
Figure 3.3. γHV68-specific memory CD8 T cells mount a recall response to γHV68 infection.
**Figure 3.4.** \(\gamma HV68\)-specific memory CD8 T cells protect to a \(\gamma HV68\) challenge.

Sub-lethally irradiated B6.PL mice received \(1 \times 10^6\) CFSE-labeled CD44\(^+\)CD8\(^+\) T cells isolated from the spleens of B6 mice that have been infected with \(\gamma HV68\) for at least 3 months (\(\gamma HV68\)-memory, grey bar) or from non-infected age-matched controls (irrelevant-memory, black bar). The recipient B6.PL mice were rested for 30 days after adoptive cell transfer and subsequently infected with \(\gamma HV68\). The bar diagram shows the number of \(\gamma HV68\)-infected cells in the spleen 14 days after infection. Bars represent the mean value of three experimental animals and error bars indicate SEM. Similar results were obtained in two independent experiments.
Figure 3.4. γHV68-specific memory CD8 T cells protect to a γHV68 challenge.
Figure 3.5. Memory CD8 T cells isolated from γHV68-infected mice survive and proliferate in the absence of cognate viral antigens.

B6.PL mice received $1 \times 10^6$ CFSE-labeled CD44$^+$CD8$^+$ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months (γHV68 memory transfer) or from non-infected age-matched controls (irrelevant memory transfer). Panel A) FACS histograms show a temporal kinetic analysis of the CFSE intensity of CD90.2$^+$ CD8$^+$ T splenocytes at days 1, 7, and 21 after adoptive transfer. Left panel: B6.PL mice that received γHV68-memory, right panel: B6.PL mice that received control irrelevant-memory CD8 T cells. Panel B) Frequency of CD8 T cells of donor origin (CD90.2$^+$) on days 1, 7, and 21 after adoptive cell transfer in the spleen (left panel) and bone marrow (right panel). The dotted lines represent B6.PL mice that received control irrelevant-memory CD8 T cells and the solid line represents B6.PL mice that received γHV68-memory. Panel C) On the day adoptive cell transfer, B6.PL recipient mice were administered BrdU in their drinking water for 14 days. Bar diagrams display the frequency of donor CD90.2$^+$ CD8$^+$ T cells and host CD90.2$^-$ CD8$^+$ T cells that incorporated BrdU in the spleen (left panel) and bone marrow (right panel). Panel D) Bar diagram shows the total number of donor CD90.2$^+$ CD8$^+$ T cells in the spleen and bone marrow that have incorporated BrdU 14 days after adoptive cell transfer. The black bars represent B6.PL mice that received γHV68-memory CD8 T cells and the white bars represent B6.PL mice that received control irrelevant-memory CD8 T cells. Bars represent the mean value of three experimental animals and error bars indicate SEM.
Figure 3.5. Memory CD8 T cells isolated from γHV68-infected mice survive and proliferate in the absence of cognate viral antigens.
Figure 3.6. Memory CD8 T cells from γHV68 persistently-infected mice survive in an environment free of cognate γHV68-antigens, and mount a proliferative response during recall to γHV68.

B6.PL mice (CD90.1) received 1 x 10^6 CFSE-labeled CD44^+CD8^+ T cells isolated from the spleens of B6 mice (CD90.2) that have been infected with γHV68 for at least 3 months (γHV68-memory, dark bars) or from non-infected age-matched controls (irrelevant-memory, empty bars). Panel A) Bar diagrams represent the frequency of CD90.2^+CD8^+ T cells in the bone marrow, MLN, and spleen 30 days after adoptive cell transfer. Panel B) Bar diagrams show the total cell number of CD90.2^+CD8^+ T cells in the bone marrow, MLN, and spleen 30 days after adoptive transfer. Recipient mice were rested for 30 days without antigen after adoptive cell transfer and subsequently infected with γHV68. Panel C) Bar diagrams represent the frequency of donor CD90.2^+CD8^+ T cells in PBLs on day 0 (before γHV68 infection) and on day 14 after γHV68 infection. Bars represent the mean value of three experimental animals and error bars indicate SEM. Similar results were obtained in two independent experiments.
Figure 3.6. Memory CD8 T cells from γHV68 persistently-infected mice survive in an environment free of cognate γHV68-antigens, and mount a proliferative response during recall to γHV68.
Figure 3.7. “Rested” γHV68-specific memory CD8 T cells mount a proliferative response upon antigen recall.

B6.PL mice received 1 x 10^6 CFSE-labeled CD44^+ CD8^+ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months (γHV68-memory) or from non-infected age-matched controls (irrelevant-memory). The recipient B6.PL mice were rested for 30 days after adoptive cell transfer and subsequently infected with γHV68. All data presented here are from day 14 after γHV68 infection. Panel A) Representative FACS plots from mice that received γHV68 memory CD8 T cells (left panel) or control irrelevant memory T cells (right panel) by adoptive cell transfer. Left plot: CD8 vs. CD90.2, shows the frequency of donor CD90.2^+ CD8^+ T cells. Middle plot: CD8 vs ORF6_{487-495}/D^b, shows the frequency of tetramer-positive cells among donor CD90.2^+ CD8^+ T cells. Right plot: CD8 vs. ORF6_{524-531}/K^b, shows the frequency of tetramer-positive cells among donor CD90.2^+ CD8^+ T cells. Panel B) Bar diagrams indicate the frequency of donor CD90.2^+ ORF6_{487-495}/D^{b+} cells among CD8 T cells. Panel C) Bar diagrams represent the frequency of donor CD90.2^+ ORF6_{524-531}/K^{b+} cells among CD8 T cells. Black bars indicate B6.PL mice that received control irrelevant-memory CD8 T cells and gray bars indicate B6.PL mice that received γHV68-memory CD8 T cells transfer. Bars represent the mean value of six experimental animals and error bars indicate SEM. Similar results were obtained in two independent experiments.
Figure 3.7. “Rested” γHV68-specific memory CD8 T cells mount a proliferative response upon antigen recall.
Figure 3.8. γHV68-specific memory CD8 T cells survive long-term without cognate antigen and mount a recall response.

B6.PL mice received 1 x 10^6 CFSE-labeled CD44^+CD8^+ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months or from non-infected age-matched controls. The recipient B6.PL mice were rested for 60 days after adoptive cell transfer and subsequently infected with γHV68. FACS plots show the frequency of CD90.2^+CD8^+ T cells in PBLs and spleen at day 60 after adoptive transfer (left panel) and at day 14 after γHV68 infection (day 74 after adoptive cell transfer, right panel).
Figure 3.8. $\gamma$HV68-specific memory CD8 T cells survive long-term without cognate antigen and mount a recall response.
Figure 3.9. “Rested” γHV68-specific memory CD8 T cells mediate a protective recall response.

B6.PL mice received $1 \times 10^6$ CFSE-labeled CD44$^+$CD8$^+$ T cells isolated from the spleens of B6 mice that have been infected with γHV68 for at least 3 months (γHV68-memory, black bar) or from non-infected age-matched controls (irrelevant-memory, grey bar). The recipient B6.PL mice were rested for 30 days after adoptive cell transfer and subsequently infected with γHV68. Bar diagrams shows the number of infected cells splenocytes 14 days after infection. Bars represent the mean value of six experimental animals and error bars indicate SEM. Similar results were obtained in two independent experiments.
Figure 3.9. “Rested” γHV68-specific memory CD8 T cells mediate a protective recall response.
CHAPTER 4

TERMINALLY DIFFERENTIATED NKG2A+ KLRG1+ CD8 T CELLS MOUNT A FUNCTIONAL MEMORY AND RECALL RESPONSE

4.1 INTRODUCTION

Immunological memory is critical for protection against recurring infections and vaccinations, yet we have very little understanding of how memory T cell responses are regulated, specifically during latent persistent viral infections. The current thought is that CD8 T memory to acute infections is generally functional whereas the memory population to chronic viral infections becomes progressively dysfunctional with increasing amounts of antigen [53, 58]. During many chronic viral infections, it has been reported that CD8 T cells up-regulate a variety of receptors that have the capacity to modulate their function, and contribute to the CD8 inhibition of function [73, 74, 165]. There are increasing reports of the expression of a variety of inhibitory receptors on CD8 T cells during chronic viral infections [166, 167]. In particular, inhibitory NK cell receptors (iNKRs) were initially described on NK cells, and function to modulate NK cell activity [168]. NK cells have a combination of inhibitory (iNKRs) and stimulatory (sNKRs) receptors on their cell surfaces, and send their respective signals to the cell
when the receptors are engaged. When iNKRs bind their respective ligands on potential target cells, they send inhibitory signals to prevent killing by the NK cell [169]. However, the expression of iNKRs is not only restricted to NK cells. Recent studies have reported the up-regulation of iNKRs on CD8 T cells during both bacterial and viral infections [170]. Killer cell lectin-like receptor G1 (KLRG1) expression on CD8 T cells is associated with repeated antigen stimulation, decreased proliferative capacity, or complete lack of proliferative function altogether [171-173]. Additionally, KLRG1 expression on CD8 T cells has been used as a marker of senescence and terminal differentiation [171, 174]. The binding of KLRG1 to its ligand E-cadherin sends a negative signal to KLRG1 expressing cells to inhibit cytotoxicity [175][176]. Additionally, KLRG1 on CD8 T cells has been shown to affect the secretion of cytokines by dendritic cells [177]. Another iNKR, NKG2A, forms a heterodimer with CD94 and has been reported to prevent cytolysis by CD8 T cells after TCR engagement and ligand binding [178, 179]. NKG2A expression on CD8 T cells has also been implicated in providing a balance between infection clearance and immunopathology [180]. The ligands for these receptors are the non-classical MHC molecule Qa-1 for NKG2A and E-cadherin for KLRG1[175], [178]. NKG2A has recently been found to assist CD8 T cells in detecting antigen processing defects via its non classical MHC ligand Qa-1b [181], which could be very important during viral infections that cause down regulation of MHC class I presentation.

γHV68 is a low level persistent viral infection, in which the CD8 T cells retain their functional capacities, however, the virus is able to persist (albeit at low levels) for the life of the host [35, 182]. The memory CD8 T cells that are generated in response to
γHV68 persistent infections are heterogeneous in phenotype, and the majority of the virus specific CD8 T cells up-regulate the inhibitory receptors NKG2A and KLRG1 during the peak of infection, and the expression is maintained during long-term viral latency. We used the unique phenotype of γHV68 specific CD8 T cells, that the majority of virus specific cells express high levels of both KLRG1 and NKG2A, to ascertain the impact of the simultaneous expression of these receptors on CD8 T cell function. Previous studies suggest that CD8 T cells that express NKG2A and KLRG1 are deemed terminally differentiated [183]. A key unanswered question is to determine how the “terminally differentiated” memory CD8 T cell subsets function in the presence of persistent herpesvirus antigens, and to define the capacity of the terminally differentiated CD8 T cells to generate memory recall responses in vivo.
4.2 MATERIALS AND METHODS

Mice and viral infection

C57BL/6J and B6.PL-Thy1a/CyJ mice were obtained from The Jackson Laboratory and Harlan Farms, or were bred at the Research Institute at Nationwide Children’s Hospital. γHV68, clone WUMS, was propagated and titered on a monolayer of NIH3T3 fibroblasts. Mice were housed in BL2 containment under pathogen-free conditions. The Institutional Animal Care and Use Committee approved all of the animal studies described in this work. Mice were anesthetized with 2,2,2-tribromoethanol and intranasally inoculated with 1000 PFU γHV68 in 30 ul of HBSS.

Flow Cytometry Analysis

Single cell suspensions were obtained from the following organs: Spleen, lung, and bone marrow. RBCs were lysed, and the number of cells per organ was determined. Cells were stained with Fc block (CD16/32) and washed and stained with a combination of the following: γHV68-specific MHC tetramers: open reading frame ORF6487–495/D^b, ORF61524–531/K^b, and antibodies against CD8 (53-6.7), CD90.2 (53.2-1), NKG2A, KLRG1, CD62L (MEL-14), CD43 (1B-11), CD44 (IM7), CD122 (TM-b1), and CD127 (A7R34). MHC tetramers were generated, as described[87], or obtained from the National Institutes of Health Tetramer Core Facility. Flow cytometry data were acquired on a BD LSR II (BD Biosciences) and analyzed using FlowJo software. Gates were set using negative controls and isotype controls. For any given cell surface marker and tetramer combination, all the analysis gates are identical in size and position.
**Intracellular cytokine staining**

Single-cell suspensions were obtained from spleens. A total of $2 \times 10^6$ cells/sample were incubated in complete medium in the presence of IL-2 (10 U/ml), brefeldin A (10 ug/ml), and purified ORF6$_{487-495}$/D$^b$ peptide and ORF6$_{524-531}$/K$^b$ for 5 h at 37°C. As positive control, cells were stimulated with PMA/ionomycin. After Fc blocking, the cells were stained with the following antibodies, anti-NKG2A, anti-KLRG1, and anti-CD8, fixed, permeabilized, and stained with anti–IFN$\gamma$ (clone XMG1.2) and anti–TNF$\alpha$ (clone MP6-XT22), or isotype control Abs. The results were gated on the CD8$^+$ IFN$\gamma^+$ TNF$\alpha^+$ population. The data show the frequency of NKG2A and KLRG1 receptor expression in the CD8$^+$ IFN$\gamma^+$ TNF$\alpha^+$ population. In a separate experiment, the intracellular cytokine assay was performed as described above, with the addition of affinity purified blocking antibodies to NKG2A, KLRG1, Syrian hamster IgG, or Rat IgG2A during the 5-hour incubation. The results were gated on CD8$^+$ cells and show in representative FACs plots the expression of IFN$\gamma$ within the groups of antibody treatment.

**Degranulation Assay**

Single-cell suspensions were obtained from spleens as described above. A total of $2 \times 10^6$ cells/sample were incubated in 1mL of complete medium with 1ug/ml of each of anti-CD28 (clone 37.51), anti- CD49d (9C10 [MRF4.B]), anti-CD107a (1D4B), and anti-CD107b (ABL-93), purified ORF6$_{487-495}$/Db peptide and ORF6$_{524-531}$/Kb, IL-2 (10 U/ml), and brefeldin A (10 ug/ml). As positive control, cells were stimulated with PMA/ionomycin. In every experiment, a negative control (anti-CD28, anti-CD49d, anti-CD107a, and anti-CD107b) was included to control for spontaneous production of
cytokine and/or expression of CD107a/b. The cultures were incubated for 1 h at 37°C, followed by an additional 5 h in the presence of the secretion inhibitor monensin. After Fc blocking, the cells were stained with Abs anti-NKG2A, anti-KLRG1, and anti-CD8, fixed, permeabilized, and stained with anti-IFNγ or isotype control Abs. The results were gated on the CD8+ IFNγ+CD107a/b+ population. The data show the frequency of NKG2A and KLRG1 receptor expression in the CD8+ IFNγ+ CD107a/b+ population. In a separate experiment, the degranulation assay was performed as described above, with the addition of affinity purified blocking antibodies to NKG2A, KLRG1, Syrian hamster IgG, or Rat IgG2A during the 6-hour incubation. The results were gated on CD8+ cells and show in representative FACs plots the expression of IFNγ within the groups of antibody treatment.

In vivo cytotoxicity assay

Single-cell suspensions obtained from naive spleens were pulsed with ORF6487-495 peptide or irrelevant influenza NP366-374 peptide (1 ug/10⁶ cells) for 2 h at 37°C. The NP-pulsed control cells were stained with 50 nM CFSE and the relevant targets with 500 nM CFSE for 15 min at 37°C. The cells were thoroughly washed, combined in a 1:1 ratio, and a total of 1 x 10⁷ cells were injected intravenously into mice. Recipient mice were injected with purified NKG2A, KLRG1, Syrian hamster IgG, or Rat IgG2A antibodies on day -3 and day -1 before transfer of target cells. The presence of CFSE positive cells was analyzed in spleen cell suspensions from the recipient mice 18 h later. The results are shown as the percent-specific killing, which was calculated according to the formula:

\[
\text{percent-specific killing} = (1 - (\text{ratio of infected recipients/} \text{ratio of naïve recipients}) \times 100),
\]
where ratio = (number of CFSE^{high} / number of CFSE^{low}).

Adoptive cell transfers

Splenocytes from C57BL/6 mice that were at least 3 months post-γHV68 infection were processed into single-cell suspensions, as described above. Cells were plated in flasks coated with anti-mouse IgG plus IgM Abs (Jackson ImmunoResearch Laboratories) for 1 h to enrich for T cells. The nonadherent cells were incubated with Fc block (CD16/32) and washed and stained with anti-CD44, anti-CD8, anti-NKG2A, and anti-KLRG1 Abs. Cells were purified using a FACSVantage with Diva option; and CD8+CD44hi cells were gated on, and purified into NKG2A+KLRG1+ and NKG2A-KLRG1- populations (purity 98%). 1x10^6 purified cells from each group were injected i.v. into recipient B6.PL mice. The presence of CD8+CD90.2+ cells was analyzed in spleen cell suspensions from recipient mice at different time points after transfer. The presence of γHV68 was routinely analyzed in the spleen of host mice after adoptive transfer by limiting dilution nested PCR [35] and infective center assays.

Virus titers

The number of latently infected cells was determined on splenic cell suspensions on day 16 after infection using a standard infectious center assay as described [30]
4.3 RESULTS

γHV68 specific CD8 T cells express both KLRG1 and NKG2A receptors on cell surface during viral latency.

We sought to gain a better understanding of the heterogeneity of CD8 T cell subsets in the lymphoid organs and the respiratory tract during γHV68 latency. The up-regulation of the inhibitory molecules on CD8 T cells have been implicated in CD8 T cell functional exhaustion during chronic infections. It was thought that the B7/CD28 class of molecules could be regulating the CD8 T cell responses during gamma-herpes viral infection. Therefore, we initially analyzed the cell surface expression of the B7/CD28 family of receptors on γHV68 specific CD8 T cells during the acute and latent phase of gamma herpesvirus infection. The B7/CD28 family of receptors including ICOS, BTLA, CTLA, and PD-1, were not consistently up regulated on γHV68 specific CD8 T cells during gamma herpes viral persistence (data not shown). To determine the phenotype of γHV68 specific CD8 T cells with respect to other receptors, we analyzed the expression of a variety of inhibitory receptors on the CD8 T cells during γHV68 latency. The expression of NKG2A and KLRG1 on CD8 T cells has been previously reported during infections with antigen persistence. The analysis of NKG2A and KLRG1 expression was performed at the peak of the CD8 T cell response, (3 weeks post infection), and during viral latency, (3 months post infection). γHV68 specific CD8 T cells up-regulate the inhibitory receptors NKG2A and KLRG1 during the peak of infection, and the expression is maintained during viral latency, Figure 4.1A. The results compare the expression levels of KLRG1 and NKG2A in the spleen on two virus specific epitopes of CD8 T cells at 3
weeks and 3 months post infection and naïve mice were used as a negative control. Due to the fact that the majority of the γHV68 specific CD8 T cells expressed both NKG2A and KLRG1, we wanted to analyze the simultaneous expression of these receptors on a per cell basis. We next analyzed the dual expression of both NKG2A and KLRG1 on virus specific CD8 T cells in the spleen, lung and bone marrow of γHV68-infected mice during latency at 3 months post infection. The majority of γHV68 specific CD8 T cells (75%) expressed both of the receptors. In order to determine if the up regulation of NKG2A and KLRG1 was specific to γHV68 infection and not just a response to a respiratory viral infection or inflammation, the phenotype of influenza specific CD8 T cells was analyzed in the spleen, lung, and bone marrow of mice that had been infected with influenza virus (x-31) for 3 months. The results indicate that the influenza specific CD8 T cells have a distinct phenotype compared to the CD8 T cells from γHV68 infected mice or naïve control mice. Figure 4.1B shows representative FACs plots of the phenotypes of virus specific CD8 T cells in the spleen during γHV68 infection, influenza infection, and non-infected naïve control mice. The pie charts in Figure 4.1C demonstrate the percentage of virus specific CD8 T cells that express one or both of the receptors during γHV68 infection, influenza infection, or non-infected naïve controls. The majority of γHV68 specific CD8 T cells (75%) expressed both NKG2A and KLRG1 in the spleen, whereas the percentage of influenza specific CD8 T cells that expressed both the receptors in the spleen was ~12%. The frequency of naïve CD8 T cells that co-expressed NKG2A and KLRG1 in the spleen is ~4%. The percentages of the NKG2A and KLRG1 subsets were similar in the lung and bone marrow. Table 4.1 displays the mean percentages of the receptor expression profile of virus specific CD8 T cells plus or minus
the standard deviation in parentheses, and compares the percentages of each NKG2A/KLRG1 subset for each organ analyzed between the 3 groups of infection models. The results indicate that γHV68 specific CD8 T cells during latency have a unique phenotype of NKG2A KLRG1 expression and that the expression profile is distinct from that of other acute respiratory infections or their naïve counterparts.

**Correlation of CD8 T cell memory subsets with NKG2A and KLRG1 expression**

Previous reports have shown that KLRG1 expression on CD8 T cells has been associated with replicative senescence. In addition, the expression of these markers is associated with an effector phenotype and function, and terminally differentiation. In essence, the cells that express these two receptors do not undergo homeostatic proliferation in response to cytokines and are usually short lived. We wanted to correlate CD8 T cell memory subsets with the expression of NKG2A and KLRG1 in our model to define the population phenotypically. The virus specific CD8 T cells were grouped into memory subsets based on their expression of CD62L and CD43 associated glycoform surface stain. CD62Llo/CD43hi are considered effector cells, (TE) they have immediate effector function and are not long lived. CD62lo/CD43lo are effector memory cells (TEM) and have an immediate function, but are longer lived than their effector counterparts and are located in the periphery. CD62L hi/CD43lo are central memory cells (TCM), which are a long-lived quiescent subset that is located mainly in the lymphoid organs. Representative FACs plot in figure 4.2A show the breakdown of memory subsets by phenotype. In figure 4.2B, the percentage of cells that express none,
one, or both of the NKG2A and KLRG1 receptors is compared between the 3 different memory subsets (effector, effector memory, and central memory) among the three different infection groups. The results are gated on CD8+ tetramer+ cells, and then divided into the corresponding memory populations as defined above. The bar diagrams indicate the percentage of NKG2A and KLRG1 receptor expression in the three memory populations, fig 4.2B. The results demonstrate that during γHV68 latency the majority of effector and effector memory phenotypes express NKG2A and KLRG1, while the majority of central memory CD8 T cells do not express NKG2A and KLRG1. Our results indicate that NKG2A+KLRG1+ CD8 T cells are of an effector and effector memory phenotype, and this result correlates to recent reports suggesting that the NKG2A+KLRG1+ cells are terminally differentiated cells.

**NKG2A+KLRG1+ CD8 T cells have sustained effector function during γHV68 latency**

One of the hallmarks of CD8 T cells is their cytotoxic effector function. CD8 T cells have the ability to secrete cytokines and release cytotoxic granules that kill virally infected cells. The function of NKG2A and KLRG1 receptor expression has been tested on NK cells, and to a much lesser extent on CD8 T cells. On NK cells, NKG2A and KLRG1 send inhibitory signals after binding to their respective ligands. After ligand binding, the SHP1/2 phosphatases are recruited to the intracellular ITIM domain on the receptors sending an inhibitory signal to the cell. However, the inhibitory function of NKG2A and KLRG1 on CD8 T cells is much less understood. Reports have demonstrated a small decrease in *in vitro* cytotoxicity of CD8 T cells that express NKG2A. In addition, KLRG1 expression has been shown to be a marker replicative
senescence. Our model of persistence allows us to explore the functions of both of these receptors simultaneously on CD8 T cells. To investigate the functional properties of these terminally differentiated CD8 T cells, their effector function was assayed. We measured IFNγ and TNFα production by intracellular cytokine staining. After in vitro stimulation with two γHV68 peptides (ORF6_{487} and ORF61_{524}), the CD8 T cells could make both IFNγ and TNFα, indicating that the CD8 T cells were polyfunctional, figure 4.3A left two panels. We observed a significant increase in the production of IFNγ and TNFα by the CD8 T cells that were stimulated with class I specific peptides compared to the non-stimulated controls, figure 4.3A third panel. PMA/Ionomycin was used as a positive control to demonstrate the potential of CD8 T cells to make both IFNγ and TNFα, figure 4.3A right panel. To define what cells were producing IFNγ and TNFα, we analyzed the expression of NKG2A and KLRG1 in the polyfunctional IFNγ+TNFα+ CD8+ T cells. Upon further investigation, the majority of the cells that were making the cytokines in response to viral specific peptides were NKG2A+ and KLRG1+ CD8+ T cells (80%). Figure 4.3B. Next, we tested whether antibody engagement of the iNKR receptors on the CD8 T cells in vitro has any impact on the ability of the CD8 T cells to make cytokines. Splenocytes from mice infected for 3 months with γHV68 were stimulated ex vivo with class I γHV68 peptides ORF6_{487} and ORF61_{524}, and were simultaneously incubated with saturating levels of either anti-KLRG1, anti-NKG2A, or the corresponding isotype controls. IFNγ production was measured by FACs analysis in an intracellular cytokine assay. Figure 4.3C shows representative FACs plots of splenocytes stimulated with ORF61_{524}, in addition to anti-KLRG1, anti-NKG2A, isotype controls, or no antibody controls. The analysis was previously gated on CD8+ T cells.
The results demonstrate that the virus specific production of IFNγ was unchanged between the five groups of antibody treatment. One possibility of this result could be that the virus-peptide signal to the CD8 T cells can override the inhibitory effect of the two receptors. The engagement of these iNK receptors on the cell surface of the ORF61\textsubscript{524} virus specific CD8 T cell was not sufficient to inhibit interferon production. Altogether these results indicate that majority of the functional virus specific CD8 T cells, are the terminally differentiated NKG2A+KLRG1+ effector/effector memory CD8 T cells. The presence of the receptors on the CD8 T cell surface did not alter the ability of the CD8 T cell to produce cytokines. Moreover, antibody engagement of the inhibitory receptors in the presence of virus specific peptide did not inhibit cytokine production. In essence, the virus specific CD8 T cells that are polyfunctional are the terminally differentiated NKG2A+KLRG1+ cells, and the expression or engagement of these receptors on the cell surface does not pose any detrimental effect to the CD8 T cell.

After demonstrating that the CD8 T cells were polyfunctional with respect to IFNγ and TNFα production, we sought to uncover whether the CD8 T cells during γHV68 latency were multifunctional with regards to IFNγ production and cytotoxicity. To address this question, we utilized the degranulation marker CD107a/b, which is a surrogate marker of cytolytic T cells [184], and performed an in vitro degranulation assay to analyze the amount of IFNγ production and CD107a/b expression. Splenocytes were isolated from mice that were infected with γHV68 for 3 months and ex vivo stimulated with the class I γHV68 specific peptides ORF6\textsubscript{487} and ORF61\textsubscript{524} or PMA/ionomycin as a positive control. The results shown in figure 4.4A show representative FACs plots of CD107a/b and IFNγ staining. The plots are previously gated on CD8+ T cells and the
numbers indicate the frequency of IFN\(\gamma\)+CD107+ cells of the total CD8 T cells. The first two panels are FACS plots depicting spleens that have been stimulated with ORF6\(_{487}\) and ORF6\(_{1524}\) peptides, and the frequency of IFN\(\gamma\)+CD107+ cells is 1% and 2% respectively. There is essentially no IFN\(\gamma\) or CD107a/b staining when the same splenocytes are not stimulated with any peptides. The fourth plot is the positive control demonstrating the total number of cells capable of producing IFN\(\gamma\) as a result of adding PMA/ionomycin to the cells. We analyzed the expression of NKG2A and KLRG1 of the polyfunctional CD8+IFN\(\gamma\)+CD107+ cells. The results indicate that the majority of cells that produce IFN\(\gamma\) and express CD107a/b on their cell surface in response to two \(\gamma\)HV68 specific peptides express both NKG2A and KLRG1 shown in figure 4.4B. These results demonstrate that the polyfunctional \(\gamma\)HV68 specific CD8 T cells express NKG2A and KLRG1.

We next directly assessed the in vivo cytotoxic capacity of ORF6\(_{487}\) specific CD8 T cells 3 months post \(\gamma\)HV68 infection. Previous reports analyzing the effects of KLRG1 or NKG2A expression on CD8 T cells have looked into the receptor-ligand interactions to address whether the engagement of the NKG2A and KLRG1 receptors may affect its cytotoxicity. In the previous experiment, antibody engagement of these two receptors did not change the production of cytokines in vitro. However, we wanted to assess cytotoxicity as an additional function of CD8 T cells in an in vivo setting. In order to address this issue, antibodies specific to NKG2A, KLRG1, or the corresponding isotype control were administered by intraperitoneal injection (I.P.) into mice at day -3 and -1 before the in vivo cytotoxicity assay. The NKG2A+ and KLRG1+ CD8 T cells were not depleted (data not shown); as the virus specific cell numbers remained the same. The
elimination of ORF6<sub>487</sub> loaded target cells was analyzed 18 hours after transfer. Figure 4.4C displays the representative histograms of CFSE stained cells in the 5 experimental groups of mice. There were no significant differences observed in percent specific killing between the mice that received anti KLRG1, anti NKG2A, or the isotype controls, Figure 4.4D. The results demonstrate that the engagement of the receptors (or the in vivo blockade of ligands) does not affect the cytolytic function of the γHV68 specific CD8 T cells during viral latency. Altogether these data indicate that terminally differentiated NKG2A+KLRG1+ γHV68 specific CD8 T cells have the capacity to mount a functional effector response with regard to IFNγ and TNFα dual production. Additionally, these terminally differentiated cells have cytotoxic functions demonstrated by CD107a/b degranulation and in vivo cytotoxicity. The take home message from this data is that NKG2A+KLRG1+ γHV68 specific CD8 T cells are polyfunctional effector/effector memory cells with the capacity for producing IFNγ, TNFα, and killing.

**NKG2A+KLRG1+ CD8 T cells mount a protective recall response**

Previous reports have discussed that CD8 T cells that express KLRG1 have undergone an extensive number of cell divisions [172, 185]. In addition, the KLRG1+ cells were severely impaired in their ability to proliferate in response to antigen, and were termed senescent [172]. KLRG1 has become a marker of a non-replicative, senescent, terminally differentiated subset of CD8 T cells, meaning that they are incapable of proliferating homeostatically or in response to cognate antigen [186]. We investigated if NKG2A+KLRG1+ CD8 T cells and NKG2A-KLRG1- CD8 T cells from γHV68 latently infected mice can mount an effective recall response to secondary γHV68, by utilizing an
adoptive transfer strategy. We FACs purified antigen-experienced CD8 T cells (CD44 hi CD8+) into a NKG2A+KLRG1+ population and NKG2A-KLRG1- population from mice that were infected with γHV68 for at least 3 months, and adoptively transferred 10^6 cells into naïve CD90.1 congenic recipients. The average frequency of virus specific cells transferred per mouse was 5,000 ORF6487+ and 50,000 ORF61524+. 24 hours after adoptive transfer, we challenged the mice with γHV68. 16 days after infection, we assessed the frequency and number of virus specific donor cells in the spleens of infected mice. Figure 4.5A are representative FACs plots demonstrating the frequencies of donor CD8 T cells (CD90.2+ cells) in the total CD8 T cell population of mice that received NKG2A+KLRG1+ CD8 T cells (left panel) and NKG2A-KLRG1- CD8 T cells (right panel). In the bottom panel of Figure 4.5A, are representative FACs plots showing the frequencies of ORF6487 specific CD8 T cells in the donor cell population in both the NKG2A+KLRG1+ and NKG2A-KLRG1- transfer groups. Bar diagrams indicate the frequencies of donor CD8 T cells (CD90.2+) in the total CD8 T cell population, ranging from 1.5-2.5% (Figure 4.5B left panel). The bar diagram in right panel illustrates the total cell number of donor CD8 T cells in the spleen, and shows no significant difference between the two transfer groups. Gray bars indicate the NKG2A+KLRG1+ transfer group, and black bars indicate NKG2A-KLRG1- transfer group. The bottom left panel demonstrates the percentage of ORF6487+CD90.2+ CD8 T cells in the NKG2A+KLRG1+ and NKG2A-KLRG1- transfer groups. Although the frequencies are variable, the bar diagrams displaying the total cell number of ORF6487+CD90.2+ CD8 T cells in the spleen of NKG2A+KLRG1+ and NKG2A-KLRG1- transfer groups shows no difference in the total number of virus specific donor cells (right bottom panel). Next we analyzed
the number of latently infected cells in the spleens of mice that had been infected with γHV68 the day after adoptive transfer via an infectious center assay. This experiment was to assess the immediate effector function of the adoptively transferred CD8 T cells to mount a protective recall response to a secondary γHV68 challenge. The number of latently infected cells/10^6 splenocytes is shown in the bar diagram in figure 4.5C. The NKG2A+KLRG1+ transfer group had significantly less latently infected splenocytes than the NKG2A-KLRG1- transfer group (p<.0008), demonstrating that ++ cells can protect against latency better than the -- transfer group. This result suggests that although the frequencies and total cell numbers of the virus specific donor CD8 T cells are similar, the terminally differentiated NKG2A+KLRG1+ CD8 T cells have a functional protective advantage over their NKG2A-KLRG1- central memory CD8 T cell counterparts. In addition, the NKG2A+KLRG1+ CD8 T cells, which have an effector/effector-memory phenotype, and are terminally differentiated have better immediate effector recall responses than the central memory NKG2A-KLRG1- CD8 T cells and thus are qualitatively more functional in an immediate recall response. Altogether these results indicate that the terminally differentiated NKG2A+KLRG1+ CD8 T cells do have the potential to mount a protective recall response when it is needed.

**Terminally differentiated NKG2A+KLRG1+ memory CD8 T cells mount a functional recall and protective memory response.**

The ability of the immune system to remember previously encountered antigens is a hallmark of the adaptive immune response. Memory CD8 T cells possess the capacity to mount responses to previously encountered intracellular pathogens, and thus provide
an important role in immunity to viruses. However, memory is not clearly defined during persistent viral infections such as γHV68. In earlier studies, we have shown that functional CD8 T cell memory is generated and maintained during γHV68 persistence. Additionally, the γHV68 memory CD8 T cells do not require antigen for their survival and proliferation. We wanted to investigate the potential of the γHV68 memory CD8 T cells to survive and proliferate in an antigen free environment using the NKG2A and KLRG1 markers to further divide the two memory populations. First we addressed whether the NKG2A/KLRG1 CD8 T cell populations express CD127 and CD122, which are the cell surface receptors for IL-7 and IL-15, respectively. It has been well described that memory CD8 T cells undergo homeostatic proliferation in response to the cytokines IL-7 and IL-15. We asked whether both NKG2A+KLRG1+ and NKG2A-KLRG1- populations expressed these two cell surface receptors. Spleens from mice at 3 months post infection with γHV68 (same time post infection as splenocytes used for adoptive transfer studies) were isolated and stained with the ORF61524 tetramer and antibodies CD8, KLRG1, NKG2A, CD122, and CD127 and analyzed by flow cytometry. Results in Figure 4.6A are shown as histograms of the expression levels of CD122 and CD127 in the different population gates. Gray filled histograms are representative of unstained controls. CD8 represents the total CD8+ T cell population; ORF61524 represents total ORF61524+ CD8+ T cells. The ++ population was gated on CD8+ ORF61524+ NKG2A+KLRG1+ cells and then their expression of CD122 or CD127. The -- population was gated on CD8+ ORF61524+ NKG2A-KLRG1- cells and then their expression of CD122 or CD127. Figure 4.6B is a bar diagram displaying the mean fluorescence intensity, (MFI) of CD122 (gray bars) and CD127 (black bars) in the
populations described above. In each of the groups described, the majority of the cell population expressed CD122 and CD127.

Next, we tested if NKG2A+KLRG1+ CD8 T cells and NKG2A-KLRG1- CD8 T cells from γHV68 latently infected mice could mount an effective memory recall response to secondary γHV68 challenge, using an adoptive transfer strategy. We FACs purified antigen-experienced CD8 T cells (CD44 hi CD8+) into a NKG2A+KLRG1+ population and NKG2A-KLRG1- population from mice that were infected with γHV68 for at least 3 months, and adoptively transferred 10^6 cells into naïve CD90.1 congenic recipients. In order to assess the memory response of the antigen experienced CD8 T cells, we allowed the adoptively transferred cells to “rest” in an antigen-free environment for at least 30 days before analyzing their frequency in the recipient animals and determining their NKG2A/KLRG1 phenotype. Figure 4.7A shows FACs plots of the NKG2A/KLRG1 phenotype of the two groups of CD8 T cells that were adoptively transferred into naïve congenic mice at day 0 (day of transfer). After resting the transferred CD8 T cells for 30 days without antigen, we then determined the transferred CD8 T cells’ NKG2A KLRG1 phenotype at day 30. Figure 4.7A second panel are representative FACS plots showing the frequency of donor CD8 T cells in the recipient’s CD8 T cell population. The transferred CD8 T cells retained their original phenotype after 30 days without cognate antigen, figure 4.7A third panel, demonstrating that in the absence of cognate antigen stimulation, the NKG2A/KLRG1 phenotype remains unchanged. The bar diagram in figure 4.7B shows the percentage of donor CD8 T cells (CD90.2) that are NKG2A+KLRG1+ in the two different groups of transfers. The results in Figure 4.7 show that both the terminally differentiated effector memory cells
(NKG2A+KLRG1+) and the central memory cells (NKG2A-KLRG1-) have the capacity to survive without antigen for at least 30 days after adoptive transfer. In addition, the transferred CD8 T cells retain their original NKG2A/KLRG1 phenotype after being “rested” in an antigen free environment.

Next, we challenged the recipient mice with γHV68, 30 days after adoptive transfer. This strategy tests the capacity of the antigen-experienced CD8 T cells to mount a memory recall response, via proliferation and protection against infection. The experimental approach selects for antigen-experienced CD8 T cells that are capable of surviving without antigen for 30 days, and thus are the long-lived memory CD8 T cells. The results shown are at day 16-post γHV68 infection. (Day 46 post AT, day 16 dpi)

Figure 4.8A top panel are representative FACs plots showing the presence of donor cells (CD90.2+) in the spleens of mice day 46 after adoptive transfer and day 16 after γHV68 secondary challenge for both mice that received NKG2A+KLRG1+ (iNK+) and NKG2A-KLRG1- (iNK-) CD8 T cells. The numbers represent the frequency of donor cells in the total CD8 T cell spleen pool. Figure 4.8A bottom panel are representative FACs plots showing the frequency of virus specific donor CD8 T cells (CD8+CD90.2+ ORF6487+) in the donor CD8 T cell population. This data shows that in both the (iNK+) and the (iNK-) adoptive transfer groups, virus specific CD8 T cells could survive 30 days without cognate antigen, and upon rechallenge mount a recall response by proliferating in response to viral antigens. The frequencies of donor cells were similar between the iNK+ and iNK- transfer groups, demonstrating that their proliferation capacities were comparable. In Figure 4.8B the bar graph indicates the number of latently infected spleen cells per 10^6 cells calculated by an infectious center assay at day 16 post
rechallenge in the iNK+, iNK-, and no transfer control groups. The results show there is no significant difference between the protection capacities of the iNK+ or iNK-adaptively transferred cells, but there is a significant difference between the adoptively transferred cells and no adoptive transfer controls.

4.4 DISCUSSION

The expression of NKG2A and KLRG1 on CD8 T cells has been reported during a variety of viral infections, in particular chronic/persistent infections [3, 114, 173, 178, 187, 188]. Recent reports indicate that repeated antigen stimulation causes the up-regulation of KLRG1 and is associated with impaired proliferative capacity [172, 173]. NKG2A has been reported to inhibit CD8 T cell cytotoxicity, when bound to its ligand Qa-1 [178]. Additionally, KLRG1 has become a marker of T cell senescence and terminal differentiation in humans and mice due to the lack of clonal expansion after activation on CD8 T cells that express KLRG1 [171, 172, 174]. However, the physiological role of the presence of NKG2A and KLRG1 on CD8 T cells is still rather unclear. Our findings show that terminally differentiated virus specific CD8 T cells are fully functional during a persistent viral infection. In addition, terminally differentiated CD8 T cells have the ability to survive long term without antigen as bona fide memory T cells do, and when challenged mount a protective antiviral recall response. Altogether, this data indicate that terminally differentiated effector memory CD8 T cells are functionally as good as the “true” central memory CD8 T cells.
We found that ~70% of the γHV68 virus specific CD8 T cells express both KLRG1 and NKG2A during γHV68 latency. Since the majority of virus specific CD8 T cells express both KLRG1 and NKG2A, we used γHV68 viral infection as a model to study these receptors on the CD8 T cell. This model allowed us to study the function of NKG2A and KLRG1 expressed simultaneously on virus specific CD8 T cells, alongside virus specific CD8 T cells that do not express these receptors.

Our results show that the NKG2A+KLRG1+ CD8 T cells have an effector/effector-memory phenotype, while their NKG2A-KLRG1- counterparts display a central memory phenotype. The CD8 T cells that express KLRG1 and NKG2A are functional effectors with respect to IFNγ and TNFα production as well as killing capacity. Moreover, of the CD8 T cells that are polyfunctional, 80% are of the KLRG1+NKG2A+ phenotype. This finding indicates that KLRG1+NKG2A+ CD8 T cells have immediate effector functions, which corresponds to their effector/effector-memory phenotype. The addition of anti-NKG2A and anti-KLRG1 antibodies to block the receptor/ligand interactions did not change the functional activity of the CD8 T cells with regard to IFNγ, TNFα, degranulation, and in vivo cytotoxicity. Previous studies have reported that KLRG1 and NKG2A expression on CD8 T cells may modulate their function in an inhibitory way [180] [189]. However, this is hard to reconcile with our previous studies demonstrating that γHV68 specific CD8 T cell memory is fully functional, providing that 70% of γHV68 specific CD8 T cells express NKG2A and KLRG1. Thus, contrary to the belief that these receptors are inhibitory on CD8 T cells, in the γHV68 infection model, there was no evidence of CD8 T cell inhibition. Our results suggest that the virus specific peptide/MHC signal sent to the CD8 T cell may override
any inhibitory effect these receptors are sending to the CD8 T cell. During times of inflammation and/or infection, the incidental role of the receptors on the cell surface may be completely superseded. There is a possibility the effect is too small to measure in our system and that to observe a physiological effect of these receptors we may need to amplify the amount of CD8 T cells, or have an excess of ligand expressing target cells. However, our findings indicate that the presence of these receptors on CD8 T cells is not necessarily inhibitory, and may be a sign of functional activation and differentiation, particularly during infection and inflammation.

Our previous studies provide evidence that fully functional antigen-independent CD8 T cell memory can be generated during persistent viral infections [35]. In addition, the CD8 T cell memory that is formed is capable of mounting a protective recall response to rechallenge [35, 190]. However, the contribution of the terminally differentiated KLRG1+NKG2A+ CD8 T cell population to the memory response has not been evaluated. It has been shown during other persistent infections that when the infection is cleared, a population of functional memory CD8 T cells forms and is stably maintained [164]. However, the role of specific subsets, or which subset is providing the protective function has not been analyzed. In this study, we analyzed the contribution of terminally differentiated effector memory cells along side the central memory population to provide an immediate recall response. Our findings demonstrate that after adoptive transfer of the two sub populations of CD8 T cells into naïve congenic animals, the terminally differentiated CD8 T cells protected as well as or better than the central memory CD8 T cells. This finding corresponds to our previous phenotypic and functional analysis demonstrating that KLRG1+NKG2A+ CD8 T cells are polyfunctional, activated cells,
and reiterates the concept that these terminally activated cells show no signs of inhibition and are capable of mounting a protective immune response. The generation of long-lived functional memory responses is crucial for protection against secondary infections and is an important goal of vaccination strategies [53, 191]. In order to target vaccines for stable CD8 T cell memory production, it is essential to know which CD8 T cells are capable of long term survival and protection. The central memory CD8 T cell subset has been shown to be critical for the memory recall response [53], whereas other studies have demonstrated the importance of effector memory cells in memory recall [57]. Depending of the type and route of infection, different memory CD8 T cell subsets contribute to protection against secondary infections [192]. Interestingly, a recent study correlates memory recall effectiveness with the expression of activation markers such as KLRG1, among others, on the CD8 T cell, rather than their central or effector memory status [193]. Our findings show that NKG2A+KLRG1+ CD8 T cells survive without cognate antigen and retain their NKG2A/KLRG1 phenotype, probably due to the expression of IL-7 and IL-15 receptors. Our finding indicates that the NKG2A/KLRG1 phenotype is not entirely due to recent antigen stimulation, and that changing the environment to a setting without antigen does not convert the inherent phenotype into a central memory cell. Additionally, after resting without antigen, both the terminally differentiated CD8 T cells and central memory CD8 T cells expand in response to viral antigens, showing that they have proliferative capacity, and have the ability to protect against secondary infection equally. Thus, these terminally differentiated NKG2A+ KLRG1+ CD8 T cells are functionally equivalent to their central memory cell counterparts in this aspect, and our results suggest a role for
both terminally differentiated CD8 T cells and central memory CD8 T cells in the overall memory recall response.

The presence of NKG2A and KLRG1 on the CD8 T cell surface may be indicative of repetitive antigen stimulation and increased proliferation, however, these receptors do not lock the CD8 T cell into a terminally exhaustive state. The idea that the CD8 T cell memory population’s fate is flexible has been addressed during an acute viral infection [52], where CD8 T cells that functioned as effectors during the primary infection, functioned in the proliferative memory recall response to a secondary infection. It is likely that CD8 T cell memory is quite flexible, and can adapt to conditions when necessary. The ability of the terminally differentiated CD8 T cells to become functional memory and mount a response is possible, during both acute and persistent infections. Our data show the plasticity of CD8 T cell memory, in that terminally differentiated CD8 T cells have the capacity to become long-lived functional memory CD8 T cells, and mount an immune response when presented with a viral challenge. Our results show that terminally differentiated effector memory cells function equally in their ability to form memory, survive without antigen, proliferate, and protect against secondary challenge as their central memory counterparts. This finding is rather novel in the field of memory to persistent viral infections and has important implications in that terminally differentiated CD8 T cells do contribute to the long-lived and protective capacities of an effective memory population. KLRG1+NKG2A+ CD8 T cells display all the functions of true memory CD8 T cells and can act as bona fide memory cells. These results raise the idea that heterogeneous memory may be the best strategy during persistent viral infections; to have activated effector memory cells that can produce IFN\(\gamma\), TNF\(\alpha\), and kill virally
infected target cells alongside central memory cells. Depending on the virus and route of infection, it may be advantageous to have a heterogeneous population of CD8 T cell memory. This information is important for the design of successful vaccination strategies; in that to achieve the most effective protection, both effector memory and central memory CD8 T cell populations should be targeted.
Figure 4.1. The majority of virus specific CD8 T cells during γHV68 latency co-express the receptors NKG2A and KLRG1. A, Representative histograms of KLRG1 and NKG2A expression in the spleens of the γHV68 virus specific CD8 T cell populations (ORF6_{487-495}/D^b and ORF61_{524-531}/K^b) at different times after γHV68 infection. The dotted line represents naïve uninfected control mice, the solid line indicates 3 weeks post infection, and gray fill indicates 3 months post infection with γHV68. B, Splenocytes from mice that were infected for 3 months with γHV68, 3 months with influenza virus (X-31), or naïve age-matched controls were used. The data are gated on CD8+ virus specific cells, and results shown are representative FACs plots of the NKG2A and KLRG1 co-expression. C, Pie charts show the distribution of NKG2A KLRG1 expression in the spleen, lung, and bone marrow in mice infected for 3 months with γHV68, influenza virus, or naïve age-matched controls. Purple indicates the percentage of cells that are NKG2A+KLRG1+, green indicates the percentage of cells that are NKG2A+KLRG1-, red indicates the percentage of cells that are NKG2A-KLRG1+, and yellow indicates the percentage of cells that are NKG2A-KLRG1- in each infection.
Figure 4.1. The majority of virus specific CD8 T cells during γHV68 latency co-express the receptors NKG2A and KLRG1
Table 4.1. NKG2A KLRG1 receptor expression profile.

Displays the NKG2A KLRG1 percentage expression in the three organs (spleen, lung, bone marrow) between the 3 groups of viral infections. The results shown in the table are the average of the frequencies of each NKG2A/KLRG1 population in the CD8+ virus specific gate ORF61\textsubscript{524-531}/K\textsuperscript{b} (n = 3), and the numbers in parentheses indicate standard deviation.
Table 4.1. NKG2A KLRG1 receptor expression profile.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Virus</th>
<th>NKG2A- KLRG1+</th>
<th>NKG2A+ KLRG1+</th>
<th>NKG2A+ KLRG1-</th>
<th>NKG2A- KLRG1-</th>
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<tr>
<td>Spleen</td>
<td>γHV68</td>
<td>17.4 (8.3)</td>
<td>76.5 (7.5)</td>
<td>2.2 (1.0)</td>
<td>3.7 (0.4)</td>
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<td></td>
<td>Influenza</td>
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<td>11.7 (2.6)</td>
<td>58.0 (2.0)</td>
<td>27.2 (2.6)</td>
</tr>
<tr>
<td></td>
<td>naïve</td>
<td>2.2 (0.9)</td>
<td>3.7 (2.2)</td>
<td>7.1 (4.9)</td>
<td>87.0 (8.0)</td>
</tr>
<tr>
<td>Lung</td>
<td>γHV68</td>
<td>15.6 (7.5)</td>
<td>79.5 (5.2)</td>
<td>2.9 (2.0)</td>
<td>2.0 (0.7)</td>
</tr>
<tr>
<td></td>
<td>Influenza</td>
<td>3.9 (3.4)</td>
<td>29.2 (2.3)</td>
<td>41.7 (16.2)</td>
<td>24.3 (15.6)</td>
</tr>
<tr>
<td></td>
<td>naïve</td>
<td>1.2 (0.3)</td>
<td>1.8 (0.2)</td>
<td>1.2 (0.3)</td>
<td>95.9 (0.4)</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>γHV68</td>
<td>21.6 (4.1)</td>
<td>58.8 (2.8)</td>
<td>5.0 (1.8)</td>
<td>14.6 (0.9)</td>
</tr>
<tr>
<td></td>
<td>Influenza</td>
<td>6.1 (2.60)</td>
<td>9.8 (3.4)</td>
<td>47.6 (7.1)</td>
<td>36.7 (3.6)</td>
</tr>
<tr>
<td></td>
<td>naïve</td>
<td>3.0 (1.5)</td>
<td>4.5 (3.8)</td>
<td>0.9 (0.5)</td>
<td>91.7 (5.2)</td>
</tr>
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Figure 4.2. NKG2A+KLRG1+ virus specific CD8 T cells are of an effector/effector memory phenotype, during γHV68 latency. A, Representative FACs plot showing the gating strategy used to distinguish effector, effector memory, and central memory populations using the markers CD62L and CD43 associated glycoform. B, Bar diagrams representing the frequencies of the NKG2A/KLRG1 subsets in the effector, effector memory, and central memory populations, using the gating strategy above. Results were gated on CD8+ virus specific cells ORF61524-531/Kb in the γHV68 and NP366-374/Db + in the influenza group.
Figure 4.2. NKG2A+KLRG1+ virus specific CD8 T cells are of an effector/effecter memory phenotype, during γHV68 latency
Figure 4.3. Analysis of cytokine production by virus specific CD8 T cells expressing or not NKG2A or KLRG1. A, Representative FACs plots of intracellular cytokine production assaying for TNF$\alpha$ and IFN$\gamma$. Splenocytes from mice that were infected for 3 months with $\gamma$HV68 were stimulated ex vivo with ORF6$^{487-495}/D^b$, ORF6$^{524-531}/K^b$, PMA/ionomycin, or no peptide controls, and results were gated on CD8$^+$ T cells. B, Bar diagram displaying the percent receptor expression of KLRG1 and NKG2A in the CD8$^+$TNF$\alpha$+IFN$\gamma$ + gate for both ORF6$^{487-495}/D^b$ and ORF6$^{524-531}/K^b$ peptides. N=3, results done in triplicate. C, Representative FACs plots showing IFN$\gamma$ production from splenocytes that were stimulated ex vivo with ORF6$^{524-531}/K^b$ in addition to blocking antibodies to KLRG1, NKG2A, or their respective isotype controls.
Figure 4.3. Analysis of cytokine production by virus specific CD8 T cells expressing or not NKG2A or KLRG1.
Figure 4.4. Analysis of effector function: CD8 T cell killing capacity in vitro and in vivo. A, Representative FACs plots of intracellular cytokine production assaying for IFNγ and cell surface stain for CD107a/b. Splenocytes from mice that were infected for 3 months with γHV68 were stimulated ex vivo with ORF6487-495/Db, ORF61524-531/Kb, PMA/ionomycin, or no peptide controls, and results were gated on CD8+ T cells B, Bar diagram displaying the percent receptor expression of KLRG1 and NKG2A in the CD8+IFNγ+CD107a/b+ gate for both ORF6487-495/Db and ORF61524-531/Kb peptides, n=3, results done in triplicate, error bars represent standard deviation. C, In vivo cytotoxicity assay, representative histograms of CFSE+ cells in the spleen 18 hours post adoptive transfer of the 5 groups of mice that received CFSE labeled splenocytes. The lower concentration of CFSE cells were pulsed with an irrelevant peptide (NP) and higher concentration of CFSE labeled cells were pulsed with ORF6487-495/Db. The recipient mice were 3 months post γHV68 infection and were previously I.P. injected 3 days previously with the corresponding antibodies indicated above the FACs plots; naïve mice were used as controls. D, Bar diagram shows the calculated specific lysis of ORF6487-495/Db loaded cells between the groups of mice, (n=3) and error bars display standard deviation.
Figure 4.4. Analysis of effector function: CD8 T cell killing capacity in vitro and in vivo.
Figure 4.5. Terminally differentiated virus specific NKG2A+ KLRG1+ CD8 T cells have an immediate functional effector recall response. NKG2A+KLRG1+ (iNK+) CD8+ T cells and NKG2A-KLRG1- (iNK-) CD8+ T cells from mice infected with γHV68 for 3 months were FACs cell sorted and adoptively transferred into naïve congenic animals and then challenged with γHV68 the next day. A, Results show the frequency of transferred cells by CD90.2 expression in the spleens of the recipient animals 16 days post infection. Lower panel, representative FACs plots showing the frequency of CD8+CD90.2+ORF6_{487-495} + cells in the NKG2A+KLRG1+ (iNK+) transfer group and in the NKG2A-KLRG1- (iNK-) transfer group. B, Bar diagrams shows the frequency of transferred cells in the total CD8 T cell population in the NKG2A+KLRG1+ (iNK+) group and NKG2A-KLRG1- (iNK-) group (left panel), as well as the total cell number of transferred cells in the spleens, (right panel). Bottom left panel shows the frequency of CD8+CD90.2+ORF6_{487-495} + cells in the spleen and bottom right panel shows the total cell numbers of CD8+CD90.2+ORF6_{487-495} + cells in the spleen, n=3. C, Results obtained from an infectious center assay. The bar diagram displays the number of latently infected spleen cells per 10^6 splenocytes 16 days after γHV68 challenge, and 17 days after adoptive transfer, n=3, in triplicate. Error bars indicate standard deviation.
Figure 4.5. Terminally differentiated virus specific NKG2A+ KLRG1+ CD8 T cells have an immediate functional effector recall response
Figure 4.6. Virus specific NKG2A+KLRG1+ terminally differentiated CD8 T cells express IL-7 and IL-15 receptors during γHV68 infection.

A, Representative histograms display the expression profiles of CD122 and CD127 in the spleens of mice that were infected with γHV68 for 3 months. Results were gated on unstained controls, total CD8+ T cells, CD8+ORF61<sub>524-531</sub>+ cells, CD8+ORF61<sub>524-531</sub>+ NKG2A+KLRG1+ cells (iNK+), and CD8+ORF61<sub>524-531</sub>+ NKG2A-KLRG1- cells (iNK-). The CD122 and CD127 expression levels were analyzed by FACs.

B, Bar diagram indicates the mean fluorescence intensity (MFI) of CD122 and CD127 within the groups described above, n=3. Error bars indicate standard deviation.
Figure 4.6. Virus specific NKG2A+KLRG1+ terminally differentiated CD8 T cells express IL-7 and IL-15 receptors during γHV68 infection
Figure 4.7. NKG2A+ KLRG1+ CD8 T cells can survive without antigen for 30 days and retain their original NKG2A/KLRG1 phenotype. NKG2A+KLRG1+ CD8+ T splenocytes (iNK+) and NKG2A-KLRG1- CD8+ T splenocytes (iNK-) were FACs cell sorted from 3 months post infection γHV68 mice, and adoptively transferred into naïve congenic animals. A, Left column is representative FACs plots showing the NKG2A/KLRG1 expression profile of the adoptively transferred cells at day 0 (before transfer). Top row is NKG2A+KLRG1+ (iNK+) adoptive transfer group and bottom row is NKG2A-KLRG1- (iNK-) adoptive transfer group. The middle column shows the frequency of CD8+CD90.2+ transferred cells, 30 days post AT. Right column presents the NKG2A/KLRG1 expression profile of the adoptively transferred cells after resting 30 days without antigen. B, Bar diagram displays the frequency of CD90.2+NKG2A+KLRG1+ cells in each of the adoptive transfer groups, n=3. Error bars indicate standard deviation.
Figure 4.7. NKG2A+ KLRG1+ CD8 T cells can survive without antigen for 30 days and retain their original NKG2A/KLRG1 phenotype.
Figure 4.8. NKG2A+KLRG1+ CD8 T cells can survive without antigen and mount an effective recall response to γHV68. NKG2A+KLRG1+ CD8+ T splenocytes (iNK+) and NKG2A-KLRG1- CD8+ T splenocytes (iNK-) were FACs cell sorted from 3 months post infection γHV68 mice, and adoptively transferred into naïve congenic animals. 30 days after adoptive transfer, the recipient mice were challenged with γHV68, and results shown are 16 days after γHV68 challenge. A, Results shown are representative FACs plots of the frequency of CD8+CD90.2+ transferred cells in the spleen, top panel, in the (iNK+) and (iNK-) adoptive transfer groups. Bottom panel shows the frequency of CD8+ORF6487-495+ cells of the adoptively transferred cells for both the(iNK+) and (iNK-) transfer groups. B, Results obtained from an infectious center assay. Bar diagram shows the number of latently infected splenocytes per 10^6 splenocytes 16 days after γHV68 challenge, and 46 days after adoptive transfer, n=3. Error bars indicate standard deviation. * p value < 0.05.
Figure 4.8. NKG2A+KLRG1+ CD8 T cells can survive without antigen and mount an effective recall response to γHV68
CHAPTER 5

GENERAL DISCUSSION

5.1 Summary of Results

We determined that virus specific memory CD8 T cells are generated and maintained long term during γHV68 persistence through a variety of phenotypic and functional assays. We found that the affinity and avidity of the CD8 TCR was unaffected by antigen persistence. We showed that virus specific CD8 T cells retained their function to produce cytokines and to lyse target cells in a virus specific manner. We found that true bona fide memory CD8 T cells are fully functional during γHV68 latency, which is contrary to other chronic viral infections characterized by high antigenic load [35]. We discovered that γHV68 specific memory CD8 T cells are capable of surviving in a naïve mouse after adoptive transfer and subsequently proliferating homeostatically in response to cytokines. Additionally, the “rested without antigen” adoptively transferred memory CD8 T cells had the ability to mount a protective recall response against secondary infection with γHV68 [190]. We analyzed the functional role of specific subsets of memory CD8 T cells in order to understand what impact each subset has on the in vivo immune response. We found that both terminally differentiated effector memory CD8 T cells and central memory CD8 T cells survive in the absence of cognate antigens and proliferate in response to viral antigens. In addition, we found that both terminally
differentiated effector memory CD8 T cells and central memory CD8 T cells contribute equally to the overall protective recall response.

5.2 Viral persistence does not necessarily result in CD8 T cell dysfunction

The regulation of CD8 T cell memory to persistent viral infections is poorly understood, and is an area of study that is actively being explored. Much of the research on CD8 T cell memory to viral infections has been done using either acute viral infections or high titer chronic viral infections. The γHV68 mouse model provides a means to study CD8 T cell memory to additional type of viral infection, viruses that establish latency in their hosts. The current two dominating principles in the field of CD8 T cell memory are: 1) acute infection followed by viral clearance and stable memory formation and 2) persistent infection where there is no viral clearance which leads to T cell dysfunction and loss of memory as well as the dependence on antigen for survival and proliferation.[61, 90]. The continuous recruitment of newly activated T cells has also been shown to contribute to the population of memory CD8 T cells during persistent viral infections, and decreased thymic output results in decreased memory T cells [62].

My data has contributed to the field of immunology by demonstrating that there is an additional option for how CD8 T cells function during persistent viral infections, where viral persistence does not always lead to memory CD8 T cell dysfunction. We found that the virus specific CD8 T cell memory population is fully functional during viral latency. Furthermore, our adoptive transfer studies show that the presence of functional CD8 T cell memory during γHV68 latency is not solely due to new CD8 T cell recruits. γHV68 is a low load viral infection that establishes latency, and we have shown
that lytic viral antigens are being presented during long term latency (up to 8 months post infection), albeit at low levels. The presence of constant cognate antigen is thought to drive the CD8 T cell memory to become dependent on antigen, however our results indicate that the γHV68 CD8 T cells have the capacity to be antigen independent. This result demonstrates that antigen persistence does not always equate to CD8 T cell dysfunction and exhaustion. The amount of antigen present during γHV68 latency may be just low enough not to trigger CD8 T cell dysfunction. Since the CD8 T cell memory becomes increasingly dysfunctional with increasing amounts of antigen, our results provide evidence for decreasing the amount of antigen by various means in order to increase the CD8 T cell response to prevent disease. Antiviral drugs are currently being used to inhibit herpesviral reactivation after organ transplantation. I think our results implicate the idea of using interferon treatment in combination with antiviral drugs as prophylactic treatments to prevent viral reactivations in patients with immune deficiencies. In particular, IFNγ treatment may be a good candidate to treat γ-herpesviruses because IFNγ has been shown to prevent the virus from reactivating from latency. In addition, a recent study has shown that boosting CD8 T cells with IL-2 and anti-IL-2 immune complexes reduced the reactivation of γHV68 in CD4 deficient mice [194]. The concept of decreasing viral antigens to indirectly increase T cell function can be applied to a variety of chronic human viral infections including Hepatitis C Virus (HCV) and HIV, with a combination therapy of antiviral drugs, interferon treatment, and CD8 T cell stimulators. Our research suggests that CD8 T cell memory function and antigen persistence occur on a continuum, where manipulating the amount of antigen can affect CD8 T cell function.
5.3 T Cell Memory is Adaptive

The plasticity of T cell memory is still unresolved in the area of immunology; posing the question, how flexible is T cell memory. Our results showed that virus specific terminally differentiated effector memory CD8 T cells can be functional (i.e. long-lived, antigen-independent, and protective) when adoptively transferred into an antigen-free environment and subsequently rechallenged with γHV68. Terminally differentiated CD8 T cells were previously thought to be unable to proliferate and respond to secondary infections. However, by changing their surrounding environment, terminally differentiated CD8 T cells can become functional memory CD8 T cells. Additionally, it has been reported that a stable protective CD8 T cell memory population can be generated after clearance of a chronic parasitic infection[164]. Providing further evidence that if you decrease the viral antigen load, the CD8 T cell population is capable of adapting and becoming fully functional. There is a study in a subset of HIV patients termed HIV non-progressors, which are people who have been infected with HIV, but do not progress to AIDS or do so very slowly. This report has illustrated that the CD8 T cells in non-progressors maintain a high level of functionality compared to progressor patients[195]. This result introduces the concept of which came first, the CD8 T cell functionality or the control of the HIV infection. Are the CD8 T cells functional because the patients control HIV infection, or do the patients control HIV infection due to their functional CD8 T cells? The conclusion I reach from analyzing our data and others in the literature, is that it probably is a little of both.
However, the flexibility of CD8 T cell memory may have a point of no return when it has reached a threshold of antigen encountered, whereby changing the environment will have no effect on the CD8 T cell. Moreover, our results suggest that CD8 T cell memory is rather flexible in its ability to adapt to changing environmental conditions. The dysfunction of T cells and memory T cells may be an adaptive mechanism to prevent immunopathology. The immune system needs to find a balance between pathogen clearance and immunopathology, and when there is such a high amount of systemic antigen, the immune system adapts to prevent tissue damage. The “dysfunction” CD8 T cells may not be an inherent quality of the CD8 T cell, but a protective mechanism by which the immune system avoids killing itself.

5.4 Effector Memory vs Central Memory for Vaccination Strategies

The purpose of this project was to increase the understanding of CD8 T cell memory and immunity, in order to gain novel therapies for infections. We want to apply the knowledge of what we learned from γHV68 to not only herpesviruses, but our overall understanding of CD8 T cell function to all chronic persistent infections. Persistent viral infections have posed a significant challenge for vaccination strategies, and understanding the complexities of memory formation and maintenance are essential to create successful vaccines. Antibodies, CD4 T cells, and CD8 T cells are targeted in vaccinations to intracellular pathogens. However, which T cell population contributes to the protection or control of viruses is under debate. Previous studies using LCMV and vaccinia viruses have shown that the different memory subsets are more beneficial
depending on the infection, where LCMV requires TCM and vaccinia virus calls for TEM [49]. Our results provide evidence that both effector memory and central memory CD8 T cells are important for protection against secondary infection with γHV68. Furthermore, our results suggest that effector memory CD8 T cells may provide a better immediate recall response, probably due to their increased activation status and localization in the periphery. The contribution of TEM and TCM populations in protective immunity will be determined by the route of infection, the site of infection, and the type of infection (i.e. acute, latent, or chronic). Our results implicate that the generation of both TEM and TCM would be advantageous to effectively control persistent infections. To have a more activated TEM population scanning the periphery with the potential to mount an effector response, in combination with a TCM population that is long lived and retains the ability to make more effectors could be the best combination of CD8 T cell immunity during persistent viral infections. The prevention of herpesviral reactivation is of clinical importance during immune suppression to prevent malignancies. Methods to boost virus specific T cells to inhibit herpesviral reactivation after organ transplantation are currently being evaluated. Expanding on our results would suggest to target therapies that are able to induce activated effector, effector memory and central memory CD8 T cells in cooperation to induce effective protection against infection and prevention of disease.
5.5 Future Directions

There are some key unanswered questions in regards to CD8 T cell memory that still require further exploration. First, what are the specifics of memory generation? There is an abundance of studies in the literature targeting this broad question, however the specifics of T cell memory generation is still unclear. Memory plays such a large role in the adaptive immune response, and understanding the exact mechanisms of memory generation will have a huge impact on human immunology. In particular, our results lead to the question of how do you target the generation of TCM vs TEM. What characteristics of antigen exposure and delivery direct CD8 T cells to either a TEM or TCM phenotype. An additional critical research question is understand the circumstances that influence the priming and recruitment of CD8 T cells to sites of infection. This could be accomplished by testing the both the timing (prolonged vs acute exposure) and dose (high and low levels) of antigen delivery. Gaining information about how the priming of CD8 T cells directs the differentiation lineages and recruitment of T cell memory would allow the design of new strategies for vaccines and boosters. Another future direction of research would be to test what infections require different populations of memory for protection, comparing infections that target systemic immunity and to those that target to mucosal immunity in order to evaluate the contributions of TEM and TCM subpopulations.
5.6 Concluding Remarks

Each day more is uncovered about CD8 T cell memory, but we are still in the learning stages. Our results show that CD8 T cell memory is heterogeneous in phenotype and function. CD8 T cell memory is adaptable, and not limited or confined to one strict path, but can be flexible with the changing environment. Persistent antigen does not necessarily equate to CD8 T cell function, and true protective CD8 T cell memory can be achieved during persistent viral infections. Our results have implications for the use of a combination of therapies to prevent virus reactivation, and for targeting specific subsets of CD8 T cell memory in designing vaccination strategies.

Herpesvirus have evolved alongside human evolution, and have found a delicate balance of successful infection without causing undue harm to their hosts. Herpesviruses are nearly ubiquitous, and thus extremely successful pathogens. There is evidence that there also may be some advantages of a persistent viral infection. Previous work with γHV68 has implicated the increased resistance to infection with other pathogens in mice that are persistently infected, and this “bystander protection” is mediated by increased levels of interferon gamma [196]. Disease from herpesviruses usually only occurs when there is immune suppression. Without the availability of vaccinations to these virus, the focus of treatment should be to limit viral reactivation and disease. As previously discussed, our results implicate that using antiviral drugs in combination with interferon treatment in order to reduce viral load, as well as boosting the T cell response could provide the necessary therapy to herpesviral infection.
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