IN VITRO AND *IN VIVO* CHARACTERIZATION OF Rh178, A RHESUS CYTOMEGALOVIRUS-SPECIFIC INHIBITOR OF ANTIGEN PRESENTATION

by

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CERTIFICATE OF APPROVAL

This is to certify that the PhD Dissertation of

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SELECTED ABBREVIATIONS

β2m: Beta-2 microglobulin

AIDS: Acquired immunodeficiency syndrome

APC: Antigen presenting cell

BAC: Bacterial artificial chromosome

BAL: Bronchoalveolar lavage

CCMV: Chimpanzee cytomegalovirus

CMV: Cytomegalovirus

EBV: Epstein-Barr virus

ER: Endoplasmic reticulum

GPCMV: Guinea pig cytomegalovirus

HC: Heavy chain (of MHC-I)

HCMV: Human cytomegalovirus

HHV-5: Human herpesvirus-5 (equivalent to HCMV)

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

HSV-1/2: Herpes simplex type 1 or type 2

IE: Immediate-early

IFN: Interferon

ISG: Interferon stimulated gene

KSHV: Kapsoi's sarcoma-associated herpesvirus

MCMV: Murine cytomegalovirus

MHC-I: Major histocompatibility complex Class I

NK cell: Natural killer cell

ORF: Open reading frame

PBMC: Peripheral blood mononuclear cell

PLC: Peptide loading complex

RCMV: Rat cytomegalovirus

RhCMV: Rhesus cytomegalovirus

RM: Rhesus macaque

RNC: Ribosome-nascent chain complex

RTN: Rev-Tat-Nef (fusion of SIV proteins)

SIV: Simian immunodeficiency virus

SP: Signal peptide

SPF: Specific pathogen free

SRP: Signal recognition particle

TAP: Transporter associated with antigen processing

T_{CM}: Central memory T cell

TCR: T cell receptor

T_{EM}: Effector memory T cell

TRF: Telomerized rhesus fibroblasts

VIHCE: Viral inhibitor of heavy chain expression

VZV: Varicella zoster virus

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Isaac Newton once said, “If I have seen further it is only by standing upon the shoulders of giants.” In this thesis, I have endeavored to present data that contribute a small slice of new knowledge to the viral immunology field, but my gratitude to those who have lent me their shoulders throughout this journey is boundless. To Klaus, thank you for providing an environment for me to grow as a scientist and independent thinker, and thank you for constantly inspiring me with your immense knowledge base and gifted scientific mind. To the Früh lab members, past and present, thank you for being the sounding board for all of my questions and ruminations, no matter how silly and undeveloped. In addition to learning a great deal of science from each of you, I have formed lasting friendships and look forward to future adventures together in the great Pacific Northwest.

A particular thank you goes to those who have contributed directly to this work. Colin Powers performed the initial characterization of Rh178, setting the stage for the work presented in Chapter Three. Colin also made the ΔV, ΔU, and ΔVΔU viruses for the *in vivo* experiments of Chapter Four. Isabel Scholz has been my partner-in-crime for the *in vitro* investigations of Rh178, and she performed the Eeyarestatin experiment. Scott Hansen performed the T cell analysis presented in Chapter Four, and the animal care was provided by Michael Axthelm and his team. Michael Jarvis, Daniel Malouli, and Emily Marshall have been invaluable in troubleshooting creation of the BAC-derived viruses. I would also like to thank my thesis advisory committee of Ann Hill, Mark

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the point of being able to explain antigen presentation, your encouragement and love are more than I could have hoped for and I thank you for being my partner in life.

ABSTRACT

Cytomegalovirus (CMV) efficiently evades host immune defenses, and encodes a number of proteins that prevent antigen presentation by major histocompatibility complex class I molecules (MHC-I) in order to escape recognition and killing of infected cells by cytotoxic CD8+ T cells. We recently identified Rh178 as a Rhesus cytomegalovirus (RhCMV)-specific protein that interferes with MHC-I expression. Here, we demonstrate that Rh178 localizes to the membrane of the endoplasmic reticulum displaying a short luminal and large cytosolic domain, and that the membrane-proximal cytosolic portion is essential for inhibition of MHC-I expression. We further observed that Rh178 does not require synthesis of full-length MHC-I heavy chains but is capable of inhibiting the translation of short, unstable amino-terminal fragments of MHC-I. The cytosolic orientation of Rh178 and its ability to target protein fragments carrying the MHC-I signal peptide are consistent with Rh178 intercepting partially translated MHC-I heavy chains after signal recognition particle-dependent transfer to the endoplasmic reticulum membrane. However, interference with MHC-I translation by Rh178 seems to occur prior to Sec61-dependent protein translocation since inhibition of MHC-I translocation by Eeyarestatin-1 resulted in a full-length degradation intermediate that can be stabilized by proteasome inhibitors. These data are consistent with Rh178 blocking protein translation of MHC-I heavy chains at a step prior to the start of translocation, thereby downregulating MHC-I at a very early stage of translation.

We have also investigated the *in vivo* role of Rh178 along with other RhCMV-encoded MHC-I inhibitors in RhCMV superinfection of rhesus macaques. RhCMV, like human CMV (HCMV) can promote secondary infection despite preexisting anti-CMV

immunity. We have identified the MHC-I inhibitors encoded by RhCMV as being solely responsible for allowing superinfection to occur. RhCMV that lacks the MHC-I inhibitors encoded within the Rh182-189 region can no longer superinfect. This is true whether or not Rh178 is present, so Rh178 alone is not sufficient for superinfection. The same is true for Rh189, the RhCMV orthologue of HCMV US11. Based on these results, a majority of MHC-I inhibitors must be encoded for RhCMV to be competent for superinfection. We also identified the same region of genes as responsible for altering the immunodominance profile of CD8+ T cells against a recombinant RhCMV carrying SIVgag. With these results, we have gained a greater understanding of immune evasion and immune stimulation by RhCMV, and these are the first *in vivo* data demonstrating the importance of MHC-I immune evasion genes in a non-human primate system. Information gained from this study will inform rational vaccine design both against CMV and for the use of CMV as a vaccine vector.

PREFACE

This thesis comprises my original work towards the PhD with the following exceptions:

- The initial codon-optimization of Rh178 was performed by Dr. Colin Powers by using services from GeneArt.
- Subcloning of codon-optimized Rh178 into pUHD10.1 was completed during my research rotation in the Früh lab with the help of Dr. Colin Powers.
- Eeyarestatin experiments (Figure 3.6) were performed by Dr. Isabel Scholz
- ΔV, ΔU, and ΔVΔU recombinant RhCMVs were generated by Dr. Colin Powers
- The original WT RhCMV-gag virus was created with the BAC system by Dr. Michael Jarvis.
- T cell stimulation assays were carried out by Dr. Scott Hansen, under the direction of Dr. Louis Picker.
- Dr. Michael Axthelm and his group at the animal facility were responsible for care of the rhesus macaques used in this study.

Data presented in Chapter Three represent a modified version of a paper that was submitted to the Journal of Virology in May, 2011, authored by Rebecca Richards, Isabel Scholz, Colin J. Powers, and Klaus Früh.

Figures 1.4, 4.4, 4.5, 4.6, and 4.7 and earlier versions of Figures 4.2 and 4.3 were published in the journal Science in April 2010 (*Science 2 April 2010: Vol. 328 no. 5974 pp. 102-106*).

Permissions for Figure 1.1 were received on 5/27/11 from Adam Hirschberg at Cambridge University Press.

CHAPTER ONE

Introduction

The virus-host relationship is complex and constantly evolving, prompting studies from every biological field in an attempt to understand this unique interface. In order to infect a host and establish persistence in an individual and in the population, viruses have developed methods to circumvent host immune responses, invoking an intriguing game of hide-and-seek. Even small viruses make the most of their limited coding capacity to gain an edge against assaults from the host. For example, human immunodeficiency virus (HIV) has a compact genome with only nine genes, yet still regulates host defenses ranging from interferon response to CD4 and CD8 T cell recognition to macrophage function [1]. Cytomegaloviruses (CMVs) encode over 200 gene products, and thus have developed a more diverse array of immune evasion mechanisms. The work presented here investigates the biological mechanism and *in vivo* role of a single rhesus CMV (RhCMV) immunoevasin, Rh178. Rh178 contributes to the inhibition of viral peptide presentation by MHC Class I (MHC-I), allowing the virus to evade cytotoxic T cell recognition and killing of infected cells. An introduction of the wide variety of immune evasion strategies employed by the herpesvirus family and by CMV specifically will present context for understanding the biological mechanism of Rh178, which will be described further in Chapter Three. Chapter Four presents data on the *in vivo* role of Rh178 and other RhCMV-encoded inhibitors of MHC-I, and discusses the implications of these data for development of a CMV vaccine and the use of CMV as a vaccine vector.

1.1 CMV is a member of the β-herpesvirus family

Human CMV (HCMV) was initially isolated by three independent groups in the mid-1950s [2-4]. The name "cytomegalovirus" was coined by Craig et al. [2] to describe the enlarged cell size and bloated cytoplasm observed in infected cells. The Universal Virus Database has designated HCMV as 'human herpesvirus 5 (HHV-5)' but it is widely referred to as HCMV because of its close genetic relatedness with CMVs from other species [5]. HCMV is the cornerstone member of β-herpesviridae, a subfamily of herpesviruses. Other subfamilies are the α-herpesviridae, which includes the familiar Simplexvirus 1 and 2 (HSV-1/2) and Varicella zoster virus (VZV), and the γ-herpesviridae, which includes Epstein Barr virus (EBV) and Kaposi sarcoma herpesvirus (KSHV). The genetic similarity of HCMV to CMVs from other species and to more distantly related herpesviruses from other subfamilies is depicted in a phylogenetic tree in Figure 1.1 (adapted from [6]).

CMVs share with all herpesviruses a large, double-stranded DNA genome encapsulated in an icosahedral capsid and a glycoprotein-rich envelope. Another distinguishing characteristic of herpesviruses is their ability to establish latency after primary infection, with the potential for periodic reactivation. The sites of latency vary among herpesvirus family members. For example, HSV-1 and -2 become latent in sensory ganglia of the nervous system [7], while EBV establishes latency in and reactivates from B-cells [8]. The sites of CMV latency are less well-defined but evidence suggests that undifferentiated myeloid cells are the most likely candidates [9, 10]. In addition to latency, CMV has a number of other important immunological characteristics.

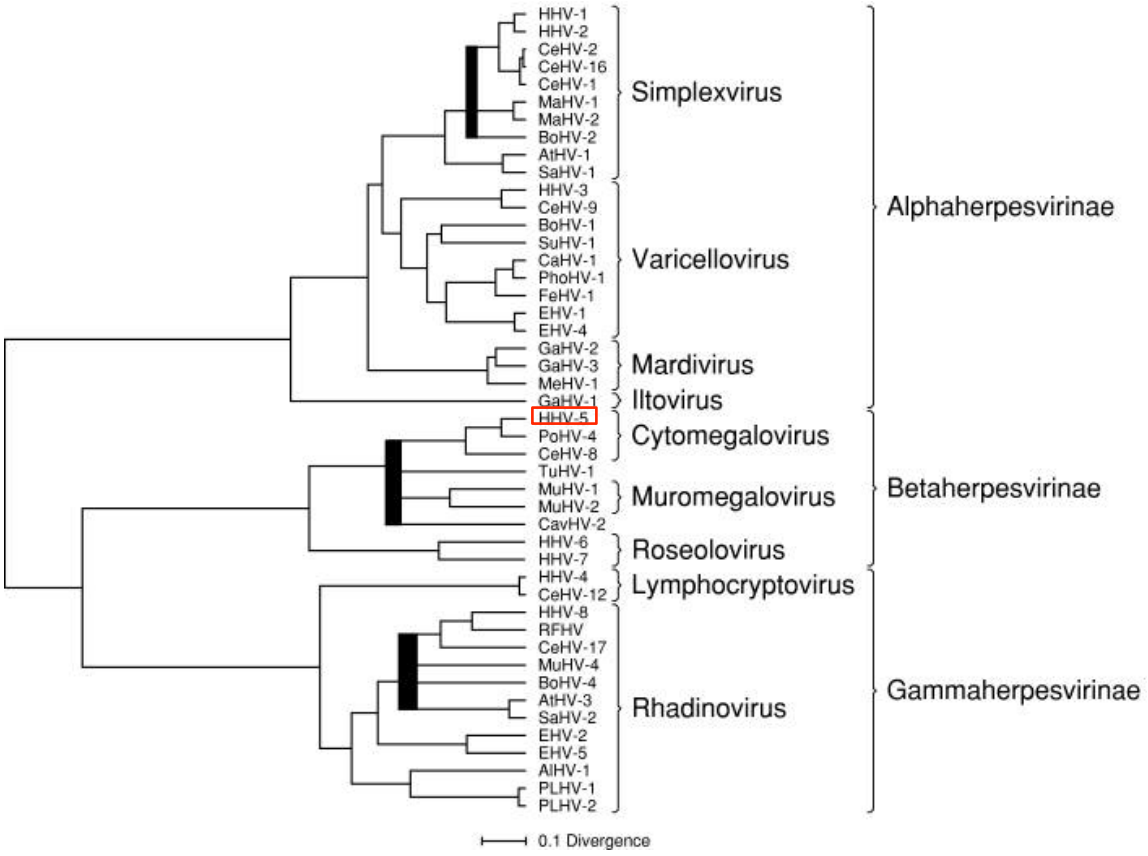


Figure 1.1: Phylogenetic tree of herpesviruses.

Branches resemble genetic relatedness among viruses based on amino acid sequence alignments of eight sets of homologous genes. The designations for formal species, genera, and subfamilies are given on the right. The location of HCMV, also called HHV-5, is highlighted by the red box.

The dedication of the immune system to HCMV epitope recognition far exceeds responses to other viruses within and outside of the *Herpesviridae* family [11-13]. In adults infected with HCMV, a remarkable 10% of circulating memory T cells are responsive to HCMV antigens [14]. Paradoxically, despite the presence of such a dedicated immune response, HCMV is able to superinfect hosts with preexisting anti-CMV immunity. Thousands of strains of HCMV likely circulate in the world population [15], and superinfection with multiple strains is a common phenomenon [16, 17] (reviewed further in section 1.7).

1.2 CMV epidemiology

Between 50-90% of adults in North America are seropositive for HCMV. The prevalence approaches 100% in underdeveloped countries, as transmission correlates with population density [18, 19]. The first opportunity for infection occurs *in utero*, though intrauterine transmission accounts for less than 3% of all infections [20]. The majority of children become infected with HCMV during the first six months of life, either from vaginal secretions during delivery or from breastmilk [21]. Those who remain seronegative after breastfeeding ceases are likely to acquire HCMV upon exposure to saliva and urine at day care centers or in pre-school [22]. Despite its ubiquitous nature, both primary and reactivating infection of HCMV are largely subclinical in healthy people. However, patients with compromised immune systems, such as pathologically immunosuppressed patients (for example, from acquired immunodeficiency syndrome [AIDS]) or artificially immunosuppressed allograft recipients, often experience significant HCMV-related morbidity and mortality. Diseases associated with HCMV

include but are not limited to interstitial pneumonitis, gastritis, colitis, renal artery stenosis, and retinitis [23-25]. HCMV can also cause devastating congenital disease after vertical transmission from mother to fetus. Notably, HCMV is a leading cause of sensorineural hearing loss in the United States [26]. Each year, an estimated 8000 infants have profound health problems linked to HCMV [27]. This is not only a personal devastation for patients and their families, but also a financial burden for the public health care system, because of the long-term care required for these patients.

1.3 RhCMV as a model for HCMV

Reliable animal models have been developed to study some human herpesviruses such as HSV and EBV [28-30]. Others (VZV and KSHV) infect laboratory animals less efficiently, but specific parameters of infection and pathology can still be studied in animals in a limited capacity [31, 32]. HCMV, however, does not replicate in any immunocompetent animal, making it more difficult to study *in vivo*. Fortunately, ortholologous CMVs have been isolated from many individual mammalian species, and the similarities of virus replication, pathology, and host immune responses have opened the door to a variety of animal models that can be used for the *in vivo* study of CMV.

Animal models have yielded important insights into CMV replication, pathogenesis and host immune response to infection. Because of the high species specificity of CMVs, studies using animal models are limited to infection with the species' corresponding CMV, and the results can at best be extrapolated to represent HCMV infection of humans. The long-established model of murine CMV (MCMV) infection has been used to investigate specific pathology and cell tropism in target organs

such as the brain or the liver [33, 34]. Because of the structural similarities of the guinea pig and human placentae [35], guinea pig CMV (GPCMV) infection has given us a greater understanding of maternal-fetal transmission and congenital CMV sequelae [36]. A rat CMV (RCMV) model has been implemented for analysis of therapeutic interventions in immunocompromised animals [37]. In addition, innovative efforts to study HCMV more directly by infecting either rats with implanted human retinae or human renal artery explants in tissue culture have been successful, but result in conclusions with a narrow focus in the absence of a whole animal model [38, 39].

The many advantages of rodent models include relatively low cost, limited variables due to inbreeding, and the ability to use genetic knockout animals. However, evidence suggests that these models may not be appropriate to study prophylactic and therapeutic interventions [40]. Additionally, comparative analyses of rodent and human CMV genomes show significant evolutionary divergence [41-43]. CMVs have co-evolved along with their hosts, so their evolutionary relatedness mirrors that of the divergent animal species [44]. Chimpanzee CMV (CCMV) is the closest genetic relative to HCMV [45], but chimpanzee research is limited in scope and would be prohibitively expensive. Therefore, a rhesus macaque (RM) CMV (RhCMV or 'cercopithecine herpesvirus 8') model has been actively developed over the last three decades [46-49]. The initial description of cytomegalic intranuclear inclusions in tissues from healthy RM was made over 80 years ago [50, 51]. Since that time, a number of CMV strains have been isolated from RM and other non-human primates [52-54]. Over the same time period, the number of primate centers that house RM and the feasibility of using RM as a reliable animal model have expanded significantly. As we continue to learn more about

the similarities between RhCMV and HCMV, the RM has become an optimal animal model for CMV research that may result in direct translation to antiviral drugs or vaccines against HCMV infection. The RhCMV model overcomes some of the obstacles associated with rodent CMV models and has given us a closer approximation of HCMV infection in humans.

The RhCMV genome is more closely related to HCMV than are those of MCMV, RCMV, and GPCMV, though significant regions of genetic divergence still exist. The RhCMV genome encodes about 230 open reading frames (ORFs), at least 60% of which have homology to genes from HCMV. Notably, most of the important gene families of HCMV are conserved in RhCMV, including those involved in viral replication, nucleotide metabolism, virion structure and stability, and immunomodulation [55]. These similarities likely contribute to important parallels that are observed in courses of infection and clinical characteristics inflicted by RhCMV and HCMV. Like HCMV in human populations, RhCMV is ubiquitous in wild rhesus monkey populations [56, 57]. One study reported that 100% of juvenile and adult RM in a breeding colony were seropositive for RhCMV. The vast majority of the animals studied had seroconverted within the first year of life [58]. Moreover, superinfection of RhCMV-seropositive RM occurs despite the presence of a specific immune response against RhCMV antigens [59]. Most healthy RM acquire RhCMV infection early in life and persistently shed low levels of virus in the urine and saliva. However, like HCMV, RhCMV can be a death sentence for an immunocompromised animal.

Specific experimental models in the RM have been developed to mimic each subgroup of humans who suffer from CMV-related disease. RM who acquire SIV

develop the simian correlate to AIDS, followed by the typical opportunistic infections that often are seen in humans with AIDS, including CMV [60-62]. Intravenous co-inoculation of RM with SIV and RhCMV has become the gold standard for studying RhCMV-induced pathogenesis in severely immunocompromised animals. The significance of a compromised immune system for giving way to RhCMV virulence has been confirmed in this model [63]. RhCMV infection is also a serious problem for non-human primates following experimental solid organ transplantation, and closely resembles disease seen in human allograft recipients [64]. Finally, a model for congenital CMV infection has been quite useful for investigations into the devastating effects of CMV infection on the immature, developing immune system [48, 65]. RhCMV does not have high rates of intrauterine transmission rates, so in this model, the fetus is infected intraperitoneally during the second trimester, and tissues are evaluated after induced delivery late in the third trimester. Pathologies closely resemble those seen in humans, including spontaneous abortion, intrauterine growth restriction, ventriculomegaly, and microcephaly [65, 66].

The continuing development of recombinant virus technology and non-human primate-specific reagents has vastly increased the feasibility of using RM as a model system *in vivo* and *in vitro*. Because over 95% of RM in breeding colonies become RhCMV seropositive early in life, it has been necessary to develop specific pathogen-free (SPF) animals. Monkeys are separated from their dams shortly after birth, and are co-housed with similarly treated animals [67]. SPF colonies are never exposed to many viruses including RhCMV, so they can be used for studies of primary infection in RhCMV-naïve animals, experiments that are impossible in a normal breeding colony.

Another vital development in RhCMV technology is the RhCMV bacterial artificial chromosome (BAC) [68]. CMV genomes are difficult to manipulate in mammalian cells because of large genome size and slow replication kinetics. Originally developed by Peter Barry and colleagues, the RhCMV BAC has a self-excisable bacterial origin of replication that allows for efficient genetic manipulation and characterization of the RhCMV genome in *Escherichia coli* before transferring the system to mammalian cells. Years of development and characterization of RhCMV infection of RM have brought this model system to the forefront of CMV research.

1.4 CMV evasion of innate immunity

Pathogens of all types have evolved methods to suppress the host immune system for their own purposes in order to survive and spread between hosts. The first description of viral immune evasion was published in 1908, when Clemens von Pirquet described how measles patients could not record a normal hypersensitivity reaction to tuberculin antigen, which in his day was used as a barometer for overall immune capacity [69]. This led to the hypothesis and later confirmation that measles virus suppressed components of the immune system [70]. CMVs encode a spectrum of genes that suppress the host immune system and allow initiation of infection and long-term survival. Immunocompetent subjects are rather successful against CMV because they manage to prevent rampant viral replication and fulminant disease. The virus can also claim victory, however, by setting up a persistent, smoldering infection for the life of the host. The large number of ORFs in the CMV genomes allows for a multi-faceted attack against all arms of the immune system. The precise immunomodulatory functions of HCMV proteins

have been addressed directly with *in vitro* studies, or indirectly by utilizing recombinant animal CMVs that contain orthologous factors and extrapolating those data to HCMV.

1.4 (a) CMV evasion of the interferon response

Innate immunity provides the first line of defense against invading pathogens. Both interferon (IFN) responses and natural killer (NK) cell defenses are essential to the success of early immunity. The general IFN pathway occurs in response to viruses that bind at the cell surface and set off a cascade of events beginning with activation of pattern recognition receptors (PRRs), followed by phosphorylation and/or dimerization of cellular transcription factors like interferon regulatory factor 3 (IRF3), CREB binding protein (CBP), p300, and NF-κB [71-73]. These transcription factors then shuttle to the nucleus and stimulate either the IFN-independent or the IFN-dependent pathway. In the IFN-independent pathway, the transcription factors directly stimulate transcription of IFN stimulated genes (ISGs). In the IFN-dependent pathway, they cooperate to initiate transcription of type I IFNs (IFNα/β), which act in an autocrine or paracrine manner via the Janus kinase/signal transducers and activators of transcription (Jak/Stat) pathway to induce transcription of a distinct set of ISGs [74, 75]. Microarray analysis of HCMV infected cells compared to mock infected cells has uncovered a number of ISGs that are upregulated during HCMV infection [76, 77]. However, the specific viral factors responsible for the activation of the IFN pathway and participating cellular receptors are largely uncharacterized. HCMV envelope glycoproteins gL and gH have been implicated in the initial signal transduction pathway because they activate transcription factors Sp1 and NF-κB when overexpressed in cell culture [78]. More recent data indicates that

HCMV co-opts a cell signaling pathway involving Z DNA binding protein 1 (ZBP1) in order to induce IRF3-dependent stimulation of IFN-β [79, 80].

A delicate balance exists between host immune response and viral immune suppression. Mice that completely lack certain components of the IFN response pathway (e.g. TLR7/TLR9, MyD88) are quickly overcome by MCMV infection and have a high mortality rate [81, 82]. In these cases, the balance is tipped in favor of the virus. In healthy animals, CMV is able to suppress the IFN response just enough to gain a foothold for low-level replication without causing widespread pathogenicity. CMVs suppress both IFN-dependent and –independent signaling, and specific mechanisms vary among HCMV, RhCMV, and MCMV. Ten years ago, a general blunting of the IFN-induced transcriptional program in response to HCMV infection was noted [83]. Subsequent work has suggested that several HCMV proteins may contribute to this phenotype. Transcription factors that act early in the IFN response (IRF3, IRF1, and NF-κB) are common targets for CMVs. HCMV tegument protein pp65 (ppUL83) has been implicated in blocking cytoplasmic phosphorylation and nuclear translocation of these proteins, though the literature does not agree on the exact mechanism [84-87]. Similarly, RhCMV and MCMV inhibit IFN production but the responsible proteins have not yet been determined [86, 88]. The other main point of interference occurs through the IFN-dependent arm, by inactivation or degradation of components of the Jak/Stat pathway. HCMV infection causes proteasomal degradation of Jak1 and Stat2 by an unknown mechanism [89, 90]. In a demonstration of redundancy, HCMV also blocks Stat2 signaling via immediate early protein IE1, which directly interacts with Stat2, thereby blocking Stat-dependent transcription of ISGs [91]. Wide gaps in knowledge remain in

the CMV-IFN field, particularly in identifying the viral factors responsible for IFN response evasion. Nevertheless, it is clearly an important mechanism of immune evasion for CMVs.

1.4 (b) CMV evasion of NK cell cytotoxicity

NK cells, a subset of lymphocytes that participate in early control of viral infection, are another component of innate immunity. NK cell surveillance is especially prominent in cancer or during infection. They are distinct from other lymphocytes (T and B cells) in molecular phenotype and target recognition. NK cells are typically identified by a $CD56^{+}$ cell surface phenotype, though the population is quite diverse [92]. There are many subpopulations of NK cells due to permutations of activating and inhibitory receptors on the NK cell surface, all of which bind to ligands on target cells and modulate the release of cytotoxic granules. For example, activating receptor CD94/NKG2D binds to cellular ligands that are upregulated during cellular stress, such as MHC Class I related genes A and B (MICA and MICB), and UL16 binding protein (ULBP) 1-6 [93-96]. Unchecked, association of one of these stress ligands with CD94/NKG2D stimulates cytotoxin release by the NK cell, ultimately resulting in apoptotic death for the offending cell. Providing a counterbalance to the stimulation of cytotoxin release are NK cell inhibitory receptors, which coexist on the NK cell surface and perform similar surveillance of host cells. Most relevant to the data presented in this thesis is the CD94/NKG2A inhibitory receptor. A healthy host cell expresses on its surface a complex of non-classical MHC-I (human leukocyte antigen-E or HLA-E in humans) bound to a 9-mer leader peptide from one of the classical MHC-I alleles (HLA-A,B,C in humans).

CD94/NKG2A recognizes this complex and initiates signaling through the NK cell to prevent lysis of the healthy cell [97]. A potential problem therefore arises when CMV infection causes downregulation of classical MHC-I molecules. Theoretically, infected cells should not be able to load classical MHC-I leader peptide onto HLA-E. Following this logic, HLA-E surface expression would decrease and NK cell inhibitory receptor CD94/NKG2A would not be engaged, leaving the infected cell susceptible to NK cell lysis.

CMV has evolved methods to overcome NK cell lysis. The virus mounts a multi-pronged defense by limiting activating receptor stimulation and simultaneously increasing inhibitory receptor stimulation. HCMV glycoprotein UL16 directly binds the cellular ULBP family members and sequesters them in the endoplasmic reticulum (ER) in order to prevent cell surface expression of these stress ligands [96, 98, 99]. Consequently, recognition by activating NK cell receptors is suppressed and NK cell-directed cytotoxicity is limited. HCMV proteins UL40 and UL18 both effect an equivalent outcome of decreased NK cell lysis, but they do so by increasing recognition by NK cell inhibitory receptors. UL40 replaces the empty binding groove of HLA-E by coding for the exact 9-mer leader peptide (VMAPRTLIL) that is lost when classical MHC-I molecules are downregulated by mechanisms described in the next section [100]. Restored expression of loaded HLA-E at the cell surface triggers the NKG2A-mediated signaling cascade that inhibits NK cell lysis [101]. HCMV UL18 encodes an MHC-I homologue that binds directly to a different NK cell inhibitory receptor, leukocyte Ig-like receptor 1 (LIR-1), in essence replacing the classical MHC-I molecules that are lacking at the surface of infected cells [102, 103]. These conclusions were all derived from

experiments in human cell lines. Though RM possess many orthologous NK cell receptors and ligands, studies of NK cells in RM have been very limited [104]. Our laboratory and others are only just beginning to broach the topic of RhCMV evasion of NK cell immunity.

1.5 CMV evasion of adaptive immunity

While the innate immune response controls pathogens at the onset of infection, the adaptive immune response is delayed but imparts life-long immunity, with the capacity for fine tuning and high specificity. The principal actors are pathogen-specific lymphocytes (B and T cells) that do not enter the picture until at least one week after infection. The interplay among these cells and others is complex but by working together, they recognize infection primarily through antigen presentation, initiate responses designed to eliminate the infected cells, and create memory cells as long-term storage for future responses against similar pathogens. Not surprisingly, CMV has developed mechanisms to defend itself against many factors that mediate adaptive immunity in addition to those involved in innate immunity. This thesis will focus on mechanisms employed by CMV to evade T cells, specifically CD8+ T cells.

1.5(a) Antigen presentation

Antigen presentation is a fundamental process of cell biology. Healthy cells and virus-infected cells present antigens on the cell surface via MHC-I or MHC-II, which are recognized by CD8+ or CD4+ T cells, respectively. During the course of an infection, antigen recognition is active during both the initiation and effector phases of T cell

immunity. Upon initial infection of a host, professional antigen presenting cells (APCs) like dendritic cells present virus-derived peptides via MHC-I at the cell surface. In this "initiation phase," a highly specific T cell receptor (TCR) interacts with the peptide-MHC complex. This constitutes T cell priming, and the population of T cells with that particular TCR expands with the help of costimulation. A subpopulation of clonal effector memory cells (T_{EM}) remains, and a smaller, longer-lived central memory T cell (T_{CM}) population will eventually emerge and persist [105]. Naïve CD8+ T cells can be primed by either direct or cross presentation. Direct presentation of endogenously produced peptide occurs if the APC is directly infected, and cross presentation results after exogenous peptide is acquired from other infected cells, for example by phagocytosis [106]. Either mode of presentation results in priming of naïve CD8+ T cells. Priming is necessary to create an anti-viral response, and can only occur in secondary lymphoid organs like lymph nodes or spleen.

Antigen presentation also governs the "effector phase" of T cell immunity. In a secondary infection, MHC-I on the surface of infected cells presents viral peptides to the now primed repertoire of CD8+ T cells. After a highly specific TCR on the surface of an effector T cell binds to the peptide-MHC-I complex, granzyme B and perforin are secreted in order to kill the infected cell [107, 108]. Simultaneously, cytokines are released to recruit other cells, including CD4+ T cells, to assist in building the adaptive immune response. In summary, recognition of MHC-I by the TCR induces naïve CD8+ T cells to differentiate into memory cells that are specific for peptide-MHC-I during the initiation phase, and stimulates memory CD8+ T cells to kill infected cells during the effector phase. CMV-encoded MHC-I inhibitors are expressed during primary and

secondary infections, and therefore may interfere with CD8+ T cell priming and/or cytotoxicity.

The CMV-derived factors that prevent MHC-I with bound peptide from reaching the cell surface are explained in detail below, but to understand their points of interference, it is first necessary to elaborate on the cellular proteins involved in peptide loading of MHC-I. MHC-I is translated on the ribosome and translocated to become a transmembrane protein in the ER. Membrane-bound calnexin and soluble calreticulin are chaperone proteins that assist in the correct localization and folding of immature MHC-I. Its well-conserved structure includes three Ig-like domains, α1, α2, and α3, followed by a transmembrane domain and a very short cytoplasmic tail [109]. The bulk of the protein resides in the ER lumen, where it forms a non-covalent bond with β2-microglobulin (β2m) [110]. Upon forming a heterodimer, it directly binds to tapasin, which prompts indirect interaction with transporter associated with antigen processing (TAP). These newly interacting partners are aptly named the peptide loading complex (PLC), because MHC-I is now competent to load peptides. Peptides (normally between 8-10 amino acids) are generated from protein degradation in the proteasome, and are fed from the cytosol into the ER by a mechanism driven by TAP. For optimal high affinity peptide loading, tapasin must be present to act as the intermediary [111]. Peptides bind to the highly variable peptide-binding groove formed by motifs within the α1 and α2 domains of MHC-I. Once peptide is bound, the MHC-I complex dissociates from the PLC and is transported to the cell surface through secretory vesicles. A simplified view of direct antigen presentation is demonstrated in Figure 1.2. Downregulation of MHC-I to prevent antigen presentation is a common strategy used by a diverse group of invading pathogens,

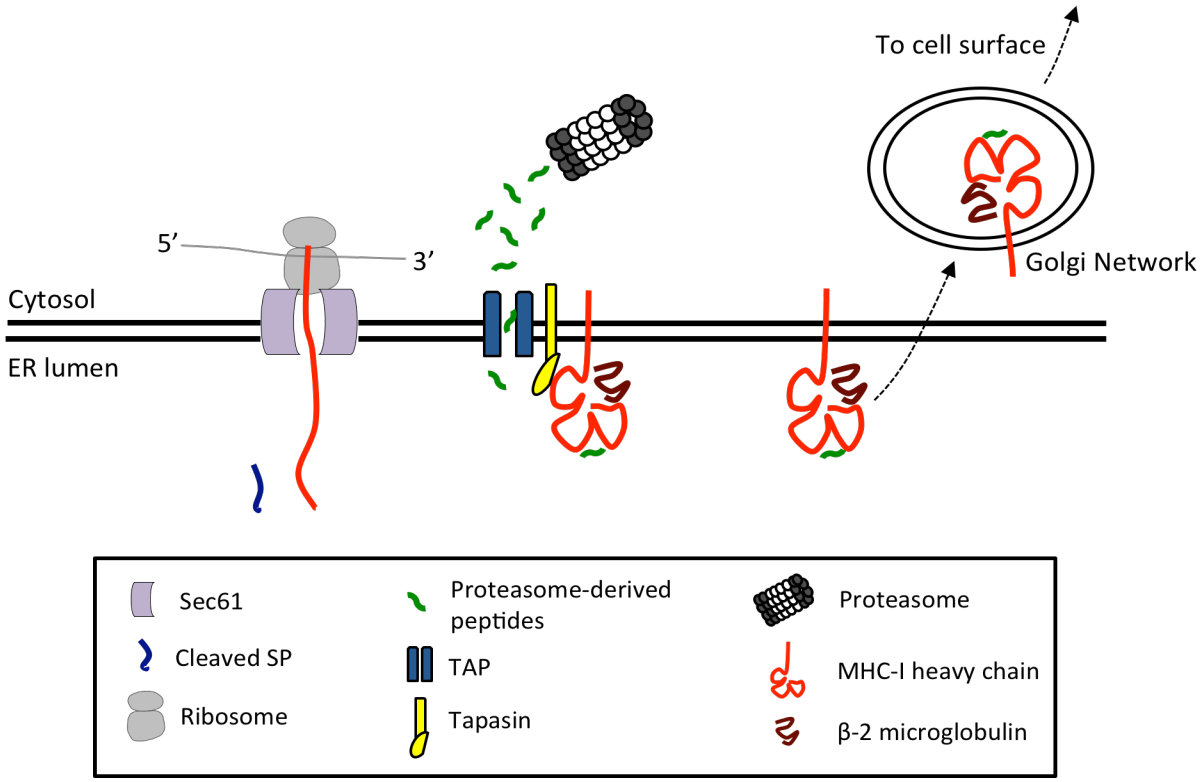


Figure 1.2: The direct antigen presentation pathway via MHC-I.

After the nascent MHC-I protein is translocated through the Sec61 translocon, the heavy chain associates with β-2 microglobulin in the ER lumen. Peptides are generated by the proteasome, and with the help of tapasin, are fed through TAP so that they can be loaded into the peptide binding groove of MHC-I. The peptide-MHC-I complex is then shuttled into the Golgi complex for transport to the cell surface, where it is recognized by CD8+ T cells.

including adenovirus, HIV-1, and many of the herpesviruses [112-114]. A select few bacteria even target this pathway for immune evasion, including *Chlamydia*, *Salmonella*, and *Yersinia* species [115].

1.5(b) General interference with MHC-I expression by CMV

The first description of HCMV downregulation of MHC-I was given by Browne et al. in 1990 [116]. They used the previous identification of an MHC-I homologue in HCMV (UL18) [102] to hypothesize that UL18 bound to and sequestered β2m, thereby preventing any mature MHC-I from reaching the cell surface. They made the seminal discovery that HCMV-infected and uninfected cells had equivalent MHC-I mRNA levels, but infected cells exhibited significantly decreased mature MHC-I protein expression at the cell surface. However, they incorrectly concluded that UL18 was the sole dictator of this phenotype. The phenotype was mapped to the US2-11 region of the HCMV genome shortly thereafter [117], and a barrage of detailed mechanistic studies quickly followed. We now know that the decrease in mature MHC-I at the cell surface is mostly driven by proteins that inhibit peptide loading and maturation of the heavy chain, but do not affect β2m. HCMV encodes four proteins, US2, US3, US6, and US11, that downregulate MHC-I in this manner. They are all leftward facing genes in the same genetic locus, and are part of the US6 family, characterized by a Type Ia transmembrane and Ig domain super-family glycoprotein structure [118]. Individual mechanisms for each of these proteins have been studied in detail, primarily in cell culture. The RhCMV genome encodes functional homologues of all four US6 family members [119], which has

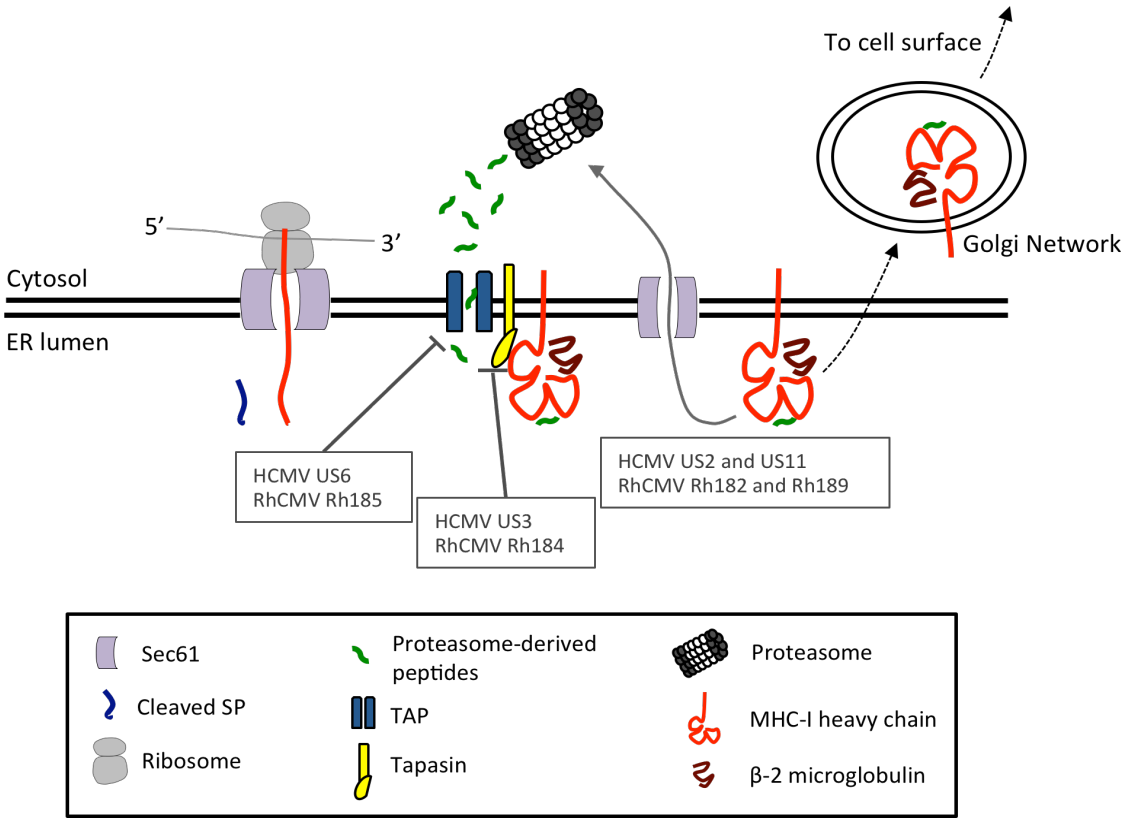


Figure 1.3: HCMV and RhCMV interference with MHC-I presentation.

All HCMV and RhCMV inhibitors of MHC-I are type I ER transmembrane proteins that exert their effects in the ER lumen. HCMV US6 (Rh185) directly associates with the TAP luminal domain to halt peptide transport into the ER. HCMV US3 (Rh184) interferes with peptide loading chaperone tapasin, contributing to ER retention of MHC-I. HCMV US2 and US11 (Rh182 and Rh189) cause retrotranslocation of MHC-I through the Sec61 translocon, targeting it for degradation in the proteasome. Working together, these four factors in HCMV and RhCMV decrease cell surface expression of MHC-I, which leads to decreased recognition by CD8+ T cells.

allowed recent investigation into their *in vivo* role in the RM model system. Figure 1.3 depicts the diverse mechanisms by which the US6 family members of HCMV and RhCMV interfere with MHC-I antigen presentation. In addition, RhCMV encodes Rh178, a non-US6 family protein that also downregulates MHC-I [120]. A summary of homology between MHC-I inhibitors encoded by RhCMV and HCMV is provided in Table 1.1. The *in vitro* and *in vivo* characterization of the only RhCMV-specific factor, Rh178, is the topic of this thesis, and is discussed in Chapters Three and Four.

1.5(c) US2 and US11

Normally, a nascent polypeptide chain that will become a cell surface protein or a secreted protein is translocated into the ER through the Sec61 translocon. In the ER lumen, the protein may be glycosylated and adopts a mature conformation before transiting through the vesicular pathway to reach the cell surface. HCMV glycoproteins US2 and US11 both stimulate MHC-I to travel this pathway in reverse, moving it from the ER lumen to the cytosol through a process called retrotranslocation. Though US2 and US11 are often mentioned together because they force MHC-I to an equivalent endpoint, they operate by distinct mechanisms that have given us considerable insight into the cellular proteins involved in retrotranslocation and proteasomal degradation.

MHC-I heavy chain (HC) in HCMV-infected cells is rapidly degraded, and thus has a very short half-life compared to uninfected cells [121]. At the time US2 and US11 were discovered, dislocation of misfolded proteins from the ER to the proteasome was known to be a normal cellular housekeeping process, and some potential cellular chaperones had been identified [122, 123]. Wiertz et al.[124] presented the first account

Table 1.1: HCMV and RhCMV inhibitors of antigen presentation.

Homology between Human and Rhesus CMV MHC-I inhibitors			
Human	***Rhesus***	***Major function***	***Reference(s)***
US2	Rh182	Targets MHC-I to proteasome	118,124,125
US3	Rh184	Causes ER retention of MHC-I via tapasin	138,139,140,141
US6	Rh185	Blocks TAP peptide loading of MHC-I	148,153,154
US11	Rh189	Targets MHC-I to proteasome	117,126,127,128
none	Rh178	Blocks SP-dependent translation of MHC-I	120

indicating that a viral protein could co-opt this system for the benefit of the virus. They gave a stepwise description of ATP-dependent, US2-mediated dislocation of MHC-I HC from the ER to the cytosol. This model begins with US2 binding directly to HC, causing retrotranslocation through the Sec61 translocon, followed by N-glycanase-mediated deglycosylation of HC. In the presence of proteasome inhibitors, the deglycosylated intermediate was not only associated with the Sec61 translocon, but also with the proteasome, which strongly implicated the proteasome as the final destination for HC. X-ray crystallography and mutagenesis studies have further confirmed direct binding of US2 to MHC-I HC, and have isolated the interaction as occurring at the HC junction of the α3 and peptide-binding domains, with particular importance given to HC residue Arg^{181} (at least in HLA-A2 alleles) [118, 125]. US2 appears to bind in a peptide-independent manner, which would be important for complete downregulation of MHC-I HC.

HCMV glycoprotein US11 had been identified prior to US2 as a mediator of HC instability and rapid degradation [117]. The original description of the US11 mechanism suggested that it caused MHC-I HC retrotranslocation in a manner exactly mirroring that of US2 [126]. However, more detailed mechanistic descriptions have emerged, and though there are many similarities between the two glycoproteins, it is now clear that US2 and US11 have unique requirements for degradation of MHC-I HC. US2 requires its short cytosolic domain to degrade HC, whereas the comparable domain of US11 is dispensable for function. In contrast, the US11 transmembrane domain is necessary for degradation of HC [127]. Lilley and Ploegh [128] further emphasized the importance of a single glutamine residue in the US11 transmembrane region, and implemented an affinity

purification system to compare cellular proteins that bound to wild-type (WT) US11 vs. mutant US11 ($US11_{Q192L}$). This strategy led to the identification of the novel cellular factor Derlin-1, which is required for US11- but not US2-mediated destruction of MHC-I HC. A similar approach was used to identify signal peptide peptidase (SPP) as essential for US2 and not US11 function [129]. The pathways directed by each of these HCMV proteins have become increasingly apparent, as a number of participating proteins have been identified over the last decade [130-134]. In summary, US2 and US11 elicit retrotranslocation and subsequent proteasomal degradation through unique cellular machinery, and we have only begun to understand why the virus has evolved such an intricate system. Some have argued that US2 and US11 may employ divergent mechanisms to degrade MHC-I at various times during infection, or to differentially regulate MHC-I in an allele-specific or cell type dependent manner [135-137].

1.5(d) US3

The only HCMV US6 family member expressed with immediate early (IE) kinetics is the 22 kDa glycoprotein US3. US3 has significant amino acid and structural similarity to US2, so it was originally hypothesized to have an analogous function. US3 downregulation of MHC-I was indeed confirmed, but it does not share the capacity of US2 and US11 to direct MHC-I HC to the proteasome for degradation. Instead, US3 causes ER retention of fully assembled MHC-I heterodimers, and accordingly prevents maturation and transport to the cell surface [135, 138]. Proteins that reside within the ER often contain one of the classical ER retention motifs, a short amino acid sequence of KDEL or KKXX. Neither of these exists within the US3 sequence, but the ER

localization phenotype is dependent on specific amino acids within the luminal domain [138]. Mutation of any of three non-contiguous residues within the luminal domain, Ser^{58}, Glu^{63}, or Lys^{64}, rendered US3 inactive. Disruption of this novel S/EK ER retention motif prevented US3-mediated retention of MHC-I, but did not interfere with the transient interaction between US3 and MHC-I [139].

Because these data demonstrated a separation between the MHC-I binding and ER retention motifs, it remained likely that other proteins cooperated with US3 to retain MHC-I in the ER. More recent studies by Park and colleagues have confirmed this, and have revealed the importance of tapasin and protein disulfide isomerase (PDI) for US3 function [140, 141]. Tapasin is a chaperone dedicated to assisting with peptide loading of MHC-I. First, it assists in optimal peptide loading by bridging the gap between TAP and MHC-I. Once peptide is loaded, it allows for release of mature MHC-I from the PLC [142-146]. To complicate matters, only a subset of human MHC-I alleles, or HLA, are dependent on tapasin for peptide loading and export to the cell surface. "Tapasin-dependent" loading is slower than the less discriminatory "tapasin-independent" loading [147]. Not surprisingly, alleleic sensitivity to US3 segregates with allelic dependence on tapasin. One study showed that tapasin-dependent alleles like HLA-B44 are efficiently retained by US3, whereas tapasin-independent alleles like HLA-B27 escape to the cell surface when US3 is present [140]. The same group also outlined an important interaction between US3 and PDI, another member of the PLC. In a healthy cell, PDI maintains oxidation of an α2 disulfide bond in the peptide binding groove of MHC-I, a necessary feature for optimal peptide loading. US3 causes degradation of PDI, which in turn leads to a change in the redox state of the MHC-I peptide binding groove [141]. Therefore,

fewer MHC-I molecules attain optimal peptide loading status, decreasing their release from the PLC. The interplay between the mechanisms of US3-mediated ER retention of MHC-I requires further study, but US3 is clearly an important player in HCMV evasion of antigen presentation.

1.5(e) US6

Description of the fourth and final HCMV inhibitor of antigen presentation came soon after the flurry of reports that uncovered the cellular targets of US2, US3, and US11. Like the other factors in its gene family, US6 acts through a unique mechanism to achieve the endpoint of MHC-I downregulation. Instead of binding to the PLC chaperone tapasin or promoting retrotranslocation of MHC-I, US6 interferes directly with TAP-dependent peptide translocation [148]. HSV immediate early protein ICP47 was also known to inhibit TAP [149, 150], but by competing with the substrate binding site in the cytosol [151, 152]. In contrast, US6, a type Ia ER transmembrane protein, binds to a luminal domain of TAP [148]. Later studies expanded on this mechanism by revealing that US6 binds only to assembled luminal domains of TAP1 and TAP2 subunits, promoting a conformational change in the TAP heterodimer, which ultimately prevents ATP-dependent peptide translocation [153, 154].

1.5(f) Interference with MHC-I by RhCMV

All of the US6 family members can individually downregulate MHC-I cell surface expression, though they each have slightly different kinetics and efficiency among specific alleles. US3 is expressed earliest in HCMV infection and promotes ER

retention of the MHC-I heterodimer. This could be viewed as a delay mechanism, to essentially "hold" assembled MHC-I in the ER until the virus can translate the other inhibitors of antigen presentation. Soon thereafter, US6 continues the prevention of peptide loading by inhibiting TAP, and works in conjunction with US2 and US11 as they force the retained MHC-I HC to the proteasome for degradation. Working together, these four proteins have created an efficient, well-oiled machine for evasion of antigen presentation.

The RhCMV genome contains a similarly structured segment of ORFs that resembles the US2-11 region of HCMV. In the initial description of the RhCMV genome, the corresponding RhCMV Rh182-189 region was identified as containing likely homologues to the US2-11 region, even though their amino acid sequences were only 33-43% identical [55]. Intracellular staining strongly suggested an ER localization for each of the Rh182-189 gene products. More importantly, the functions of Rh182-189 proteins almost exactly mimicked those of US2-11. Rh182 and Rh189 initiated ER dislocation and proteasomal degradation of MHC-I, identifying them as US2 and US11 homologues, respectively. Rh184 shared the ability of US3 to retain MHC-I in the ER, although it was less efficient and only temporarily delayed ER exit. This minor discrepancy may be explained by a decreased efficiency of Rh184 in retaining the specific alleles expressed in human HeLa cells that were used in this experiment. Rh184 may be just as proficient at retaining RM MHC (called "Mamu" alleles) in a relevant RM cell line as US3 is at retaining human HLA molecules. A TAP transport assay completed the functional phenotype comparison by identifying Rh185 as the US6 homologue [119].

The cooperation of US2, US3, US6, and US11 to downregulate MHC-I is remarkably efficient during HCMV infection. Transient transfection and often overexpression of individual gene products helped to determine their molecular mechanisms. The creation of an HCMV BAC streamlined the viral mutagenesis process, giving way to experiments in which effects of WT and recombinant HCMV infections could be compared *in vitro* [155]. Studies of HCMV infection cannot be extended *in vivo*, however, because of species specificity and the obvious ethical problems with experimentally infecting humans. Herein lays the importance of RhCMV infection of RM as the *in vivo* model for HCMV infection.

Whereas MHC-I is rapidly degraded in cells infected with WT HCMV, cells infected with HCMV lacking the US2-11 genomic region display steady-state levels of MHC-I on the cell surface [117]. With the close homology of the US2-11 and Rh182-189 regions, one would expect a similar result in RhCMV-infected cells. However, a recent study showed that deletion of Rh182-189 from RhCMV did not restore MHC-I levels in infected cells. In fact, pulse-chase analysis determined that newly synthesized MHC-I was almost nonexistent, even in the RhCMV Rh182-189 mutant (ΔU). Therefore, the authors concluded that RhCMV operates distinctly from HCMV in two ways. First, there is an additional factor outside of the Rh182-189 genomic region that downregulates MHC-I. Secondly, this factor is able to either very quickly degrade or prevent altogether new synthesis of MHC-I HC. This activity was named viral inhibition of heavy chain expression (VIHCE). Expression of other cellular proteins and β2m remained at or near expression levels in uninfected cells, indicating that the RhCMV factor responsible for VIHCE specifically acts on MHC-I HC [120].

1.5(g) Identification of Rh178 as the mediator of VIHCE

Once the VIHCE phenotype of RhCMV was identified, a series of deletion mutants within the Rh158-180 region was created in order to isolate the responsible ORF(s). Cells were infected with each mutant virus and then subjected to pulse-chase experiments to assess new synthesis of MHC-I HC. A lack of any new HC synthesis served as the indicator of VIHCE function. Using this method, RhCMV ORF Rh178 was identified as the single ORF required for VIHCE [120]. During infection with a double knockout of Rh178 and Rh182-189 (ΔVΔU) MHC-I expression was indeed restored to steady state levels. Cells infected with RhCMV lacking only Rh178 (ΔV) initially express MHC-I before it is degraded by the Rh182-189 gene products. Therefore, along with the four homologues to the HCMV US2-11 region, RhCMV encodes an additional factor that is responsible for VIHCE activity. Rh178 joins with Rh182-189 in downregulating MHC-I, but it utilizes a distinct mechanism to do so.

Initial characterization of Rh178 yielded some important clues about its mechanism. Rh178, unlike all the US6 family members, does not have structural homology to the Ig-like superfamily. PNGase treatments and immunofluorescence studies determined that it is a 24 kDa, unglycosylated protein that localizes to the ER. Even though Rh178 has significant structural differences from the US6 family members, their shared subcellular localization indicates that they all target MHC-I prior to its exit from the ER. A number of different hypotheses were generated to explain the pulse-chase data showing that Rh178 caused a dramatic reduction in newly synthesized MHC-I. First, Rh178 could block translation initiation by preventing the ribosome from binding to the MHC-I HC mRNA. Alternatively, it could block completion of translation. Finally, it

might cause such rapid degradation of MHC-I HC that normal detection techniques would miss the transient expression. This initial study concluded that RhCMV-infected cells transcribed normal levels of MHC-I mRNA that were bound to actively translating ribosomes. Therefore, translation initiation did not seem to be affected. Interestingly, Rh178 downregulation of MHC-I was found to be dependent on the HC signal peptide (SP), a short, N-terminal amino acid sequence that directs translocation into the ER [120]. To date, there are no other descriptions of one protein targeting the SP of another to mediate its downregulation.

1.6 Translation, translocation and the importance of the signal peptide

MHC-I peptide loading and transport to the cell surface was explained in detail in section 1.5(a), but the initial conclusions about Rh178 function indicate that VIHCE activity prevents expression of full length MHC-I HC at any point during infection. Therefore, the search for a mechanism for Rh178 shifted to events that occur early in MHC-I translation and translocation. Steps that precede MHC-I peptide loading include translation initiation at the ribosome, SP recognition, and recruitment to and translocation across the ER membrane. To understand how Rh178 targets MHC-I, it is necessary to introduce the details of the biological processes of protein translation and translocation.

1.6(a) The signal hypothesis

ER membranes and membranes of other cellular organelles are barriers that prevent free diffusion of proteins, allowing compartmentalization of cellular functions. However, since all protein translation begins on ribosomes in the cytosol, the cell must

have a way to direct proteins to their final destination. The mechanism by which proteins are targeted to specific locations throughout the cell was something of a black box until the articulation of the signal hypothesis in the 1970s. Two landmark reports by Blobel and Dobberstein presented a remarkably accurate description of nascent protein targeting to cellular organelles, and their findings have provided a mechanistic framework for what we now know about SP-mediated targeting [156, 157]. To study intracellular targeting, Blobel and Dobberstein evaluated translation of IgG light chain mRNA from murine myelomas. IgG is a secreted protein, and therefore must temporarily access the ER lumen before transiting through the secretory pathway and into the extracellular space. Earlier studies had demonstrated discrepancies between the lengths of the authentic light chain product translated in whole cells as compared to the *in vitro* translation product, suggesting the possibility of a light chain precursor [158, 159]. Blobel and Dobberstein fractionated rough and free ribosomes from murine myeloma cells, and then performed cell-free, *in vitro* translations of the light chain mRNA. IgG light chain translated on the rough ribosomes had a smaller molecular weight than protein translated on free ribosomes. The difference in MW was about two kDa, which corresponds to about 20 amino acids. They hypothesized that early in translation, those 20 amino acids acted as a signal that directed the mRNA-ribosome-nascent protein complex (RNC) to the ER membrane, where translation continued across the ER membrane and into the ER lumen.

Blobel and Dobberstein assembled these results into the signal hypothesis, which begins with mRNA binding to a ribosome and ends with a full-length and properly folded mature protein at the appropriate cellular location. The signal hypothesis states that all ribosomes begin as “free” ribosomes in the cytosol. Only after the first ~20 N-terminal

amino acids of a nascent protein emerge from the large ribosomal subunit can the ribosome become associated with an organellar membrane. They acknowledged that various unique SP sequences likely exist in order to target proteins to diverse cellular compartments. In the case of ER targeting, the RNC complex moves to the ER membrane and translocation through an ER membrane pore commences. Other chaperone proteins in the cytosol and in the membrane assist in crosslinking the ribosome to this putative translocation pore, through which the linear nascent protein is cotranslationally translocated. Cleavage of the SP is mediated by a luminal protein after a sufficient length of protein has entered the ER lumen. The protein then acquires its tertiary, folded structure in the lumen, at which point the ribosome dissociates from the ER membrane and returns to the cytosol to repeat the process. They described the ribosomal-membrane association as "functional, specific, and transient" [157]. They further suggested that the signal hypothesis applies to secreted and transmembrane proteins, though the details of how a protein was retained within the membrane remained unclear.

1.6(b) Recent additions to the signal hypothesis

The last thirty-five years of research have rounded out the story, filling in some of the details that were missing from the original signal hypothesis. A consistent length of ~20 amino acids, a highly hydrophobic core, and possession of a bulky residue at the cleavage site are all important characteristics for the SP of any ER-destined protein [160]. As the SP of a nascent protein emerges from the ribosome, it is bound by the signal recognition particle (SRP), comprised of six polypeptide subunits and a small 7S RNA [161]. The SRP then brings the entire complex to the ER membrane, where it binds to the

membrane-bound SRP receptor [162]. After docking, GTP hydrolysis regulates affinities of SP for SRP and of SRP for the SRP receptor. Upon GTP hydrolysis, the SRP is released from the SP, and the RNC complex is transferred to the translocon [163]. The Sec61 heterotrimer is the major component of the mammalian translocon, and is necessary for translocation of both secreted and transmembrane proteins [164-166]. Electron microscopy and X-ray crystallography have confirmed the original proposal of a continuous channel from the translating ribosome through the ER membrane into the lumen [167, 168]. The Sec61 translocon remains in an open conformation as long as the ribosome is bound and actively translating, and closes upon translocation completion and ribosome dissociation [169]. Another factor, translocating chain-associating membrane protein (TRAM), acts as a chaperone during cotranslational translocation by associating with the nascent chain and bringing it into close proximity with the translocon [170].

The standard pathway for secreted proteins is translocation through the Sec61 translocon followed by processing of the precursor protein, first by cleavage of the SP by signal peptidase and then further breakdown by signal peptide peptidase [171, 172]. Cleavage of the signal peptide accounts for the original observation by Blobel and Dobberstein about variations in IgG light chain size that led to the development of the signal hypothesis. Transmembrane proteins are translocated via a similar mechanism, with one important difference. They encode at least one stop-transfer sequence, which interrupts translocation and precludes the rest of the protein from entering the ER lumen. The result is a bitopic protein with three distinct domains: an ER luminal domain, a short transmembrane domain derived from the stop-transfer sequence, and a cytoplasmic domain. Multi-spanning transmembrane proteins require a series of signal sequences and

stop-transfer sequences. The order of these signals within the primary sequence ultimately determines the number of membrane spanning sections and the orientation of the protein within the membrane (e.g. whether the N-terminus is luminal or cytosolic) [173].

1.6(c) MHC-I SP conservation

MHC-I is an example of a single-spanning transmembrane protein that undergoes cotranslational translocation. As described in section 1.5(a), the α1, α2, and α3 domains reside in the ER lumen and, after secretion, in the extracellular space. They are N-terminal to a single transmembrane segment and a very short cytosolic C-terminus. MHC-I localization to the ER is dependent on the SP, which is one of the most conserved regions across all MHC-I alleles [174]. The relative conservation of the SP is significant when considering the intricacy of the MHC-I loci. High allelic diversity and frequent polymorphisms make the MHC-I locus one of the most complicated regions of the genome across all mammalian species. The human MHC-I locus (human leukocyte antigen, or HLA) encodes six loci (HLA-A,B,C,E,F,G) with thousands of allelic variants. The RM MHC-I locus (*Macaca mulatta*, or Mamu) is even more convoluted due to gene duplications within some of the orthologous loci (Mamu-A,B,E,F,G) [175, 176]. The conservation and diversity of MHC-I alleles is an important consideration in the study of viral immune evasion. If viruses are to effectively target MHC-I for downregulation on a population-wide basis, they need to either target conserved regions or to develop multiple mechanisms to attack various alleles.

1.7 Advantages and limitations of CMV superinfection

After primary infection with a pathogen, a successful host immune response will first control the initial infection and then promote long-lasting immunity by producing antigen-specific memory B and T cells. Memory B cells can persist indefinitely [177], whereas memory T cell populations drop more precipitously, with a half-life in the range of a decade [12, 178]. In most cases, this enduring immunological memory prevents secondary infection with an equivalent or related strain, also called superinfection. "Superinfection" is a term fraught with confusion in the literature, and is often mislabeled "reinfection" or "coinfection." Reinfection occurs after a primary infection has been cleared by the immune system. Coinfection and superinfection both fall into the category of dual infection, in which a host is infected with two different but related strains. In the case of coinfection, the two infections happen either simultaneously or in the window of time before an immune response can develop. This thesis will adopt from the HIV field a definition of superinfection as "infection with a second strain after the initial infection and the immune response to it has been established [179]." Using this definition, superinfection has been documented for chronic viruses like hepatitis C virus (HCV) and HIV [180-184]. The high genetic diversity and pathogenic potential of these viruses are the main contributing factors to their ability to superinfect. The RNA-dependent RNA polymerase of HCV lacks a proofreading mechanism, and the nucleotide sequences of different HCV strains differ by up to 30% [185]. The variability in the HIV envelope protein, with 15-35% amino acid sequence diversity within and among clades, partially accounts for HIV superinfection [186, 187]. In addition, superinfection with both HCV

and HIV occurs in hosts with a virally-induced compromised immune system, after the immune defenses have already been damaged.

HCMV is another chronic virus for which superinfection in the context of a pathologic state has been documented, for example in immunocompromised individuals and STD clinic attendees [188, 189]. Notably, infection with multiple strains of HCMV also seems to occur frequently in healthy individuals, including children in day care and HCMV seropositive women of childbearing age [16, 190]. In addition, HCMV does not have near the proportional genetic diversity of HCV or HIV. Localized regions of the 230 kb genome have high sequence divergence among different strains, but overall the nucleotide sequences remain relatively stable [191-195]. Therefore, two major differences exist between superinfection with HCV or HIV and superinfection with HCMV. First, HCV and HIV create a disease state, so the superinfecting virus is confronting an already weakened immune system. In contrast, HCMV superinfection occurs in healthy individuals. Second, superinfection by HCV or HIV is postulated to rely on high variability of one or more viral gene products. HCMV strains have less diversity among their genomes, so an alternate explanation for HCMV superinfection may exist. Superinfection seems to have been conserved throughout CMV evolution, and has been detected in the wild or experimentally proven in both mice and RM [59, 196-198].

In summary, CMV has the unique and remarkable ability to superinfect in the face of a healthy, robust, and specific immune response. The quality of HCMV superinfection creates difficulties for HCMV vaccine design, but it may also present opportunities to develop it as a vaccine vector for other diseases.

1.7(a) Disadvantages of HCMV superinfection in vaccine development

Mimicking the natural long-term immunity of primary infection without also stimulating associated pathology is the signature of an effective vaccine. This principle was first exploited over two centuries ago by Edward Jenner when he protected a healthy eight year old boy from smallpox challenge by inoculating him with discharge from a lesion containing the related cowpox virus [199]. His experiments, however crude by current standards, laid the groundwork for all current vaccines. A report in 2000 by the Institute of Medicine placed an HCMV vaccine in the "highest priority" category based on the impact that HCMV-related disease has on quality of life and health care spending [200]. Prior to this declaration, early attempts at creating an HCMV vaccine had been largely unremarkable. Reports in the 1970s and 1980s indicated that vaccination with live, attenuated HCMV in various target populations stimulated cellular and humoral immune responses that were equivalent to natural infection [201-203]. There were no serious safety issues that arose from these trials, but they failed to demonstrate protection from subsequent HCMV infection. Because we now know that natural immunity to HCMV is insufficient to protect against superinfection, it seems unlikely that a vaccine could offer complete protective immunity. Natural immunity does, however, protect against HCMV-related disease in allograft recipients and neonates [204, 205], indicating that prevention of disease rather than infection is a reasonable goal for an HCMV vaccine [206, 207].

More recently, a number of HCMV vaccines have entered preliminary clinical trials. Formulations and delivery mechanisms have been diverse, including live-attenuated virus, recombinant subunit gB-based vaccines, pp65-derived T cell epitopes,

and gB and pp65 containing dense bodies [208-213]. Each of these vaccine candidates has shown some promise, but none have progressed beyond a Phase II clinical trial. No study has demonstrated full protection from secondary infection, further confirming the significance of superinfection and the necessity for a shift in strategy on HCMV vaccine development. It is almost certain that an HCMV vaccine will not eradicate the virus, as seen with the polio vaccine, or even fully prevent infection, as does the measles vaccine. Instead, if formulated and targeted correctly, an HCMV vaccine could establish natural immunity in a healthy individual that would prevent later development of HCMV-mediated disease. Ideal target populations for such a vaccine would include women of childbearing age and patients who are seronegative before receipt of an organ transplant.

1.7(b) Harnessing the power of CMV superinfection – CMV as a vaccine vector

Superinfection may hinder the development of a CMV vaccine, but it may also provide the foundation for introducing CMV as a vaccine vector. A recent study theorized that CMV has a number of characteristics that make it an ideal vaccine vector for certain pathogens [198, 214]. CMV is a relatively innocuous virus for healthy individuals, and therefore would not pose significant dangers to most patients. In addition, it could be attenuated to address concerns in the remainder of patients with compromised immune systems. As discussed in section 1.1, CMV stimulates staggeringly strong cell-mediated immunity, both in number and diversity of CMV-specific CD4+ and CD8+ T cells [14]. Presumably, this principle would also apply to foreign antigens carried by a CMV vector. An important consideration for a vaccine that stimulates cell-mediated immune responses is the nature of the memory CD4+ and CD8+ T cells. Many

vaccines designed to induce T cell immunity initially result in antigen-specific central and effector memory T cells (T_{CM} and T_{EM}, respectively). T_{CM} are the more durable subset and reside in lymphoid tissue, ready to proliferate and differentiate upon exposure to antigen. T_{EM} have a shorter life span and exist in peripheral tissues. Because they have taken up residence at the site of a possible future infection, they will provide the first line of defense with immediate effector functions including antigen recognition, IFN-γ secretion, and cytotoxicity [215, 216]. Nonpersistent vaccine vectors only produce antigen for a short time, and the T_{EM} population fades out with the antigen, leaving only a T_{CM} compartment. In contrast, CMV consistently produces low levels of antigens, so the T_{EM} compartment is retained, and a more rapid response against an invading pathogen can be expected [217]. Finally, the superinfection capability of CMV allows for multiple rounds of vaccination with different antigens, even in a CMV-positive animal. Based on all of the above qualities, CMV as a vaccine vector has the potential to be a safe and widely available vaccine that rapidly and specifically targets a broad set of pathogenic antigens.

This principle was recently investigated by using RhCMV as a vaccine vector for simian immunodeficiency virus (SIV) [198, 214]. SIV infection of RM is a common animal model for the study of HIV infection of humans, and vaccines are often tested in this model before progressing to human trials. The search for an HIV vaccine has been a worldwide priority for almost thirty years, since its discovery and identification as the causative agent of AIDS in 1983 [218, 219]. AIDS is a worldwide menace; UNAIDS estimates that of the 58 million people infected with HIV since the early 1980s, 25 million have died. Great strides have been made in controlling AIDS with the

development of an antiretroviral cocktail, highly active antiretroviral therapy (HAART), which allows HIV-infected individuals to live a long, relatively normal life. However, many underserved nations lack access to these expensive drugs and many with access to HAART will eventually progress to AIDS. Clearly, a vaccine against HIV is still desperately needed.

The report by Hansen et al. suggests that a RhCMV vaccine vector that generates a vigorous T_{EM}-based response may solve some of the problems that have vexed the HIV field for years. In the original study, twelve RM were vaccinated with RhCMV vectors containing the SIV proteins gag, a rev-tat-nef fusion (rtn), and env in three separate doses, then challenged about two years later with multiple intrarectal doses of highly pathogenic SIVmac239 [198]. Compared to an unvaccinated control group in which all RM acquired progressive SIV infection, the vaccinated animals required more doses on average to become infected. Even more significantly, after thirteen SIVmac239 challenge doses, four of twelve RM had undetectable or transient viral load in the plasma. A more recent study has corroborated this result in a larger group of RM and demonstrated a 50% protection rate [214]. Therefore, at least in a subset of RM, RhCMV-SIV vaccine vectors have completely prevented progressive SIV infection. This is in contrast to other SIV/HIV vaccine trials that have only succeeded in decreasing viral replication and therefore reducing the disease progression or transmission risk [220-224]. The preliminary success of RhCMV as a vaccine vector for SIV provides hope that a CD8+ T cell-based HIV vaccine strategy may be capable of providing complete protection against viral replication. However, it will be necessary to dissect components of the immune response in more detail before a CMV vaccine vector could be tested in the human

population. Chapter Four will describe *in vivo* investigations into the role of RhCMV-encoded US6 family members and Rh178 on superinfection and CD8+ T cell evasion, and the implications for the use of RhCMV as a vaccine vector.

1.8 Hypotheses

Chapter Three describes the use of an *in vitro* expression system to further investigate the effects of Rh178 on MHC-I HC expression. The initial characterization of Rh178 identified it as an ER-localized RhCMV-specific factor that specifically downregulates MHC-I HC. This Rh178-mediated VIHCE activity is dependent on the MHC-I SP [120]. SP-dependent interference is a novel mechanism not only for viral inhibition of antigen presentation but also for general protein translation. **Our hypothesis is that Rh178 interrupts MHC-I expression by targeting the SP of the nascent protein very early in translation.**

Chapter Four describes results from experiments investigating the *in vivo* role of Rh178 in the context of the other RhCMV viral inhibitors of antigen presentation, Rh182-189. Experimental infections with recombinant CMVs can help to determine virally encoded factors that are responsible for a particular phenotype. This principle can be applied to the superinfection phenotype but ethical considerations prevent experimental infection of humans with recombinant HCMV. Therefore, we use RhCMV infection of RM to study superinfection *in vivo*. Superinfection of RhCMV-seropositive RM has recently been confirmed [59]. In this model, superinfection is possible with subcutaneous inoculation as low as 100 plaque forming units (pfu) (Figure 1.4). It is well known that the US6 family of genes works together through a variety of molecular mechanisms to

downregulate MHC-I and prevent presentation of viral peptides to CD8+ T cells. Therefore, this region of the genome is an attractive candidate for allowing superinfection by HCMV. RhCMV ORFs Rh182-189 encode proteins that are equivalent to US2, US3, US6, and US11. RhCMV also encodes Rh178, which by VIHCE activity, inhibits translation of MHC-I HC. Therefore, when using RhCMV infection of RM, Rh178 must be considered when examining the *in vivo* effects this class of immune evasion genes has on superinfection. **We hypothesize that all or a subset of RhCMV factors that downregulate MHC-I are responsible for superinfection.**

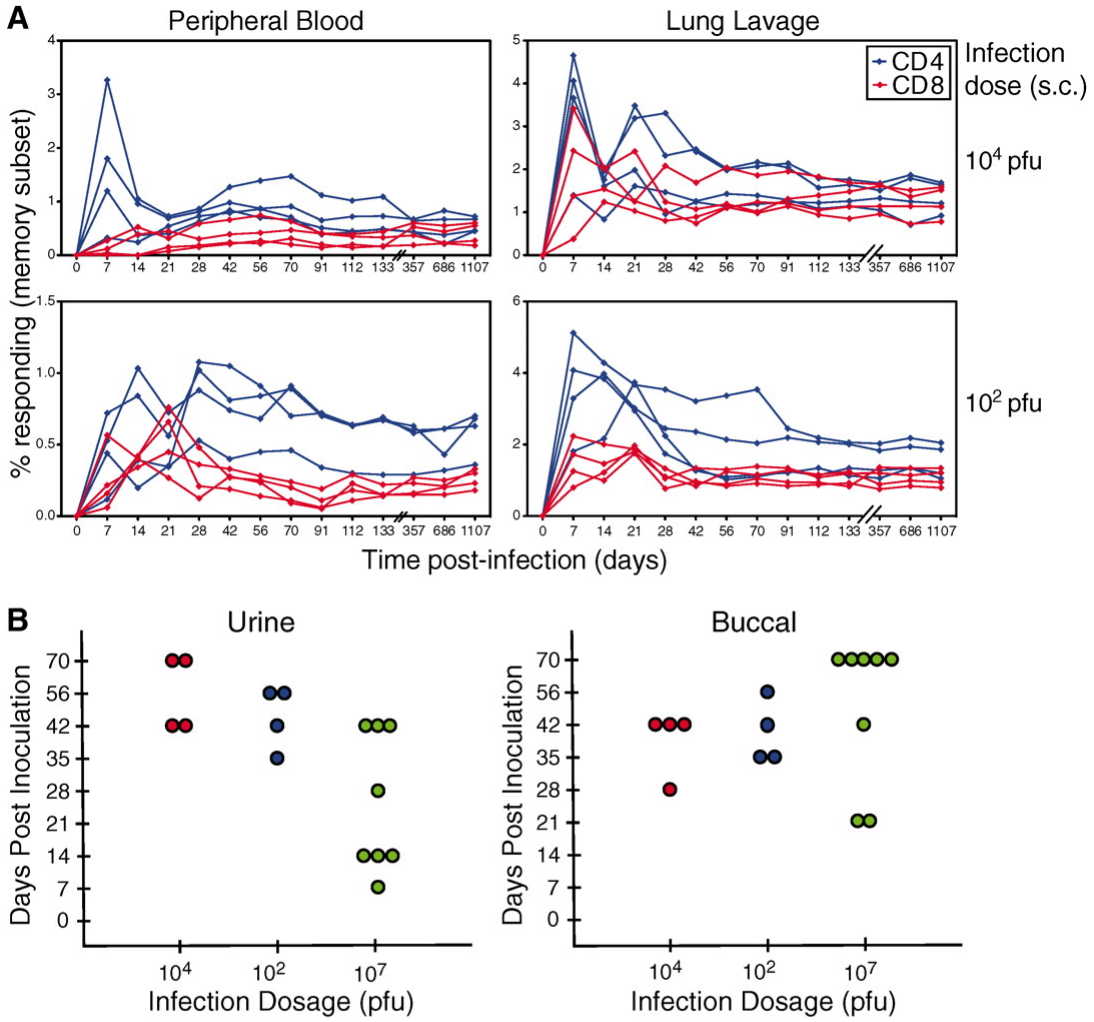

A
Peripheral Blood
Lung Lavage
CD4
CD8
Infection dose (s.c.)
10^4 pfu
10^2 pfu
% responding (memory subset)
Time post-infection (days)
B
Urine
Buccal
Days Post Inoculation
Infection Dosage (pfu)

Figure 1.4: Superinfection of RhCMV-positive animals is independent of viral dose. (A) At day 0, two cohorts of four RhCMV-seropositive animals each were infected subcutaneously with 10^2 or 10^4 PFU of RhCMV-gag. The SIVgag-specific T cell responses in PBMC or in BAL were monitored by flow cytometric analysis of ICCS for CD69 and tumor necrosis factor–α (TNF-α). (B) Day of first detection of SIVgag-expressing virus in the urine or buccal swabs collected at the indicated intervals from each animal in the two cohorts shown in (A). Also included are results from a third cohort of eight $RhCMV^+$ animals inoculated with 10^7 PFU of RhCMV-gag. Expression of SIVgag was determined by immunoblot using antibody to SIVgag from viral cocultures. Each circle represents an individual animal.

CHAPTER TWO

Materials and Methods

2.1 Cells, virus, and antibodies

HeLa-Tet-Off cells were obtained from Clontech and telomerized rhesus fibroblasts (TRF) [225] were obtained from Dr. Jay Nelson. TRF-178 cells were made by cloning rh178-FLAG into pRevTre (Clontech), transfecting the resultant vector into Phoenix-AMPHO packaging cells (provided by Dr. Ashlee Moses) and infecting telomerized rhesus fibroblasts (TRFs) with the resulting retrovirus, followed by selection in 200 μg/ml Hygromycin (Invivogen). HeLa cells, TRFs, and stable transfectant TRF-178 cells were maintained in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/ml streptomycin. All cells were grown at 37°C in humidified air with 5% CO_2. Adenovirus containing the tet-transactivator (Ad-tTA) was obtained from Dr. David Johnson. Mouse anti-Flag M2, anti-Flag-FITC conjugate, and mouse anti HA (clone HA-7) antibodies were purchased from Sigma. Mouse anti-GAPDH (clone 6C5), mouse anti-Integrin αV (clone P2W7), and goat anti-mouse-HRP conjugate antibodies were purchased from Santa Cruz Biotechnology. Goat anti-mouse Immunoglobulin/RPE antibody was purchased from DAKO. Alexa Fluor 594 goat anti-mouse and Alexa Fluor 647 chicken anti-mouse Immunoglobulins (H+L) were purchased from Invitrogen. Anti-MHC Class I monoclonal W6/32 antibody was purchased from Abcam.

2.2 Plasmid construction

Rh178 was originally PCR amplified from RhCMV strain 68-1 (provided by Dr. Scott Wong). It was Flag tagged and codon-optimized for expression in mammalian cells. Codon optimization services were provided by GeneArt. See Figure 2.1 for a comparison of the nucleotide sequences of the WT Rh178 compared to the codon-optimized Rh178. VIHCE-Cfl and all VIHCE mutants were constructed by PCR and inserted into EcoRI and BamHI sites of the cloning vector pUHD 10-1 [226]. The sense primer for all mutants in this series is VIHCE-F, and the antisense primers for VIHCE-CFl, VIHCE Δ10, VIHCE Δ20, VIHCE Δ30, VIHCE Δ40, VIHCE Δ50, VIHCE Δ60, VIHCE Δ70 are, respectively, VIHCE-Fl-R, Δ10-R, Δ20-R, Δ30-R, Δ40-R, Δ50-R, Δ60-R, and Δ70-R. Sequences of all oligonucleotides are provided in Table 2.1. VIHCE TM SytII was cloned into BamHI and HindIII sites of pUHD10-1 by using three sense primers in sequence: TMS1-F, TMS2-F, TMS3-F, and antisense primer TM-R. HLA-A3 truncations and A3-CD8 fusion constructs were also cloned into pUHD10-1 by PCR into EcoRI and BamHI sites. HLA-A3 truncations with C-terminal HA-tags were each created with the same sense primer, A3-F. Antisense primers for A3-HA, A3 294, A3 206, A3 160, and A3 114 are A3-HA-R, A3 294-R, A3 206-R, A3 160-R, and A3 114-R, respectively. Control plasmid CD8-HA was created by inserting CD8α from CD8α-pRMHA [227] into the EcoRI and BamHI sites of pUHD10-1. Primers used for creation of CD8-HA were CD8-HA-R and CD8-F. HLA-A3-CD8 fusions and Rh67-CD8 fusion were created by a triple ligation of HLA-A3 or Rh67 truncations, CD8-HA (without the CD8 signal sequence), and pUHD10-1. The HLA-A3/Rh67 and CD8-HA PCR products were fused with an added XbaI site. The CD8 sequence, minus SP, was generated by using primers

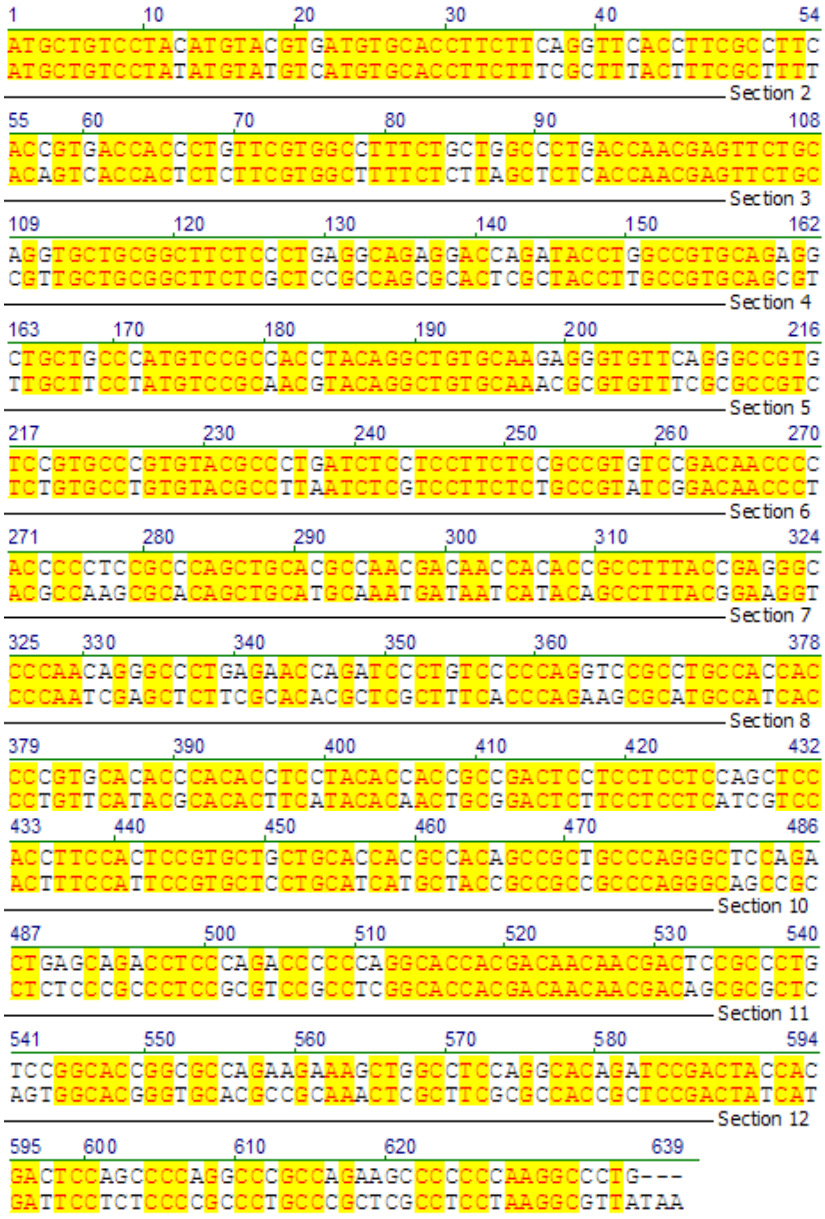


Figure 2.1: Codon-optimized Rh178

A codon-optimized version of Rh178 was created to increase expression for in vitro studies. A comparison of the nucleotide sequences of the original Rh178 cloned out of RhCMV 68.1 (top row) and the codon-optimized version (bottom row, services provided by GeneArt) depicts conserved nucleotides in yellow and optimized nucleotides in white.

Table 2.1: Oligonucleotides used in this study (5'-3').

Primers for Rh178 and HLA-A3 mutant constructs	
Rh178-F	CTGGAATTCATGCTGTCCTAC
Rh178-Fl-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCCAGGGCCTTGGGGGGGCT
Δ10-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCGGGGCTGGAGTCGTGGTA
Δ20-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCCCTGGAGGCCAGCTTTCT
Δ30-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCGCCGGACAGGGCGGAGTC
Δ40-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCGTGCCTGGGGGGTCTGGG
Δ50-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCTCTGGAGCCCTGGGCAGC
Δ60-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCGTGCAGCAGCACGGAGTG
Δ70-R	CTGGGATCCTCATCACTTGTCGTCGTCGTCCTTGTAGTCGGAGGAGGAGGAGTCGGC
TMS1-F	GGGCTCCTGCTTCTCACCTGCTGCTTCTGCATCTGCGAGTTCTGCAGGTGC
TMS2-F	TTCTTCAGGGCACTGATCGCCATTGCTGTGGTTGCTGGGCTCCTGCTTCTC
TMS3-F	CTGGGATCCATGCTGTCCTACATGTACGTGATGTGCACCTTCTTCAGGGCACTG
TM-R	CTGAAGCTTAGTCATCACTTGTCGTC
A3-F	CTGGAATTCATGGCCGTCATGGCGCCCCGAAC
A3-HA-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTATGGGTACACTTTACAAGCTGTG
A3 294-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTATGGGTAGAGGGGCTTGGGCAGAC
A3 206-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTATGGGTACGTGCGCTGCAGCGTC
A3 160-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTATGGGTACGCCGCGGTCCAAGAGC
A3 114-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTATGGGTAGGCCTCGCTCTGGTTGTAG
A3tr-F	CTGGAATTCGCCGCCACCATGGCCGTCATGGCGCCC
114-R	CTGTCTAGAGGCCTCGCTCTGGTTGTA
73-R	CTGTCTAGACGCCCGCGGCTCCATCCT
50-R	CTGTCTAGAGCCCACGGCGATGAAGCG
24-R	CTGTCTAGACGCCCAGGTCTGGGTCAG
CD8-F	CTGGAATTCGCCGCCACCATGGCCTTACCAGTGACC
CD8noSS-F	CTGTCTAGAAGCCAGTTCCGGGTGTCG
CD8-HA-R	CTGGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTAGACGTATCTCGCCGAAAG
Rh67-F	CTGGAATTCGCCGCCACCATGCTGCTCAGCGTGGCG
Rh67-31-R	CTGTCTAGATACCCCCAAAGCCACCGT
Rh67-Fl-F	CTGGAATTCATGCTGCTCAGCGTGGCGATGGTG
Rh67-Fl-R	CTGGGATCCTCACTTGTCGTCGTCGTCCTTGTAGTCTGGAATGGTTATCATTTC
SSFlA3-F1	GACTACAAGGACGACGACGACAAGGGCTCCCACTCCATG
SSFlA3-F2	CTGCTACTCTCGGGGGCCCTGGCCCTGACCCAGACCTGGGCGGACTACAAGGACGAC
SSFlA3-F3	CTGGAATTCATGGCCGTCATGGCGCCCCGAACCCTCCTCCTGCTACTCTCGGGG
SSFlA3-R	CTGGGATCCTCACACTTTACAAGCTGT

CD8noSS-F and CD8-HA-R. A3 114, A3 73, A3 50, and A3 24 fragments were made using sense primer A3tr-F and antisense primers 114-R, 73-R, 50-R, and 24-R, respectively. The Rh67 fragment was made using sense primer Rh67-F and antisense primer Rh67-31-R. Rh67-Fl was created with sense primer Rh67-Fl-F and antisense primer Rh67-Fl-R. SSFlA3 was created with sequential sense primers SSFlA3-F1, SSFlA3-F2, and SSFlA3-F3 and antisense primer SSFlA3-R.

2.3 *In vitro* Transcription, Translation, and Proteinase K Digestion

mRNA was transcribed with SP6 RNA polymerase (Promega) using 500 ng of plasmid DNA in a 10-µl volume at 40 °C for 1 h in reactions containing 40 mM Tris-HCl (pH 7.5), 6.0 mM magnesium acetate, 2 mM spermidine, 0.5 mM each of ATP, CTP, UTP (all from Promega), 0.1 mM GTP, 0.5 mM GpppG (Promega), 10 mM DTT, 0.75 U/ml RNAse inhibitor (Promega), and 0.4 U/ml SP6 RNA polymerase. Rabbit reticulocyte lysate (RRL) was prepared as previously described [228, 229]. Translation was performed at 25°C for 1.5 hours in reactions containing 20% transcript mixture, 40% nuclease-treated RRL, with additional 1 mM ATP, 1 mM GTP, 12 mM creatine phosphate (Promega), 40 µM each of 19 essential amino acids (Promega), except methionine, 1 µCi/µl of [^{35}S]-label (Express Protein labeling mix, Perkin-Elmer), 40 µg/ml creatine kinase (Promega), 0.2 U/µl RNAse inhibitor (Promega), 10 mM Tris-HCl (pH 7.5), 100 mM potassium acetate, 2 mM DTT, and 2 mM $MgCl_2$. Canine pancreas microsomal membranes(0.3 µl) (Promega) were added at the start of translation. For protease protection experiments, Proteinase K (Fermentas) was added (2 mg/ml) in the presence or absence of 1% Triton X-100. Samples were incubated at 4˚C for 1h, and

residual protease was inactivated by rapid mixing with phenylmethylsulfonyl fluoride (10 mM) and heating to 100 °C in 10 volumes of 1% SDS, 0.1 M Tris, pH 8.0, for 5 min. Samples were then added directly to SDS loading buffer and electrophoresed on a 16.5% Tris-tricine gel, and visualized by autoradiography.

2.4 Transfection and Nucleofection

For expression and co-transfection experiments, 500 ng Rh178 mutants were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions. Rh178 mutants, HLA-A3 truncations, and HLA-A3-CD8 fusions were electroporated into TRFs [225] using the AMAXA Nucleofector II (AMAXA Biosystems) and cell line solution L (Lonza AG). 500 ng to 2 μg of plasmid DNA was mixed with $0.5x10^6$-$2x10^6$ TRF, suspended in 100 μl AMAXA solution, and electroporated under parameters defined by program T-030. Cells were then recovered in 500 μl RPMI (Gibco) for 45 min at 37°C, followed by plating in complete DMEM. Co-transfection with GFP vector revealed transfection efficiency was consistently between 50-80%.

2.5 Streptolysin O and Immunofluorescence

HeLa cells were plated to ~70% confluence in 24-well dishes on coverslips, and either mock transfected (500 ng control plasmid) or transfected with 500 ng C- or N-Flag tagged Rh178. SLO permeabilization and immunofluorescence was performed 48h post-transfection as described [230]. Briefly, SLO (1μg/ml) is pre-activated by incubating at 37°C for 10 min with 4mM dithiothreitol (DTT) (Fisher Scientific) in a sodium-free

buffer (25 mM Hepes, 2.5 mM MgCl, 25 mM KCl, 25 mM sucrose, pH 7.4). Activated SLO was then added to the cells at 4°C for 10 min. Cells were washed 2x with cold sodium-free buffer, and then permeabilized at 37°C for 15 min followed by fixation in 1% formaldehyde (Fisher Scientific) at room temperature (RT) for 10 min. Cells were blocked with 1% bovine serum albumin (Fisher Scientific) and 5 mg/ml glycine (Fisher Scientific) in sodium-free buffer at RT for 15 min. Cells were then incubated for 1.5 h with a 1:200 dilution of anti-Flag antibody in either sodium-free buffer (for SLO permeabilized cells) or 0.25% saponin (Calbiochem) in sodium-free buffer (for saponin permeabilized cells). Cells were rinsed 3x with sodium-free buffer, then incubated for 1h with a 1:500 dilution Alexa Fluor 594 goat anti-mouse antibody. Cells were rinsed 3x with sodium-free buffer, then mounted onto glass slides (Fisher Scientific) with VECTASHIELD® (Vector Laboratories). Slides were visualized on a Zeiss Axioskop 2 Plus fluorescent microscope and images were produced with AxioVision v4.6 software (Zeiss).

2.6 Immunoblot

HeLa cells were harvested 48 hours post transfection or AMAXA nucleofection and lysed directly in 2x Laemmli buffer [231]. Samples were run through QIAshredder columns (QIAGEN) to decrease viscosity, and 2-mercaptoethanol (Sigma) was added to 5%. Samples were separated on 10% polyacrylamide gels by SDS-PAGE (BioRad), and transferred to PVDF membranes (Millipore). After blocking for 30 min in 10% powdered milk, PBS/0.1% Tween-20 (Fisher Scientific) (PBST), membranes were incubated with primary antibodies directed against either Flag (1:5000) or HA (1:2000) epitope tags,

GAPDH (1:10000), or integrin αV (1:5000) in 5% milk in PBST. Membranes were washed 3x with PBST then incubated with secondary antibody goat anti-mouse HRP (1:5000) in PBST. After washing 3x with PBST, membranes were incubated with Supersignal West Pico Chemiluminsecent substrate (ThermoScientific) for 5 min, and developed on chemiluminescent film (GE Healthcare).

2.7 Flow Cytometry

To monitor surface expression of MHC-I in Rh178-positive cells, TRFs were harvested 48h after AMAXA nucleofection, trypsinized, and resuspended in 10% fetal bovine serum (FBS) (Hyclone)/PBS. Cells were washed 2x with PBS followed by a 30 min incubation with a 1:500 dilution of W6/32 antibody. Cells were washed 3x with PBS and then incubated with a 1:500 dilution of Alexa Fluor 647 chicken anti-mouse antibody for 30 min in the dark. Cells were then fixed in 2% paraformaldehyde (Electron Microscopy Sciences) at RT for 15 min, and permeabilized with 1% w/v saponin in 10% FBS/PBS to allow for intracellular staining. Cells were then incubated with a 1:200 dilution of anti-Flag M2 FITC conjugated antibody at RT for 30 min, washed 3x, and resuspended in PBS. All antibody incubation and wash steps were performed at 4°C. Surface expression of MHC-I and intracellular expression of Rh178 were quantified using flow cytometry (FACS Calibur, BD Biosystems). Data analysis was performed using FlowJo software v7.6 (Treestar Inc.).

2.8 Eeyarestatin treatments

Eeyarestatin I (ES_1) and eeyarestatin R35 (ES_{R35}) were kind gifts from Stephen High (University of Manchester, Manchester, UK). Eeyarestatin treatments were based on methods as previously described [232]. TRFs in culture were treated with DMSO or 10μM MG132 for 4 h, followed by treatment with DMSO, 10μM ES_1, or 10μM ES_{R35} for 1 h in the continuing presence of DMSO or MG132. Subsequently, cells were starved for 30 min in methionine- and cysteine-free medium in the presence of previously applied compounds, followed by 30 min of metabolic labeling in the presence of drugs with [^{35}S]-label (Express Protein labeling mix, Perkin-Elmer). Cells were rinsed three times in PBS and either collected and lysed or chased for 90 min before collection. Samples were lysed in PBS+1% NP-40+protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, Thermo Scientific) for 30 min. All steps were performed at 4˚C. Samples were then pelleted for 10 min at 16,100 rpm, pre-cleared with Protein A/G Plus Sepharose beads (Santa Cruz) for 30 min, incubated with primary antibody (W6/32 and HC-10) for 1h, followed by addition of Protein A/G Plus Sepharose beads and incubation for 1 h. Bound samples were washed three times in PBS and eluted with 2X Laemmli buffer, followed by SDS-PAGE and autoradiography.

2.9 Construction of recombinant RhCMV

All recombinant viruses used for RhCMV *in vivo* studies were derived from strain RhCMV 68-1 [55] and are depicted in Figure 4.1. RhCMV(gagL) was generated by replacing the loxP-flanked enhanced green-fluorescent protein (EGFP) in RhCMV-EGFP [233] with a loxP-flanked expression cassette for SIVmac239-gag under control of the

EF1α-promoter by *in vivo* recombination in tissue culture. All other recombinant viruses were created using the RhCMV bacterial artificial chromosome (RhCMV-BAC) [68] (Figure 1.4). The BAC-cassette was inserted between the RhCMV homologs of US1 and US2 and self-excises via Cre-recombinase [68]. Recombinant virus RhCMV(gag) contains a codon-optimized, FLAG-tagged SIVmac239-gag sequence under control of the EF1α-promoter inserted between ORFs R213 and R214 [198]. Deletion of the US2-11 region by homologous recombination (ET cloning) with an FRT-flanked Kanamycin-resistance (KanR) cassette was described previously [120]. ΔUS2-11(gag) was created by replacing the entire Rh182-189 region (base pairs 184489-191243) using the same primers and mutagenesis strategy as before [120] except that the inserted fragment harbored both the KanR-cassette and the codon-optimized, FLAG-tagged SIVgag-cassette. The KanR-cassette was removed by arabinose-induced FLP-expression [120]. ΔVIHCEΔUS2-11(gag) was created by subsequent deletion of Rh178 (VIHCE; base pairs 181320-182060). Since ΔUS2-11(gag) contains a single FRT recombination site from KanR-excision, we used a KanR cassette flanked by the F5-mutant FRT sequence for deletion of VIHCE. This prevents potential recombination between new and existing FRT sites when creating dual-recombinants. The mutant FRT-flanked KanR cassette was obtained from plasmid pOri6K-F5 [234] using primers 5'- TAAAAGTGTCG GATGAATGTGCGGCGCCAACACGCAGACCGAAAAGTGCCACCTGCAGAT-3' and 5'-GCCTGACTGATGACTAGTCATCGCACGCCTCTTCCCGCCCCAGGAACACTTAA CGGCTGA-3'. ΔVIHCE was created by replacing base pairs 181320-182060 with the SIVgag expression cassette using primers 5'-

TTTGTTCGTATAAAAGTGTCGGATGAATGTGCGG CGCCAACACGCAGACCGTAAAACGACGGCCAGT-3' and 5'-CGCTCCCTCG GCCTGACTGATGACTAGTCATCGCACGCCTCTTCCCGCCCGTATGTTGTGTGG AATTGTGAG-3'. ΔRh186-8(retanef) was created from previously described V5-tagged RhCMV(retanef) [198] by deletion of base pairs 187934-190031 using the KanR-cassette flanked by the F5-mutant FRT sites. All recombinant BACs were verified for correct deletions by restriction digest, southern blot and sequence analysis of the insert-borders. RhCMV virus was reconstituted by electroporation of TRFs [235].

2.10 Characterization of recombinant viruses by RT-PCR

Resulting viruses were plaque-purified and characterized for gene expression of deleted and flanking genes by RT-PCR. TRFs were infected at MOI=1 and total RNA was collected at 24hpi using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. 4μg of RNA was treated with DNAse I (Applied Biosystems) for 30 min at 37°C. 1μg of DNAse-treated RNA was used in a 20μl reverse transcription reaction containing 50ng random hexamers, 0.5mM dNTPs, 10mM DTT, and 1μl superscript III RT in 1x RT buffer (Invitrogen) for 1 hour at 37°C. 1μl of the RT reaction was used for semi-quantitative PCR with Platinum taq polymerase (Invitrogen) under the following conditions: 1x platinum taq buffer, 1.5mM MgCl2, 0.2mM dNTPs, 0.5μM each primer, and 1.5U polymerase. 35 cycles of amplification was performed under the following conditions: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 15 sec.

The following primer pairs were used: SIVgag 5'-ACCCACAACCAGCTCCACAA-3' and 5'-ATCCACTGGATCTGTTCGTCAA-3';

Rh156 5’-CAATGAGGATAGGTTCCCAGTTG-3’ and 5’-GCCAGTGGGATGTCAGTACCA-3’; Rh175 5’-CTAGCAGTACTGAGAGCTAG-3’ and 5’-TCACGCCAATCGACAGTGCACG-3’; Rh178 5’-CGCATACTGACAAGCCAGGGC-3’ and 5’-GCGAAAGAAGGTGCACATGAC-3’; Rh181 5’-CCTTACGGAGTCGCTCGTT GAC-3’ and 5’-TGTGTCGTCTCTTTCTCCGCAG-3’; Rh182 5’-GATTTTCGTTGAACAT GTCCGAC-3’ and 5’-GTTATGTGTCAGAAAGTCCG GCT-3’; Rh189 5’-TGCTTC GTCCTGGTGCTGT-3’ and 5’-TTAGCAGTTTCATGGTTG CGA-3’; Rh190 5’-GAA ATGGATAGCGGTGCTCAC-3’ and 5’-CAGACAACAGGTTG TTCAGG-3’; GAPDH 5’ 5'-GCACCACCAACTGCTTAGCAC-3' and 5'-TCTTCTGGGTGG CAGTGATG-3’. For characterization of the RhΔ186-8(retanef) virus, RT-PCR was performed as described above with the following primer pairs: Rh185 5’-AGCGTAGCTCCTCCATACCG CT-3’ and 5’-ATCCGCGGACTGTTTGGGTGT-3’; Rh186 5’-GCTTCTTCCAGAAGTTGCA TAGGATGA-3’ and 5’-CGACTTTCCGGATCCTACGTGGC-3’; Rh187 5’-CCATAGCCATG CAATGGTCGCA-3’ and 5’-GCGCCATCCCGTGTTACCCC-3’; Rh188 5’-AGAGCT CTGGTCGTCGGCGT-3’ and 5’-TGGCTGGCCACCAGATGGATGT-3’; Rh189 5’-AACCAGTAGGAGCGCCCGGT-3’ and 5’-CGACTCCTGCATGCTTACTGGGGA-3’; β-actin 5’-TCACCCACACTGTGCCCATCTACGA-3’ and 5’-CAGCGGAACCGCTCATTGCCA ATGG-3’.

2.11 Characterization of recombinant viruses by comparative genome sequencing

To confirm that the genetic manipulation of the RhCMV genome did not introduce unwanted mutations outside the regions targeted for deletion, we used Comparative Genome Sequencing (CGS) to compare the deletion viruses against RhCMV-BAC. Single nucleotide differences between reference and test strains of herpesviruses can be identified with CGS [236, 237]. CGS of viral DNA was performed using a microarray hybridization-based technique with services provided by NimbleGen Systems, Inc. (Madison, WI). A RhCMV comparative genomic hybridization array was created using the published sequence for RhCMV 68.1 [55]. Oligonucleotides that comprised this array were designed to be between 29 and 32 bp, with overlapping sequences of at least 7 bp, with coverage of both strands of the RhCMV 68.1 genome. Viral DNA was isolated using standard methods from a) parental RhCMV-BAC [68], b) ΔVIHCEΔUS2-11(gag), c) ΔUS2-11(gag), or d) ΔVIHCE(gag). Briefly, virus was produced in TRFs, supernatants were collected and, after proteinase K treatment, DNA was isolated by cesium chloride gradient centrifugation.The resulting viral DNA was ethanol precipitated and brought to a final concentration of 1μg/μl. Viral DNA was fragmented and labeled with Cy3 (RhCMV-BAC as reference) or Cy5 (deletion viruses). Labeled reference and test viral DNA probes were co-hybridized to the tiling arrays and the Cy3 and Cy5 signals were scanned. SignalMap software (NimbleGen Systems, Inc.) was used to analyze all CGS data.

2.12 Rhesus macaques

A total of 30 purpose-bred juvenile and adult male rhesus macaques (RM) (*Macaca mulatta*) of Indian genetic background were used in this study, of which four animals were specific-pathogen-free (SPF) animals and lacked RhCMV-specific T cells and antibodies. All other animals used in the study acquired RhCMV naturally while in the colony. The presence or absence of RhCMV-specific T cell responses was confirmed by intracellular cytokine staining of RhCMV Ag-stimulated PBMC. All RM were free of cercopithicine herpesvirus 1, D-type simian retrovirus, simian T-lymphotrophic virus type 1 and SIV infection. Animal protocols were approved by the Oregon National Primate Research Center Animal Care and Use Committee, under the standards of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were inoculated with 10^2-10^7 PFU of recombinant virus subcutaneously. For CD8+ cell depletion, RM were treated with 10, 5, 5 and 5 mg per kg body weight of the humanized monoclonal antibody cM-T807 [238] one day before viral infection and on days 2, 6, and 9 post infection, respectively.

2.13 Virological analysis of rhesus macaques

Isolation and co-culture of virus from urine and buccal swaps was performed as described previously [198]. Briefly, virus was concentrated from cleared urine and co-cultured with rhesus fibroblasts and cell lysates were collected after cytopathic effects were observed on or after 42 days.

2.14 Immunological analysis of rhesus macaques

Collection of BAL was performed as described previously [216]. CD4+ and CD8+ T cell responses were measured by flow cytometric intracellular cytokine analysis of PBMC and BAL cells, as previously described [198]. For T cell stimulation assays RhCMV lysates (68-1 strain) or overlapping 15mer peptides representing the SIVmac239 Gag, Rev/Tat/Nef proteins or the RhCMV Immediate Early-1 and 2 proteins (overlap = 11 amino acids), were used in the presence of co-stimulatory mAbs CD28 and CD49d (BD Biosciences). Co-stimulation in the absence of antigen served as a background control. Cells were incubated with antigen and the co-stimulatory molecules alone for 1 hr, and then in the presence of the secretion inhibitor Brefeldin A (10μg/ml; Sigma Aldrich) for an additional 8 hrs. After surface and intracellular staining with conjugated mAbs, polychromatic (6 to 8 parameter) flow cytometric analysis was performed on an LSR II Becton Dickinson instrument. List mode multi-parameter data files were analyzed using the FlowJO software program (version 8.8.6; Tree Star, Inc.). Using this software $CD3^+$ cells were divided into $CD4^+$ and $CD8^+$ T cells subsets, and then analyzed for a subset manifesting up-regulation of the activation marker CD69 and cytokine, either TNFα alone, or TNFα and/or IFN-γ (see Figure 2.2 for a schematic). For PBMCs, this background-subtracted value was divided by the fraction of total memory cells (determined as described below) to achieve the reported "memory corrected" response frequency [216]. For BAL, the reported responses were background response (no antigen) subtracted only, as BAL T cells are entirely memory cells. [216]. To determine the memory fraction of circulating T cells, memory and naive T cell subset populations were delineated based on CD28 and CD95 expression patterns, as described in [216].

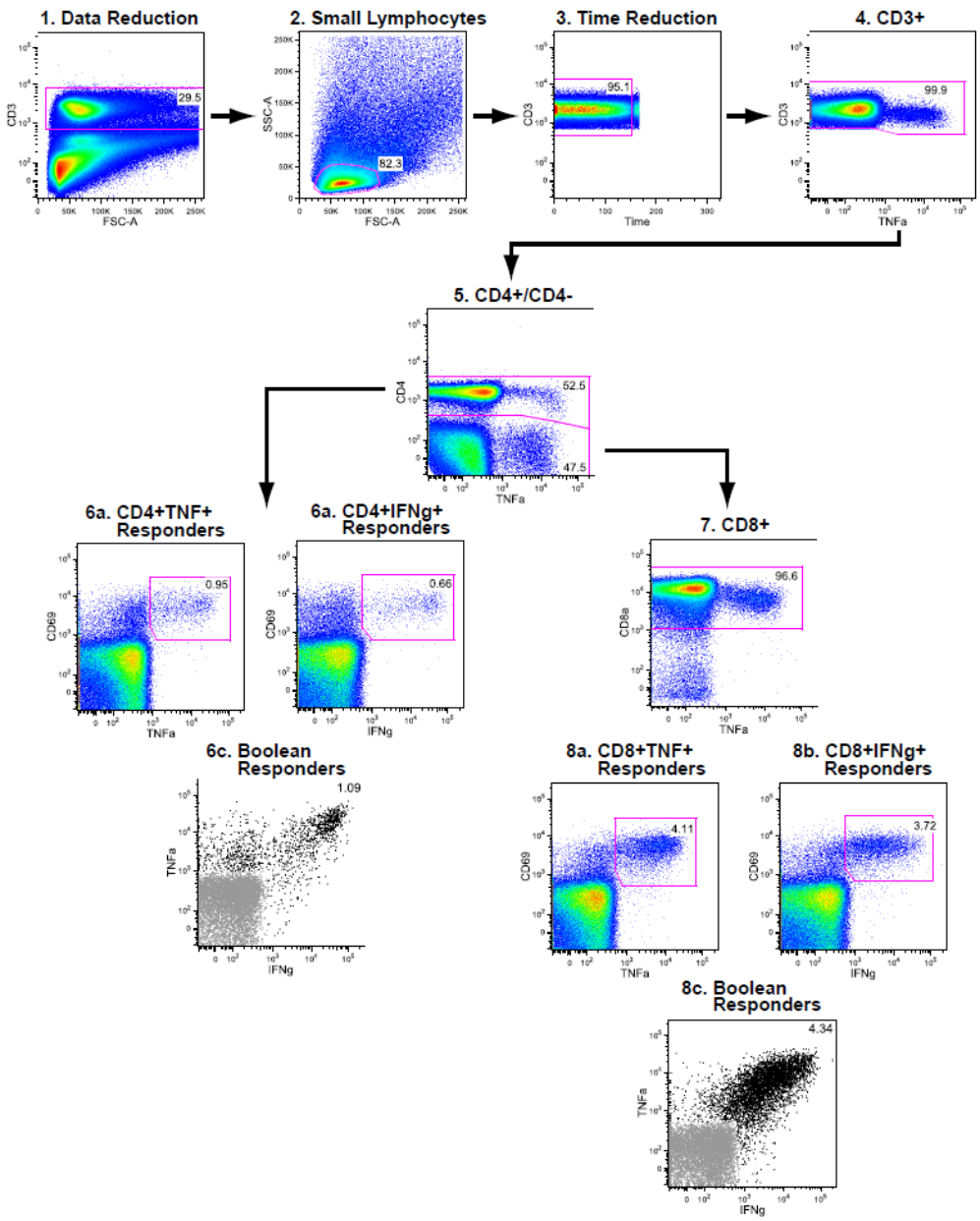

1. Data Reduction
29.5
CD3
FSC-A
2. Small Lymphocytes
82.3
SSC-A
FSC-A
3. Time Reduction
95.1
CD3
Time
4. CD3+
99.9
CD3
TNFa
5. CD4+/CD4-
52.5
47.5
CD4
TNFa
6a. CD4+TNF+ Responders
0.95
CD69
TNFa
6a. CD4+IFNg+ Responders
0.66
CD69
IFNg
7. CD8+
96.6
CD8a
TNFa
6c. Boolean Responders
1.09
TNFa
IFNg
8a. CD8+TNF+ Responders
4.11
CD69
TNFa
8b. CD8+IFNg+ Responders
3.72
CD69
IFNg
8c. Boolean Responders
4.34
TNFa
IFNg

Figure 2.2: Response frequency gating strategy.

Lymphocytes originating from PBMC and BAL were stimulated with Ag, and then stained and collected on a flow cytometer. Data were analyzed using a hierarchical gating strategy to delineate Antigen-responding subsets. Gates are depicted here in pink, with corresponding subset names numbered and displayed above the cytometric plots. Response values for all figures were determined using Boolean gating to delineate cells that are CD69+ and TNFα+/IFNγ-, TNFα-/IFNγ+, or TNFα+/IFNγ+. Gating strategy as follows: 1. Separation of CD3+ lymphocytes. 2. Gating for small lymphocytes based on forward and side scattering. 3. Time reduction to dispose of cell debris. 4. CD3+ gate. 5. Separation of CD4+ lymphocytes from CD4- lymphocytes (CD8+). 6a. Isolation of activated CD4+ lymphocytes that secrete either TNFα or IFNγ. 6c. Isolation of the final population of CD4+ lymphocytes that respond to stimulation by the SIV antigen peptide pool, as determined by Boolean gating for TNFα and/or IFNγ secretion. 7. Gating for CD8+ lymphocytes. 8a. and 8b. Similar to 6a., isolation of activated CD8+ lymphocytes that secrete either TNFα or IFNγ. 8c. Similar to 6c., Boolean gating for CD8+ lymphocytes that respond to stimulation by the SIV antigen peptide pool.

CHAPTER THREE

The cytoplasmic domain of Rhesus cytomegalovirus Rh178 interrupts translation of MHC-I leader peptide-containing proteins prior to translocation

3.1 Introduction

Cytomegaloviruses (CMV), members of the β-herpesviridae, are masters at evading the host immune system. The CMV genomes encode over 200 open reading frames (ORFs), many of which are dedicated to escaping various mechanisms of immune defense [239]. CMV-encoded immunomodulators function to circumvent cell-autonomous defenses such as apoptosis and the interferon (IFN)-response, as well as to prevent innate and adaptive immune responses by natural killer (NK) cells and T cells [240-243]. These proteins allow the virus to establish primary infection, maintain persistent infection, and support repeated superinfection of chronically infected hosts. The study of cytomegaloviral immunomodulatory proteins has not only underscored the important and delicate relationship between virus and host, but has also revealed novel proteins of the host immune system like the UL16-protein binding family of NKG2D ligands and the UL18-binding NK cell inhibitory receptor LIR-1 [96, 244].

In addition, CMV immunomodulators have been employed to decipher basic cell biological principles such as protein quality control. Glycoproteins within the US6 family of human CMV (HCMV) [245] block endoplasmic reticulum (ER)-associated degradation of MHC Class I (MHC-I) proteins and thereby prevent antigen presentation to CD8+ T cells. Specifically, US2 and US11 facilitate rapid retrotranslocation of MHC-I from the ER to the cytoplasm followed by proteasomal degradation [124, 126]. Despite

their similar type I transmembrane topology and luminal Ig-like folds [118], US2 and US11 achieve the endpoint of MHC-I dislocation from the ER by distinct mechanisms. US2-mediated retrotranslocation requires signal peptide peptidase (SPP), protein disulfide isomerase (PDI), and p97 ATPase [129, 133, 134]. In contrast, US11 utilizes its TM domain to recruit Derlin-1 and Sel1L by a presumably independent yet complementary pathway [128, 130].

Additionally, US6 (Rh185) binds directly to the transporter associated with antigen processing (TAP) in the ER lumen [148, 246], causing a conformational change and subsequent inhibition of peptide loading and maturation of MHC-I heterodimers [154]. US3 (Rh184) interferes with the functions of peptide loading complex chaperones tapasin [140] and protein-disulfide isomerase [141], thereby complementing US6 abrogation of MHC-I peptide loading and causing MHC-I retention within the ER. When the US2-11 region (including US2, US3, US6, and US11) is deleted from HCMV, MHC-I heavy chain (HC) surface expression in infected cells reverts to steady state levels. However, when we deleted the homologous region (Rh182-189) from RhCMV, MHC-I levels at the cell surface recovered only slightly, which led to the discovery of a RhCMV-specific mechanism of MHC-I inhibition, termed viral inhibition of heavy chain expression (VIHCE). The process of VIHCE was determined to be mediated by a RhCMV protein encoded by Rh178 [120].

Rh178 encodes for a 212 aa protein that is ER-localized and has no known homology to the US6 family of MHC-I inhibitors or any other viral or cellular protein. Interestingly, Rh178 seems to prevent HC expression by a unique post-transcriptional pathway. Based on the finding that Rh178 function is specifically dependent on the

MHC-I signal peptide (SP) [120], we hypothesized that Rh178 specifically prevents early events during translation or translocation of MHC-I HC.

The HC of all MHC-I alleles consist of N-terminal cleavable SP, α1, α2, and α3 domains, a transmembrane (TM) domain, and a short C-terminal cytosolic domain. The HC forms a heterodimer with β2-microglobulin [247]. Like other type I membrane proteins, nascent MHC-I is targeted to the ER as the SP emerges from the ribosome, and binds to the signal recognition particle (SRP) [162]. As SRP binds to its receptor on the ER membrane, the SP is released, the ribosome is transferred to the Sec61 translocon, and the nascent chain is cotranslationally translocated into the ER lumen [248]. Translocation is terminated by synthesis of the TM segment (stop transfer sequence) to establish a type I topology with the C-terminal domain residing in the cytosol. This interaction at the ER membrane allows translocation to be initiated and the nascent MHC-I protein to be fed through the Sec61-translocon, allowing the bulk of the protein to reside in the ER lumen, anchored only by the stop transfer sequence that becomes the C-terminal transmembrane domain [249]. Despite the polymorphic nature of MHC-I molecules within and among different animal species, the SP is highly conserved [174, 176]. The determination that Rh178 relies on the SP sequence for MHC-I downregulation indicates that RhCMV may take advantage of the conserved SP sequence among MHC-I alleles for immune evasion.

One obstacle in understanding the function of Rh178 is that it is poorly expressed in the absence of viral infection. To overcome this, we used a codon-optimized version that is highly expressed in transfected cells. Using an *in vitro* system, we also determined that Rh178 downregulates MHC-I HC during early stages of translation and showed that

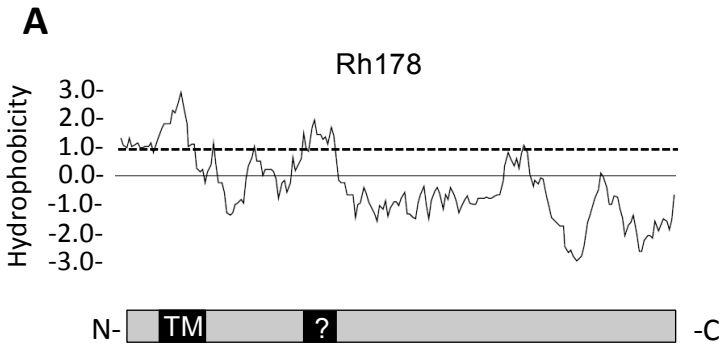
the MHC-I SP is not only necessary but also sufficient for VIHCE. In contrast to small molecule inhibitors that block MHC-I HC translocation, Rh178 acts prior to this step by inhibiting translation of the full length protein.

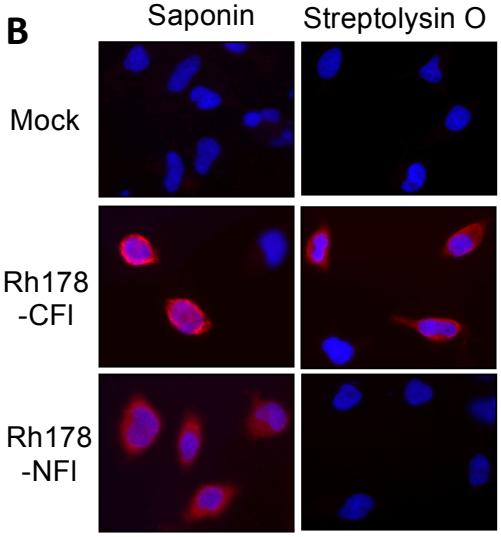
3.2 Results

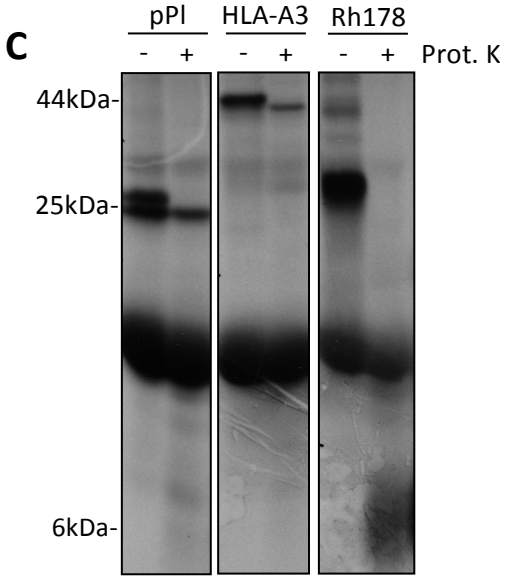
3.2(a) Rh178 is a type Ib transmembrane protein that is anchored in the ER-membrane and faces the cytosol

Our previous experiments determined that Rh178 is localized in the membrane of the endoplasmic reticulum (ER) [120], but the number of transmembrane domains and the orientation within the ER-membrane were unknown. A Kyte-Doolittle hydrophobicity plot [250] indicates one highly likely transmembrane domain close to the N-terminus and at least one other highly hydrophobic region that represents a potential transmembrane or membrane-associated domain (Figure 3.1A).

To determine the membrane orientation of Rh178 we used a codon-optimized version of Rh178 to improve expression in tissue culture cells. HeLa cells were transfected with a control plasmid or with C- or N-terminal Flag-tagged versions of Rh178 (Rh178-Cfl and Rh178-Nfl, respectively), followed by immunofluorescence of saponin- or streptolysin O (SLO)-treated cells. Saponin permeabilizes all cellular membranes whereas SLO permeabilizes only the plasma membrane. As expected, the Flag epitope tag was detected in both Rh178-Cfl and Rh178-Nfl transfected cells upon saponin treatment (Figure 3.1B). In SLO-treated cells, only Rh178-Cfl was detectable, indicating that the C-terminus of Rh178 extends into the cytosol, whereas the N-terminus of Rh178 is not cytosolically accessible. This result indicates that Rh178 spans the ER-


A
Rh178
Hydrophobicity
3.0-
2.0-
1.0-
0.0-
-1.0-
-2.0-
-3.0-
N-
TM
?
-C


B
Saponin
Streptolysin O
Mock
Rh178
-CFI
Rh178
-NFI


C
pPI
HLA-A3
Rh178
- + - + - +
Prot. K
44kDa-
25kDa-
6kDa-

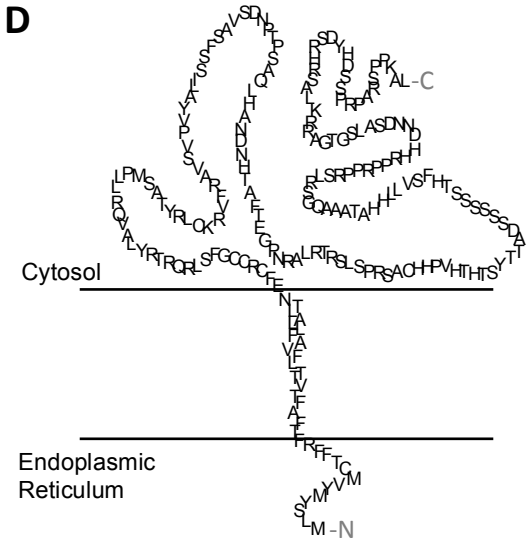

D
Cytosol
Endoplasmic
Reticulum
-C
-N

Figure 3.1: Rh178 is a type Ib ER-resident transmembrane protein.

(A) Kyte-Doolittle hydropathy plot of Rh178. Indexes above one (dashed line) represent hydrophobic or transmembrane domains. VIHCE is represented below the x-axis of the plot, and the most likely transmembrane domain (TM) and another possible transmembrane or membrane-associated domain (?) are outlined by black squares. (B) HeLa cells were either mock transfected or transfected with a vector containing Rh178 with an N- or C-terminal Flag tag. After 48h, cells were treated with either saponin, to permeabilize all cellular and organelle membranes, or with Streptolysin O (SLO), to permeabilize only the cell membrane. Immunofluorescence was performed to visualize the Flag-tagged Rh178 with anti-Flag epitope tag antibody M2, followed by an AlexaFluor anti-mouse 547 antibody. Nuclei are stained with VectaShield (blue). (C) mRNAs were translated in rabbit reticulocyte lysate (RRL) in the presence of canine microsomal membranes, and resultant proteins were digested with Proteinase K and analyzed by SDS-PAGE and autoradiography. pPl, preprolactin control. (D) Diagram showing the predicted orientation of Rh178.

membrane once with its N-terminus in the lumen and C-terminus in the cytosol. Since the hydrophobicity plot indicated a high probability TM-domain at the N-terminus, it is likely that this region functions as a Type I signal anchor to translocate the N-terminus and span the membrane.

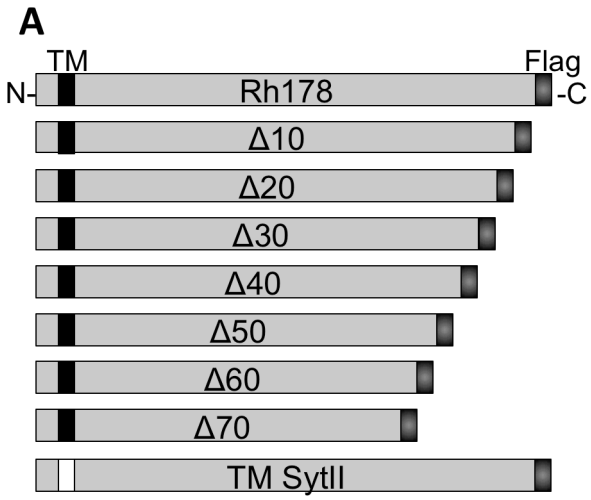
To confirm this hypothesis we performed a protease K protection assay ^{35}S-labeled Rh178 translated *in vitro* in the presence of microsomal membranes. Proteinase K (PK) digests portions of proteins outside the microsomes, whereas protein domains inside the microsomes are protected. For control, we used preprolactin (PPL) and HLA-A3, which both undergo SP cleavage [251]. As expected, only cleaved prolactin was protected from PK digestion whereas the cytosolic precursor was degraded (Figure 3.1C). Similarly, HLA-A3 was also protected from PK but shifted in size due to clipping of the short cytoplasmic tail (Figure 3.1C). In contrast, almost all of Rh178 was digested by PK, leaving a small ~6 kDa fragment (Figure 3.1C), demonstrating that most of the protein resides outside the microsomal lumen. Taken together, these results indicate that Rh178 is a single-spanning transmembrane protein with a type I topology (Figure 3.1D).

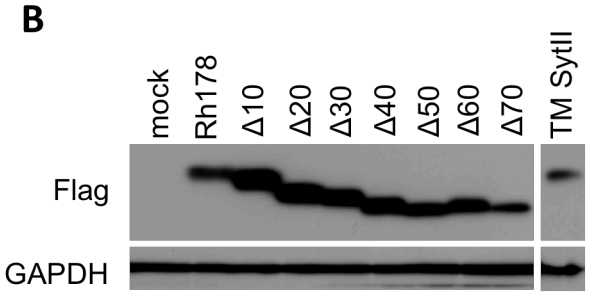
3.2(b) Membrane and membrane-proximal domains of Rh178 are indispensable for VIHCE

The type I topology of Rh178 suggests a prominent role of the cytosolic domain for VIHCE. BlastP-searches with the C-terminal portion of Rh178 did not reveal homology to any known protein. We therefore mapped the determinants within the C-terminus of Rh178 that were required for VIHCE by constructing a series of C-terminal deletion mutants that contained a C-terminal Flag epitope tag (Figure 3.2A). To test

whether the transmembrane (TM) domain of Rh178 is required for VIHCE we replaced the TM domain with that of another type I TM protein, Synaptotagmin II [252] (TM SytII) (Figure 3.2A). Expression in HeLa cells demonstrated that the corresponding proteins had the expected molecular weight (kDa) and were expressed at similar levels (Figure 3.2B). Immunofluorescence of transfected HeLa cells further confirmed equivalent expression of all constructs (data not shown).

To examine the ability of these constructs to downregulate MHC-I, we used the AMAXA nucleofection system to express each Rh178 mutant in telomerized rhesus fibroblasts (TRFs). By gating for Flag-positive cells using flow cytometry, we compared MHC-I surface expression of Rh178-negative and Rh178-positive cell populations (Figure 3.2C). Compared to non-transfected cells, MHC-I surface levels were significantly reduced in TRFs expressing full-length Rh178. A similar degree of MHC-I downregulation was observed for Δ10, Δ20, and Δ30. Cells expressing Δ40 showed a slight downregulation of MHC-I, indicating some residual activity. However, MHC-I levels were unchanged in cells expressing Rh178 lacking 50 amino acids or more. These results indicate that the membrane-distant 30 amino-acids of Rh178 are not essential for VIHCE. Interestingly, cells expressing the TM SytII construct still showed a reduction of MHC-I surface levels, indicating that the TM domain predominantly serves as a membrane anchor. We also constructed a mutant lacking the entire N-terminal TM and luminal domain. However, the resulting protein was highly unstable (data not shown). These data demonstrate that the TM-domain and membrane-proximal cytoplasmic domain represent the functional core of Rh178 for VIHCE.


A
TM
Flag
N-
Rh178
-C
Δ10
Δ20
Δ30
Δ40
Δ50
Δ60
Δ70
TM SytII


B
mock
Rh178
Δ10
Δ20
Δ30
Δ40
Δ50
Δ60
Δ70
TM SytII
Flag
GAPDH

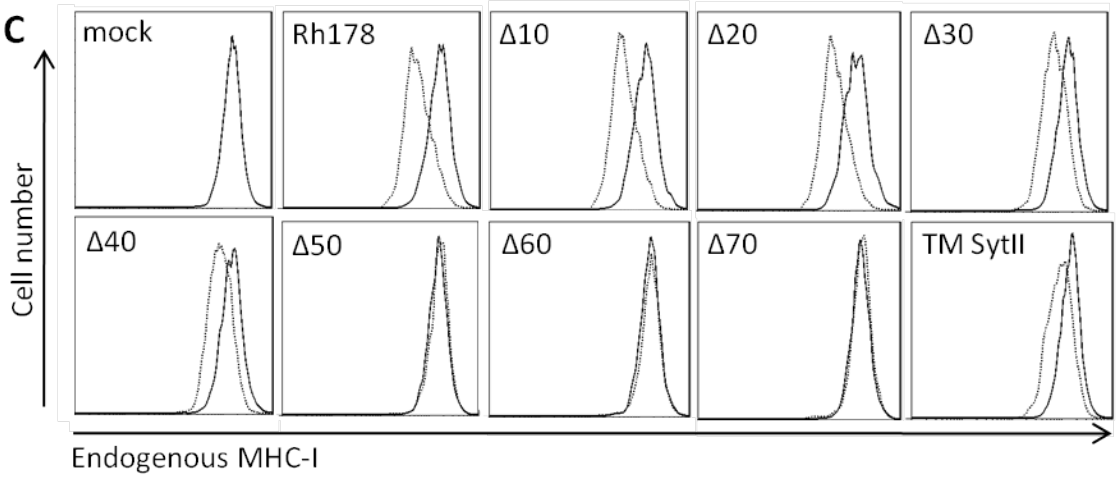

C
mock
Rh178
Δ10
Δ20
Δ30
Δ40
Δ50
Δ60
Δ70
TM SytII
Cell number
Endogenous MHC-I

Figure 3.2: Membrane and membrane-proximal domains of Rh178 are important for downregulation of MHC Class I.

(A) Diagrams of Rh178 C-terminal deletions, each containing a C-terminal Flag tag. The transmembrane domain of Rh178 was replaced with the transmembrane domain of Synaptotagmin II (TM SytII) (B) HeLa cells were either mock transfected or transfected with the Rh178 mutants. After 48h, cells were lysed in an SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-Flag epitope tag antibody M2 followed by an anti-mouse HRP. (C) Analysis of Rh178 downregulation of MHC Class I. Rh178 mutants were electroporated into telomerized rhesus fibroblasts (TRFs) using the AMAXA nucleofection system. Flow cytometry was used to quantify cell surface expression of total MHC Class I in Flag negative and Flag positive populations. Flow cytometry using anti-Flag antibody conjugated to FITC separated Flag negative and positive populations. Surface staining with MHC Class I antibody W6/32 followed by anti-mouse APC antibody to quantify surface MHC Class I expression. Each panel demonstrates mean fluorescence intensity of Flag negative cells (black line) compared to the Flag positive cells (dotted line).

3.2(c) Rh178 downregulates truncated versions of HLA-A3

We previously demonstrated that replacement of the MHC-I SP with an unrelated SP renders MHC-I resistant to Rh178 [120]. This finding strongly suggested that Rh178 requires the translation of the MHC-I SP. However, despite this circumstantial evidence that MHC-I is translated we cannot detect a translation or degradation intermediate of the MHC-I HC, even in the presence of proteasome inhibitors [120]. Thus, we hypothesized that only a very short translation product is generated in the presence of Rh178 followed by rapid, proteasome-independent degradation. One corollary of this hypothesis is that Rh178 should prevent expression of very short amino-terminal fragments of MHC-I. To test this assumption, we created a series of C-terminally truncated versions of HLA-A3, each named for the number of amino acids remaining and containing a C-terminal HA epitope tag to facilitate detection by Western blot (Figure 3.3A). The human MHC-I allele was chosen for these experiments because we have previously shown that HLA-A3 expression is inhibited by Rh178 in a SP-dependent manner [120]. Like all classical MHC-I molecules, HLA-A3 is comprised of a signal sequence, α1, α2, and α3 domains, in addition to a C-terminal transmembrane domain and short cytoplasmic tail. While the longer constructs were stably expressed upon transfection, the shortest construct, A3 114, was rapidly degraded unless proteasome inhibitors were added (Figure 3.3B).

To determine whether expression was inhibited by Rh178 we generated a stable cell line under control of the tet-off system (TRF-178) [253], in which Rh178 is induced upon transduction with an adenovirus expressing the tetracycline-responsive transcriptional activator (Ad-tTA) (Figure 3.3B). Using this system we compared VIHCE for each of the HLA-A3 constructs with or without induction of Rh178. Upon expressing

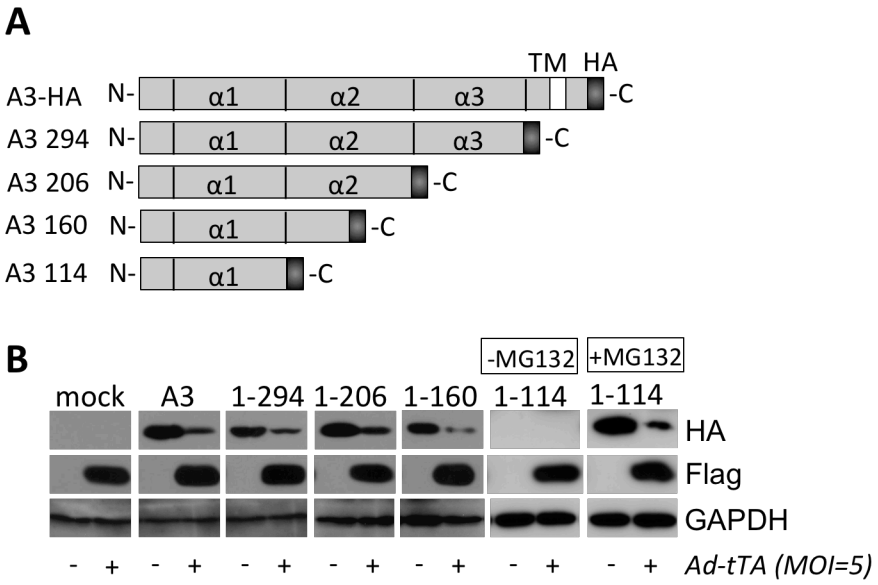


Figure 3.3: Rh178 downregulates truncated versions of HLA-A3.

(A) Diagram of C-terminal HLA-A3 mutants. A3 294 lacks the transmembrane domain and C-terminus, A3 206 lacks the α3 domain, A3 160 lacks the α3 and half of the α2 domain, and A3 114 lacks the α3 and α2 domain. All constructs contain a C-terminal HA epitope tag. (B) Truncated HLA-A3 constructs are downregulated by Rh178. TRF-178 is a telomerized rhesus fibroblast cell line that stably expresses VIHCE under control of the tet-off system. Control adenovirus (Ad-RTA) (-) or adenovirus with the tet-transactivator (Ad-tTA) (+) was added at an MOI of 5 to TRF-178, followed by AMAXA nucleofection of truncated constructs of HLA-A3 24 h.p.i. Cells were lysed 72 h.p.i. in SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-HA epitope tag antibody, followed by anti-mouse HRP antibody. Blots were also probed with anti-Flag epitope tag antibody M2 and anti-GAPDH for a loading control. MG132 was added to stabilize the A3 114 construct, which was not expressed well in the absence of MG132.

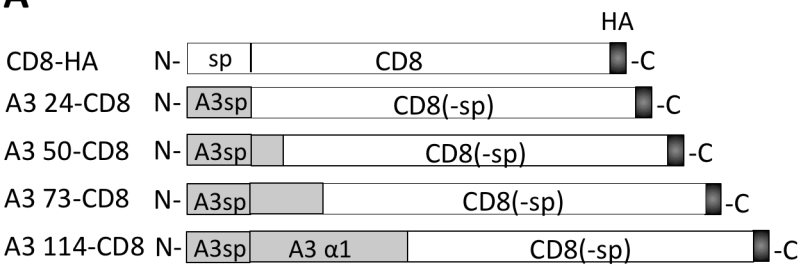
Rh178 by Ad-tTA transduction, significantly reduced levels of full-length HLA-A3 were observed (Figure 3.3B). Interestingly, expression of all other HLA-A3 truncation mutants was also inhibited upon induction of Rh178 (Figure 3.3B). Moreover, even the shortest fragment, A3 114, was inhibited in the presence of Rh178 even though proteasome inhibitors were needed to see any expression at all. Expression of HLA-A3 fragments that were shorter than 114 amino acids could not be stabilized by proteasome inhibitors (data not shown). From these data, we conclude that Rh178 is able to inhibit MHC-I translation prior to completion of fewer than the first 114 amino acids.

3.2(d) The signal peptide of HLA-A3 is sufficient for VIHCE

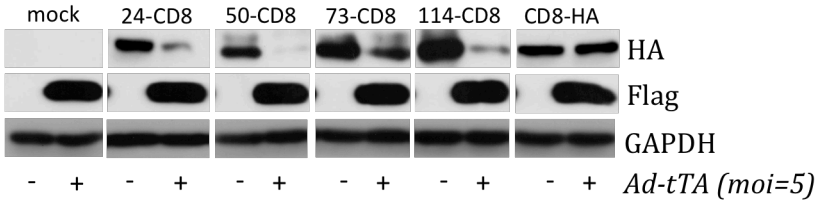
Since the N-terminal 114 amino acids of HLA-A3 were sufficient for VIHCE we wondered whether their transfer to another protein would confer susceptibility to inhibition by Rh178. To address this question we replaced the N-terminal SP of CD8 with the N-terminal 114 amino acids of Rh178 (Figure 3.4A). Upon transfection into TRF-178 cells, HA-tagged full-length CD8 was not affected by induction of Rh178 (Figure 3.4B). Similarly, expression of endogenous protein integrin α5 was not inhibited by Rh178 (Figure 3.4C). In contrast, expression of the A3 114-CD8 chimeric protein was strongly reduced upon induction of Rh178. Because HLA-A3 protein products with fewer than 114 amino acids could not be stabilized by MG132, this finding allowed us to further narrow down the minimal sequence required for VIHCE.

Therefore, we fused even shorter portions of the HLA-A3 N-terminus to the CD8 molecule, including a construct in which only the first 24 amino acids, comprising only the leader peptide of HLA-A3, were transferred (Figure 3.4A). Interestingly, expression

A


HA
CD8-HA
N-
sp
CD8
-C
A3 24-CD8
A3sp
CD8(-sp)
A3 50-CD8
A3 73-CD8
A3 114-CD8
A3 α1

B


mock
24-CD8
50-CD8
73-CD8
114-CD8
CD8-HA
HA
Flag
GAPDH
- + - + - + - + - + - +
Ad-tTA (moi=5)

C

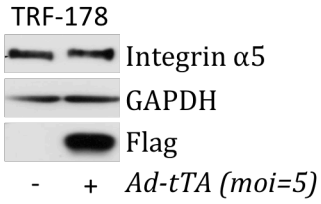

TRF-178
Integrin α5
GAPDH
Flag
- +
Ad-tTA (moi=5)

Figure 3.4: The signal peptide of HLA-A3 is sufficient for Rh178-directed downregulation.

(A) Diagram of A3-CD8 fusions. HLA-A3-CD8 fusions were created by replacing the signal peptide of CD8 with an N-terminal portion of HLA-A3. The smallest fusion, A3 24-CD8 is a direct swap of the CD8 signal peptide with the HLA-A3 signal peptide. Each fusion mutant contains a C-terminal HA epitope tag. (B) A3-CD8 fusions are downregulated by Rh178. Control adenovirus (Ad-RTA) (-) or adenovirus with the tet-transactivator (Ad-tTA) (+) was added at an MOI of 5 to TRF-178, followed by AMAXA nucleofection of A3-CD8 fusions 24 h.p.i. Cells were lysed 72 h.p.i. in SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-HA epitope tag antibody, followed by anti-mouse HRP antibody. Blots were also probed with anti-Flag epitope tag antibody M2 and anti-GAPDH as a loading control. (C) Endogenous integrin α5 is not downregulated by Rh178. Levels of integrin α5 were examined by immunoblot with anti-integrin αV antibody followed by anti-mouse HRP.

of all of the shorter HLA-A3-CD8 fusions was also inhibited by Rh178 (Figure 3.4B). This result was surprising since we previously observed that expression of a chimeric protein of CD4 with the HLA-A3 SP was not inhibited in RhCMV-infected TRFs [120]. A possible explanation is that expression levels of Rh178 are lower in virally infected cells than upon transfection of a codon-optimized gene product. Nevertheless, these data clearly demonstrate that the SP is not only necessary but also sufficient for VIHCE.

3.2(e) The UL40-homologue Rh67 is resistant to VIHCE

To further narrow down the target sequences required for VIHCE we took advantage of another RhCMV protein, Rh67, which has an N-terminal predicted SP with homology to the HLA-A3 SP. In HCMV, the glycoprotein UL40 contains a 9-mer peptide within its SP that is highly similar to 9-mer peptides within the SP of MHC-I molecules. These SP-derived peptides are loaded into the binding groove of non-polymorphic HLA-E, thereby upregulating surface HLA-E and engaging inhibitory CD94/NKG2A natural killer (NK) cell receptors [100]. Thus, UL40 mimics the normal function of peptides derived from MHC-I SPs to prevent NK cell lysis of infected cells in which the classical, polymorphic MHC-I molecules were destroyed by HCMV [254]. Rh67 likely represents the functional homologue of UL40 since its predicted SP contains a 9-mer peptide (VMAPRTLLL) that differs by only one amino acid from the UL40 9-mer (VMAPRTLIL), whereas the remaining protein is not conserved. Interestingly, the Rh67 9-mer is identical to 9 amino acids within the HLA-A3 SP that is sufficient for Rh178-mediated downregulation of MHC-I (Figure 3.5A).

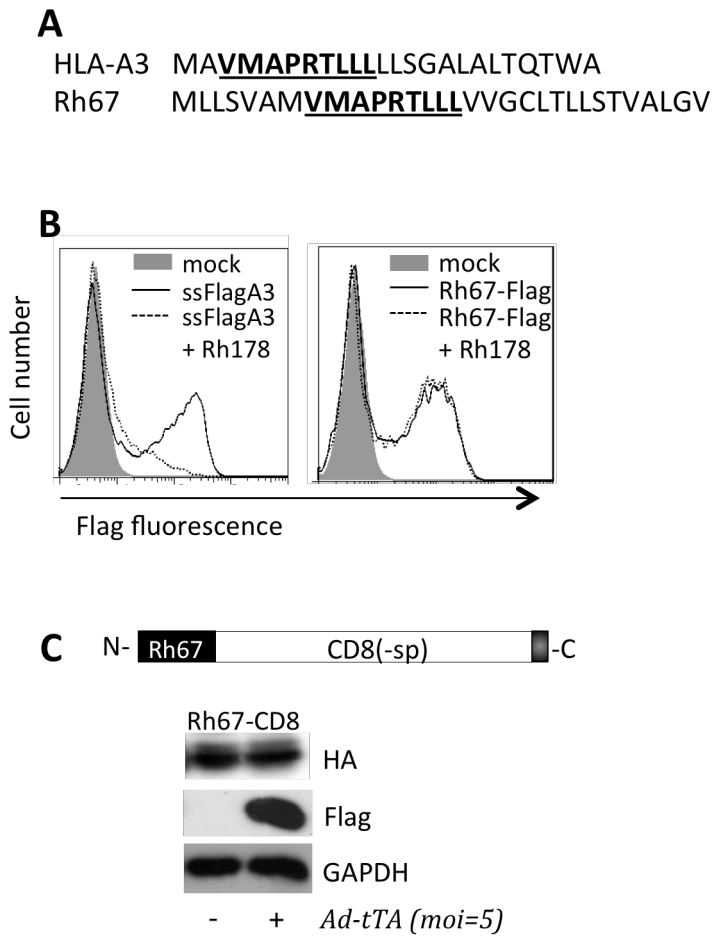


Figure 3.5: The RhCMV UL40 homologue Rh67 is not targeted by Rh178.

(A) Alignment of the SP of HLA-A3 and Rh67, which are 24 and 31 amino acids in length, respectively. The two 9 amino acid stretches of exact homology are underlined. (B) Full length Rh67 is not targeted by Rh178. HeLa cells were co-transfected with untagged Rh178 and either ssFlagA3 or Rh67-Fl. Cells were harvested 48 h post transfection and stained intracellularly for Flag expression. (C) Rh67-CD8 fusion is not downregulated by Rh178. Nucleofection of Rh67-CD8 into TRF-178 cells was performed as described in Fig 4b, followed by immunoblot for HA, Flag, or GAPDH.

This homology raised the interesting question whether Rh67 would be susceptible to VIHCE and gave us the opportunity to further map the susceptible sites recognized by Rh178. However, expression of Rh67 was not affected upon co-transfection with Rh178 into HeLa cells, unlike a Flag-tagged version of HLA-A3 (ssFlagA3), which, as expected, was downregulated by Rh178 to near control levels (Figure 3.5B). To further confirm that the Rh67 SP is not sufficient to convey VIHCE susceptibility to CD8, we created a fusion of the Rh67 SP and CD8, tagged with HA (Rh67-CD8) (Figure 3.5C). In Rh178-expressing cells, there was no downregulation of Rh67-CD8. These results suggest that sequences outside this conserved 9-mer region are targeted for VIHCE. Moreover, these results are consistent with Rh67 supporting expression of Mamu-E, the highly conserved rhesus homologue of HLA-E, despite viral interference with MHC-I expression by Rh178 as well as US6-family proteins.

3.2(f) VIHCE occurs prior to HC translocation

The finding that the MHC-I SP is sufficient for VIHCE implied that Rh178 targets the nascent HC after initiation of translation, but before translation is completed. Translation of SP-containing proteins is coupled to translocation via the signal recognition particle (SRP), which directs the ribosome-nascent chain complex (RNC) to the SRP receptor (SR) followed by transfer to the Sec61 translocon [255]. Since Rh178 is a cytosol-facing, ER-associated transmembrane protein it is reasonable to assume that Rh178 can only interfere with HC translation once the SRP has directed the RNC to the ER membrane. Thus, Rh178 could inhibit a) the interaction of the RNC-SRP with the SR, b) the transfer of the RNC to the Sec61 translocon, or c) Sec61-mediated HC

translocation across the ER-membrane. Each of these steps could presumably prevent the completion of HC translation, thus resulting in rapidly degraded translation intermediates.

Recently, the small molecule inhibitor Eeyarestatin 1 (ES_1) [255] was shown to efficiently inhibit protein translocation and prevent transfer of the RNC from the SR to Sec61 [232]. In contrast, ES_1 did not prevent docking of the RNC-SRP to the SR. Since Rh178 seems to interfere at a similar step with HC translation/translocation, we wondered whether the effect of ES_1 on HC translation would be similar to that of Rh178. TRFs were treated with DMSO, 10μM of ES_1 or the inactive analog ES_{R35} prior to metabolic labeling and immunoprecipitation of endogenously expressed MHC-I. Compared to DMSO-treated cells, recovery of MHC-I was strongly reduced in the presence of ES_1, whereas ES_{R35} did not inhibit MHC-I translation (Figure 3.6A). In contrast, MHC-I was immunoprecipitated from lysates obtained from TRFs treated with proteasome inhibitor MG132 prior to and during ES_1-treatment (Figure 3.6A). Thus it seems that inhibiting RNC-transfer to Sec61 and Sec61-dependent protein translocation does not prevent the translation of full-length HC which is then degraded by the proteasome. In contrast to ES_1-treated cells, cells that express Rh178 have no restoration of MHC-I expression even in the presence of MG132 (Figure 3.6B and [120]). Taken together, these data suggest that Rh178 acts upstream of the inhibitor ES_1, possibly by preventing the docking of SRP/nascent HC complex to the SR.

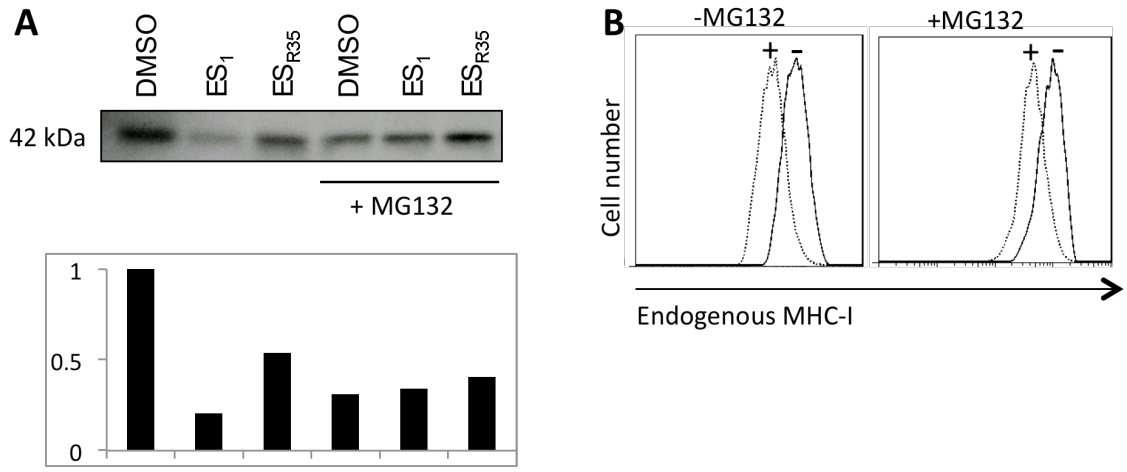


Figure 3.6: Rh178 acts at a different stage of translation than small molecule inhibitor eeyarestatin.

(A) TRFs were treated with either DMSO or MG132 for a total of 6 h. DMSO, ES_1, or ES_{R35} was applied for 1h, followed by a 30 min starvation period and a 30 min metabolic label, all in the presence of drugs as indicated. Cells were collected and lysed immediately and subjected to MHC-I immunoprecipitation, SDS-PAGE, and autoradiography. Bands were quantified using ImageJ, with DMSO-treated MG132 negative samples set to one. (B) The AMAXA system was used to nucleofect TRFs with Rh178-Flag, in the absence or presence of MG132. Flow cytometry was used to quantify cell surface expression of endogenous MHC-I in Flag negative and Flag positive cell populations. Each panel demonstrates the mean fluorescence intensity of Flag negative cells (black line) compared to the Flag positive cells (dotted line).

3.3 Discussion

Among the many host immune defense mechanisms counteracted by CMV [256] the interference with MHC-I-dependent antigen presentation and subsequent subversion of CD8+ T cell recognition is particularly complex and multi-faceted involving multiple genes in every CMV species that has been studied so far [257]. In addition to their importance for immune evasion *in vivo* [59], mechanistic studies of US2, US3, US6, and US11 have given us important insights into basic cell biological principles [258]. Here we explore a novel mechanism of interference with MHC-I expression by Rh178, which our previous observations suggest is a non-US6-related, RhCMV-specific protein that interferes with MHC-I expression in a SP-dependent manner [120]. US2 and US11 and their RhCMV homologues downregulate MHC-I by re-directing assembled immature complexes to the cytosol, where they are degraded by the proteasome. US3 and US6 block peptide loading of MHC-I in the ER by interfering with tapasin and TAP, respectively. Thus, all of the US6-related proteins exert their effects after MHC-I has been successfully translated and translocated into the ER lumen. This post-translational interference mechanism is also reflected in the membrane topology of the US6 family since all members are type Ia TM proteins, comprised of a large ER-luminal portion, a single TM domain, and a short cytoplasmic tail [118, 153, 259, 260]. While TM domains and the short cytoplasmic portion have been implicated in the function of some US6-family members [260, 261], the large luminal domains are thought to be responsible for their substrate-specificity, i.e. their ability to directly interact with MHC-I alleles or with components of the peptide loading complex [118, 148, 259-261]. In stark contrast to the US6 family members, the data presented here clearly show that Rh178 has a type Ib

orientation, and that the bulk of this 222 amino acid protein is cytoplasmic. Functional analysis of C-terminal deletion mutants of Rh178 demonstrates the importance of its cytosolic, membrane-proximal cytoplasmic core. In contrast, a Rh178 mutant with a substituted TM domain retained some functionality, indicating a less important role for the TM domain. Taken together, these data indicate that Rh178 intercepts nascent HC at the cytosolic face of the ER membrane.

The cytosolic orientation of the functional part of Rh178 also implies that, unlike US6-related proteins, Rh178 should be able to recognize its substrate, the HC, prior to its complete translation. This hypothesis is strongly supported by our studies of progressively truncated versions of HLA-A3. We observed that Rh178 successfully interfered with the expression of even the shortest N-terminal fragment of HLA-A3 that we were able to express, a 114 amino acid (aa) fragment that was only detectable in the presence of proteasome inhibitors. Thus, Rh178 clearly does not need complete MHC-I translation to recognize its substrate. Strikingly, chimeric proteins containing only the 24 aa SP of HLA-A3 fused to the N-terminus of CD8 were targeted by Rh178, confirming that Rh178-mediated VIHCE is dependent upon the HLA-A3 SP. Since the native CD8 SP was not affected we conclude that Rh178 is able to discriminate between HC-derived and non-HC-derived SPs. Additionally, Rh178 can distinguish between highly related SPs. The SP of Rh67, which contains 9 aa identical to 9 aa in the HLA-A3 SP, was not inhibited by Rh178. Since there is strong conservation of SP among MHC-I alleles (probably partially due to selective pressure by HLA-E), RhCMV has thus identified an "Achilles heel" of the MHC-I pathway. However, because SP conservation is not perfect and since RM have a more complex MHC-I locus than humans, with up to 10-fold higher

sequence divergence [174], it is still possible that Rh178 preferentially targets some but not all MHC-I alleles, but this possibility requires further investigation.

Attempts to express even shorter fragments of HLA-A3 (73 aa) failed even when proteasomal degradation was inhibited (data not shown), suggesting that HC-derived poly-peptides shorter than ~100 aa are degraded by a proteasome-independent process (assuming that the SPase is acting on such a truncated product, the resulting poly-peptide could even be further shortened by ~25 aa). Similarly, it is possible that we are unable to detect prematurely truncated HC translation intermediates even in the presence of proteasome inhibitors due to proteasome-independent degradation. Indeed short cytosolic peptides are known to be rapidly degraded by a number of proteases, e.g. amino-peptidases, Tripeptidyl-Peptidase II, Thimet oligopeptidase, Dipeptidyl-peptidase-4, and others [262-265]. Therefore, we propose that Rh178 targets the nascent HC after the SP emerges from the ribosome and the nascent HC is targeted to the ER-membrane by SRP. The length of the HC at this point of the translation process is estimated to be approximately 50 aa, based on earlier *in vitro* observations with pre-prolactin [266]. Importantly, binding of the SRP to nascent polypeptide chains slows down translation [267]. Thus, a possible mechanism for VIHCE would be that Rh178 prolongs this translational arrest resulting in a short, incomplete translational product of approximately 50 aa in length that is degraded by proteases other than the proteasome (Figure 3.7).

This model is also consistent with our conclusion that VIHCE occurs upstream of Sec61-mediated translocation because inhibition of translocation by ES_1 results in a full-length HC that is degraded by the proteasome. Eeyarestatin I and II were originally discovered in a screen for small molecule inhibitors of US11-mediated ER-associated

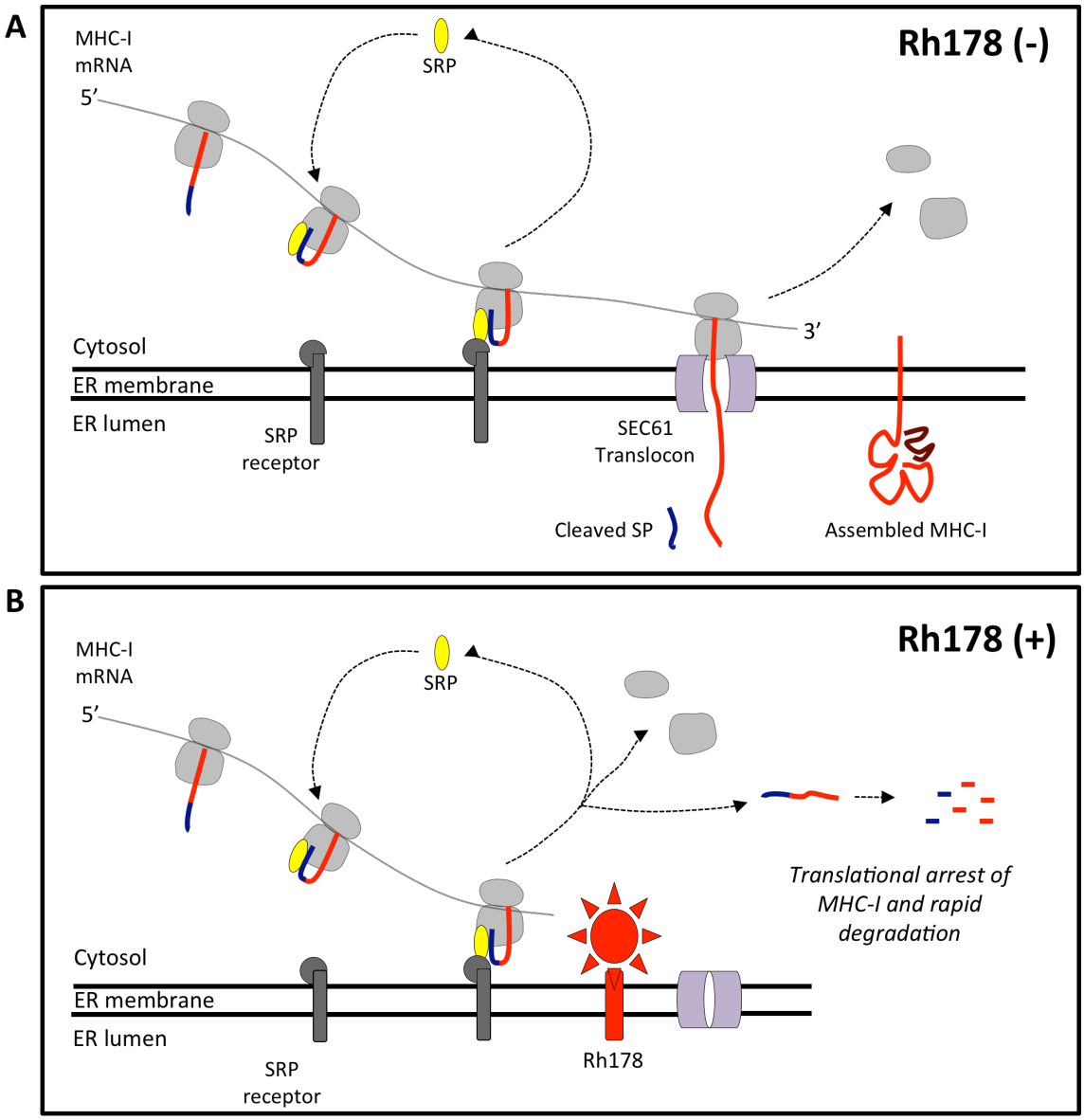

A
MHC-I
mRNA
5′
SRP
Rh178 (-)
3′
Cytosol
ER membrane
ER lumen
SRP
receptor
SEC61
Translocon
Cleaved SP
Assembled MHC-I
B
MHC-I
mRNA
5′
SRP
Rh178 (+)
Translational arrest of
MHC-I and rapid
degradation
Cytosol
ER membrane
ER lumen
SRP
receptor
Rh178

Figure 3.7: Proposed model for Rh178-mediated VIHCE activity.

(A) In the absence of Rh178, translation and translocation of MHC-I occurs as normal. The signal peptide of the nascent chain emerges from the ribosome and is recognized by SRP, which guides the entire complex to the SRP receptor in the ER membrane. The ribosome-nascent chain complex is transferred to the SEC61 translocon and the SRP is released back into the cytosol. MHC-I is translocated co-translationally in a vectorial manner from the continuous pore formed by the ribosome and the translocon, and the signal peptide is cleaved in the ER lumen. Once MHC-I is fully translated, the ribosome subunits are released into the cytosol. (B) Rh178 is a type Ib ER-transmembrane protein that blocks this process very early in translation. This blockage is dependent on the MHC-I signal peptide and occurs before translocation. Whatever portion of MHC-I is made before translational arrest is likely degraded quickly in the cytosol.

degradation (ERAD) of MHC-I [268]. This ERAD-stabilizing function correlates with the ability of ES_1 to bind to the AAATPase p97, a cytosolic chaperone that is essential for the extraction of misfolded proteins from the ER [269, 270]. In addition, however, ES_1 was shown to inhibit protein translocation - both total protein secretion as well as that of model substrates [232]. While it is not exactly known how ES_1 inhibits translocation, Cross et al. concluded that ES_1 most likely directly targets the Sec61 complex. Similar to our finding with MHC-I HC, ES_1-inhibited substrates were shown to be degraded by the proteasome. Proteasomal degradation upon inhibition of protein translocation was also reported for other small molecule inhibitors of Sec61 translocation (cotransin/CAM471) that specifically target a subset of SP-containing proteins [271, 272]. We therefore interpret the absence of a full-length HC in Rh178-containing cells treated with proteasome inhibitors as evidence that Rh178 prevents synthesis of full-length HC at a step prior to transfer to and/or translocation through the Sec61 translocon.

What then is the basis for Rh178 selectivity for the MHC-I HC? Structural studies of the SRP/SP complex suggest that, despite its hydrophobic nature, the SP is not completely buried within the SRP protein, but is bound in a cleft-like structure with some amino-acids from the SP protruding from this cleft [273]. Thus, we speculate that Rh178 might recognize specific HC-derived amino-acids that protrude from the SRP complex, somewhat reminiscent of the T cell receptor recognizing peptide epitopes bound to the MHC-I groove. Alternatively Rh178 may interfere with GTPase-mediated release of the SP from the SRP-SR complex, thereby blocking productive transfer of the RNC to Sec61.

In summary, this work further supports our previous conclusion that VIHCE represents a unique mechanism of viral interference with antigen presentation. Our results

presented here suggest that Rh178 specifically interferes with SP-dependent HC translation at a point that precedes translocation but likely requires SRP-dependent SP recognition and transfer of the RNC to the ER membrane. Rh178 thus joins the growing number of viral proteins that are useful cell biology tools to dissect cellular pathways.

CHAPTER FOUR

The in vivo role of Rh178 and Rh182-189 in RhCMV superinfection

4.1 Introduction

The ability of CMV to superinfect seropositive hosts is a rare quality among viruses. Superinfection in immunocompetent hosts has been demonstrated for MCMV, RhCMV, and HCMV [59, 196-198]. To date, these observations regarding CMV superinfection have been made in nature, but have not been extensively tested in controlled laboratory experiments using whole animal models. MCMV infection of mice has addressed the role of immune evasion molecules in CD8+ T cell control of viral transmission, establishment of primary infection, and long-term survival after an immune response has been established [274]. However, mouse models have not yet experimentally addressed the interplay between immune evasion molecules and CD8+ T cells during superinfection. The recent introduction of RhCMV infection of RM as a model system that closely resembles the comparable HCMV infection of humans has laid the groundwork for careful investigation of the mechanism by which the virus establishes superinfection.

Identifying the factors responsible for superinfection is not only important for gaining a better understanding of CMV immunology, but will also be essential when considering vaccines against CMV or vaccines carried by CMV. Superinfection poses a problem when designing a vaccine against CMV, since natural immunity against CMV does not prevent infection with a second strain. For this reason, CMV vaccine strategy is shifting toward preventing CMV-related disease rather than infection. On the other hand,

CMV superinfection is a useful quality for a CMV vaccine vector. RhCMV vectors carrying SIV antigens gag, retanef, pol, and env were used to inoculate healthy RM before challenging with highly pathogenic SIVmac239. Between 30-50% of the RM cohorts were completely protected from SIV infection in two similar studies [198, 214], an outcome that had not been previously observed with other SIV vaccines. Stimulating a broad immune response to multiple RhCMV-encoding SIV antigens would not have been possible without taking advantage of RhCMV superinfection. Greater knowledge of the factors involved in RhCMV superinfection may help in the future design of even more effective CMV vaccine vectors.

We hypothesized that RhCMV homologues to HCMV US6 family members controlled HCMV superinfection. Rh182, Rh184, Rh185, and Rh189 work together to downregulate MHC-I at the cell surface. Recent data has also shown that Rh178 contributes to this phenotype *in vitro* [120]. Data presented in this chapter represent the first study of the relative *in vivo* importance of these five RhCMV immune evasion factors. Downregulation of MHC-I at the surface of infected cells translates to resistance to killing of infected cells by CD8+ T cells in cell culture [275]. Whether the same is true *in vivo* is a crucial and currently unanswered question. To address this, we have constructed recombinant RhCMV lacking all or some subset of the MHC-I inhibitory genes, and infected RhCMV-seropositive RM to assess the contribution of RhCMV MHC-I inhibitors to superinfection.

This chapter describes the effect of immune evasion genes encoded within Rh182-189 and Rh178 on superinfection of RM with preexisting immunity against RhCMV. BAC recombineering was implemented to design mutant viruses lacking either

all or some subset of these genes, and each mutant contained an SIV antigen, either gag or retanef. Since most of the animals used in this study were already RhCMV-seropositive, the expression of these exogenous antigens allowed us to track superinfection either directly by looking for shedding of the new virus in the urine, or indirectly by monitoring antigen-specific T cell responses.

Primary infection of RM was not determined by the five RhCMV immune evasion genes, as viruses lacking either Rh182-189 (ΔU), Rh178 (ΔV), or both regions (ΔVΔU) infected CMV-naïve RM. However, CMV-seropositive RM could not be superinfected with ΔU or ΔVΔU. Therefore, the Rh182-189 region is necessary and Rh178 alone is not sufficient to confer superinfection capability. Further, superinfection is a direct consequence of CD8+ T cell evasion as CD8+ T cell depletion restored the ability of the recombinant viruses to superinfect. The same immune evasion genes also cause a shift in the immunodominance profile of SIVgag peptides. Instead of the typical CM9-dominated response that is seen in natural SIV infection, animals infected with ΔU generate CD8+ T cells directed against a distinct set of gag peptides.

Finally, we queried whether we could isolate responsibility for the superinfection phenotype to one or more ORFs within the Rh182-189 region. This question was answered by creating smaller, more targeted recombinant RhCMVs to further isolate the factors necessary for superinfection. This work is ongoing, but preliminary data suggest that a recombinant virus lacking Rh186-189 (Δ6-9) can superinfect RhCMV-seropositive RM. This work has given us a greater understanding of the immune response initiated after RhCMV infection, and will be important to consider when creating vaccines against CMV or using CMV as a vaccine vector.

4.2 Results

4.2(a) BAC recombineering strategy

To investigate the importance of RhCMV inhibitors of MHC-I antigen presentation in superinfection and SIVgag immunodominant peptide generation, we used BAC recombineering to delete either Rh182-189 (ΔU), Rh178 (ΔV), or Rh182-189 and Rh178 (ΔVΔU). The Rh182-189 region encodes homologues of all HCMV US6 family members, including US8 and US10, which do not downregulate MHC-I. We hypothesized that only the factors that downregulated MHC-I and affected antigen presentation were responsible for superinfection, so we also engineered a virus lacking the US8-10 homologous region (ΔRh186-188 or ΔR) to be used as a control virus. For further investigation of sub-regions within the Rh182-189 region, we made a virus lacking Rh186-189 (Δ6-9). To promote detection of ΔU, ΔV, ΔVΔU, and Δ6-9 a heterologous antigen (SIV gag) was inserted into the mutant viruses. This allowed for virological and immunological detection of superinfection in four RM that were already RhCMV positive. The same four RM later received the control virus, ΔR, after they had been superinfected with an SIV gag containing virus. Therefore, again to allow detection of a new virus, SIVretanef (rtn) was inserted as a distinct marker to allow detection of ΔR superinfection. Specific details about BAC recombineering can be found in Chapter Two.

The general strategy for construction of these viruses is outlined in Figure 4.1. The process begins with recombination in *E. coli* between the RhCMV strain 68-1 BAC and a PCR product containing the SIV gag/rtn marker and a kanamycin resistant (Kan^R) cassette. The Kan^R cassette is flanked by FRT sites, and the ends of the PCR product include between 40-60 base pairs of homology to the ORF to be deleted. Recombinants

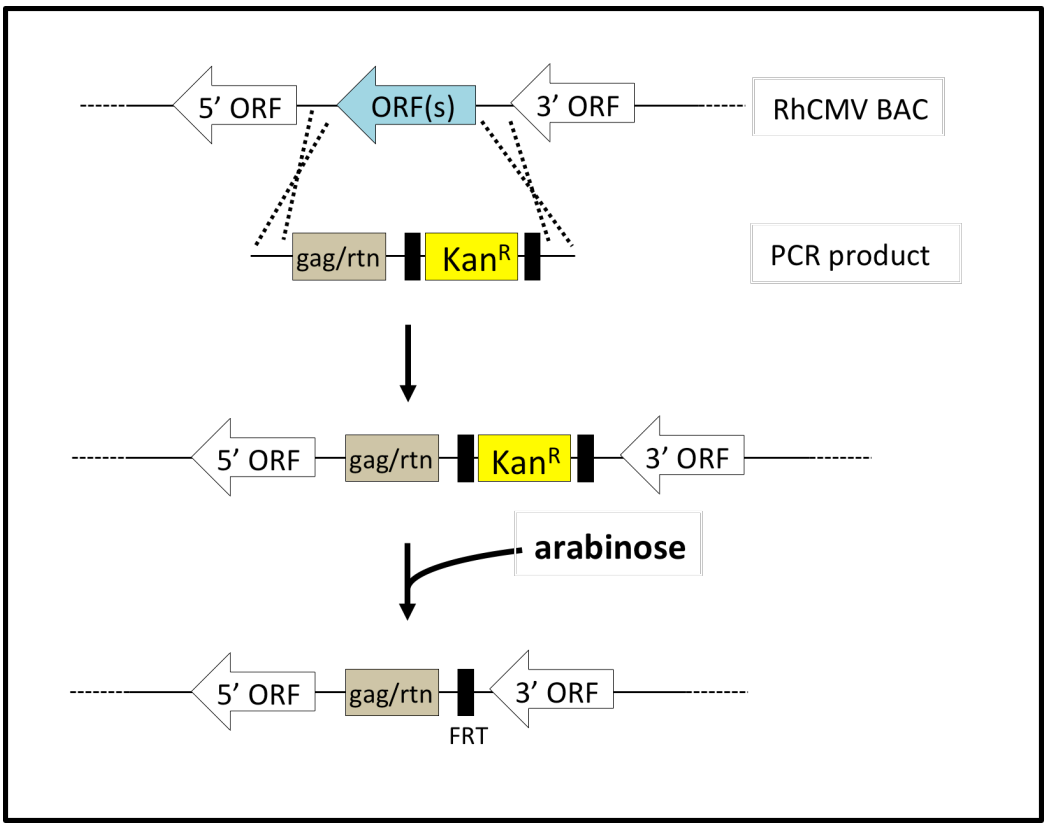


Figure 4.1: RhCMV BAC recombination strategy

The RhCMV BAC is derived from the RhCMV 68.1 strain, and includes a self-excising bacterial origin of replication between RhCMV ORFs Rh181 and Rh182. The BAC also includes a cassette with chloramphenicol resistance. A recombination is initiated between the RhCMV BAC and a PCR product with an SIV immunological marker (gag or RTN) and a kanamycin resistance cassette (Kan^R), with flanking homology to the ORF(s) of interest to be deleted. Recombinants are selected on kanamycin and chloramphenicol, followed by arabinose induction to remove the Kan^R gene. A second selection step for recombinants that are resistant to chloramphenicol but not kanamycin yields the final product that lacks the region of interest. The SIV immunological marker and a single FRT recombination scar remain in the targeted region.

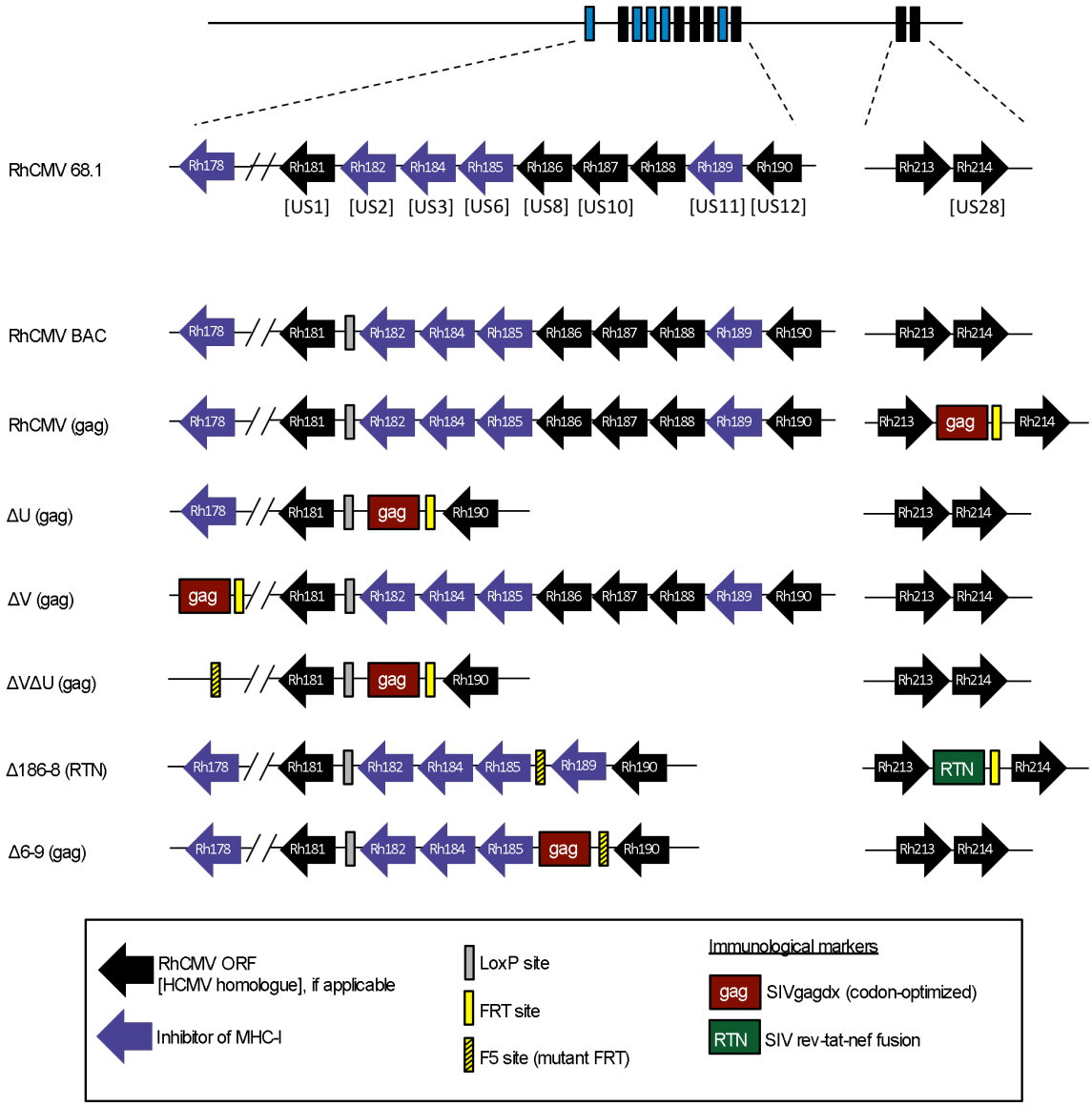

RhCMV 68.1
Rh178
Rh181
Rh182
Rh184
Rh185
Rh186
Rh187
Rh188
Rh189
Rh190
Rh213
Rh214
[US1]
[US2]
[US3]
[US6]
[US8]
[US10]
[US11]
[US12]
[US28]
RhCMV BAC
RhCMV (gag)
gag
ΔU (gag)
ΔV (gag)
ΔVΔU (gag)
Δ186-8 (RTN)
RTN
Δ6-9 (gag)
RhCMV ORF
[HCMV homologue], if applicable
Inhibitor of MHC-I
LoxP site
FRT site
F5 site (mutant FRT)
Immunological markers
SIVgagdx (codon-optimized)
SIV rev-tat-nef fusion

Figure 4.2: Diagram of viruses used in this study

The deletion strategy is described in the materials and methods. Regions of the genome that were altered to create mutant viruses are shown here in detail. All RhCMV ORFs are depicted as arrows that correspond to the direction of the ORF within the genome. Blue arrows represent genes that downregulate MHC class I. Designated RhCMV nomenclature is used for all ORFs. For ORFs with homology to HCMV genes the name of the corresponding HCMV homologue is shown in brackets. Also depicted are SIV immunological markers SIVgag and RTN, and recombination sites LoxP, FRT, and F5 FRT.

are selected with kanamycin, and are then subjected to arabinose-induced recombination of the FRT sites to delete the Kan^R cassette. Therefore, only a gag/rtn marker and a single FRT scar remain in place of the deleted ORF. This final BAC product is electroporated into rhesus fibroblasts, from which the recombinant virus is harvested. All viruses produced by this method and included in this study are diagrammed in Figure 4.2.

4.2(b) Characterization of recombinant RhCMVs used in this study

All viruses were thoroughly characterized *in vitro* before they were used to infect RM. All recombinant BACs were screened by restriction digest to demonstrate an intact viral genome. BACs were also screened by PCR to ensure that the correct ORFs were missing (data not shown). Once viruses had been reconstituted from cell culture, their gene expression profiles, SIV protein marker expression, and growth kinetics were assayed. Semi-quantitative RT-PCR confirmed that the knockout strategy had deleted the appropriate ORFs without affecting surrounding transcripts or cellular controls GAPDH or β-actin (Figure 4.3A). In addition, Western blot of infected cell lysate confirmed expression of either SIVgag or SIVrtn (tagged with Flag or V5, respectively). All infected cell lysates expressed viral protein IE-1 or IE-2 (Figure 4.3B). The ΔU, ΔV, and ΔVΔU RhCMVs showed no apparent growth defects compared to WT RhCMV, as displayed in the multi-step growth curves (Figure 4.3C). Similar growth curves for the other recombinant viruses have not yet been performed, but ΔR and Δ6-9 do not have any overt growth deficits in cell culture. As an alternative to creating revertants to complement each knockout virus, we used Comparative Genomic Sequencing (Nimblegen) to compare the genomic content of WT RhCMV to the ΔU, ΔV, and ΔVΔU

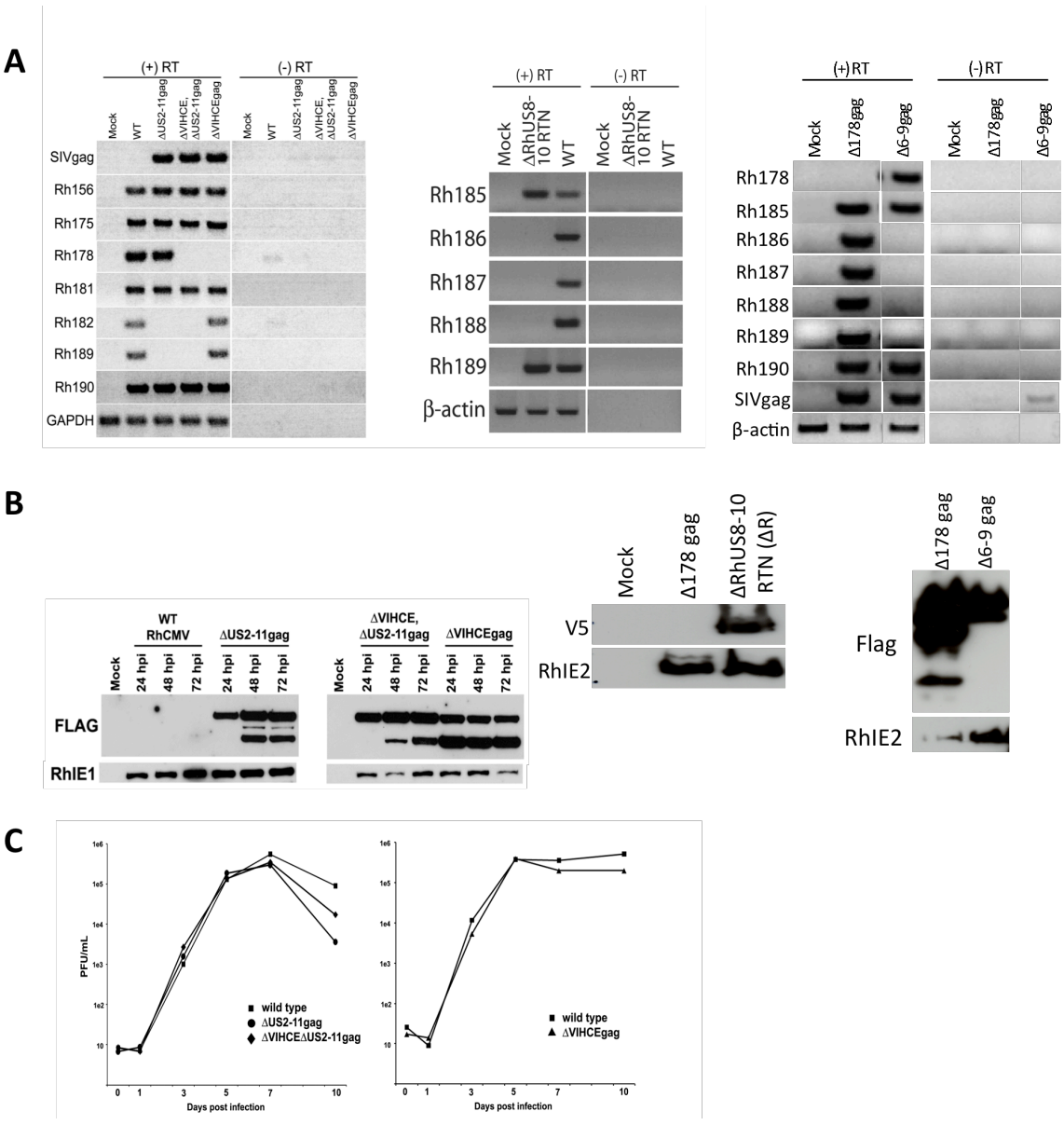

A
(+) RT
(-) RT
Mock
WT
ΔUS2-11gag
ΔVIHCE, ΔUS2-11gag
ΔVIHCEgag
SIVgag
Rh156
Rh175
Rh178
Rh181
Rh182
Rh189
Rh190
GAPDH
ΔRhUS8-10 RTN
Rh185
Rh186
Rh187
Rh188
Rh189
β-actin
Δ178gag
Δ6-9gag
Rh178
Rh185
Rh186
Rh187
Rh188
Rh189
Rh190
SIVgag
β-actin
B
WT RhCMV
ΔUS2-11gag
ΔVIHCE, ΔUS2-11gag
ΔVIHCEgag
24 hpi
48 hpi
72 hpi
FLAG
RhIE1
Δ178 gag
ΔRhUS8-10 RTN (ΔR)
V5
RhIE2
Δ6-9 gag
Flag
C
PFU/mL
wild type
ΔUS2-11gag
ΔVIHCEΔUS2-11gag
ΔVIHCEgag
Days post infection

Figure 4.3: Characterization of recombinant RhCMVs in vitro.

(A) RT-PCR. TRFs were infected at MOI=1 with the indicated virus and total RNA was harvested at 24hpi. cDNA was synthesized by random hexamer priming, and transcripts were amplified with primers specific for the ORFs indicated on the left. Genes flanking the deleted regions were included to detect possible changes in transcription due to the deletions. WT=BAC-derived wild type RhCMV. RT=reverse transcriptase. (B) Expression of SIVgag and SIV RTN by recombinant viruses. Immunoblot analysis of FLAG-tagged SIVgag and V5-tagged SIV RTN was performed at the indicated times after TRFs were infected at MOI=1 and total lysate was harvested. Antibodies are described in Chapter Two. CRT=calreticulin. (C) Multi-step viral growth. TRFs were infected at MOI=0.1 and supernatant was titered by plaque assay at the indicated times. Growth is compared to BAC-derived wild type RhCMV.

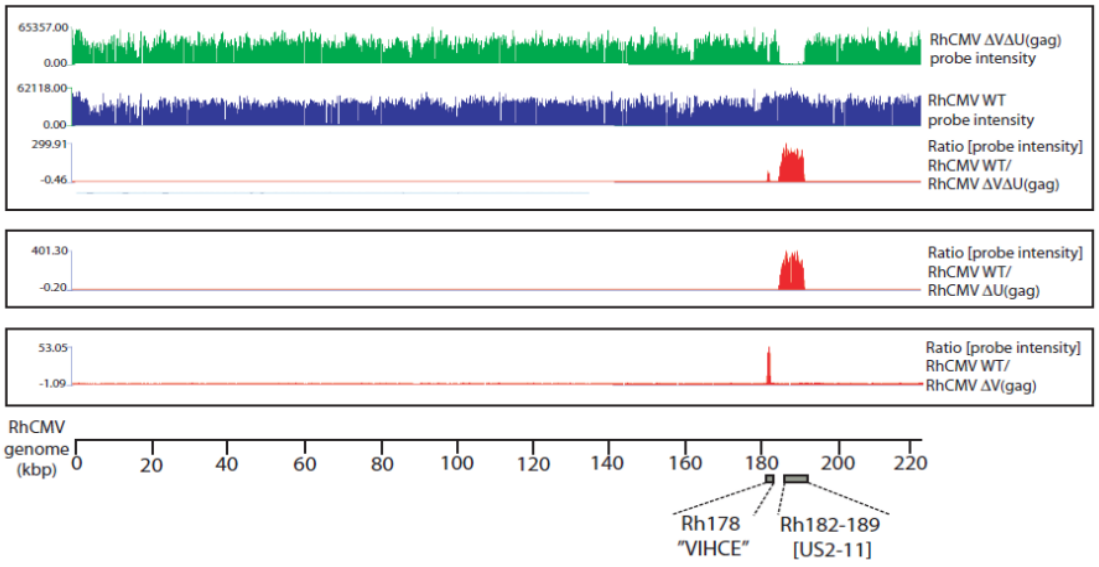


Figure 4.4: Comparative genome sequencing of recombinant viruses.

The top panel shows the probe signal intensities for labeled genomic DNA fragments obtained from the co-hybridization of ΔVIHCEΔUS2-11(gag) (ΔVΔU, Cy5 channel, green) and BAC-derived RhCMV (WT, Cy3 channel, blue) to the RhCMV-DNA-microarray of overlapping oligonucleotides. Differences in hybridization signals between the reference and test genomes are shown in red as the ratio of probe intensities for WT versus ΔVIHCEΔUS2-11(gag). The second and third panels show the ratios in probe intensities for WT versus ΔUS2-11(gag) (ΔU) and WT versus ΔVIHCE(gag) (ΔV). The bottom panel shows the nucleotide numbers of the RhCMV genome, depicted in 20 kbp increments. Also indicated are the positions of the VIHCE and US2-11 deletions. Positive red spikes represent signals that are present in the reference, but absent in the deletion viruses. These spikes correspond to the expected location of the deletions. Note that significant differences outside the deleted regions were not observed, indicating that the genomes of the deletion viruses are identical to that of the parental BAC in all but the deleted regions.

RhCMVs. This sensitive technique can detect single nucleotide differences between the two viruses being compared. The only discrepancies between WT and recombinant RhCMV genomes occurred, as expected, in the corresponding regions of genetic deletion (Figure 4.4). This eliminates the possibility that mutation(s) outside of the deleted region could be responsible for any altered phenotypes induced by the mutant viruses.

4.2(c) Viral immune evasion genes are not required for primary infection of RM

All recombinant viruses in this study contain either SIVgag or SIVrtn, which allow for detection of new infection by two methods, both of which are used throughout this study. Immunological detection of SIV markers provides an indirect yet highly sensitive way to determine whether primary infection or superinfection has occurred. Lymphocytes are isolated either from peripheral blood (PBMCs) or from bronchoalveolar lavage (BAL), followed by *ex vivo* stimulation with RhCMV IE or gag/rtn antigen peptide pools. By sorting the cells with multi-parameter FACS, activated CD4+ and CD8+ T cells that are specific for RhCMV IE, gag, or rtn can be identified. Even small percentages of antigen-specific T cells, often between 0.2% and 2% of all T cells, are representative of an immune response specific for these antigens. To corroborate new infection, a more direct virological detection method is also used. RhCMV is consistently shed at low levels in the urine of infected RM [276], so virus can be co-cultured from the urine and antigen detected by Western blot. Western blot specific for RhCMV IE-2, SIVgag, and SIVrtn from urine co-cultures is the second readout for new infection presented throughout this chapter.

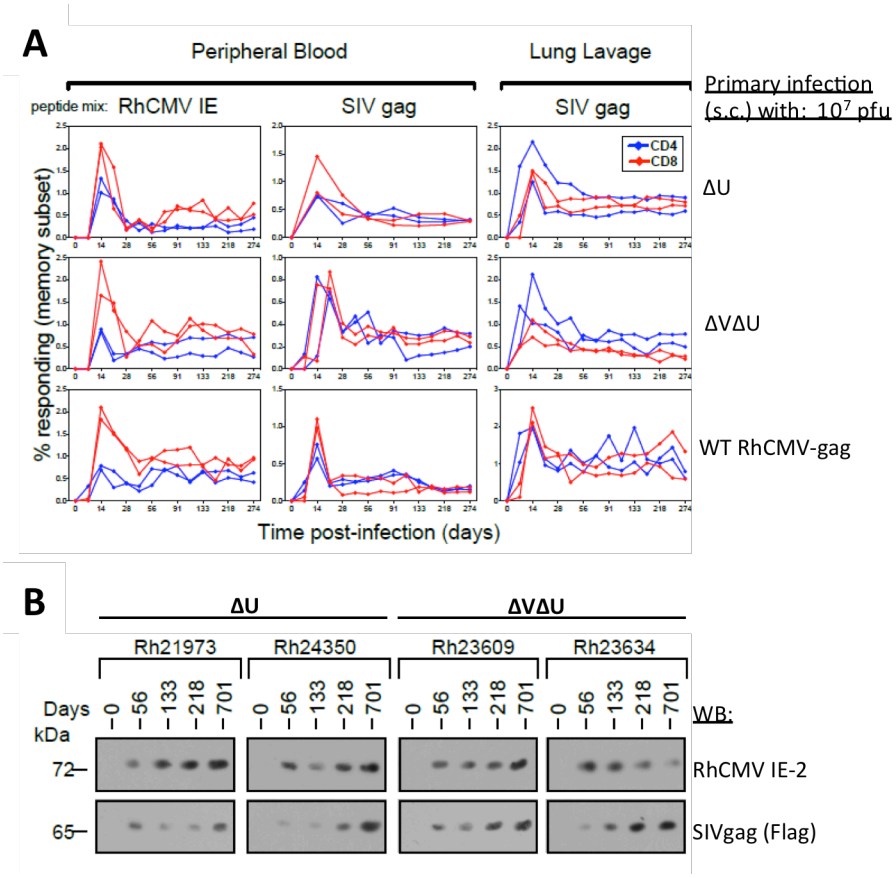


Figure 4.5: Interference with MHC-I assembly is not required for primary infection of CMV-naïve animals.

Three cohorts of two RM each were inoculated s.c. with 10^7 PFU of recombinant ΔUS2-11(gag), ΔVIHCEΔUS2-11(gag) or RhCMV(gag). (**A**) The RhCMV-specific T cell response in PBMC and the SIVgag-specific T cell response in PBMC and BAL were determined at the indicated days post infection using overlapping peptides to RhCMV immediate early genes IE1 and IE2 (IE) or SIVgag by flow cytometric analysis of ICCS for CD69, TNFα and IFNγ. (B) Immunoblot of RhCMV-IE2 or SIVgag expressed in co-cultures of urine samples obtained from animals infected with ΔU(gag) or ΔVΔU(gag). The IE2-blot confirms that the animals were negative for RhCMV prior to infection consistent with results from T cell assays.

Before testing the importance of the immune evasion genes for superinfection in RhCMV-seropositive RM, it was first necessary to show that RhCMV lacking most or all MHC-I inhibitors could establish a primary infection. To test this, four RhCMV-seronegative RM were injected subcutaneously, two each with 10^7 pfu of either the single knockout lacking most of the immune evasion genes (ΔU) or the double knockout that was also deficient for Rh178 (ΔVΔU). Two control RM were similarly infected with 10^7 pfu of WT RhCMV-gag. No RhCMV IE- or SIVgag-specific T cells were detected in any of the animals prior to infection, at day 0 (Figure 4.5A), nor was any virus detected in the urine (Figure 4.5B). After 7-14 days post infection with either SIVgag-containing WT RhCMV or recombinant viruses, there was an initial spike followed by a steady detectable number of CD4+ and CD8+ T cells that were specific to RhCMV IE and SIVgag. Antigen-specific T cells were detected in PBMCs and BAL (Figure 4.5A). In addition, Western blots from urine co-cultures demonstrate persistent viruria in RM infected with any of the three recombinant viruses at every time point after infection (Figure 4.5B). Therefore, deletion of some or all of the MHC-I immune evasion genes from RhCMV does not interfere with establishment of initial infection or long-term persistence of the virus in RhCMV-seronegative RM.

4.2(d) MHC-I evasion molecules are essential for RhCMV superinfection of RM

We next tested the requirement of the Rh182-189 and Rh178 ORFs for superinfection of RM. For this experiment, two cohorts of four RM who were RhCMV-seropositive after natural infection were injected subcutaneously with 10^7 pfu of WT or recombinant viruses lacking some or all of the MHC-I inhibitors. Antigen-specific T cells

from peripheral blood or BAL and direct detection of virus from the urine were monitored as described above. All animals displayed consistent levels of RhCMV IE-specific CD4+ and CD8+ T cells throughout the study (Figure 4.6A), which was expected because all eight RM were RhCMV-seropositive at the onset of the experiment. Four RM were challenged with WT RhCMV-gag, and the other four were challenged in succession with two doses each of ΔVΔU and ΔU, and one dose of ΔV, all of which harbored SIVgag for detection by immunological and virological methods. As seen before [198], RM challenged with WT RhCMV-gag acquired persistent SIVgag-specific T cells and shed SIVgag-containing virus in the urine (Figures 4.6A and 4.6C). However, the RM challenged with the recombinant viruses ΔVΔU or ΔU had no SIVgag-specific T cells in peripheral blood (Figure 4.6A) or BAL (Figure 4.6B) or SIVgag-expressing virus in the urine (Figure 4.6C), even after two challenge doses of each virus. In contrast, when the same cohort of RM was challenged with ΔV, SIVgag-specific T cells were detected in peripheral blood (Figure 4.6A) and BAL (Figure 4.6B), and the corresponding virus was detected in secretions (Figure 4.6D). Taken together, these results indicated that the Rh182-189 region is essential for superinfection. Rh178 is not necessary for superinfection, at least when all other MHC-I inhibitors are absent.

The Rh182-189 region encodes RhCMV homologues to HCMV US2, US3, US6, and US11 (Rh182, Rh184, Rh185, and Rh189, respectively), but also includes a set of three genes with unknown function. Rh186 and Rh187 show limited homology to HCMV proteins US8 and US10, respectively, both of which may bind to MHC-I but do not decrease its expression [119, 277, 278]. Rh188 is a RhCMV-specific gene with no known homologue in HCMV. To confirm that only the MHC-I inhibitors were responsible for

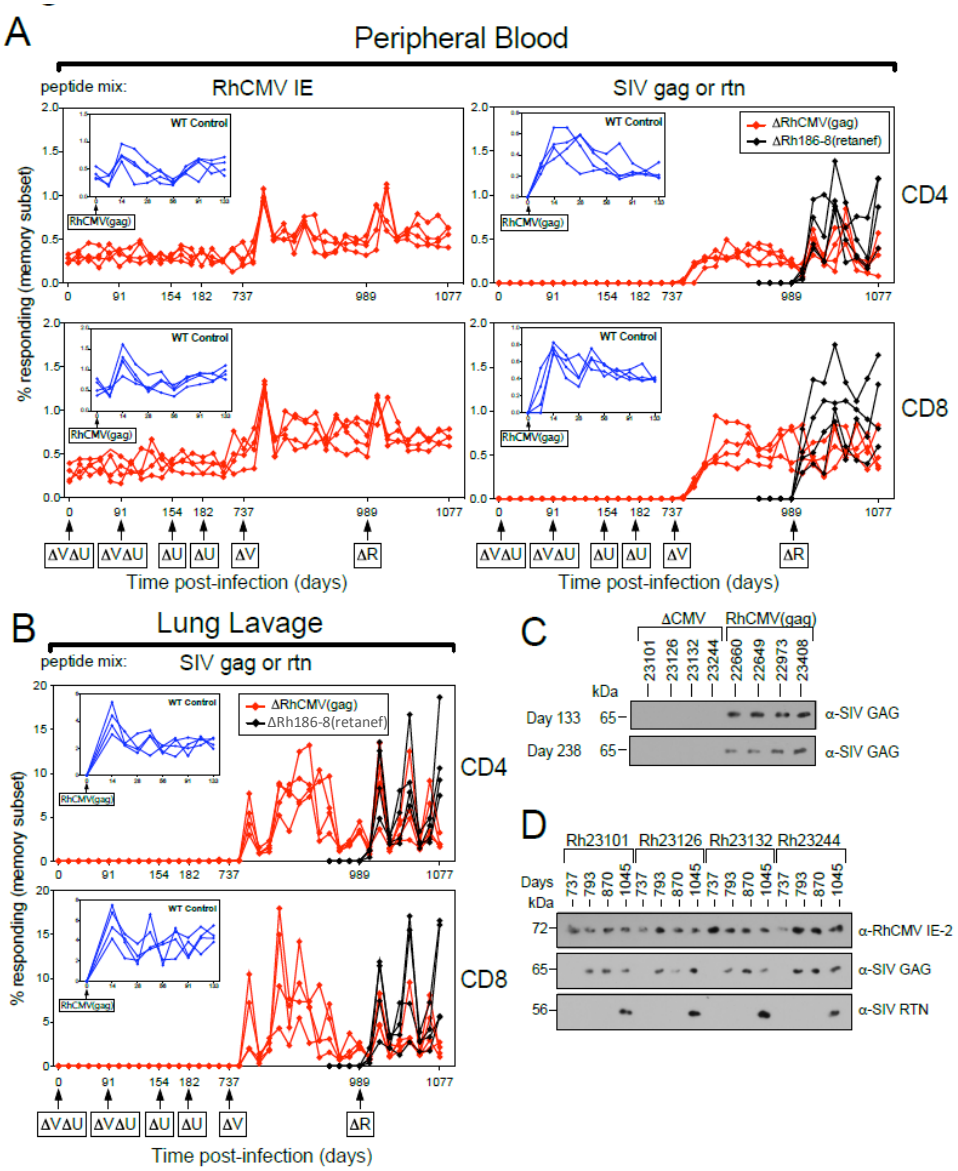

A
Peripheral Blood
peptide mix:
RhCMV IE
SIV gag or rtn
WT Control
RhCMV(gag)
ΔRhCMV(gag)
ΔRh186-8(retanef)
CD4
CD8
% responding (memory subset)
ΔVΔU
ΔVΔU
ΔU
ΔU
ΔV
ΔR
Time post-infection (days)
B
Lung Lavage
peptide mix:
SIV gag or rtn
C
ΔCMV
RhCMV(gag)
23101
23126
23132
23244
22660
22649
22973
23408
kDa
Day 133
Day 238
65
α-SIV GAG
D
Rh23101
Rh23126
Rh23132
Rh23244
Days
737
793
870
1045
72
65
56
α-RhCMV IE-2
α-SIV GAG
α-SIV RTN

Figure 4.6: US2-11 deleted RhCMV is unable to super-infect RhCMV+ rhesus macaques.

(A) A cohort of four RhCMV+ RM was inoculated s.c. with 10^7 PFU of ΔVΔU(gag) at days 0 and 91. The CD4+ and CD8+ T cell response to SIVgag or RhCMV-IE was monitored by flow cytometric analysis of ICCS for CD69, TNFα and IFNγ in PBMC. The percentage of the responding, specific T cells within the overall memory subset is shown for each time point. At day 154 and again on day 224, the same cohort was inoculated with 10^7 PFU of ΔU(gag) and RhCMV-IE and SIVgag-specific T cell responses were monitored bi-weekly. At day 737, the cohort was inoculated with ΔV(gag) and the T cell response was monitored as before. At day 989 the cohort was inoculated with ΔRh186-8(retanef) (ΔR). Besides SIVgag, a T cell response to SIVrev/nef /tat was detected by ICCS in all four animals (black lines) using corresponding overlapping peptides. Inset: A separate cohort of four animals was infected with WT RhCMV(gag) and the RhCMV-IE and SIVgag-specific CD4+ and CD8+ T cell response was monitored as described above at the indicated time points for 133 days. (B) The CD4+ and CD8+ T cell response to SIVgag in BAL was measured in parallel to the PBMC T cell responses shown in (A). (C) RhCMV secreted in the urine collected from the cohort infected with RhCMV(gag), or deletion viruses ΔVΔU(gag) or ΔU(gag), labeled ΔCMV. Virus was isolated at the indicated days by co-culture with telomerized rhesus fibroblasts (TRFs) and cell lysates were probed for expression of SIVgag by immunoblot. (D) Expression of RhCMV-IE2, SIVgag and SIVretanef by virus secreted in urine collected at the indicated days. Note that all animals were IE2-positive at the onset of the experiment confirming their RhCMV-positive T cell status.

the superinfection phenotype, and that it could not be attributed to these other genes of unknown function, we created a control virus lacking the Rh186-188 region (ΔR). Inserting SIVrtn instead of SIVgag into this virus allowed us to assess superinfection in the same cohort of RM that had been superinfected with SIVgag-expressing virus. ΔR stimulated SIVrtn-specific T cells in peripheral blood (Figure 4.6A) and BAL (Figure 4.6B), and SIVrtn-expressing virus secretion in the urine (Figure 4.6D). This result confirmed that Rh186-188-encoded proteins are dispensable for superinfection.

4.2(e) CD8+ T cells prevent superinfection in the absence of RhCMV MHC-I inhibitors

We hypothesized that evasion of existing RhCMV-specific CD8+ T cells by the RhCMV US6 family of MHC-I inhibitors was the mechanism by which superinfection occurs. Another cohort of four seropositive RM was used to test this theory. The animals were depleted of CD8+ T cells with a humanized anti-CD8 monoclonal antibody prior to superinfection with ΔU or ΔVΔU, the two recombinant viruses that failed to superinfect RM with functioning CD8+ T cells. The CD8-depleting antibody was administered one day prior to and two, six, and nine days after infection. Flow cytometric analysis demonstrated complete depletion of CD8+ T cells during this time period and CD8+ T cells were not replenished until 21 days post infection, almost two weeks after the last dose of anti-CD8 antibody (Figures 4.7A and 4.7B). Significantly, both ΔU and ΔVΔU caused superinfection in CD8+ T cell-depleted RM, as seen by SIVgag-specific T cell responses (Figure 4.7C) and SIVgag-expressing virus detected in the urine (Figure 4.7D). These signs of superinfection are evident beginning at day 7 post infection, and remain relatively constant for the duration of the experiment, mirroring responses seen in a WT

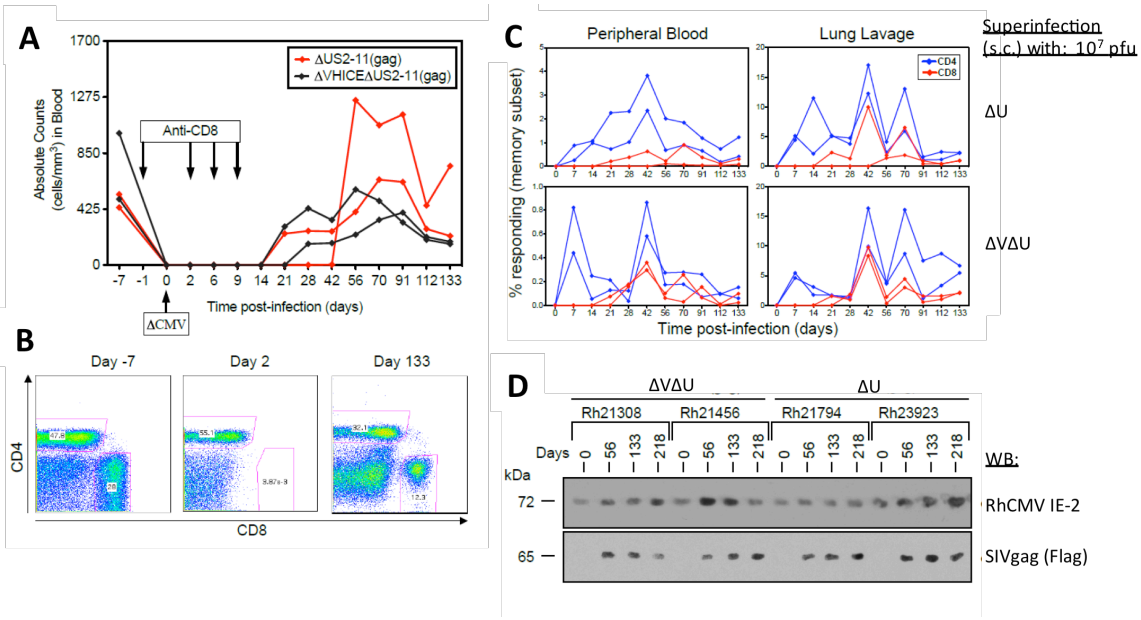


Figure 4.7: CD8+ T cells protect rhesus macaques from infection by RhCMV lacking MHC-I inhibitors.

(A) Four CMV-positive RM were treated at the indicated days with the anti-CD8 antibody CM-T807 prior to and after inoculation with 10^7 PFU of ΔVΔU(gag) (2 animals, black line) or ΔU(gag) (two animals, red line). The absolute counts of CD8+ T cells in the blood of each animal are shown over time. (B) The presence of CD4+ and CD8+ T cell populations in PBMC of one representative animal is shown for the indicated days. (C) SIVgag-specific CD4+ and CD8+ T cell responses in PBMC and BAL of CD8+ T cell-depleted animals were monitored by ICCS for CD69, TNFα and IFNγ and are shown as a percentage of total memory CD4+ or CD8+ T cells. Note the delayed appearance of SIVgag-specific CD8+ T cells. (D) Expression of SIVgag or RhCMV IE by RhCMV secreted in the urine of animals infected upon CD8+ depletion.

RhCMV superinfection. Neither ΔU nor ΔVΔU superinfected CD8+ T cell-competent RM, so this result verifies that RhCMV-encoded MHC-I inhibitors allow superinfection of a seropositive animal by evading preexisting CD8+ T responses.

*4.2(f) RhCMV-encoded MHC-I evasion molecules alter the immunodominance profile of Mamu-A*01-restricted SIVgag IDDs*

Superinfection is only one consideration in the development of RhCMV as a vaccine vector. Another potentially important concept in designing a T cell-based vaccine against a foreign pathogen is immunodominance. Immunodominance is determined by a number of factors, including but not limited to antigen processing by proteases, MHC-I affinity, MHC-I allele specificity, and the available CD8+ T cell repertoire that can recognize the peptides. The SIVgag-specific CD8+ T cell response in an animal with a Mamu-A*01 MHC-I allele is universally dominated by a 9-mer peptide called CM9 (named for the first and last amino acid and the number of total residues) [279, 280]. For the duration of this thesis, this CM9-dominated CD8+ T cell response will be referred to as the "CM9 response." SIVgag-derived peptides that bind the RM allele Mamu-A*01 have been clearly documented in the normal course of SIV infection, and are often used as a proof of principle to investigate immunodominance, because it is impractical to study every peptide from every protein, and would become even more complicated to try to apply this across all MHC-I alleles.

Viral genes that interfere with MHC-I antigen presentation could conceivably alter the peptides that are presented to CD8+ T cells, and thus change the immunodominance profile. The ΔU vector carrying SIVgag presented us with the

opportunity to test whether Rh182-189 played any role in determining immunodominant peptides from SIVgag in Mamu-A*01-positive animals. We were interested in determining if the significant decrease of MHC-I on the surface of infected cells induced by RhCMV Rh182-189 might affect presentation of peptides derived from the vaccine vector carrying SIV antigens. We chose to investigate Mamu-A*01-restricted peptides derived from SIVgag because the spectrum of CD8+ T cell responses to individual SIVgag peptides generated after SIV infection of Mamu-A*01 positive RM have been established and they fall into a defined immunodominance hierarchy. Established Mamu-A*01-restricted peptides that represent immunodominant determinants from SIVgag include CM9, LW9, VL8, QI9, VT10, LF8, and LA9 [279], all of which are identified in Figure 4.8 based on their location within SIVgag (personal communication with S. Hansen and L. Picker, unpublished data).

PBMCs were harvested from three Mamu-A*01 positive RM that had been immunized with WT RhCMV-gag. These cells were then stimulated with overlapping peptides spanning the entire length of the SIVgag protein. Cells that stained positive for cell surface CD8 and intracellular IFNγ and TNFα represented CD8+ T cells that were specific for Mamu-A*01-restricted individual peptides. This assay is similar to that used to assess superinfection by SIVgag-containing RhCMVs, but here we tested T cell responses to individual peptides rather than against full-length SIVgag protein. RM infected with RhCMV-gag did not generate CD8+ T cells specific for the typical repertoire of CM9-dominated immunodominant determinants. Rather, they possessed CD8+ T cells specific for a different set of SIVgag peptides (Figure 4.8). We suspected that the MHC-I inhibitors encoded by RhCMV prevented the typical SIVgag peptides

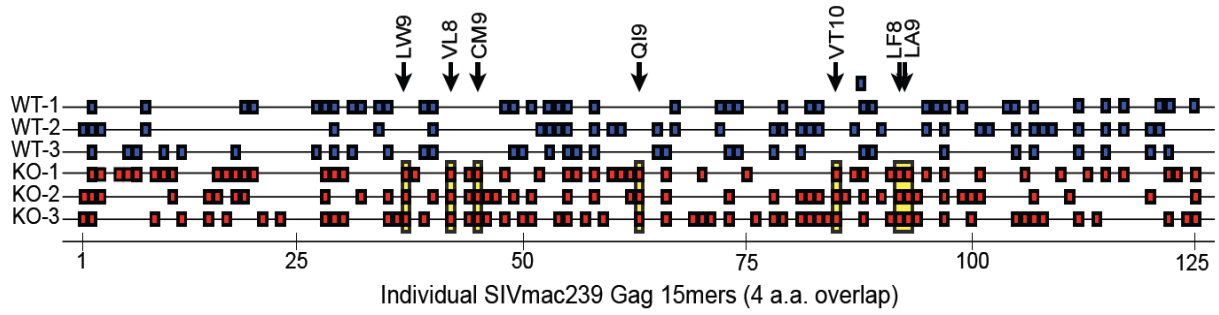


Figure 4.8: RhCMV US2-11 homologues affect the immunodominance profile of the CD8+ T cell response in Mamu-A*01-positive RM.

Two groups of three Mamu-A*01-positive RM were infected with either WT RhCMV-gag or ΔU (gag). Beginning at 154 days post infection, PBMCs were collected and stimulated with 125 individual 15-mer peptides that span the entire SIVgag protein and overlap by four amino acids. Multiparameter FACS was performed as described above, and responses were deemed positive if greater than 0.15% CD8+ T cells responded to the peptide. Each box represents a positive response to an individual peptide from the N- to C-terminus of SIVgag. Immunodominant peptides associated with the typical SIVgag CM9 response are listed at the top of the panel and highlighted in the yellow boxes.

from being presented. To test this theory PBMCs from RM infected with ΔU, which also carries SIVgag, were also surveyed for their specificity to Mamu-A*01-restricted SIVgag peptides. Strikingly, CD8+ T cells from these animals responded to the classical SIVgag determinants (Figure 4.8), leading to the surprising conclusion that RhCMV MHC-I inhibitors alter the immunodominance profile of a foreign antigen carried by a RhCMV vector, at least in RM carrying the Mamu-A*01 allele.

4.2(g) RhCMV lacking Rh186-189 causes superinfection.

The immediate conclusion drawn from Figure 4.6 attributed the superinfection phenotype to the US6 family homologues, Rh182, Rh184, Rh185, and Rh189. We wondered whether we could isolate one of these factors as solely responsible for superinfection. To begin to address this question, we infected two RhCMV-seropositive RM with a virus containing a targeted deletion within the Rh182-189 region that lacked ORFs Rh186-Rh189 and contained SIVgag (Δ6-9) (Figure 4.2). This virus still contains the majority of the MHC-I inhibitors, including Rh178 and homologues to HCMV US2, US3, and US6. Interestingly, Δ6-9 was able to overcome preexisting immunity to RhCMV and superinfect both RM, as determined by multiparamter flow cytometry of PBMCs collected from the two animals (Figure 4.9). Thus, we have begun to refine the definition of factors within the Rh182-189 region responsible for superinfection. We have now determined that the proteins encoded by the Rh186-189 region are not required for superinfection, much like the result seen with ΔV that relieved Rh178 of responsibility for superinfection.

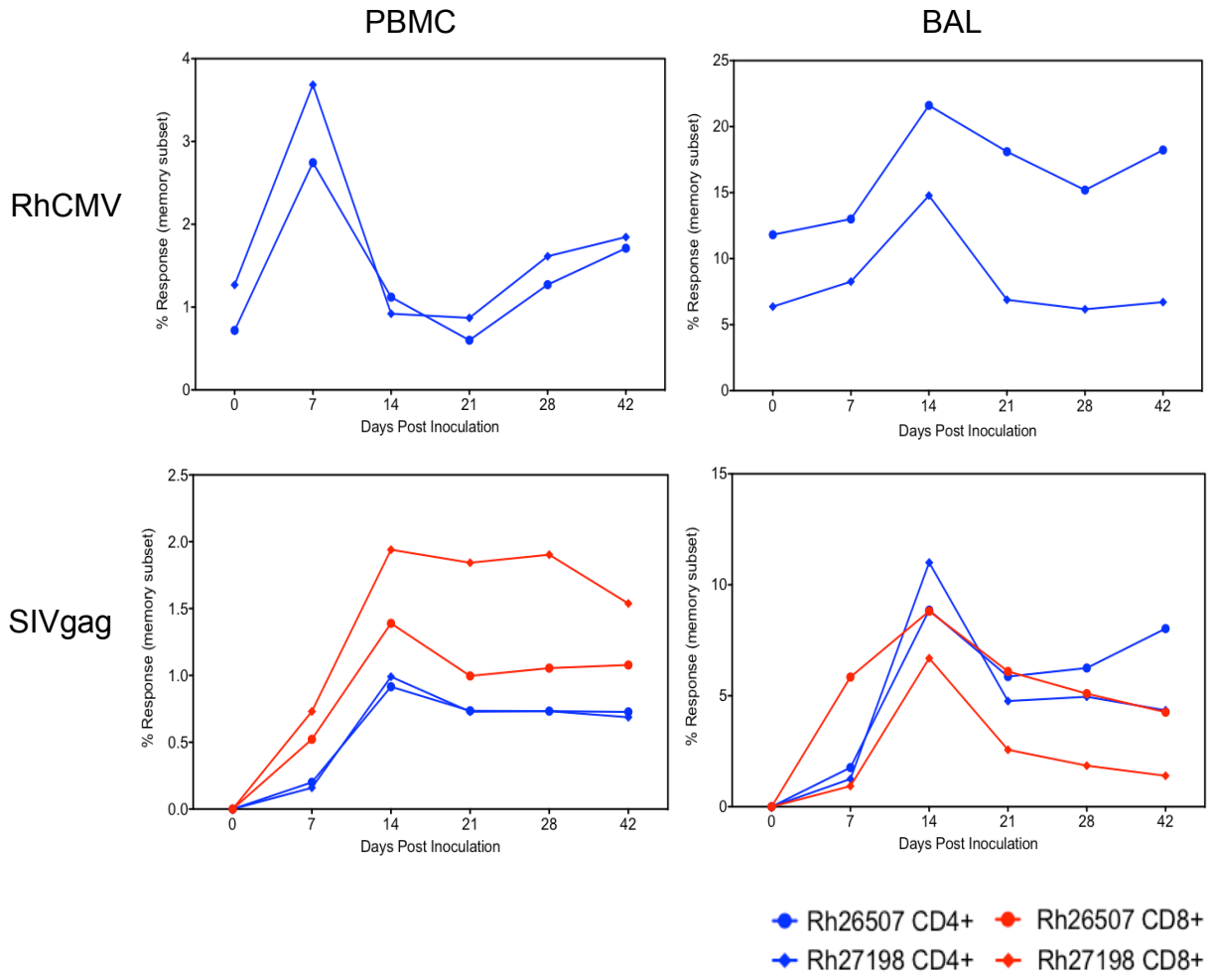


Figure 4.9: Superinfection by a recombinant RhCMV lacking Rh186-189 is possible.

A cohort of two RhCMV+ RM was inoculated s.c. with 10^7 PFU of Δ6-9 (gag) at day. The CD4+ (blue lines) and CD8+ (red lines) T cell response to SIVgag or RhCMV-IE was monitored by flow cytometric analysis of ICCS for CD69, TNFα and IFNγ. Responses were measured in PBMCs and BAL. The percentage of the responding, specific T cells within the overall memory subset is shown for each time point. Note that both animals were RhCMV-seropositive at the time of infection, as measured by a preexisting response to RhCMV IE.

4.3 Discussion

Data presented in this chapter present a framework for understanding some of the key immunological properties of CMV. Comprehension of the mechanisms by which CMV simultaneously stimulates and evades the host immune response is critical as we make progress toward the creation of a vaccine against CMV and exploitation of CMV as a vaccine vector against other pathogens. Therefore, we investigated the role of MHC-I inhibitors in regards to two parameters of the immunological response to RhCMV infection: superinfection and immunodominance.

We have identified RhCMV MHC-I inhibitors to be the exclusive gatekeepers of the CMV superinfection phenomenon. RhCMV that lacks the Rh182-189 region is fully competent to establish a primary infection and persist for years, similar to WT RhCMV infection. However, the loss of Rh182-189 gene products completely prevents superinfection of RhCMV-seropositive RM. Because of the close functional homology of Rh182, Rh184, Rh185, and Rh189 to US2, US3, US6, and US11, respectively [119], the US6 family of HCMV MHC-I inhibitors likely plays a similar role in human patients *in vivo*. A control virus lacking the intervening region Rh186-188 did establish superinfection, as did a virus lacking the only non-US6 family MHC-I inhibitor, Rh178. The most obvious conclusion from these superinfection-competent viruses is that Rh186-188 and Rh178 are dispensible for superinfection. However, an alternate explanation may exist. Superinfection could also be governed by the number of functional MHC-I inhibitors present in the virus, thus requiring a threshold of MHC-I downregulation to be reached for the virus to overcome pre-existing immunity. This possibility is bolstered by the finding that RhCMV lacking Rh186-189, which similarly to ΔV lacks only one

MHC-I inhibitor, also causes superinfection. In both cases, when four out of five RhCMV MHC-I inhibitors are encoded by the virus, superinfection can occur. When four (ΔU) or five (ΔVΔU) are absent, superinfection is blocked. If this alternate explanation is true, further investigation would be needed to determine what the threshold is for superinfection in terms of quantity and/or quality of MHC-I inhibitors, and to determine whether individual knockouts of Rh182, Rh184, and Rh185 would yield equivalent results.

Determining the relative roles of MHC-I inhibitors in CMV superinfection is further complicated by the extreme polymorphism of the MHC-I locus. The RM Mamu locus is at least as complex as the corresponding human HLA locus [174, 281], and because RM are outbred, haplotypes vary among any experimental population. Most of the HCMV US6 family members have been shown to differentially target HLA alleles [140, 282-284], and the same is likely true for their RhCMV homologues and for Rh178, though the RhCMV factors have not yet been studied in this context. The data presented in this chapter reveal unambiguous answers about superinfection when certain genes are missing from RhCMV. Though the cohorts were relatively small at only two or four RM, limited knockout viruses (ΔV, ΔR, and Δ6-9) superinfected all RM tested, and larger deletions (ΔU and ΔVΔU) superinfected none. Because of genetic polymorphisms and MHC-I inhibitor allelic specificity, we concede that absolute "yes" or "no" conclusions may not be possible with the intermediate deletions that have not yet been tested. Larger RM cohorts and continuation of this study with all combinations of MHC-I inhibitors present or missing are needed to unravel the specific requirements for each RhCMV-encoded MHC-I inhibitor in determining superinfection.

Our results help to explain why the search for a CMV vaccine has thus far been unsuccessful in preventing infection [285, 286]. It is becoming increasingly clear that the goal for a CMV vaccine should shift away from generating sterilizing immunity and toward promoting post-infection immunity that reduces the incidence of disease in groups like neonates and solid organ transplant recipients [287-289]. RhCMV-seropositive RM used in this study possessed a similar post-infection immunity, which is not protective against WT RhCMV superinfection but does prevent superinfection by ΔU or ΔVΔU. One could envision taking advantage of this knowledge to design a test for future CMV vaccines before they enter large-scale clinical trials. If the vaccine were able to elicit the desired WT-like protective immunity, superinfection with ΔU or ΔVΔU would be prevented.

The use of RhCMV as a vaccine vector for SIV has recently been reported, and based its initial success [198, 214], this may represent another creative way to exploit the superinfection capability of CMV. Superinfection allowed for administration of multiple doses of RhCMV carrying three different SIV antigens. This delivery method elicited a strong and durable effector memory T cell response, and 30-50% of the RM in two separate studies never developed productive SIV infection after challenge. Reported in this chapter is an interesting observation that infection with the vector WT RhCMV-gag leads to presentation of immunodominant determinants of SIVgag that are distinct from those presented on Mamu-A*01 in RM who are naturally infected with SIV. Thus, RhCMV causes a shift in immunodominance by eliciting a non-CM9 response. Interestingly, the same MHC-I inhibitors that are responsible for superinfection (Rh182-189) also control this shift in immunodominance. We have proven this by comparing the

CD8+ T cells generated after infection with WT RhCMV and ΔU, both expressing SIVgag. While the CM9 response never appears in RM infected with WT RhCMV, it is restored in RM infected with ΔU.

A possible explanation for Rh182-189 control over immunodominance is that the ability of these genes to strongly downregulate MHC-I may force antigen presentation to be facilitated by the alternative to direct presentation, the cross-presentation pathway. Cross-presentation occurs on MHC-I at the surface of antigen presenting cells (APCs) after they take up exogenous antigens from other infected cells [290]. A long-standing paradox in the CMV field is the development of a surprisingly strong CMV-specific CD8+ T cell response despite efficient inhibition of MHC-I in infected cells. Cross-presentation is one factor that could contribute to this apparent contradiction. Studies of MCMV have indicated that cross-presentation is the primary means of generating a CD8+ T cell response, whether or not virally-encoded MHC-I inhibitors are present [291, 292]. Unlike what we demonstrate here with RhCMV, the MHC-I inhibitors of MCMV do not alter the immunodominance profile of the immune response [291]. In contrast, when HCMV US2 and US11 are grafted into a recombinant vaccinia virus (rVV), a distinct subset of rVV-specific CD8+ T cells are seen [293]. The authors of this study concluded that cross-priming allows the virus to override viral interference with MHC-I antigen presentation. Similarly, factors within the Rh182-189 region of RhCMV may change the method by which RhCMV-specific CD8+ T cells are primed. This discrepancy may suggest an important difference between MCMV and primate CMVs in the manner in which they stimulate the immune response.

The data described in Figures 4.6 and 4.8 reveal the importance of RhCMV-encoded Rh182-189 in both superinfection and determination of immunodominance in an infected animal. These factors are both worthy of consideration for rational design of a RhCMV-based SIV or HCMV-based HIV vaccine. The ability of a RhCMV vaccine vector to superinfect is valuable because it allows for repeated inoculation with a broad spectrum of viral antigens, all delivered by the same vaccine vector [198, 214]. Creation of CD8+ T cell responses against a variety of viral antigens is widely understood to be a beneficial feature of an SIV/HIV vaccine. Less clear is whether it is desirable to mimic the immune response to natural infection by directing vaccine-induced CD8+ T cells against viral determinants that will be presented during acute infection [294]. The current RhCMV-SIV vaccine can superinfect but causes a shift in immunodominance compared to natural SIV infection, at least in Mamu-A*01 positive RM. A recombinant RhCMV-gag vector (ΔU) that restores the CM9 response fails to superinfect. Can we separate these two phenotypes and create a RhCMV-SIV vaccine vector that superinfects without changing immunodominance? If we could, would this improve efficacy of the current RhCMV-SIV vaccine vectors? These are both important questions to consider as we evaluate possible CMV-based vaccine vectors, and will require further exploration of individual MHC-I inhibitor contributions and large scale studies in RM with diverse haplotypes.

CHAPTER FIVE

Discussion and Future Directions

This thesis has described a number of consequences of CMV immune evasion. Our group has been particularly interested in studying factors that downregulate MHC-I, both investigating mechanisms at a cellular level as well as determining their effects on the host immune response, using RhCMV infection of RM as an animal model. This work began with a two-fold objective: first, to describe the molecular mechanism of a newly identified RhCMV-specific MHC-I inhibitor, Rh178; and second, to assess the contribution of Rh178 along with other US6 family MHC-I inhibitors to a developing immune response in infected RM. In Chapter Three, we present data that has brought us closer to a detailed understanding of MHC-I downregulation by Rh178. We have determined that Rh178 targets MHC-I in a signal peptide-dependent manner, blocking translation of the full-length MHC-I HC before the nascent protein begins the process of translocation. In Chapter Four, we illustrate that Rh178 has either no role or at most a contributory role in RhCMV superinfection, but that the other MHC-I inhibitors are essential for both superinfection and for determining certain aspects of immunodominance. This project has given us great insight into the immunobiology of RhCMV-infected RM, and will have an impact in the vaccine field on both development of a CMV vaccine and utilization of CMV as a vaccine vector.

5.1 Perspectives on the molecular mechanism of Rh178

Viruses rely on their host for replication and spread, but must simultaneously avoid detection by responses designed to eliminate foreign pathogens. The intricate complexity of this virus-host relationship is abundantly apparent in the wide variety of immune evasion molecules that have been discovered and continue to be studied in an attempt to better understand both pathobiology of viruses and immunobiology of their hosts. The strategies employed by viruses to evade immunological surveillance are as ancient and diverse as viruses themselves. However, only relatively recently have we gained the tools and background knowledge to investigate individual viral immune evasion molecules and determine their cellular mechanisms of action. CMV is an expert at evading the immune system, likely because it establishes long-term persistence, and its large genome encodes many immune evasion molecules in order to facilitate this long-term survival. Data presented in Chapter Three along with our recent report [120] add Rh178 to the growing list of immune evasion molecules encoded by the CMV family of viruses.

Where does the Rh178 mechanism fit in among those of other immune evasion molecules? Rh178 downregulates a very specific target, MHC-I, and this action is dependent on the N-terminal 24 amino acid SP. Preventing MHC-I from being expressed at the cell surface is a common strategy among CMVs. The other MHC-I inhibitors exert their effect after the full-length protein has been translated and has acquired its tertiary three-dimensional structure, a process mediated by the milieu of the ER lumen. HCMV US3 and US6 work by distinct mechanisms to hold MHC-I in the ER, and prevent transport to the cell surface [135, 138, 148]. This stalling and resulting ER retention open

the door for US2 and US11 to re-direct assembled MHC-I to the proteasome for degradation [117, 126, 128]. The RhCMV genome encodes functional homologues to each of these inhibitors within the Rh182-189 region [119]. The only other CMV in which immune evasion genes have been carefully studied is MCMV. Similar to US6 family members, MCMV proteins m04, m06, and m152 all affect MHC-I expression only after protein translation is complete, though they are not functional homologues to the HCMV proteins [295-298].

Similarly, interference with fully translated MHC-I occurs in many other virus families. HSV ICP47 was the first TAP inhibitor to be identified, and like US6, it prevents loading of assembled MHC-I [149, 150]. Adenovirus E3-19K has a dual function of binding directly to MHC-I as well as inhibiting tapasin, with the end result of ER retention [299]. Cowpox virus also causes ER retention of MHC-I, while concurrently blocking TAP [300, 301]. HIV nef functions slightly later, by interfering with intracellular trafficking of the full-length protein to decrease cell surface expression of MHC-I [302]. Herpesvirus proteins KSHV K3 and K5 do not act until MHC-I is assembled, loaded, and expressed at the cell surface, and then perform as E3 ubiquitin ligases to promote endocytosis and rapid degradation of MHC-I [303]. Clearly, interference with MHC-I expression is a ubiquitous and well-conserved viral phenomenon. Whether encoded by a herpesvirus or non-herpesvirus, a DNA or RNA virus, or a virus with a large or small coding capacity, each of these factors downregulates MHC-I after completion of translation and protein assembly in the ER lumen. Rh178 is therefore highly unique in its ability to interfere well before this stage.

Rh178 appears to prevent synthesis of a single host protein, MHC-I, by blocking translation in the early stages, before translocation across the ER membrane commences. Many RNA viruses also block host protein translation, but do so non-specifically by targeting machinery that is necessary for host but not viral protein synthesis [304]. This was first reported for poliovirus, a member of the picornavirus family, in 1964 [305]. Poliovirus encodes proteins that cleave and dismantle members of the translation initiation complex. An internal ribosome entry site allows translation of viral positive stranded RNA despite the lack of an effective host translation initiation complex [306]. Likewise, general host translation shut-off has been observed in cells infected with rotavirus, influenza virus, and adenovirus [304, 307].

The downregulation of MHC-I by Rh178 is in stark contrast to the highly generalized methods of the host translation shut-off described above. Rh178 targets the HLA-A3 allele only if the N-terminal 24 amino acid SP is intact [120], and adding the SP sequence to the N-terminus of CD8, a non-targeted protein, can transmit susceptibility to Rh178. Furthermore, the RhCMV Rh67 protein encodes a SP that is 38% similar to that of HLA-A3, including a stretch of 9 identical amino acids, and its expression is not affected by Rh178. We have also determined the orientation of Rh178 within the ER membrane and isolated the membrane-proximal cytosolic portion of Rh178 as the functional core of the protein. The convergence of information about important regions of Rh178 as well as the targeted portion of MHC-I allow us to speculate about its molecular mechanism.

In modeling protein-protein interactions, structure typically determines function. Though we do not know the detailed three-dimensional structure of Rh178, we can

confidently localize it to the ER membrane, with the bulk of the protein protruding into the cytosol. Therefore, we theorized that Rh178 either blocks MHC-I translation directly, or interacts with a cytosolic or ER transmembrane protein that is active in SP-directed translation and translocation. Since Rh178 does not prevent association of MHC-I mRNA with polyribosomes [120], the translational block must occur after translation initiation. We therefore hypothesized that Rh178 inhibited MHC-I expression by interfering either early in translation of the nascent polypeptide or during the completion of co-translational translocation. A known small molecule inhibitor of protein translocation across the ER membrane, ES_1, was implemented to discriminate between these two possibilities. ES_1 and the related compound ES_2 were originally identified in a large-scale screen for compounds that inhibited dislocation of misfolded proteins or proteins otherwise targeted for proteasomal degradation from the ER, a process also known as ER-associated degradation (ERAD) [268]. By interacting with a component of the Sec61 complex, ES_1 also inhibits the complementary mechanism by which proteins enter the ER, protein translocation [232]. This co-translational translocation inhibition was demonstrated on a wide-ranging group of proteins, indicating a non-specific, global decrease in translocation.

We compared the effects of ES_1 and Rh178 on MHC-I translation and translocation. Similar to all other proteins tested, ES_1 strongly inhibited expression of MHC-I. When MHC-I (or any other protein) is translocated across the ER membrane, the ER luminal environment allows for proper folding and modifications such as glycosylation [308]. If translocation is blocked, the most likely outcome for an improperly folded protein in the incorrect cellular compartment is ubiquitination followed

by proteasomal degradation [309]. Consistent with this speculation, the addition of proteasomal inhibitors stabilized MHC-I in the presence of ES_1. If Rh178 were to function in an equivalent manner to ES_1, we would expect to see similar stabilization with proteasomal inhibitors. In fact, we have shown the opposite to be true, whether Rh178 is stably expressed in cell culture (Figure 3.6B), or brought in by RhCMV infection [120]. This distinction between Rh178 and ES_1 led us to conclude that Rh178 does not inhibit translocation of MHC-I across the ER; rather, it likely acts before translocation initiation through the Sec61 translocon but after translation initiation on the ribosome.

To discriminate the MHC-I SP from those of all other concurrently translating proteins, Rh178 most likely recognizes and interacts, either directly or indirectly, with part of the 24 amino acid string. In order to interact directly, Rh178 would have to be in close proximity to the nascent amino acid chain in complex with the ribosome, which could only occur once the RNC-SRP complex had arrived near the SRP receptor at the ER membrane. If this were the case, an additional hurdle to overcome would be recognition of the SP amongst a large polyprotein complex. The S domain of SRP, mostly comprised of the 7S RNA and subunit SRP54, has dual responsibilities in binding to SP and promoting transfer of the RNC-SRP to the translocon [310]. Structural studies indicate a tight association between the SP and a cleft within the S domain [311], which introduces an obstacle for a protein such as Rh178 that is trying to access the SP while it is bound by the RNC-SRP. This difficulty is underscored by the presence of the other five SRP subunits nearby, as well as SRP receptor and Sec61 translocon subunits as the complex approaches the ER membrane.

Considering these potential steric obstacles, an indirect translational block may be the more feasible option for Rh178. Recruitment of accessory factors to form an inhibitory complex or to initiate a cascade of events leading to MHC-I downregulation could remove the restriction of close proximity for Rh178. In this case, one could imagine an interaction with the SP at a further distance from the ER membrane, in the cytosol, with fewer surrounding proteins with which to contend. We have shown that expression of a truncated version of HLA-A3 as short as 114 amino acids can be inhibited by Rh178. This result supports the conclusions of early translational inhibition by Rh178, possibly before the RNC-SRP complex even reaches the SRP receptor at the ER membrane.

The most common model for SRP-mediated targeting to the ER begins when the SP begins to protrude from the ribosome. At this stage of translation, the nascent polypeptide emerges in a linear fashion through the pore of the large ribosomal subunit. The ribosome shields at least 30 amino acids in this extended conformation from the cytosolic environment [312], so the 24 amino acid SP of HLA-A3 does not exit the ribosome until at least 54 amino acids have been translated. The RNC-SRP exhibits decreased affinity for the SRP receptor when the nascent chain length reaches about 140 amino acids [313], probably because of nascent chain interference with SRP receptor binding. The elongation rate for a new polypeptide is about nine amino acids per second [314], so the window of opportunity for SRP binding to an emerging SP before the nascent chain extends too far is fewer than ten seconds [315]. Because of this short time period and tight regulation of SRP binding to SP and later, of RNC-SRP binding to SRP receptor, even a transient association with the SP by an interfering protein could disrupt

efficient targeting to the ER. To further complicate matters, one recent study suggested that a nascent chain SP can be recognized within the environment of the ribosomal tunnel, resulting in allosteric inhibition of downstream interaction between RNCs and SRP [316]. Therefore, we cannot dismiss the possibility that Rh178 initiates recognition of the SP before it even emerges from the ribosome.

Another possible opportunity for interference by Rh178 or Rh178-directed co-factors is during the translational stall that occurs after SRP binds the SP. Soon after SRP recognition of an emerging polypeptide, an Alu domain within the 7S RNA of SRP, along with portions of the SRP9/14 subunits, promotes elongation arrest [317, 318]. These RNA and protein domains compete directly for the elongation factor binding site, causing a pause in translation while the entire RNC-SRP complex is recruited to the SRP receptor at the ER membrane. A recent report suggests two reasons for this translational stall: first, it necessarily slows the kinetics of translation due to a rate-limiting supply of SRP; second, it may allow for preferential ER-directed targeting of sequences with variable affinities for the SRP [267]. Presumably, this "preferential targeting" could be regulated during the translational pause by cellular proteins or by viral proteins that have evolved to take advantage of this window of opportunity. Rh178 could regulate MHC-I translation during this pause, which would be consistent with the significant reduction in full-length protein translation. Whether Rh178 downregulates MHC-I directly or indirectly, near the ER membrane or further away in the cytosol, it has developed a mechanism to do so in a highly specific manner, dependent on the first 24 amino acids of MHC-I. We have certainly narrowed the search, but further investigation is required to fill in the rest of the mechanistic details.

5.2 Future directions for Rh178

The above speculations leave many unanswered questions about the detailed mechanism of Rh178. A conspicuous gap in our spatial knowledge lies between the ER membrane, where Rh178 is located, and the MHC-I nascent chain-containing translation initiation complex that begins on a cytosolic ribosome. We have now dissected and identified important functional domains of the actor and its target, but the intermediate players remain elusive. Two main goals of future explorations will be to identify any cellular or viral protein interactions with Rh178, and to pinpoint the location of MHC-I targeting (e.g. does it occur in the cytosol or within range of the ER membrane?).

We have begun the search for proteins that interact with Rh178, but these efforts have yet to be fruitful. A yeast-two-hybrid screen did not reveal any promising cellular proteins with which Rh178 may interact. A limitation of the yeast-two-hybrid approach is that the screen must be performed with a soluble bait protein, in this case Rh178. An N-terminally deleted version of Rh178 that lacks the transmembrane domain was therefore constructed and screened against a library of cellular proteins. In the future, it may be more useful to implement similar large-scale screens with a more native form of Rh178, one that includes the transmembrane anchor. Many such interactions have been explored using the split-ubiquitin system, an analog to the yeast-two-hybrid system used to identify interactions between transmembrane proteins [319]. Another approach we have initiated in an attempt to find interacting partners is co-immunoprecipitation with prospective cellular proteins. By narrowing the search to proteins known to be involved with SP-dependent co-translational translocation, we increase the potential of this shotgun approach.

Discovery of a protein-protein interaction between Rh178 and a known cellular protein would also bring us closer to answering the second question, of the exact location of MHC-I targeting. If Rh178 interacted with a strictly cytosolic protein involved in the initiation of protein translation, we would conclude that translational arrest likely occurs before the RNC-SRP is directed to the SRP receptor at the ER membrane. However, if Rh178 were to bind another protein anchored in the ER membrane, we would deduce that continued translation of the MHC-I nascent chain is blocked further downstream, perhaps coinciding with the beginning of translocation.

Detection of a translation intermediate would also be helpful in answering the targeting locale question. If MHC-I is partially translated and Rh178 terminates this translation, a partial translation product would be released from the ribosome followed by a brief existence within the cytosolic compartment. Since expression of an unstable, short truncation of HLA-A3 (114 amino acids in length) was inhibited by Rh178, the translation intermediate is likely shorter than 114 amino acids, and therefore would be quickly degraded by cellular proteases. Thus far, we have been unable to stabilize translation intermediates with proteasome inhibitors or inhibitors of other cellular proteases. Therefore, any MHC-I translation intermediate may be degraded by a protease we have not yet investigated, or it may be precluded from visualization by standard detection techniques.

We may be able to take advantage of HLA-E presentation of MHC-I leader peptides to circumvent some of the problems with detecting a translation intermediate. HLA-E is a non-classical MHC-I molecule of humans that exists primarily to inhibit NK cell lysis of healthy cells. Similar to classical MHC-I presentation of peptides, HLA-E

has a similar structure with a groove that binds to short peptides derived from the SP of classical MHC-I molecules. Once bound, the peptide-HLA-E complex is shuttled to the cell surface, where it engages CD94/NKG2A inhibitory receptors on NK cells, dampening any NK cell cytotoxic activity [97]. This provides a routine mechanism for healthy cells with an intact antigen presentation system to avoid NK cell killing. MHC-I molecules, including HLA-A3, all encode a highly conserved 9-mer within their SP that serves as the peptide loaded onto HLA-E for this purpose. Thus, we speculate that if the SP of HLA-A3 is translated in the presence of Rh178, this 9-mer would still be loaded onto HLA-E. In this way, detection of peptide-loaded HLA-E at the cell surface would act as a sensor for SP translation. Antibodies to assembled HLA-E exist, so detection of complexes at the cell surface is feasible. This experiment could be performed in human cell lines with the caveat that all MHC-I alleles expressed were susceptible to Rh178. Otherwise, it would be impossible to distinguish between HLA-E complexes loaded with SP derived from susceptible vs. resistant classical HLA alleles. The HLA-E antibodies have been demonstrated to bind to the orthologous Mamu-E alleles [320], so a similar study could also be done in a more biologically relevant RM cell line. If this strategy worked as expected, we would be able to determine if the SP is translated, and then quickly degraded, but whether more of the protein beyond the SP was also translated would remain a mystery.

Another unresolved topic of Rh178 activity is allele specificity. Selective downregulation of various human HLA alleles has been illustrated for US6 family members US2, US3, and US11 [140, 282-284]. It is reasonable to expect similar results for the functional homologues of the RhCMV Rh182-189 region, but reagents and cell

lines to study individual Mamu-I alleles of RM are currently limited. With the increased use of RM as a model for so many diseases, these tools are sure to become more readily available in the near future, and at that time, it may be of interest to study Rh178 allele specificity.

5.3 Perspectives on the role of MHC-I inhibitors in RhCMV superinfection

Investigations into the detailed mechanisms of Rh178 and other CMV-encoded MHC-I inhibitors have lent significant insight into the interaction between the virus and host cells. The importance of MHC-I downregulation is highlighted not only by the number of genes dedicated to this action within each strain of CMV, but also by the conservation of this phenotype from MCMV to RhCMV to HCMV. By employing *in vitro* expression studies and infections in cell culture, we and other groups have described specific mechanisms of action for all known MHC-I inhibitors. However, to fully understand the consequences of their evolution, CMV infection in a whole animal model is essential. Anything less produces pieces of the puzzle, but fails to provide the full picture of the virus-host relationship.

Our work on RhCMV superinfection is the first description of the *in vivo* effects of the US6 family of primate MHC-I inhibitors. These data build on years of investigations into MCMV viral pathogenesis and host immunological response in a murine model. These *in vivo* MCMV studies laid the groundwork for our study and for future experiments in RM. The three MCMV-encoded inhibitors of MHC-I antigen presentation, m4, m6, and m152 are not exact functional homologues of US6 family members of HCMV and RhCMV, but by working together they facilitate the same end

result: downregulation of MHC-I at the cell surface [321]. MCMV m6 seems to have the strongest direct effect on MHC-I expression *in vitro* [322], but other groups have shown that m152 may be more important in certain circumstances *in vivo* [323]. Regardless, the three factors work together cooperatively and competitively to decrease the overall expression of MHC-I. Some important immunological parameters that have been studied in the murine model are establishment of primary infection, survival of the virus once the adaptive immune response develops, and transmission efficacy. Each of these experiments involved comparing infection of mice with WT MCMV and MCMV lacking some combination of one, some, or all three of m4, m6, and m152 (Δm4+m6+m152). Each of these viruses is BAC-derived, similar to our RhCMV BAC-derived mutant viruses.

An *in vitro* CD8+ T cell killing assay revealed that the coordinated action of m4,m6, and m152 made infected cells significantly more resistant to lysis by 16 different MCMV epitope-specific T cell lines [324]. This effect translated to an *in vivo* model in immunocompromised animals that have been manipulated to rely mostly on CD8+ T cells for viral control. In irradiated mice, MCMV Δm152 was better controlled by CD8+ T cells than was WT MCMV [323]. Surprisingly, in immunocompetent animals, there is little evidence for a functional phenotype due to m4, m6, and m152. There was no significant difference between mice infected with WT MCMV and Δm4+m6+m152 in terms of primary infection or generation of a strong and durable CD8+ T cell response [325, 326]. Additionally, the viral load in lung, liver, and kidney was equivalent over the first two weeks after infection, regardless of whether or not MHC-I evasion genes were present [326]. In these cases, MHC-I inhibitors did not have much influence on

establishing an infection in the face of a competent immune system. The only descriptions of differences between WT MCMV and Δm4+m6+m152 infection in healthy mice are in the salivary glands and in neonates [323, 327]. In both of these cases, CD8+ T cells can control the mutant viruses better than WT MCMV.

Conclusions drawn from the data presented in Chapter Four mirror some of the results from the murine model, but also go beyond what has been attempted in mice to demonstrate a significant role for RhCMV MHC-I inhibitors. Like MCMV, RhCMV can establish primary infection whether or not Rh182-189 and/or Rh178 are present (Figure 4.5). The CD8+ T cell response to RhCMV and the inserted SIV antigens has persisted for over three years, so the MHC-I inhibitors do not affect the duration of CD8+ T cell responses. That downregulation of MHC-I does not prevent MCMV or RhCMV from initiating infection is not shocking. Innate immunity, including IFN responses and NK cell recognition, plays a much larger role in eliminating viruses in the early stages of infection. As discussed in Chapter One, CMV encodes a number of inhibitors of innate immunity, and one would assume that these would play a more significant role than inhibitors of the adaptive immune response in controlling the initial onslaught by CMV. Future studies in our lab will focus on this line of inquiry.

The main objective of the work presented in Chapter Four was to determine the importance of RhCMV MHC-I inhibitors in superinfection, or infection in the face of a healthy, robust anti-CMV immune response. Experimental assessment of superinfection with MCMV in mice has not been completed to our knowledge, though it has been documented to occur in nature [196]. Therefore, this was the first *in vivo* experiment to test CMV superinfection of an animal with an existing naturally occurring anti-CMV

immune response. The results were unambiguous. When 4/5 or 5/5 MHC-I inhibitors are missing from RhCMV, the preexisting immune response is competent to prevent superinfection (Figure 4.6). So far, we have tested two RhCMVs lacking only one MHC-I inhibitor (either Rh178 or Rh189), and both can superinfect seropositive RM. The original publication describing this phenomenon described Rh178 as dispensable for superinfection [59]. However, our newer data with the Δ6-9 superinfection-competent virus demonstrate that Rh178 may be simply one of five factors working together to downregulate MHC-I, evade CD8+ T cells, and establish superinfection. It is possible that any single mutation within the Rh182-189 region would behave similarly. If there were a single knockout virus that did not superinfect, the missing ORF would be strongly implicated as a dominant factor in determining superinfection.

Another complicating factor when considering the relative importance of each CMV-encoded MHC-I inhibitor in superinfection is the polymorphic nature of their targets. As of 2010, the IMGT/HLA database had identified almost 5000 MHC-I alleles in the worldwide human population, many of which are encompassed by HLA-A, -B, and –C loci [281]. Investigations into differential targeting of HLA alleles by US6 family members have been limited in scope, but have shown definite preferences for certain alleles, some of which are determined by variation in only a few amino acids. US3 has been shown to downregulate alleles that are loaded in a tapasin-dependent manner [140]. US2 preferentially targets HLA-A2, -B27, and –G, and mutations in only a few residues within the α2/α3 domain near the peptide binding groove can decrease susceptibility [282, 284]. US11-mediated downregulation is largely dependent on the length of the cytoplasmic tail of MHC-I, and adding as few as two residues to the cytoplasmic tail of

the normally resistant HLA-E can lead to targeting by US11 [282, 283]. These determinations of specificity were made *in vitro*, often by co-transfecting a single MHC-I inhibitor into a cell line that expresses a single MHC-I allele. Presumably RhCMV-encoded MHC-I inhibitors, including US6 family members and Rh178, are similarly discriminatory in their target selection. The complexity increases exponentially in a CMV-infected animal that simultaneously possesses multiple MHC-I alleles, all of the CMV-encoded inhibitors, and differential temporal and cellular expression of both groups of molecules. The difficulties in controlling for all variables within a cohort of animals are significant, so we strive to take into account the many complexities as we analyze data from *in vivo* RM studies.

These results help us to understand the naturally occurring phenomenon of superinfection that seems to be conserved across all CMVs [16, 17, 188, 196, 197]. Unlike other viruses that superinfect in hosts with severely compromised immune systems, or viruses that rapidly evolve to escape normal immune defenses, CMV superinfects healthy patients with strains that are quite similar to the original infection. There are clear evolutionary advantages for CMV superinfection. It allows a greater number of viral strains to survive in a broad range of hosts. Therefore, even if over long periods of time, the host evolves the ability to clear one strain, some strains may be just different enough to be resistant to host attack. Superinfection may allow for increased viral fitness, particularly if multiple strains can recombine portions of their genome to their advantage. Trans-complementation between strains resulted in an increased viral load in spleen, lung, and salivary glands when mice were experimentally co-infected with different MCMV strains [328]. Though the kinetics of co-infection and superinfection

differ, similar complementation may occur in animals that are superinfected in the wild. Recombination among strains in one animal could have a similar effect.

The role of MHC-I inhibitors in RhCMV superinfection has given us new perspective on the immunobiology of the response to CMV infection. This result also has implications for vaccine design. For years, researchers have questioned whether a sterilizing vaccine to CMV is possible because of its ability to superinfect. Many have concluded that a more reasonable goal is a vaccine that provides a WT-like immunity, which would ultimately prevent CMV disease. Studies that demonstrate the protective effect of preexisting immunity reinforce this suggestion. For example, mothers of children in day care were much more likely to acquire CMV infection from their children if they were CMV-seronegative at the beginning of the study [208]. Also, vertical transmission between mother and fetus was higher in women who acquired primary CMV during pregnancy than in women who began pregnancy with a CMV-seropositive status [329]. Accordingly, inducing immunity to CMV that mirrors natural immunity may be beneficial. When Rh182-189 is deleted from RhCMV, the virus cannot overcome the natural immunity already established in the seropositive RM. A comparable mutation in the HCMV US2-11 region will likely replicate these results. After confirmation of functional equivalency, challenge with HCMV ΔUS2-11 could serve as a good barometer for whether a CMV vaccine establishes an immune response that is similar to natural immunity. It is difficult to assess CMV vaccine efficacy because of the infrequent and sometimes vague clinical features [330]. To underscore this difficulty, a recent article by Dekker and Arvin estimated that with symptomatic congenital infection as the endpoint for a vaccine trial, over 50,000 women would need to enroll [331]. A possible way to

overcome this obstacle in human trials would be to challenge vaccine recipients with HCMV lacking the US2-11 region, assuming that those genes behave like the Rh182-189 region in RhCMV.

Though the quality of superinfection frustrates the search for an effective, sterilizing CMV vaccine, it has been advantageous in the novel approach of using CMV as a vaccine vector for other pathogens. The concept of using replication-competent viruses as vaccine vectors for HIV has been prevalent for years. Attempts at using live attenuated SIV showed early promise in the monkey model [332], but the subjects eventually reverted to viral replication and disease so this strategy has been deemed too dangerous for humans [333, 334]. Despite failing to meet safety standards, these studies highlighted the importance of generating a broad immune response with a robust cellular immunity component. Other live or live-attenuated vaccine vectors against SIV or HIV have been created for this purpose and have been tested with limited success [335-337]. Two recent studies with RhCMV-SIV vaccine vectors have demonstrated unprecedented efficacy of this vector in controlling viral load to almost undetectable levels [198, 214]. In the most recent, larger-scale study, 13/24 RM who received the replication competent RhCMV-SIV vaccine vectors had undetectable plasma virus initially, and 12/24 maintained this state one year later [214]. This remarkable immune control over the pathogenic SIV challenge was attributed to the armed T_{EM} cells that reside at the site of infection and can therefore respond quickly upon challenge. Another important component to the success of this trial was the breadth of the response. Because RhCMV can superinfect, the RhCMV-SIV vaccine can be administered as a series of injections, using the same background strain of RhCMV carrying different SIV antigens. In addition,

CMV superinfection allows this vaccine strategy to be tested and implemented in a largely CMV-positive population.

A surprising result obtained with the SIVgag-containing WT-RhCMV and ΔU viruses forces us to also consider the role of MHC-I inhibitors on the priming stage of adaptive immunity, which occurs soon after the initial primary infection. The adaptive immune response is added to the immunological arsenal after about one week, at which time T cells are primed and a memory population is initiated. T cell priming is vital in determining the nature of the long-term T cell repertoire that will not only help the host maintain a homeostatic state with a persistent virus such as CMV, but also will react upon encountering a secondary infection with the same virus. Priming can occur by direct or cross presentation, and our data combined with results from MCMV studies indicate that in a WT CMV infection, T cells may be primed mostly by cross-presentation [291-293]. As mentioned before, cross-presentation occurs when non-infected APCs acquire antigen from dead or compromised cells [290]. A natural hypothesis generated from these data is that the inhibitors of MHC-I antigen presentation may be responsible for shunting priming of T cell into the cross-presentation pathway. However, when tested in the MCMV infection model, this was not the case. Munks et al. [291] tested CD8+ T cell responses to 26 defined H-2^b-restricted CMV epitopes from mice infected with WT MCMV and Δm4+m6+m152. They found that over the course of two years after infection, the MHC-I inhibitors had little effect on the immunodominance repertoire of CD8+ T cells. This was a surprising result because logically, the lack of MHC-I inhibitors should allow for direct presentation of CMV epitopes, which would presumably elicit CD8+ T cell responses to a distinct subset of epitopes.

Based on the above results in the murine model, we were surprised to find that RhCMV-encoded MHC-I inhibitors influence immunodominance (Figure 4.8). Our RhCMV vectors carried SIVgag to facilitate identification of superinfection on top of a natural RhCMV infection. This gave us the opportunity to test CD8+ T cell responses to well-defined SIVgag epitopes in a similar manner to the MCMV study described above. In natural SIV infection, the CD8+ T cell response is directed against a number of SIVgag peptides, universally dominated by the CM9 epitope. We found that CD8+ T cells generated against SIVgag in the context of RhCMV did not respond to CM9 or any of the other typically dominant SIVgag peptides. Interestingly, these responses reverted to the natural SIVgag, CM9-dominated response, when RM were infected with RhCMV lacking Rh182-189. It is still unclear whether this is due to a transition to direct presentation from cross-presentation of peptides, but the shift in immunodominance is clear.

Why might our results differ so strikingly from the MCMV study? First, a few experimental differences would need to be ruled out as possible contributory factors. Both results are based on epitopes that are restricted by a single MHC-I allele - the mouse H-2^b allele and the RM Mamu-A*01 allele. Since many allele-specific differences have been cited for MHC-I inhibitors of MCMV and HCMV, it would be useful to confirm both of these results on animals of a different haplotype background. Tools for such a broad study are currently inadequate, however. Another limitation to be considered is that our results surround a foreign antigen carried by RhCMV, and the MCMV study looks only at MCMV-native epitopes. For this reason, it would be interesting to repeat the experiment in RM looking for CD8+ T cells specific to RhCMV epitopes, to more

closely mirror the MCMV results. Looking beyond these unlikely factors, however, differences in the mechanisms of action of MHC-I inhibitors may explain the discrepancy. None of the MCMV immunoevasins block TAP, which is an essential player in loading specific peptides onto MHC-I. RhCMV homologues to US3 and US6 both interfere directly with the machinery that loads peptides into the variable groove of MHC-I molecules in the ER lumen. It is conceivable that this direct interference could have a greater and more specific effect on immunodominance than MCMV inhibitors that block MHC-I at later stages such as ER to Golgi transport. No matter what the reason for this inconsistency is, these studies will certainly prompt future research that will give us a better understanding of CMV immune stimulation, antigen presentation, and immunodominance.

5.4 Future directions for RhCMV MHC-I inhibitors' role in superinfection

We have isolated a single class of RhCMV-encoded molecules as the facilitators of an important immunological phenotype, superinfection. Rh178 is at best a contributory factor, and the Rh182-189 region is essential for superinfection to occur. The ΔR virus, that lacked non-MHC-I inhibitors Rh186-188, confirmed that it is indeed the downregulation of MHC-I and subsequent evasion of CD8+ T cells that allows superinfection of RhCMV-seropositive RM. The most pressing question remaining is whether we can identify one of the contributory ORFs as being dominant or even solely responsible. We have already determined that neither Rh178 nor Rh189 can control superinfection alone, since superinfection can still occur in their absence. Assessing the superinfection ability of single RhCMV knockout viruses lacking Rh182, Rh184, and

Rh185 will definitively answer the question of whether any of these US6 family homologues plays a larger role than the others. The more likely scenario is that the five MHC-I inhibitors work together to block antigen presentation with different specificities for alleles and cell types. Therefore, when all five are present, overall antigen presentation is low and superinfection can occur in all RM tested. So far, the same is true when four of five are present. The results may become more complicated as we make different permutations of these recombinant viruses. For example, if Rh184 and Rh185 efficiently downregulated Mamu-I allele X and Rh189 preferentially affected Mamu-I allele Y, a RM infected with RhCMV ΔRh182-185 might allow superinfection in RM with Mamu-I Y but not in RM with Mamu-I X. Because experiments in RM are performed in an out-bred population, data such as these could be difficult to interpret and would require extensive characterization of the RM haplotypes.

Also interesting will be the application of this knowledge to vaccine development, as has been mentioned throughout this thesis. We speculate that superinfection with HCMV lacking US2-11 could be used as a sensor for WT-CMV-like immunity stimulated by an HCMV vaccine designed to prevent future disease. Before this could be tested in humans, confirmation is needed that ORFs within the HCMV US2-11 region exert control over superinfection in the same way as the RhCMV factors. Only then could it be considered for application as a sensor for the type of immune response generated by HCMV vaccines.

Finally, CMV superinfection is one quality that will need to be maintained as the CMV vaccine vector field advances. Approval of such a vaccine vector for human studies will likely require some attenuation of the virus and proof that the vector will not cause

serious disease. To accomplish this, many strategies are being considered, including CMV vectors that undergo only a single cycle of replication or those that are spread-deficient. Our data regarding a CMV-mediated shift in immunodominance of SIVgag will also need to be further explored to determine if it would be desirable to create a CMV vector that can superinfect and elicit an immune response to SIV epitopes that are seen in natural infection. This question will be probed by the creation of shorter deletions within the Rh182-189 region.

5.5 Conclusions

In this thesis, we have elaborated on the unique cellular mechanism of Rh178, and investigated its role in superinfection of RM *in vivo*. We have discovered that Rh178 interferes with MHC-I expression by facilitating an early translational block, prior to translocation into the ER membrane. Combining these results with our earlier initial identification of Rh178, we confirm a novel mechanism that in the future may give us new insights into the process of SP-directed translation/translocation. This is the first documentation of a viral protein interfering with expression of a specific host protein at this early stage of translation. We determined that Rh178 is not necessary for RhCMV primary infection or superinfection in seropositive RM, at least when the other RhCMV-encoded MHC-I inhibitors are present. A majority of these US6 family homologues must be encoded by the virus in order for it to overcome preexisting immunity, and this is dependent on CD8+ T cell control. Many questions remain to be answered regarding both of these projects, but we have gained a much greater comprehension of the interactions of RhCMV and its host immune system. This increased understanding of viral-host

interactions will serve us well as we move forward with the use of the RM model to further explore RM immunological responses to CMV, and to design vaccines that will someday translate to humans.

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