CHARACTERIZATION OF RHESUS CMV AS AN IN VIVO MODEL FOR CYTOMEGALOVIRUS INHIBITION OF MHC I ANTIGEN PRESENTATION

by

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List of abbreviations

β2-m: β2 microglobulin, light chain of MHC-I

BAC: Bacterial artificial chromosome

BAL: bronchoalveolar lavage

CMV: cytomegalovirus

dsDNA: double-stranded DNA

ER: endoplasmic reticulum

HC: heavy chain (of MHC-I)

HCMV: human cytomegalovirus

HIV: human immunodeficiency virus

HLA: human leukocyte antigen, human MHC

IE: immediate early

KDa: kilodalton

Kbp: kilobase pair

MCMV: murine cytomegalovirus

MHC-I: major histocompatability complex type I

MOI: multiplicity of infection

NHP: non-human primate

NK: natural killer (cell)

ORF: open reading frame

PBMC: peripheral blood mononuclear cell

PLC: peptide loading complex

RhCMV: rhesus cytomegalovirus

SIV: simian immunodeficiency virus

SP: signal peptide

SRP: signal recognition particle

TAP: transporter associated with antigen presentation

TRF: telomerized rhesus fibroblast

US6-family: the US2-US11 MHC-I inhibitors of HCMV

UTR: untranslated region

VIHCE: viral inhibitor of heavy chain expression

VIPR: viral inhibitor of antigen presentation

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Abstract

Viruses are obligate intracellular parasites that cause a wide range of disease in all forms of life. In order to protect the host organism from the damaging effects of virus infection, the immune system has evolved a wide array of mechanisms that aim to prevent viruses ability to replicate. Consequently, viruses have developed countermeasures against the immune system. In many cases, this cat and mouse game has progressed over millions of years as viruses co-evolved with their hosts, making the virus extremely well adapted to its particular replicative environment. Cytomegaloviruses (CMVs) are a fine example of this co-evolution, where each mammalian species has its own specific CMV that is unable to replicate in other species. As a result, each CMV can contain immune evasion mechanisms both conserved with other CMVs infecting animals of near evolutionary ancestry, and unique immune evasion mechanisms that have evolved after an evolutionary split of two animal species. In this dissertation, we document the discovery of a unique immunomodulatory protein encoded by Rhesus CMV that we have termed viral inhibitor of heavy chain expression (VIHCE). VIHCE is unique both in terms of its lack of sequence similarity to any other known protein, and in terms of the mechanism by which it acts. We show that VIHCE is able to specifically block the biosynthesis of major histocompatability complex type I heavy chains by targeting their signal peptides. Additionally, we identify a role for inhibitors of MHC-I antigen presentation during Rhesus CMV infection *in vivo*, showing that these immune evasion genes are necessary for CMVs surprising ability to reinfect an already seropositive host.

CHAPTER 1

Introduction

I. Herpesviruses

Herpesviridae is a family of large, double-stranded DNA viruses that infect and cause disease in a wide range of hosts. Thus far, the family contains over 200 members, of which eight infect humans [1]. Three distinct branches of herpesviruses have emerged; one infecting mammals, birds, and reptiles; one infecting fish and amphibians; and the third containing a single non-vertebrate herpesvirus [2]. This suggests an ancient ancestry among the family. They have since co-evolved with their hosts and have become extremely well adapted to replication and persistence within each host species. All herpesvirus infections are lifelong. Immunity to the infection is typically sufficient to prevent or suppress disease, but the viruses are never cleared from the infected animal. They remain latent for the life of the host and can reactivate multiple times.

Herpesviruses are divided into three subfamilies: alpha, beta, and gamma [3]. This subdivision is based upon the speed of their replication cycle, their host specificity, their tissue tropism, and their ability to transform host cells. The alphaherpesviruses infecting humans include Herpes simplex virus types 1 and 2 (HSV-1, HSV-2), Varicella-Zoster virus (VZV), and Herpes B-virus, a virus endemic in macaque monkeys that can zoonotically infect humans. These viruses can replicate in a variety of hosts, progress through their replication cycle rapidly in culture, and primarily establish latency in sensory ganglia. The betaherpesviruses infecting humans includes Human cytomegalovirus (CMV), Human herpesvirus-6, and Human herpesvirus-7. These viruses

are very species specific, replicate very slowly in culture, and have broad tissue tropism. Human gammaherpesviruses include Epstein-Barr virus (EBV) and Human herpesvirus-8, also known as Kaposi's Sarcoma associated Herpesvirus (KSHV). Infection with these viruses is limited to the natural host and to closely related species. They typically infect lymphocytes, establish latency in lymphoid tissue, and can cause lymphoproliferative disorders.

The symptoms associated with Herpesvirus infection in humans range from asymptomatic to life-threatening. For HSV-1, infection is typically asymptomatic, although common are lesions at the site of infection, usually in and around the mouth. HSV-2, which shares 83% sequence homology of protein coding regions with HSV-1, is the main cause of genital herpes. More severe disease complications due to HSV infection are seen in neonates, where infection can be fatal, in eye infections causing conjunctivitis, and in HSV encephalitis, the most common form of sporadic, fatal encephalitis in the U.S. [4]. After initial replication in the mucosal layer, HSV enters sensory nerve endings and is transported to the neuronal cell body where latency is established [5]. Reactivation can lead to recurrent lesions at the site of infection in both HSV-1 and HSV-2 infection. Varicella-Zoster virus (VZV) is the causitive agent of chicken pox, which is associated with fever, malaise, and a rash. Primary VZV can also be associated with pneumonia in healthy adults [6], encephilitis [7], and can be complicated by secondary bacterial skin infections [8-10]. After primary infection, the virus goes into latency in sensory nerve ganglia and can reactivate decades later manifesting itself as a painful rash, Herpes-Zoster (aka, shingles). Zoonotic infection with

Herpes B-virus, while rare, is frequently fatal. Infection causes a severe encephilitis and myelitis, which is fatal in 75% of cases [11].

Among the β-herpesviruses, Human herpesvirus-6 (HHV-6) and HHV-7 are both causitive agents of exanthem subitum (ES, aka roseola). These characteristically T-lymphotropic viruses are ubiquitous, with nearly all children seropositive for HHV-6 by age 2 and slightly later for HHV-7 [12]. Primary infection with both HHV-6 and -7 is typically asymptomatic, with a febrile rash being the most common symptom. Rarely convulsions or encephalopathy are associated with infection [12]. The third human β-herpesvirus, cytomegalovirus, and its associated disease is discussed in detail below.

Kaposi's sarcoma associated herpesvirus (HHV-8, KSHV) is one of two identified human gammaherpesviruses, both of which establish latency in B cells and have been implicated in lymphoproliferative disorders. Three main disease components have been associated with KSHV infection, the most significant of which is Kaposi's sarcoma (KS). KS is a multi cell type tumor whose frequency is significantly increased in AIDS patients [13]. Also associated with KSHV are two B-cell proliferative disorders, multicentric castleman's disease (MCD) and primary effusion lymphoma (PEL) [14, 15]. Epstein-Barr virus (EBV) is the etiologic agent of infectious mononucleosis [16], whose symptoms can range from mild fever to several weeks of pharyngitis, lymphodenopathy, and general malaise [17]. EBV establishes latency in B cells and has been implicated in several lymphoproliferative disorders.

The structure of the herpesviruses is conserved across all subfamilies (Fig 1.1A). At the core of the particle is the dsDNA genome. The viral genome is encased by an icosohedral protein capsid of the same size in all herpesviruses [1]. Surrounding the

capsid is a generally amorphous group of viral and cellular proteins, as well as RNAs, known as the tegument. The tegument can vary in size between different subfamilies and is used by the virus as a way to carry into a newly infected cell pre-made proteins that can then immediately perform their function. Surrounding the tegument is a glycoprotein-studded lipid bilayer envelope, derived from modified cellular membranes. The envelope is necessary for viral attachment and entry into host cells.

All Herpesviruses have large (120-230Kbp), linear, double-stranded DNA genomes with conserved packaging sequences at the termini [18]. The number of genes expressed ranges from 70 to over 200 depending on the virus. There are approximately 40 genes that are conserved across all herpesvirus subfamilies [1]. Within each subfamily there is also conservation of various subsets of genes particular to that group. Gene expression during infection occurs as a cascade of three distinct subsets of viral genes: immediate early (IE or α), early (E or β), and late (L or γ). IE genes are immediately expressed upon entering the cell, and expression of IE genes does not require de novo viral protein synthesis as their RNA can be detected in the presence of the translational inhibitor cyclohexamide. Functions of some IE genes include transactivation of other viral genes, immune evasion, and perturbation of the cell cycle. Expression of IE proteins is required for the transcription of the next subset of viral transcripts, E genes. Although E genes are involved in multiple manipulatory processes, the most important function of E genes is DNA replication. The final subset in the cascade is the L gene group, which can be further divided into L1 and L2 categories. In general, very little L gene expression occurs in the absence of viral DNA replication. Some L genes (L1) are expressed at low levels in the absence of viral DNA replication, and their expression is significantly

increased by DNA replication. L2 genes are strictly expressed only after viral DNA replication. L genes mainly encode structural proteins and the main function of L genes is assembly and egress of the virus particle.

II. Cytomegaloviruses

Overview

Cytomegalovirus (CMV) is the best studied of the betaherpesviruses. Human CMV (HCMV) is a widespread pathogen. Immunological and PCR-based assays revealed that 60% to 100% of the adult population is infected with the virus, with frequency of infection highest in urban areas [19]. Infection is generally asymptomatic in healthy individuals, but the virus is a major cause of morbidity and mortality in immunocompromised hosts who are less able to control primary infection or reactivation of latent virus. In particular, HCMV represents a dangerous opportunistic infection during AIDS and a common life-threatening post-transplant complication in allograft recipients [20]. Congenital infection is also a serious problem with HCMV being the largest infectious cause of birth defects in the United States [21]. Total health care costs related to HCMV disease in the U.S. alone exceed $4.4 billion annually with costs for CMV-induced sensorineural hearing loss and mental retardation alone exceeding $1 billion [22].

The name cytomegalovirus stems from cytomegaly, an increased volume of the cytoplasm and bloating of cells typical with CMV infection. Intranuclear inclusions and cytomegalic cells were first observed in 1881 in kidney cells from a stillborn by German scientists who thought they represented protozoa [23]. Virus was later identified by

electron microscopy in intranuclear inclusions of pancreatic cells from a cytomegalic inclusion disease (CID) stillborn [24], and CMV was finally isolated and cultured in 1957 [25].

HCMV disease

In the immunocompetent host, the majority of individuals have no symptoms associated with primary CMV infection. Of those who do develop symptoms, they are typically similar to that of EBV infectious mononucleosis. Potential complications from CMV mononucleosis can include pneumonia, hepatitis, and meningitis. Other than mononucleosis, the most frequent major complication from CMV infection of immunocompetent hosts is inflammation within the gastrointestinal tract [26]. Other issues can include central nervous system or vascular complications. After initial infection CMV persists for the life of the host in a latent state. However, reactivations can lead to viral shedding years after exposure in multiple body fluids including saliva, tears, urine, genital secretions, and breast milk.

Persistent HCMV infections have been linked to certain conditions including vascular disease and immune senescence. Cardiovascular disease is the leading cause of death in humans, and atherosclerosis accounts for the majority of those deaths. Vascular damage, specifically to the endothelial cells, is the initial step in the condition. This is followed by the release of cytokines and chemokines which promote migration of immune cells and cause platelet adhesion to the wound. Smooth muscle cell migration to the wound also occurs, all of which leads to the formation of a fibrous plaque and subsequent narrowing of the vessel. Epidemiological studies have indicated an increased

percentage of vascular disease in CMV-positive individuals [27]. There is also a significant amount of evidence documenting the presence of CMV antigens and DNA in vascular lesions and diseased vessels, along with evidence of CMV infection of vascular endothelial cells and smooth muscle cells [28]. Animal models, such as the rat carotid-injury model, provide evidence that CMV can increase the incidence of vascular disease following angioplasty [29]. And infection with rat CMV has been shown to accelerate transplant vascular sclerosis, leading to graft failure [30, 31]. The molecular mechanism behind CMV-induced vascular disease likely involves virus-host-cell interactions which can lead to cell migration and subsequent plaque enlargement, induction of inflammatory cytokines, inhibition of apoptosis, or increased expression of surface receptors.

Immune senescence is the progressive decline of immune system function with age. A component of this phenomenon is an increased percentage of highly differentiated effector memory cells within the T cell compartment. The total number of T cells within the compartment generally stays the same throughout life. However, thymic involution leads to the production of fewer naïve T cells, so the compartment fills by division of existing T cells. Some of these highly differentiated cells are less functional and do not replicate well. CMV infection seems to accelerate this extreme differentiation in CD8+ T cells. Infection also leads to an extremely large anti-CMV T cell response, with 40% or more of CD8+ T cells CMV-specific in some individuals [32]. Combined with this high percentage of CMV-specific CD8+ T cells generated against infection, the CMV-induced differentiation can lead to a large number of non-reactive CMV-specific T cells in an elderly individual. These then take up space within the compartment. This in turn leads to less “room” for T cells of other specificities [33]. A possible cause of this increased rate

of progression to senescence is frequent antigenic stimulation. Frequent reactivations or antigen production [34] may lead to persistent CD8+ T cell stimulation and a rapid progression to the highly differentiated state. CMV seropositivity is now considered an immune parameter when defining healthy elderly patients as having an immune risk phenotype [33]. This CMV-induced memory inflation is discussed further below.

In general, CMV infection is much more problematic in patients with weak immune systems. Immunocompromised patients are at risk not only from primary CMV infection, but also reactivation of latent virus or reinfection with a new CMV. Given the high percentage of CMV seropositive individuals, this makes CMV a significant risk in immunocompromised patients. Complications from CMV infection have been well documented in patients undergoing solid organ transplantation. In heart transplants, CMV infection is associated with a greater incidence and severity of atherosclerosis and a higher rate of rejection [35]. Primary infection in solid organ transplant recipients is linked to multiple organ specific problems: renal impairment in kidney transplants, hepatitis in liver transplants, coronary stenosis and rejection in heart transplants, and pneumonitis after lung transplants [36]. In general, primary CMV infection due to a CMV-positive donor organ is more severe than reactivation of latent CMV in the seropositive recipient. Additionally, CMV infection seems to correlate with an increased risk of other opportunistic infections after transplant. In hematopoetic stem cell transplant recipients, CMV has been a frequent cause of pneumonitis and gastrointestinal disease.

CMV infection in patients with acquired immunodeficiency syndrome (AIDS) has been a major cause of morbidity and mortality. Prior to the development of highly active antiretroviral therapy (HAART), 20-40% of adults with AIDS developed CMV disease.

The most frequent manifestation of CMV disease in AIDS patients has historically been retinitis. Less frequent is esophagitis and colitis. The use of HAART, which helps prevent loss of immune function, now has significantly reduced CMV disease in AIDS patients [37, 38].

CMV is the number one infectious cause of birth defects in the U.S. The incidence of CMV infection at birth worldwide is between 0.5 and 3% of all births [39]. Transmission of CMV from the mother can occur either transplacentally, during birth, or via breast milk to the neonate. Primary infection during pregnancy carries the largest associated risk. About 1-4% of expectant mothers will have primary CMV infection during pregnancy, and about one third of those will pass the virus to the child [40]. This rate of passage is much higher than passage by a latent or reactivated virus. While preconceptional immunity provides significant protection from intrauterine transmission of CMV [41], it is only partial protection [42, 43]. Although less frequent, reinfection of CMV-positive women with a different strain of HCMV can lead to intrauterine transmission and symptomatic CMV disease in the neonate [44]. Additionally, infections occurring during the first trimester are associated with a higher rate of symptomatic disease [45].

Most CMV positive infants, about 90%, will be asymptomatic at birth. However, of those babies who show symptoms, 80-90% will have problems within the first few years of life. This can include hearing loss, vision loss, and varying degrees of mental retardation. The most frequent symptom associated with congenital CMV disease is sensorineural hearing loss. This occurs in 40-50% of all symptomatic children and in 10% of non-symptomatic children [46]. Strangely, a significant number of these cases are

of delayed onset, such that hearing loss progressively becomes worse into childhood [47, 48]. While the rate of hearing loss in congenital CMV infection is the same regardless if infection was primary or non-primary, children born to mothers with pre-existing CMV immunity have less severe hearing loss that is also less likely to progress [46]. While the precise cause behind CMV-induced hearing loss is not known, a higher viral titer in the newborn is more likely to lead to the condition [49]. Exposure to CMV by neonates also carries a potential risk of morbidity, especially in premature infants.

CMV tropism

Person to person spread of HCMV is through contaminated bodily fluids during primary infection, reinfection, or during virus shedding from reactivation. Infection initially occurs through the epithelial cells in the respiratory, gastrointestinal, and genitourinary tracts. In the murine CMV model, dissemination of the virus throughout the host has been shown to occur by infected immature myelomonocytic leukocytes [50]. Viral DNA can subsequently be detected in peripheral blood mononuclear cells (PBMC) in healthy hosts. Systemic infection leads to virus replication in the urinary tract and the epithelial cells within salivary gland, and subsequent shedding in the urine and saliva. Eventually cellular immunity gradually reduces viremia as virus replication is limited. The site of virus latency is considered hematopoietic progenitor cells. Monocytes, in particular, are now recognized as a major site of carriage of HCMV DNA in healthy individuals [51]. Sporadic reactivation can occur normally in the healthy host. Additionally, a compromised immune system can be a specific cause of reactivation. At the cellular level, reactivation appears to be driven by the state of the latently infected cell

and may be directly affected by the differentiation state of the cell. Specifically it is proposed that activation of the major immediate early promoter of CMV by cytokines or transcription factors such as NF-κB may lead to CMV gene expression and subsequent reactivation [36].

CMV has a wide tissue tropism *in vivo*. Tissue sections from persons with acute HCMV infection has indicated the presence of viral proteins in fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, and macrophages [52]. Additionally, infection has been noted in stromal cells, neuronal cells, and hepatocytes. Histopathological analyses of autopsy material from HCMV-infected patients has shown that almost any organ can be infected with the virus [53].

CMV replication

The structure of CMV is typical of all herpesviruses (Fig 1.1A,B). Of note is the larger size of CMV compared to other herpesviruses, specifically due to the increased size of the tegument. At 200-300nm in diameter, the virus particle is the largest of the herpesviruses. The CMV replication cycle begins with attachment to a host cell (Fig 1.1C). The viral glycoprotein gB is crucial for attachment as it is the major heparan sulfate binding protein. The viral heterodimer gH:gL, sometimes with gO or gpUL128/pUL130/gpUL131 [54], mediates fusion of the viral and host membrane, which for most cell types occurs at the cell surface. In epithelial and endothelial cells however, CMV entry is through endocytosis and subsequent low pH mediated fusion in the endosome [55]. Fusion results in release of the viral capsid and tegument into the cytosol. The capsid is then transported to the nuclear pore via microtubules, mediated by the

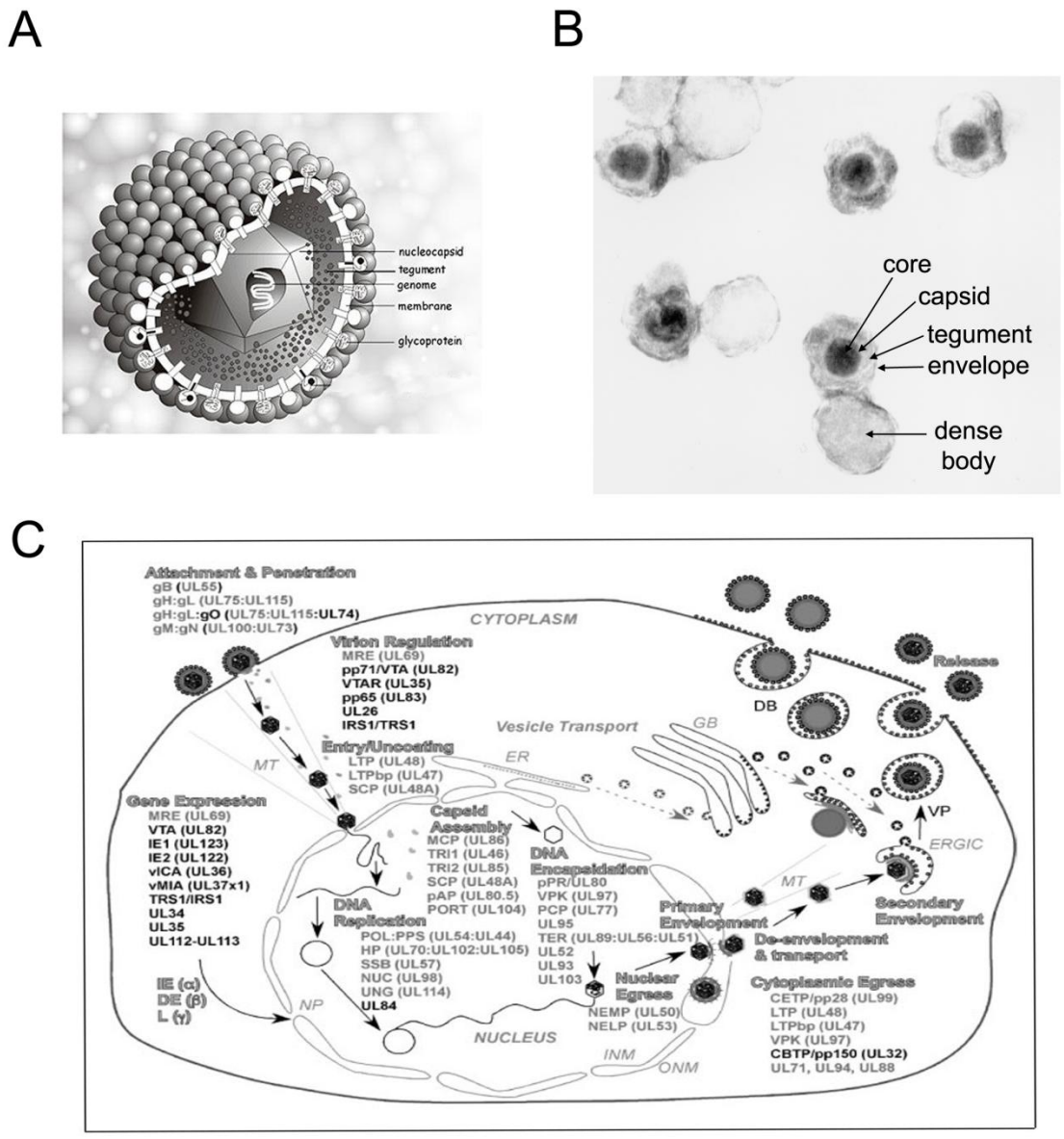


Fig 1.1 Structure and replication of cytomegalovirus. A) The basic structural components of cytomegaloviruses (Marko Reschke, 1997). B) Transmission electron microscopy image of HCMV virus particles. Noted are the structural components and a dense body, a defective particle with an envelope and tegument but without a nucleocapsid (C Powers, 2000). C) The replication cycle of HCMV with viral ORFs involved noted at each step (from ref [36]).

proteins encoded by the ORFs UL47 and UL48, where the genome is subsequently injected into the nucleus and circularizes for replication. Gene expression follows the three tiered cascade like all herpesviruses and is robust during infection in tissue culture. However, viral gene expression *in vivo* and in infection competent replication-restricted cells is much more limited. The immediate early 1 and 2 (IE1/IE2) gene products are transcriptional transactivators are critical for viral gene expression. Expression of IE1/IE2 is enhanced by the tegument proteins pp71 and pUL35. Other IE genes include the immune evasion proteins gpUS3 and pIRS1/TRS1, and the anti-apoptotic genes pUL36 and pUL37. After transcription and translation of early viral gene products, viral DNA replication begins. Herpesviruses encode a large number of their own enzymes for DNA replication and nucleic acid biosynthesis. For CMV this includes pUL54, the DNA polymerase, the pUL44 DNA polymerase processivity subunit, three DNA helicase subunits (pUL70, pUL102, pUL105), pUL98 deoxyribonuclease, pUL114 uracil-DNA glycosylase, and the pUL84 dUTPase. After replication of the genome takes place, L genes can be synthesized and capsid assembly takes place. The capsid is composed of five conserved herpesviral proteins. Assembly of the capsid and DNA encapsidation occurs inside the nucleus. Evidence suggests that tegument proteins function in directing the completed nucleocapsid through egress. The initial envelopment occurs at the inner nuclear membrane and requires the conserved UL50 and UL53 gene products which localize to the nuclear rim and cause a rearrangement of the nuclear lamin [56]. This rearrangement is also aided by recruitment of protein kinase C, which along with the viral kinase pUL97 phosphorylates nuclear lamins and promotes dissolution of the nuclear rim [57, 58]. This is subsequently followed by a deenvelopment at the outer nuclear

membrane, releasing the capsid and its associated proteins into the cytosol. Secondary envelopment then occurs at the ER-golgi intermediate compartment, whereby exiting virus obtains a double envelope. The ERGIC membrane contains a multitude of viral glycoproteins, as CMV itself is predicted to encode more than 50 proteins predicted to be glycosylated or have transmembrane domains [36]. Finally fusion at the plasma membrane releases the infectious particle from the cell. The total number of virally encoded proteins within the complete HCMV particle has been shown to be upwards of 59 [59]. A defining aspect of CMV and other betaherpesviruses is the slow speed at which replication takes place. Generally a complete replication cycle for the virus takes about 48 hours.

CMV replication also leads to the production of non-infectious particles, specifically dense bodies (DBs) and non-infectious enveloped particles (NIEPs). DBs are composed of a tegument surrounded by a membrane, with no complete viral nucleocapsid or DNA. The protein content of dense bodies differs from the standard tegument composition, in that the UL83 encoded pp65 protein is present at a much higher concentration, as is the phosphoprotein pUL25 [59]. NIEPs are composed of a tegument, envelope, and a defective nucleocapsid that does not contain DNA. It is unclear whether DBs or NIEPs play a role during CMV infection in vivo.

HCMV genomics

The genome of HCMV (strain AD169) is 229,354bp in size. This is the largest genome among herpesviruses. The genome is divided into two covalently attached segments known as the unique long (U_L) and unique short (U_S) regions. Each segment is

flanked by terminal and internal repeats. These are denoted terminal repeat long (TRL) or short (TRS), and internal repeat long (IRL) or short (IRS). Flanking those is the conserved packaging sequence (pac). Thus, the overall organization of the genome follows a pac-TRL-U_L-IRL-pac-IRS-U_S-TRS-pac structure. However, the two segments can isomerize, meaning that four different genomic organizations can be detected in a virus population. These are present in equimolar amounts and each is equally infectious.

Upon the sequencing of the HCMV genome by Chee and colleagues [60], a nomenclature for HCMV genes was developed that used the genomic segments as references. For example, the predicted ORFs within the U_L region are denoted U_L1 through U_L150, and the predicted ORFs within the U_S region denoted U_S1 through U_S34. HCMV is predicted to encode 192 ORFs [61]. This number is based on the sequence of the HCMV strain AD169. This particular strain, along with the Towne strain, were attenuated in culture and had been developed as vaccine candidates [62]. As a result, it was later discovered that AD169 is missing about 15Kbp of the genome that clinical isolates of HCMV still contain [63]. This region is denoted U_Lb'. There is also significant variation within the U_Lb' region between experimental strains of HCMV. While AD169 lacks it all together, the Towne strain is only missing a portion of U_Lb', while another portion is present but inverted. Clinical isolates of HCMV are thus predicted to encode around 220 ORFs. However, clinical isolates themselves seem to lack an IRL segment, suggesting a deletion of U_Lb' and a duplication of RL sequence in AD169 during years of passage in culture.

Since it is not required for growth in tissue culture, the U_Lb' region likely contains genes associated with *in vivo* virus-host interactions, such as immune evasion. In fact, an

analysis of the predicted proteins encoded within U_Lb' includes predicted CXC chemokines, an MHC-I homolog that inhibits NK cells, a TNF receptor homolog, and two other NK cell inhibitors.

Animal models of CMV

Since CMVs are very species-specific in terms of infection, HCMV will not replicate in an immunocompetent animal model. Because of this limitation, research on CMV replication, pathogenesis, and immunity *in vivo* has been performed using the CMVs of other mammals. These animal models of CMV include murine (MCMV), rat (RCMV), guinea pig (GPCMV), and rhesus (RhCMV) . Since CMVs have co-evolved with their hosts for millions of years, the relatedness of each CMV to another generally parallels the relatedness of their hosts[64]. Therefore, the genomes of the primate CMVs (human[60], chimp[65], and rhesus[66]) are much more closely related to each other than to rodent CMVs (mouse[67], rat[68], guinea pig[69]).

The most widely used animal model for CMV has been MCMV. Small animal models such as MCMV have many advantages, including the availability of inbred mouse strains, the availability of genetic knockout animals, lower cost, and a better characterized immune response. However, since they are more distantly related to HCMV, the limited homology of the viral genomes limits the functional analysis of homologous gene products. The closest relative to HCMV is Chimpanzee CMV (CCMV). Chimps, though, are a protected species of very limited availability and very expensive. Thus, they are not a viable animal model for CMV. In contrast, rhesus macaques are a more widely used experimental animal species and, while more distant

than CCMV, RhCMV contains most of the HCMV gene families thus allowing the study of their role in acute and latent CMV infection. RhCMV has thus become a valuable animal model for many questions in HCMV biology [70].

III. Rhesus Cytomegalovirus

Overview

Rhesus macaques were first identified as carrying their own species of CMV in 1974 when the virus was detected in the urine of healthy animals [71]. Since then, RhCMV research has largely been focused on questions that are not readily testable in the small animal models. This includes the role of CMV infection during immunosuppression by SIV (the rhesus equivalent of HIV) and the role of CMV in causing congenital birth defects. It has also been useful as a bridge from small animal models to humans in the development of both CMV vaccines and anti-CMV drugs.

RhCMV genomics

A significant attraction to the RhCMV model is the relatedness of its genome with HCMV (Fig 1.2). The RhCMV genome (strain 68.1) is 221,459 bp in length, slightly smaller than HCMVs 229,354 bp [66]. The genomes are colinear and share a similar structure, although unlike HCMV, RhCMV does not appear to isomerize [66]. Initially 230 open reading frames (ORFs) were predicted within strain 68.1, although this number was later proposed to be modified to 260 [72]. Of these, 135 are homologous to known HCMV proteins. These homologues include members of almost all of the labeled

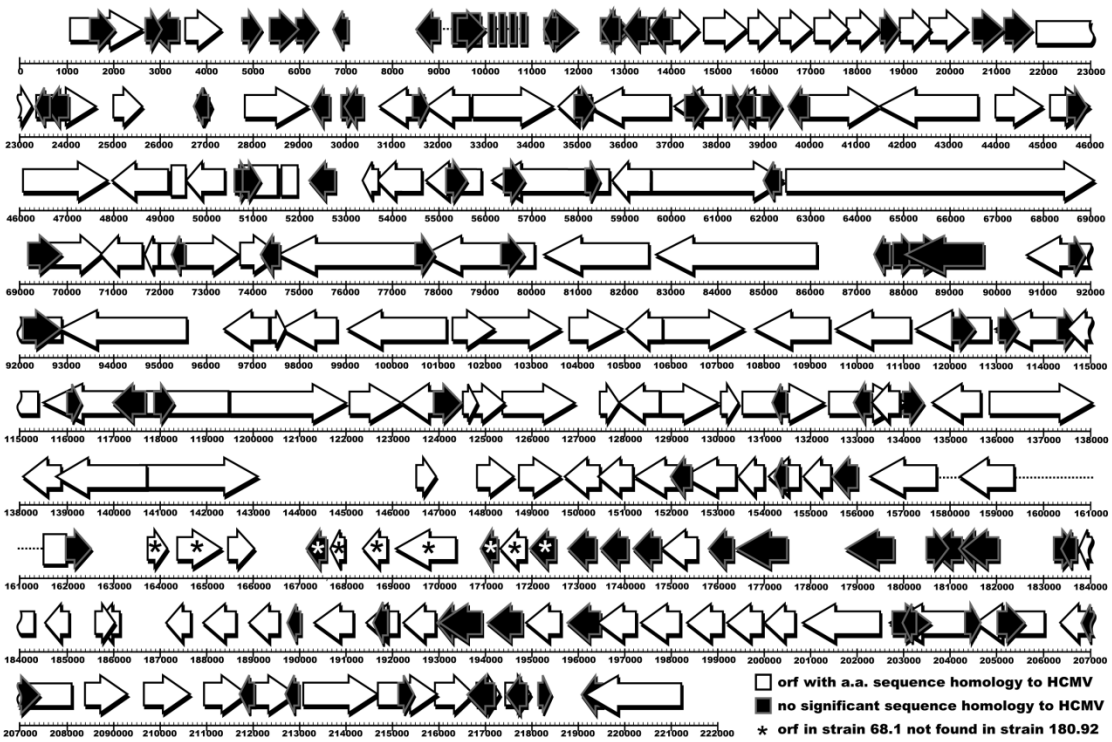


Fig 1.2 RhCMV open reading frame homology to HCMV. This diagram details the predicted open reading frames (ORFs) in the RhCMV genome (strain 68.1). ORFs in white have significant amino acid sequence homology to HCMV proteins, while ORFs in black have no significant homology to HCMV. Asterisks indicate open reading frames in strain 68.1 not found in strain 180.92. Rectangles indicate known exons. Dashed lines indicate known introns. (A. Townsend and C. Powers)

HCMV gene families, such as RL11, UL25, UL82, US1, US6, US12, US22, and seven-transmembrane protein families. Large loci of RhCMV-specific genes are located at the ends of the RhCMV genome and in the rh165-180 ORFs, a region at the same position as the U_L/b' region of HCMV [63]. There are currently two full-length RhCMV genomes sequenced [66, 72]. The two genomes are 97% identical at the nucleotide level, although strain 180.92 is 5,781 nucleotides shorter than strain 68.1, is missing ten ORFs found in strain 68.1, and contains eight ORFs not identified in strain 68.1 [72]. Just as the largest amount of variability between different strains of HCMV is observed in the U_Lb' region of the genome, the variability seen between the different strains of RhCMV is mainly localized to a similar corresponding region. Along with the identification of orthologues in CCMV, the overall similarity between RhCMV and HCMV has helped redefine the coding potential for HCMV [61, 65]. In fact, the IL-10 homolog encoded by primate CMVs was first identified in RhCMV [73]. And while there is a large amount of coding potential seemingly unique to RhCMV, many RhCMV proteins may have maintained functional homology to HCMV proteins despite limited sequence homology, such as in the US6 family of genes [74].

Characterization of RhCMV genes and gene products

Several of the RhCMV proteins have already been characterized in comparison to their HCMV counterparts. Early studies on the RhCMV immediate early 1 and 2 (IE1/IE2) gene and promoter region showed a conservation of gene structure, transcription, and protein sequence with HCMV IE1/IE2 [75, 76]. gB, a major target of anti-HCMV antibodies, is also well conserved in RhCMV. The cloned RhCMV gB is

proteolytically processed similarly to HCMV gB, portions of RhCMV gB cross-react with anti-HCMV gB antibodies, and RhCMV gB antibodies can be detected during RhCMV infection [77, 78]. Phosphoprotein 65 (pp65) is another dominant target for the immune response to HCMV. RhCMV encodes two pp65 homologs. Much like HCMV pp65, Rh-pp65-2 localizes to the nucleus, is contained within the virion, and is the target of both humoral and cellular immunity [79]. The CMV inhibitors of apoptosis are also functionally conserved in RhCMV. Both the UL36 (viral inhibitor of caspase-8-induced apoptosis, vICA) and UL37 (mitochondria inhibitor of apoptosis, vMIA) homologs in RhCMV were able to prevent Fas-mediated apoptosis in HeLa cells [80]. The IL-10 homolog of both human and rhesus CMV was shown to be expressed *in vivo*, targeted by the humoral immune response, and to have immunosuppressive properties [73, 81]. RhUS28.5, a 7-transmembrane domain chemokine receptor homolog to HCMV US28, has been shown to have a similar ligand binding profile as US28 [82]. Lastly, but most important for this dissertation, is the US6 family of proteins. These are inhibitors of MHC-I antigen presentation and the genes and their mechanisms of action are conserved in RhCMV [74]. These genes and their functions will be described in further detail below.

RhCMV pathogenesis

In addition to the genomic similarities, the clinical manifestations, epidemiology, and pathogenesis of RhCMV parallel that of HCMV. The majority of the population of rhesus macaques (RM) are CMV positive, typically measured by serology. For instance, 95% of RM at “monkey temples” in India tested positive for RhCMV [83]. Similarly,

close to 100% of RM in primate centers are CMV positive [84]. Most monkeys seroconvert during the first year of their life [85]. Once infected, non-human primates (NHP) continue shedding virus for the rest of their lives in urine and saliva [71, 86]. Experimental infection of naïve animals (oral or i.v.) was shown to result in initial viremia in the blood and virus could be detected in various organs, particularly the spleen [87]. All infections were asymptomatic, similar to primary CMV infection of adult humans.

RhCMV immunity

The immune response to RhCMV, while not nearly as well characterized as that against HCMV, does show similar important features. One of the more striking aspects of anti-CMV immunity in general, and one that is true for both HCMV and RhCMV infection, is the high percentage of both CD4+ and CD8+ T cells that are directed against the virus (see next section). RhCMV has largely been used as a tool by immunologists to help provide insight into the macaque immune response that provides critical information for the development of the rhesus macaque model in general. This includes the characterization of the $CD4^+$ $CD8^+$ T cell population [88], the characterization of the cytolytic properties of $CD8^+$ T cells [89], defining of the memory T cell population [90], and the description of useful techniques such as polychromatic flow cytometry analysis of immune cells during infection [91].

Another focus of immunological study using RhCMV has been characterizing the response to RhCMV during SIV infection. This helps to establish the model for CMV pathogenesis during AIDS. In the simian AIDS model, both cellular and humoral

immunity are critical in suppression of CMV disease [92]. In this study it was observed that while a loss of CMV-specific T lymphocytes was associated with reactivation and low-level viremia, it was only in animals who also had a decline in CMV-specific antibodies that showed extremely high viral titers. While the CD8+ T cell responses to RhCMV were similar in both SIV-negative animals and those infected with pathogenic and attenuated SIV strains [93], the frequency of CMV-specific $CD4^+$ T cells was found to be reduced in SIV-positive macaques [94]. This reduction of CMV-specific CD4+ T cells correlated with an increase in viral titers. Lastly, the induction of the regulatory T cell response to RhCMV has been characterized, providing some insight into the regulation of the anti-CMV immune response. It has been found that during primary infection with RhCMV, the induction of the regulatory T cell response is delayed when compared to acute infection with a different virus, SIV [95].

IV. Anti-viral immunity

Overview

The immune response to viral infection can be generally broken down into two components: innate immunity and adaptive immunity. The innate response to viral infection includes cells and immune molecules that are not specific to individual pathogens, but rather recognize or activate based on general signals of infection or cellular damage. The adaptive immune response includes cells of the immune system that undergo germline rearrangement to encode receptors that recognize specific antigens. These include T and B lymphocytes. Both types of immunity are critical in protecting the host from viral infection.

While this dissertation focuses on viral inhibition of MHC-I antigen presentation and subsequent prevention of CD8+ T cell recognition, it is worth noting other important components of anti-viral immunity that are targeted for inhibition by many viruses, and CMV specifically. These will be discussed in the subsequent section on viral immune evasion. Here we focus on the CD8+ T cell response in general, and specific components of anti-CMV cellular immunity.

General CD8+ T cell response to virus infection

Cytotoxic CD8+ T cells are mediators of cellular immunity that recognize specific antigenic peptides in the context of major histocompatibility complex type I (MHC-I) molecules. Since the peptides presented by MHC-I to CD8+ T cells are generally generated from intracellular sources (with the exception of cross presentation), this makes CD8+ T cells extremely important in the recognition of virally infected cells. Binding of MHC-I by CD8+ T cells is through the T cell receptor (TCR). Each CD8+ T cell expresses TCRs with only a single specificity. Yet because of somatic recombination of different segments of the TCR, diversity in human TCRs is estimated to be as high as 10^{18} different possible receptors [96].

Binding of a CD8+ TCR to MHC-I also involves the co-receptor CD8, which binds to an invariant portion of the MHC-I molecule. CD8 binding to MHC-I is necessary for signaling and subsequent activation of the T cell. However, this TCR/CD8 recognition of MHC-I is not sufficient to induce activation of a naïve T cell. Co-stimulatory molecules on the antigen presenting cell, such as B7.1 and B7.2 which bind to CD28 on T cells, are also required for activation. Once activation and proliferation of a

specific CD8+ population happens, these effector cells activate much more readily, with less dependence on co-stimulation.

Recognition of a viral peptide in the context of MHC-I leads to both the production of cytokines from the activated T cell and direct lysis of the target cell. Cytokine release in general results in inflammation, proliferation of immune cells, and recruitment of other immune cells. IFN-γ is a particularly important cytokine secreted during virus infection as it leads to the creation of an antiviral state within surrounding cells, which includes increasing the expression of MHC-I and other antigen presentation components. Direct cell killing by CD8+ T cells can be mediated in more than one way. Induction of apoptosis in the target can be achieved by Fas-Fas ligand interaction. However, primarily killing is through the release of cytotoxins including perforin and granzymes. Cytotoxins are released by T cells through modified lysosomes and are not active until released from the cell. Perforin is a protein that polymerizes to form pores within the target cell membranes. Granzymes are proteases which can cleave target cell proteins leading to the induction of apoptosis. Thus, in order to avoid CD8+ T cell mediated killing, viruses must not only attempt to prevent recognition by the T cells but also find ways to prevent host cell killing if it is targeted.

The MHC-I antigen presentation pathway

All nucleated cells express MHC-I molecules. The way in which cells process antigen and express it on MHC-I molecules for CD8+ T cell recognition is known as the MHC-I antigen presentation pathway. In general this is a way for cells to signal passing CD8+ T cells as to the intracellular contents of the cell. Although the MHC-I pathway is

generally thought to be involved in direct presentation of peptides derived from intracellular components, in phagocytic cells such as dendritic cells and macrophages exogenous antigen can be endocytosed and enter the MHC-I pathway. This process is known as cross-presentation.

The MHC-I molecule is a heterotrimer consisting of a 44kDa heavy chain (HC), a 12kDa light chain β2-microglobulin (β2-m), and a peptide of typically 8-11 amino acids in length. The HC protein itself is divided into three domains termed α1, α2, and α3 (Fig 1.3A). Between the α1 and α2 domains lies the peptide-binding cleft. While the majority of the HC sequence is conserved across alleles, the primary amino acid sequence within the α1 and α2 domains that make up the peptide-binding cleft are extremely variable. This polymorphism allows for the recognition of a high number of antigenic peptides within a population. Associating with the α1 and α3 domains is the light chain, β2-m. β2-m is necessary for the stability of the MHC-I molecule, as is an optimal peptide. MHC-I molecules without β2-m or without a well-fitting peptide are not stable, and are either reloaded with peptide or turned over by the cell. Only stable MHC-I molecules progress to the cell surface.

The translation and translocation of the HC is typical of other transmembrane proteins (Fig 1.3B). The HC contains a highly conserved N-terminal signal peptide (SP) that targets the molecule for the secretory pathway. Upon the initial translation of the SP, it is bound by the signal recognition particle (SRP). The SRP is a ribonucleoprotein composed of six peptide subunits and a single RNA molecule. The SRP recognizes the stretch of 8 or more non-polar amino acids within the signal peptide. Upon SP binding, the SRP also associates with the large ribosomal subunit and stalls translation of the

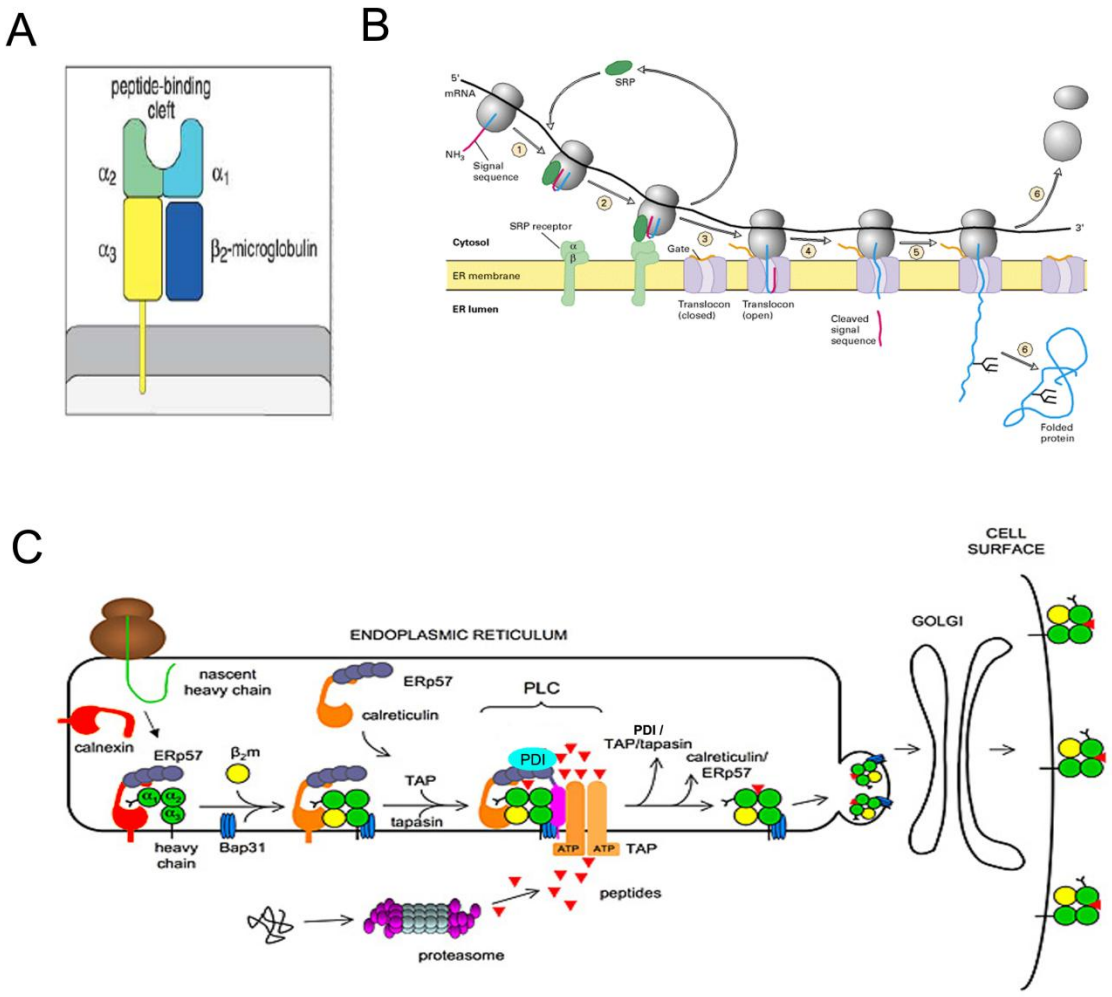


Fig 1.3 Synthesis and assembly of MHC-I. A) Basic structure of the MHC-I heterodimer (Garland Science, 2001). B) The translocation pathway. Signal peptides are recognized by the signal recognition particle (SRP) that binds to the large ribosomal subunit and stalls translation. The SRP-ribosome complex then docks at the ER by binding the SRP receptor, followed by association with the Sec61 translocon through which translocation takes place (WH Freeman and Co., 2000). C) Assembly of MHC-I. Upon translocation into the ER, MHC-I is properly folded and assembled with the light chain and a peptide with the help of several ER chaperone molecules (see text for details) (Modified from David Williams, 2008).

mRNA. Translation remains stalled until the SRP-ribosome-mRNA complex docks at the ER membrane, where the SRP binds to the SRP receptor, an integral membrane protein exposed on the cytosolic side of the ER. This entire complex then associates with the ER translocon, and the SRP and SRP receptor disassociate. Translation then commences through the translocon.

The ER translocon, also known as the Sec61 complex, is composed of the core Sec61αβγ heterotrimer and several associated components (TRAM, TRAP$\alpha\beta\gamma\delta$, oligosaccharyltransferase (OST), and signal peptidase complex). The complex forms a pore in the ER membrane which allows for the passage of proteins into the ER lumen. The pore itself is passive and must associate with either ribosomes or chaperones which mediate the passage of the polypeptide into the lumen. For co-translational translocation such as MHC-I HCs, this passage occurs with the ribosome docked at the Sec61 translocon. After the dissociation of the SRP and SRP receptor from the translocon, the ribosome resumes translation, pushing the HC polypeptide into the ER lumen. The HC SP is clipped off cotranslationally by signal peptidase, and the remainder of the protein is translated with the majority of it within the ER lumen. Only a ~33 amino acid tail resides in the cytosol.

After completion of translation, MHC-I assembly within the lumen begins. New HC in the ER is not properly folded and requires chaperones in order to completely assemble (Fig 1.3C). This process begins with HC binding to calnexin. Calnexin mediates proper folding of the HC and intrachain disulfide bond formation also occurs at this step. HC then dissociates from calnexin and binds to β2-m. The MHC-I heterodimer

then joins the peptide loading complex (PLC), a macromolecular association of several ER proteins. The PLC includes MHC-I, calreticulin, tapasin, Erp57, TAP, and PDI.

After dissociating from calnexin, MHC-I quickly binds calreticulin. Calreticulin is a soluble ER chaperone that binds N-linked glycans on the MHC-I HC and stabilizes HC binding to β2-m. At the center of the PLC is the transporter associated with antigen presentation (TAP). This is a heterodimeric protein complex that forms a channel that actively moves peptides from the cytosol into the ER lumen. Joining the MHC-I/calreticulin complex to TAP is tapasin. Aside from bridging MHC-I with TAP, tapasin also forms disulfide bonds with the oxidoreductase ERp57. ERp57 plays a role in maintaining the oxidation state of the MHC-I HC. Lastly, protein disulfide isomerase (PDI) has recently been suggested to be a part of the PLC. PDI oxidizes the α2 disulfide bond within the peptide binding groove. Oxidation of this bond facilitates optimal peptide binding, and peptide loading itself can be facilitated by PDI. In the absence of PDI and subsequent reduced state of the α2 bond, the bond is either reoxidized for peptide binding or the HC is dislocated and destroyed [97]. After loading of optimal peptide and subsequent release from the PLC, the stable MHC-I molecule progresses from the ER into the golgi, where its sugars are further modified prior to expression on the cell surface where it is able to be recognized CD8+ T cells.

The immune response to CMV

CMV is a potent immunogen and triggers multiple components of immunity. Primary CMV infection in the immunocompetent host is eventually controlled by a combination of both innate and adaptive immune responses. One particular indication of

the importance of various aspects of the immune response to CMV is how much effort CMV puts into inhibiting those immune components. These immune evasion mechanisms are discussed in the next section. This section looks at some of the aspects of anti-CMV cellular immunity that help in controlling infection.

Anti-CMV cellular immunity

It is generally thought that cellular immunity is the major immune mechanism that controls CMV infection. The loss of cellular immunity has been shown in both animal models and in humans to lead to uncontrolled virus replication and viral dissemination within the host. In the MCMV model, both CD4+ T cells and CD8+ T cells are proposed to prevent dissemination [98, 99]. CD8+ T cells specifically are critical in controlling CMV infection. Studies in mice have indicated that depletion of CD8+ T cells leads to a reactivation of latent CMV [100]. Additionally, adoptive transfer of CD8+ T cells protects mice from infection or lethal challenge [101-105]. In humans, adoptive transfer of CMV-specific CD8+ was shown to provide protection against virus reactivation [106] and reduce viral loads in patients who had undergone bone marrow transplantation [107]. Also, long-term protection in bone marrow transplant recipients required CMV-specific CD4+ T cells [108].

The diversity of the T cell response to CMV has been studied in both mice and humans. In humans, CMV infection was shown to elicit an incredibly broad T cell response. Of 213 predicted HCMV ORFs tested, 151 had a significant percentage of either CD4+ or CD8+ T cells generated against HCMV peptides in at least one infection [32]. The median number of ORF-specific responses per individual was 12 for CD4+ T

cells and 8 for CD8+ T cells. Thus, while a large number of HCMV ORFs are immunogenic, the response is typically focused on a narrow subset that varies between individuals. In the MCMV model, CD8+ T cell responses from acutely infected mice were detectable against 27 of 170 tested ORFs [109]. An important difference between these studies was the T cells measured against HCMV were from persistently infected individuals, while the MCMV study analyzed the response in acute infection. The CD8+ T cell response in mice infected long term was dominated by only five epitopes [110].

As mentioned earlier, one of the more striking features of CMV immunity is the robust CD8+ and CD4+ T cell response that is directed towards the virus. While limited information is available on this response in primary infection in humans, much more is known about the memory cells. The total number of CMV-specific CD8+ T cells in infected individuals can rise well into the double digits in older individuals [111, 112]. Even in younger individuals, the median response to CMV has been documented to be 4-5% of the total CD4+ and CD8+ T cells, and 9-10% of the memory compartment [32]. These large percentages of CMV-specific T cells are the result of "memory inflation". Memory inflation occurs when certain antigen-specific memory T cells continue to replicate long after resolution of primary infection, likely due to continued antigenic stimulation, and fill up more and more of the T cell compartment. Typically after a primary infection and subsequent expansion of specific T cells, the antigen-specific T cells dramatically reduce in number, leaving a small percentage of central memory cells that can quickly respond to antigen if encountered later in life. In CMV infection, these cells continue to expand over time rather than maintaining the typical low percentages. This expansion seems to be of a narrow subset of CMV-specific cells. In fact, one study

reported several instances where over 90% of the clonal expansion was a single T cell [112]. Phenotypically this population contains a large portion of dysfunctional cells. They are unable to respond to specific antigen *ex vivo*, and are therefore considered anergic [113, 114]. Very few of these clonal CMV-specific cells express the co-stimulatory receptor CD28, and most express KLRG1. This phenotype is associated with end-stage differentiation and apoptotic resistance [115]. The data seem to suggest a model whereby CMV infection induces the clonal expansion of a select few CD8+ T cell clones, many of which progress to a functionally anergic state. It has even been estimated that this memory inflation due to CMV infection "ages" the immune system by approximately 35 years [116].

While the CD4+ T cell response to CMV is not as well characterized as the CD8+ response, CD4+ T cells are important in controlling CMV infection. CD4+ T cells can play a role in initiating CD8+ T cell expansion after stimulation by dendritic cells [117, 118]. In one study during primary asymptomatic infection, CMV-specific CD4+ T cells appear first, followed by the presence of anti-CMV antibodies and CD8+ T cells [119]. Conversely, the CD4+ T cell response is considerably delayed in primary symptomatic infection. Another study indicated the importance of CD4+ T cells by demonstrating that functional CD8+ T cells were not sufficient to control virus replication, and that IFN-γ expressing CD4+ T cells were required for recovery [120]. Additionally, the loss of CD4+ T cells in HIV infected patients is associated with end-organ disease [121]. And CMV-specific CD4+ T cell deficiency was associated with persistent virus shedding in children [122]. Similar to CD8+ T cells, very high frequencies of CMV-specific CD4+ T

cells are observed with infection [123, 124], although this expansion has not been nearly as well characterized as that for the CD8+ T cells.

V. Viral Immune Evasion

Overview

In an effort to avoid or limit detection by the immune system, virtually all viruses have evolved mechanisms that interfere with immune signaling functions. This includes interference with all aspects of anti-viral immunity, ranging from innate immunity such as the interferon response and NK cells, to humoral immunity and antibodies, to cellular immune functions of T cells. The methods that viruses utilize to avoid immune detection vary widely. Often times critical components of anti-viral immunity are targeted multiple ways by a single virus, and different viruses often evolve separate proteins that target the same immune molecules in interesting descriptions of convergent evolution.

While this dissertation focuses on the inhibition of MHC-I antigen presentation, the presence and importance of other modes of viral immune evasion cannot be overlooked. Most viruses, herpesviruses especially, contain multiple mechanisms for inhibiting the immune response. These mechanisms act in concert during an infection to promote replication and thus one must consider the role of other genes at play, especially when studying the function of one subset in the context of whole virus infection in an *in vivo* setting. Here we first describe other especially relevant viral immune evasion strategies before discussing inhibition of MHC-I.

Viral inhibitors of MHC-II antigen presentation

CD4+ T cells recognize antigen in the context of MHC-II molecules. MHC-II expression differs significantly from MHC-I, in that MHC-II is only expressed on lymphoid or myeloid tissue. Additionally, the antigen presented by MHC-II molecules is primarily generated from intravesicular pathogens and through endocytosis of extracellular material. Activation of CD4+ T cells proceeds down one of two paths: T_H1, which leads CD4+ T cells to generate cytokines that promote cellular immunity, or T_H2, which causes promotion of humoral immunity. The T_H1 response helps generate a more anti-viral state with the production of IFN-γ, IL-2 that promotes T cell proliferation, and TNF-β. Since CMV infects cells that express MHC-II, such as macrophages and Langerhans dendritic cells, and since CD4+ T cells can assist in the production of an anti-viral state, they are important to keep in mind with regard to this work.

HCMV encodes three proteins that have been shown to interfere with expression of MHC-II. US2, along with its function in MHC-I downregulation (see below), is able to target the HLA-DRα chain of MHC-II molecules for degradation [125]. US3 also targets MHC-II by stably binding MHC-II in the ER, causing a displacement of the invariant chain and a mislocalization of the molecule [126]. Lastly, UL83 (pp65) mediates accumulation of MHC-II within lysosomes, leading to degradation of the MHC-II α chain [127]. In HCMV-infected Langerhan dendritic cells, it has also been shown that binding and entry were sufficient to induce a relocalization of MHC-II within the cell, causing a reduction of MHC-II on the cell surface [128]. Additionally, MCMV has been shown to reduce cell surface expression of MHC-II on infected macrophages by inducing IL-10 production [129]. Rat CMV is also known to eliminate MHC-II from the surface by an

unknown, IL-10-independent mechanism [130]. It is unknown as of yet if the RhCMV homologs of US2 and US3 have an effect of MHC-II expression.

Viral inhibitors of NK cell function

Natural killer cells (NK cells) are lymphocytes that are considered a part of the innate immune response since they do not undergo recombination to select for particular ligands. They are thus important during the early stages of infection where they mediate cytotoxicity and secrete cytokines and chemokines prior to the onset of the adaptive immune response [131]. Evidence to the importance of NK cells during viral infection includes that humans with defects in their NK cell response are extremely susceptible to infections by herpesviruses [132].

NK cells are controlled by a system of positive and negative regulatory signals sent by surface receptors upon binding to ligand. Among other ligands, NK cell receptors recognize MHC-I on other cells. This makes them important for consideration when analyzing the role of viral proteins that downregulate surface expression of MHC-I. The inhibitory receptors of NK cells that bind MHC-I include the killer-cell immunoglobulin-like receptors (KIR) and the c-type lectin-like CD94:NKG2A heterodimer [133]. A principle of NK cell biology is that they do not kill cells that express the full set of autologous MHC-I molecules, but do kill cells lacking MHC-I. This is known as "missing self". Consequently, MHC-I downregulation by CMV should lead to NK cell lysis of the infected cells. However, CMV has evolved multiple mechanisms to inhibit NK cell activity.

In order to prevent NK cell activation because of "missing self", CMV encodes an MHC-I homolog, UL18 [134]. UL18 binds β2-microglobulin [135] and endogenous peptides [136]. It is a ligand for the leukocyte immunoglobulin-like receptor 1 (LIR-1), an inhibitory receptor that is widely expressed in macrophages, dendritic cells, subsets of NK cells, and T cells [137-139].

The HCMV UL142 protein inhibits NK cell killing by downregulating MIC-A, a polymorphic, stress-induced MHC-I-like protein that is the ligand for the NK cell activating receptor NKG2D [140, 141].

The NK cell inhibitory CD94/NKG2A receptor recognizes the non-polymorphic human HLA-E molecule [142]. The peptide presented by HLA-E is a portion of the MHC-I HC signal peptide. This allows for NK cells to monitor the normal production of MHC-I occurring inside the target cell. Since HCMV causes MHC-I destruction and prevents peptide loading, there is no cellular source of peptide to load onto HLA-E. Thus HCMV UL40 has usurped the same MHC-I signal peptide sequence which can be loaded onto HLA-E independent of TAP [143, 144]. This allows for normal HLA-E expression and prevention of NK cell activation.

The HCMV proteins UL14 and UL141 share significant homology and are considered a part of the same protein family. UL141 has been shown to reside in the ER and retain CD155, a ligand for the activating NK receptors DNAM-1 (CD226) and TACTILE (CD96) [145]. UL14 is also an ER resident capable of inhibiting NK cell mediated killing, but despite the homology with UL141 appears to act by a different mechanism [146].

HCMV UL16 binds MIC-B and two GPI-linked proteins, ULBP1 and ULBP2 [147] [148]. Similar to MIC-A and -B, ULBP1 and ULBP2 bind to NKG2D and stimulate NK cell as well as T cell activity [149]. UL16 inhibits this binding by retaining the NKG2D ligands in the ER [150-153].

Viral inhibition of interferons

Interferons (IFN) are a group of anti-microbial cytokines. They induce expression of interferon-stimulated genes (ISG) that actually carry out effector functions to create an anti-viral state within the cell. CMV itself is susceptible to the effects of ISGs and IFN-induced cellular states *in vitro* [154-158]. Despite this, HCMV is unable to completely block the induction of IFN or ISGs [159-161]. HCMV does however reduce expression of these genes, since infection without viral gene expression leads to an even higher activation of IFN and ISGs [158, 162, 163]. The IE2 encoded protein IE86 has been implicated in this effect, in that it is able to inhibit transcription of IFNβ and RANTES by interfering with NFκB activation [164]. Interestingly, RhCMV fails to induce ISGs even when viral gene expression is blocked [165]. Additionally, upon co-infection of RhCMV with HCMV, RhCMV is able to actively block HCMV-induced ISG induction. This suggests that RhCMV carries into the cell with it a gene product able to block this cellular pathway.

Viral inhibitors of apoptosis

The induction of programmed cell death, or apoptosis, in virally infected cells is a mechanism used by the host to limit viral replication. Cells can be induced to undergo

apoptosis by external stimulus, such ligation of Fas from a CD8+ T cell, or from internal sources that are activated upon specific intracellular signals. HCMV encodes at least two proteins that block apoptosis in infected cells. The UL36 ORF encodes a viral inhibitor of caspase-8 induced apoptosis (vICA) [166]. vICA is able to suppress apoptosis triggered by the ligation of death receptors such as Fas, but less able to block apoptosis induced by intracellular signals [166]. It binds to pro-caspase-8, thus preventing its association with FADD and subsequent cleavage and activation. HCMV UL37 exon 1 encodes viral mitochondria-localized inhibitor of apoptosis (vMIA) that is able to suppress apoptosis induced by death receptors and intracellular stimuli. vMIA associates with the outer mitochondrial membrane and binds to Bax, thus sequestering it and preventing Bax-mediated permeabilization of the mitochondrial membrane [167, 168].

Secreted viral mediators of immunity

Along with virally-encoded immune evasion proteins that act intracellularly or at the cell surface, CMVs also encode several secreted factors that act to manipulate the host response. This includes homologs to chemokines, small, host cell-cell signalling mediators. Chemokines are chemoattractant cytokines that induce inflammation by recruiting leukocytes to the site of infection. They can also stimulate adhesion and extravasation. On the surface, inducing leukocyte migration to the site of infection seems like something the virus would rather avoid. However, it has been postulated and even demonstrated in some systems that this helps the virus with dissemination within the organism [169, 170]. HCMV encodes three chemokine homologs: UL128, UL146, and UL147. The UL128 gene product is a CC-chemokine-like protein that, along with its

neighbors UL130 and UL131, has been implicated in cell type tropism and viral entry and egress. Specifically it has been demonstrated to be essential for entry into epithelial and endothelial cells [55, 171, 172], as well as for cell-cell spread in polymorphonuclear leukocytes and monocytes [171]. Thus far, UL128 has not been shown to have any chemoattractant or inflammatory abilities. However, m131, which is the MCMV homolog of UL128, is capable of inducing calcium flux and chemotaxis of macrophages [169]. Additionally, MCMV lacking this ORF showed reduced dissemination ability in vivo [169], as did Rat CMV lacking its UL128 counterpart, r131 [170]. Interestingly, only one of two RhCMV strains contains a UL128 homolog [72], but it has not yet been studied.

HCMV also encodes two CXC chemokine-like proteins, UL146 and UL147. These appear to more recently evolved in HCMV, as they are not present in the rodent CMVs [173]. RhCMV contains a homolog to UL146, but not UL147. However, RhCMV also encodes a second CXC chemokine-like protein [72]. Homologs to both UL146 and UL147 are found in chimpanzee CMV, along with two novel CXC chemokine-like proteins [65]. While there is a high degree of hypervariability in UL146 and UL147 between HCMV clinical isolates, there appears to be no correlation with disease based on the different genotypes [174].

HCMV UL146 has been shown to be a functional chemokine. In culture, it is able to induce calcium flux and migration of neutrophils by binding the CXCR2 receptor [175]. Despite sharing only 22% identity at the amino acid level, the chimp CMV UL146 was also able to induce calcium release and migration in human neutrophils [176]. Additionally, both HCMV and CCMV UL146 viral chemokines were able to decrease

apoptosis of neutrophils and slightly upregulate surface expression of integrins [176]. The decreased amount of apoptosis may help in viral dissemination by prolonging survival of neutrophils, which typically undergo apoptosis within 24-48 hours [176]. Lastly, HCMV lacking UL146-147 had reduced ability to transfer to polymorphonuclear leukocytes, but not monocytes, from infected endothelial cell culture in a transwell assay [171]. Taken together, data from studies of CMV chemokine homologs suggest a situation in vivo where the virus secretes chemoattractant proteins to recruit leukocytes to the site of infection which are then infected by CMV and disseminated throughout the host. This is one example of how CMV manipulates the immune response not to avoid detection, but to specifically promote spread.

Inhibiting MHC-I antigen presentation

CD8+ T cells are critical in the host defense against viruses. Perhaps the best evidence for this is the great lengths viruses go to in order to prevent CD8+ T cell recognition of infected cells. While there are a variety of ways viruses can inhibit CD8+ T cell function, the most exploited way noted thus far is to inhibit the MHC-I antigen presentation pathway. Most viral mechanisms for inhibiting the MHC-I pathway have been identified in large DNA viruses such as Herpesviruses, Adenoviruses, and Poxviruses. Some exceptions include HIV, SIV, and papillomaviruses. Without question though, Herpesviruses are the masters of MHC-I inhibition. Cytomegaloviruses in particular express a wide array of MHC-I inhibitors (Table 1). The sheer number of genes encoded by these viruses directed at blocking this pathway is a clear indication of the value they bring to virus replication.

Table 1. CMV interference with MHC-I

Virus	ORF	Function
HCMV	US2	ER localized protein. Causes retrotranslocation and degradation of MHC-I HCs
HCMV	US3	Enters the peptide loading complex. Causes degradation of PDI, thus preventing optimal peptide loading and retaining MHC-I in the ER.
HCMV	US6	Binds to TAP and inhibits ATP hydrolysis, thus preventing peptide transport into the ER.
HCMV	US11	ER localized protein. Causes retrotranslocation and degradation of MHC-I HCs
MCMV	m04	Binds to MHC-I in the ER and at the cell surface, preventing T cell mediated lysis.
MCMV	m06	Binds MHC-I and redirects it to the lysosome, where both are destroyed.
MCMV	m152	Causes retention of MHC-I in the ER-golgi intermediate compartment.
RhCMV	Rh182	Causes rapid proteasomal degradation of MHC-I HCs.
RhCMV	Rh184	Delays MHC-I trafficking to the cell surface.
RhCMV	Rh185	Inhibits peptide transport into the ER.
RhCMV	Rh189	Causes rapid proteasomal degradation of MHC-I HCs.
RhCMV	Rh178	Prevents the biosynthesis of MHC-I HCs.

Virtually every step of the MHC-I antigen presentation pathway has been targeted by a viral protein. Transcription of the cellular components of the pathway is downregulated by adenovirus [177], HIV [178], and EBV [179]. EBV and HCMV have evolved to prevent or limit antigen processing of certain proteins, thus preventing their direct presentation from infected cells [180, 181]. The TAP transporter is targeted by multiple viruses [74, 182-187]. Retention of MHC-I in the ER by direct interaction and/or preventing efficient peptide loading is accomplished by adenovirus [188], HCMV [189], MCMV [190], RhCMV [74], and Cowpox [191]. MHC-I heavy chains are

retrotranslocated from the ER into the cytosol for degradation by HCMV [192], RhCMV [74], MHV-68 [193], and HIV [194]. MCMV m152 causes MHC-I retention in the ER cis-golgi [195]. Lastly, if MHC-I is successfully expressed on the cell surface, some viruses are able to directly remove it, including MCMV m6 [196], HIV/SIV nef [197], and KSHV K3 and K5 [198].

HCMV encodes multiple viral inhibitors of antigen presentation (VIPRs). All of these are located within the same genetic locus in the virus, encoded by the ORFs between US2 and US11. Collectively the HCMV VIPRs can be referred to as the US6-family of proteins because of a similar structure of single transmembrane, immunoglobulin (Ig) domain super-family glycoproteins [199]. Four of these proteins have been shown to reduce cell surface expression of MHC-I: US2, US3, US6, and US11. US8 and US10 interact with MHC-I but they are less well characterized and thus far do not seem to inhibit antigen presentation [200-202].

HCMV US2 and US11

The most studied of the HCMV VIPRs are US2 and US11. This is likely because their mechanism of retrotranslocation provides insights not just into viral immune evasion, but an important cellular pathway. Interestingly, despite the same end result of retrotranslocation and proteasomal degradation of MHC-I HCs, US2 and US11 use different pathways and have different requirements to cause this. Also, despite an overlap in specificity, there is a difference in the MHC-I alleles they can target. Both are able to cause degradation of certain HLA-A, HLA-B, and HLA-C alleles, but not others [192,

203-208]. US2 can additionally degrade the mouse MHC-I molecules D^b and D^d, but not K^b or L^d. Whereas US11 can degrade all four of these [209].

US2 is readily observed in association with assembled and unfolded MHC-I heavy chains by coimmunoprecipitation, but very little US11 is observed by this method [210], suggesting a more transient or less stable association by US11. Both US2 and US11 bind MHC-I heavy chains in the ER lumen. The crystal structure of US2-bound HLA-A2 suggests that US2 binds to the junction of the α3 and peptide-binding domain [199], an observation consistent with mutagenesis studies [211]. Dislocation, however, requires the cytosolic tail of US2 [212]. In contrast, US11 does not require its cytosolic tail for dislocation. US11 mediates dislocation through its transmembrane domain which contains a Gln residue essential for dislocation, but not for the interaction with MHC-I [213]. Screening for cellular proteins interacting with US11 but not with the Gln-mutant identified Derlin-1, whose yeast homolog is required for the degradation of a subset of ER proteins [214]. Independently, Derlin-1 was identified as a multiple transmembrane domain protein responsible for recruiting to the ER the cytosolic ATPase p97, a protein required for retrotranslocation [215]. Both studies further proposed that Derlin-1 is a component of the retrotranslocation channel.

Interestingly, a dominant negative Derlin-1 failed to prevent dislocation by US2 [214]. A screen for cellular proteins interacting with wild-type but not dislocation defective US2 implicated signal peptide peptidase (SPP) in HC dislocation by US2, but not US11 [216]. While the cytosolic tail of US2 is required for SPP-binding, it is not sufficient for dislocation since US2 containing the CD4 transmembrane domain was unable to cause dislocation. This indicates a necessary interaction between the US2

transmembrane domain and either SPP or some other protein [216]. Thus, US2 and US11 might have evolved independently to achieve MHC-I destruction by different molecular means. This also illustrates how US2 and US11 have been used to better understand a complex cellular pathway.

HCMV US3

The 22kDa US3 gene product of HCMV binds to and causes ER retention of MHC-I molecules [182, 189]. Like US2 and US11, US3 is a single transmembrane Ig domain superfamily ER resident. US3 does not utilize either of the classical ER-retention motifs KDEL or KKXX, thus its ability to remain in the ER is likely through protein-protein interactions with other ER resident proteins. In fact, mutational analysis has shown the lumenal domain of US3 is sufficient for its ER retention [217]. However, the ability of US3 to associate with the MHC-I molecule is dependent not only on the lumenal domain but also on its transmembrane domain [218].

The mechanism for ER retention of MHC-I molecules was recently elucidated by Ahn and colleagues [97, 219]. Their studies revealed that US3 prevented the optimization of peptide loading onto MHC-I heterodimers. Peptide loading is optimized by tapasin which forms a transient complex with empty MHC-I and TAP and releases MHC-I peptide complexes [220-224]. The availability of MHC-I binding peptides regulates the duration of this transient complex resulting in fast ("tapasin-independent") and slowly exiting ("tapasin-dependent") MHC-I alleles [225]. US3 was shown to preferentially retain tapasin-dependent MHC-I alleles by inhibiting their acquisition of high-affinity peptides whereas tapasin-independent alleles were not affected [219]. The same group

recently identified a critical role of PDI in stabilizing the peptide-receptive site of MHC-I by regulating the oxidation of the α2 disulfide bond in the peptide-binding groove [97]. Interestingly, PDI protein levels were decreased in the presence of US3 and a complex between US3 and PDI is stabilized by proteasome inhibitors. By degrading PDI, US3 inhibits the binding of high affinity peptides to tapasin-dependent alleles of MHC-I. Since PDI and tapasin are part of the peptide loading complex and can be co-immunoprecipitated it is likely that previously observed interactions between US3 and MHC-I or tapasin are the result of US3 entering the peptide loading complex [97].

HCMV US6

The HCMV US6 glycoprotein inhibits the transporter associated with antigen presentation (TAP) [226, 227]. TAP is a member of the ATP binding cassette family of transporters, and thus couples ATP hydrolysis to the translocation of peptides into the ER lumen. TAP is a heterodimer, composed of two single transmembrane subunits, TAP1 and TAP2. Each of these contains a transmembrane domain responsible for peptide binding [228], and a nucleotide binding domain responsible for ATP binding and hydrolysis [229]. US6 does not prevent peptide binding to TAP, but rather it functions by binding directly to TAP in the ER lumen and preventing ATP-hydrolysis [230]. This contrasts with the TAP inhibitory mechanism of HSV ICP47, which binds TAP on the cytosolic side and competes for peptide-binding [184, 231, 232]. The result of TAP inhibition is a lack of peptides in the ER lumen, and thus incomplete assembly of MHC-I molecules and a reduction of MHC-I on the cell surface.

MCMV m04, m06, and m152

The main value in studying the VIPRs of CMVs other than human is being able to characterize them in an *in vivo* model. The MCMV VIPRs have provided tremendous insight in this regard. While MCMV does not encode homologs of the HCMV US6 family, it does contain three of its own VIPRs [104, 233]. The gp34 protein encoded by m04 does not reduce MHC-I surface levels but forms a tight association in the ER and accompanies MHC-I to the cell surface [190, 234] where it is able to inhibit cytotoxic T cell lysis by an unknown mechanism [233]. The m06-encoded gp48 associates with MHC-I and directs this complex to the lysosomes where both are destroyed [196]. Lastly, m152 encodes gp40 which retain MHC-I in the ERGIC [195]. Interestingly, MHC-I retention occurs in the absence of a detectable biochemical interaction [195, 233].

Several studies using MCMV have now tried to answer the question of what do VIPRs do for the virus *in vivo*. Initial studies in immunocompromised mice have suggested a role for m152/gp40 in controlling the CD8+ T cell response to the virus and being responsible for increased viral titers [235]. It was further demonstrated that m152/gp40 protected MCMV from adoptively transferred epitope-specific T cells [236], the first (and so far only) experiment showing a VIPR preventing viral peptide presentation *in vivo*. In contrast, the m152/gp40-deleted virus induced a CD8+ T cell response to the immunodominant M45 epitope that was similar to WT-MCMV in C57BL/6 mice [237]. Moreover, and probably most surprisingly, the CD8+ T cell response to MCMV lacking all three VIPRs was very similar to that induced by WT [238, 239]. An important conclusion from these studies suggests that VIPRs do not seem to influence the induction of CD8+ T cell responses. This suggests that passive presentation

of infected cells (cross-presentation), and not direct presentation of virus-derived peptides by infected cells, induces the T cell response. It is also surprising to see genome copy numbers of the triple-deleted virus comparable to WT MCMV during the acute phase of infection, although this could be explained by NK cell control early in infection prior to the robust CD8+ T cell response [238]. Even more surprising was the ability of the triple knockout to establish infection for at least 6 weeks, after which it was able to reactivate upon immunosuppression the mice [238]. Perhaps the redundancy of immune evasion mechanisms, most notably the modulation of T cell and NK-cell activating signals, enables this deletion virus to survive. It is also possible that VIPRs facilitate transmission since salivary gland titers of the triple-deleted virus were lower than WT virus [240]. It is further conceivable that VIPRs are required for superinfection of CMV-immune individuals [44, 241, 242]. Finally, the use of inbred laboratory mice might not accurately reflect the infection and spread of CMV in an outbred population. Thus, additional studies of animal CMVs in outbred populations, such as rhesus macaques, might help to establish the role of VIPRs *in vivo*.

Chapter 2

RhCMV contains an additional inhibitor of MHC-I antigen presentation outside the US6-family of genes

Introduction

Human cytomegalovirus is a widespread and extremely successful pathogen. Infection with HCMV is lifelong, as the virus is able to persist in its host despite a strong immune response that is generated against the infection. An important part of HCMVs strategy to avoid immune recognition and thus infect and persist in the host is its ability to inhibit antigen presentation. Infected cells use antigen presentation pathways in order to signal infection to the immune system. CMVs in general are masters of interfering with the MHC class I antigen presentation. HCMV encodes four proteins that block this pathway: US2, US3, US6, and US11. These are collectively referred to as the US6-family of proteins or US2-11.

These HCMV inhibitors of MHC-I antigen presentation have been well characterized in tissue culture. However, because CMV infection is species specific, the role these proteins play for viral infection *in vivo* has not been characterized. The previous research analyzing the function of CMV inhibitors of MHC-I antigen presentation *in vivo* has been limited to studies using the murine CMV model. While this model has addressed many important questions, MCMV does not contain the US6-family of genes, but rather three of its own MHC-I inhibitors that act by mechanisms different than the US6-family. Additionally, the use of inbred laboratory strains of mice may not

accurately represent the role of MHC-I inhibitors during virus infection of an outbred genetically variable population.

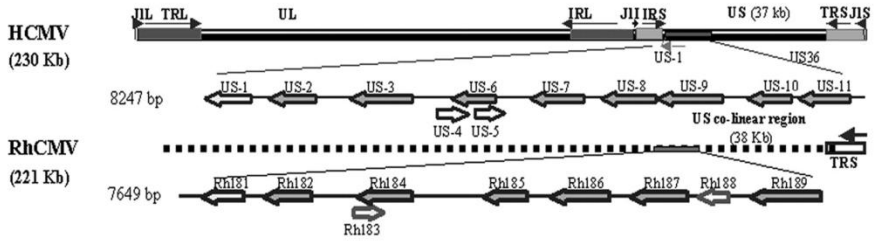
Non-human primate models such as the rhesus macaque offer a more closely related animal model to study important questions in infectious disease. Rhesus macaques also harbor their own species of CMV, RhCMV. In an effort to utilize RhCMV as an *in vivo* model for HCMVs ability to block MHC-I antigen presentation, we have characterized RhCMVs ability to block this pathway. In this chapter we show that RhCMV, along with containing homologs to the HCMV US6-family, contains a single other RhCMV-specific ORF encoding an inhibitor of MHC-I heavy chain biosynthesis.

Results

Identification of the RhCMV homologs of HCMV US2-11

Given the high degree of homology between RhCMV and HCMV, and the ability to study RhCMV in an outbred primate population, we began a characterization of the VIPRs encoded by RhCMV. The genomes of RhCMV and HCMV are colinear, and an analysis of the RhCMV genomic region homologous to the HCMV US2-US11 region found a similar gene alignment (Fig 2.1A). This region in RhCMV lies between the ORFs Rh182-189. The primary amino acid sequence homology between RhCMV ORFs Rh182-189 and HCMV US2-11 is marginal. The identity between the sequences ranges from 17.9% to 19.8%, with similarities from 28.3% to 38.7% (Fig 2.1B). Although there is a conservation of important cysteine residues bracketing Ig-like domains [74]. Also of note is that while HCMV encodes a predicted 10 ORFs between US2-11, RhCMV only encodes a predicted 8 ORFs between Rh182-189. However, these 8 ORFs seem to

A

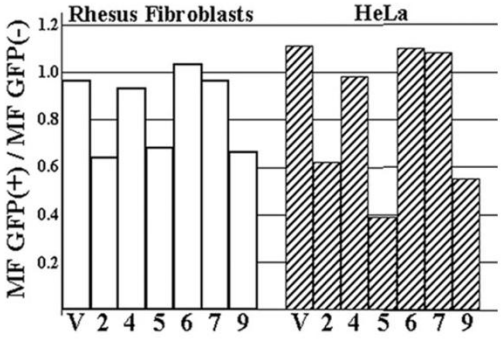


B

	US2	US3	US6	US7	US8	US9	US10	US11
Rh182	19.1/38.7	17.9/34.8	12.7/22.8	9.7/22.6	11.4/22.3	9.7/18.5	10.2/24.9	17.5/31.3
Rh183	10.0/26.9	17.9/31.1	13.4/24.1	11.9/24.7	12.7/21.1	13.1/22.2	10.8/21.1	13.0/26.4
Rh185	8.5/21.0	12.8/23.0	19.8/28.3	8.8/21.7	10.5/16.7	11.3/19.0	12.1/20.5	12.2/19.8
Rh186	13.8/28.7	14.0/23.8	13.6/21.1	14.5/26.6	10.3/23.0	12.9/19.6	13.6/20.9	12.8/24.3
Rh187	10.5/23.7	8.8/22.5	13.7/21.6	16.0/29.4	14.8/26.6	13.1/27.5	12.7/25.4	16.7/27.9
Rh189	11.3/22.4	10.5/19.3	12.4/20.9	10.0/22.7	11.3/21.0	16.6/28.9	11.0/18.1	19.5/33.2

% Identity/Similarity Highest Identity

C



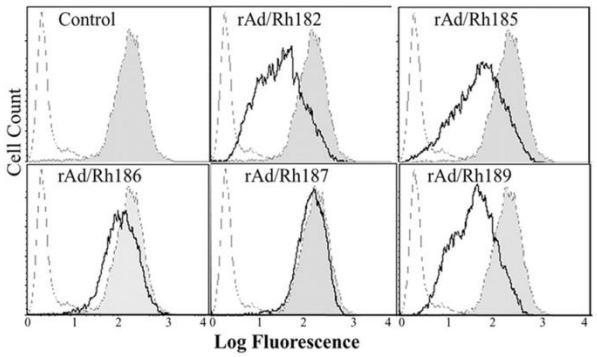


Fig 2.1 RhCMV contains homologs to the HCMV US6-family of proteins. A) Genomic organizations of the HCMV US1-11 region and the RhCMV Rh181-189 region. B) Primary amino acid sequence homology between HCMV US2-11 proteins and RhCMV Rh182-189 proteins. The first number indicates identity, the second number similarity. In yellow are significant identities for US2, US3, US6, and US11. C) Left panel: Surface expression of MHC-I in rhesus fibroblasts and HeLa cells after transfection with GFP and empty vector (V) or Rh182 (2) through Rh189 (9). Displayed as the ratio of mean fluorescence of GFP positive to GFP negative cells. Right panel: Representative surface expression of MHC-I after transfection with Rh182-189 proteins.

include the homologs of US2, US3, US6 and US11, all of the known MHC-I inhibitors of HCMV. It therefore seemed that the US6-family of genes within CMV predates the evolutionary split of humans and old world primates. It also again highlights the importance of these genes since both HCMV and RhCMV have maintained them despite millions of years of separation and evolution.

RhCMV ORFs Rh182-189 are functional homologs to HCMV US2-11

In order to determine if Rh182-189 ORFs were functionally conserved with HCMV US2-11, each ORF was cloned into the mammalian expression vector pUHD10.1 [243] and tested for effects on the surface expression of MHC-I by FACS. Upon transfection of each RhCMV ORF into either telomerized rhesus fibroblasts (TRFs) or HeLa cells, MHC-I surface expression was seen reduced by Rh182, Rh185, and Rh189 (Fig 2.1C). Thus, at least three of these RhCMV ORFs were able to block MHC-I expression. Further analysis of Rh182-189 ORFs was performed in collaboration with Pande and Ahn [74]. As is the case for HCMV US2 and US11, both Rh182 and Rh189 were able to cause rapid degradation of MHC-I HCs which could be stabilized with proteasomal inhibitors. The HCMV US3 homolog Rh184, although not able to significantly decrease MHC-I surface expression in transfection experiments, did cause a transient retention of MHC-I in the ER. And as with HCMV US6, Rh185 prevented peptide loading onto MHC-I molecules by inhibiting TAP peptide transport. Thus, RhCMV contains functional homologs to the HCMV US6-family of proteins and provides the opportunity to study their function in an *in vivo* setting.

Isolation and characterization of a RhCMV lacking Rh182-189

Like HCMV, RhCMV reduces MHC-I expression during infection of fibroblasts in cell culture. Upon deletion of the US2-11 region of the HCMV genome (strain AD169), HCMV is no longer able to prevent expression of MHC-I heavy chains [205]. Given the high degree of homology between HCMV and RhCMV, and specifically the conservation of the US6 family of genes, we hypothesized that deletion of the RhUS2-11 region would likewise lead to a restoration of MHC-I expression in RhCMV-infected cells. To determine if deletion of RhUS2-11 restored MHC-I synthesis, we created a recombinant RhCMV lacking the US2-11 homologs, Rh182-189 (Fig 2.2). The mutagenesis was performed using a RhCMV bacterial artificial chromosome (BAC). This technique allows for performing recombination and mutagenesis of large viral genomes in bacteria, a much simpler task than in cell culture.

The RhCMV BAC is composed of the entire circularized RhCMV viral genome with a BAC cassette inserted between the Rh181 and Rh182 ORFs. BAC insertion at this point does not affect transcription of either of those ORFs upon reconstitution of the virus. The BAC cassette contains a chloramphenicol resistance (Cm^r) gene, an OriS bacterial origin of replication, partitioning factors, and a cre recombinase expression cassette with a eukaryotic promoter. Additionally, the BAC cassette is flanked by loxP recombination sites. The entire RhCMV BAC is then maintained within bacteria as an F plasmid, at single or very low copy number.

Recombination is performed by first inducing λ-phage recombination genes in the bacteria by incubation at 42ºC. Secondly, we electroporated the cells with a PCR product containing a kanamycin resistance (Kan^r) cassette flanked by FRT recombination sites.

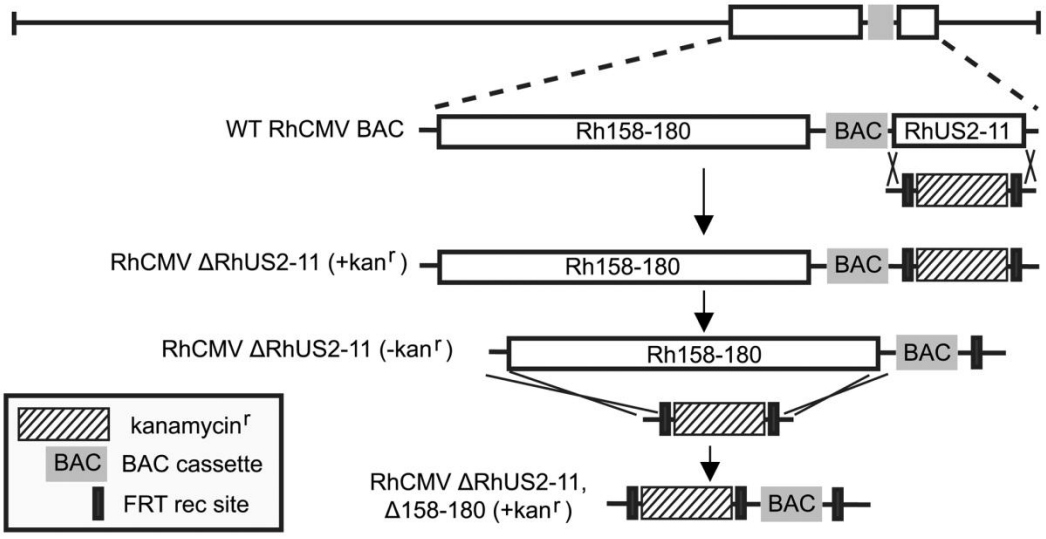


Fig 2.2 Strategy for RhCMV BAC recombination. Deletion of the genomic regions occurs by homologous recombination with a PCR product containing a kanamycin resistance cassette flanked by FRT recombination sites and RhCMV homologous sequences. The process is repeated for double deletions. See text for details.

The entire Kan^r cassette is also flanked by 50bp of RhCMV sequence that is homologous to the sequence surrounding the region of the RhCMV genome you wish to remove. After recombination takes place, a Kan^r cassette is left in place of the region for deletion and selection is performed with Cm and Kan. After removing the Kan^r cassette by FLP recombinase induced with arabinose, only a ~80bp FRT site remains, thus minimizing the amount of foreign sequence introduced into the virus. RhCMV can then be reconstituted by electroporation of the recombinant RhCMV BAC into rhesus fibroblasts. Upon electroporation, the cre recombinase in the BAC cassette is expressed and the BAC cassette is self-excised from the viral genome. This leaves a single loxP recombination site where the BAC cassette had been, again minimizing the amount of foreign DNA sequence in the virus genome.

Prior to reconstitution of virus, we characterized the recombinant RhCMVΔRh182-189 BAC. In order to determine if the recombinant BAC contained any large unwanted deletions, recombinations, or other genomic instabilities, we performed restriction digest. As predicted, restriction digest of the recombinant BAC showed the loss of a 24Kbp EcoRI fragment and the gain of 12.7Kbp and 6.3Kbp fragments compared to the wild-type BAC (Fig 2.3A, lanes 1-2). Upon removal of the Kan^r cassette the 12.7Kbp fragment shifted to an 11.2Kbp fragment, as predicted (Fig 2.3A, lane 3). Southern blotting of the EcoRI digest of the recombinant BAC DNA determined that the Kan^r cassette was inserted only into a single fragment as expected, and was removed by the FLP recombination (Fig 2.3B). It also confirmed the loss of the Rh182 and Rh189 ORFs. Direct sequencing of the region of deletion in the recombinant BAC indicated that

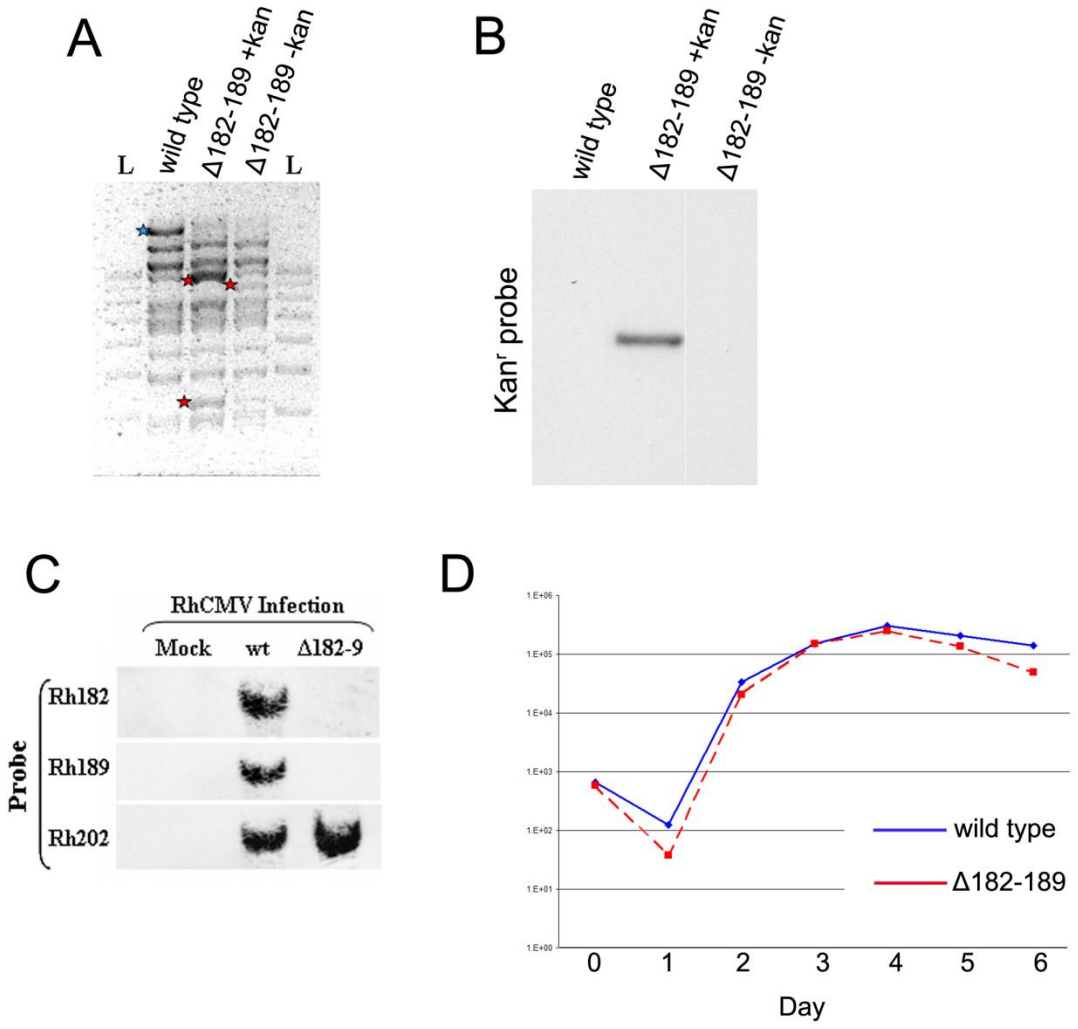


Fig 2.3 Characterization of RhCMVΔ182-189 BAC and virus. A) Restriction digest of RhCMV BAC DNA. Stars indicate expected EcoRI fragment shifts in the Δ182-189+kan and Δ182-189-kan recombinant BACs. L, ladder. B) Southern blot of recombinant BAC DNA using a Kanamycin resistance cassette probe. C) Southern blot of DNA from the recombinant virus Δ182-189, with mock-infection and wild type (wt) RhCMV infection as controls. D) Single-step growth curve comparing wild type RhCMV to RhCMVΔ182-189. Rhesus fibroblasts were infected at an MOI=3 and virus harvested from supernant as days 0-6.

recombination had taken place where expected, and the Rh182-189 genes had been removed (data not shown). We conclude that we had successfully created a RhCMV BAC lacking the US2-11 homologous region Rh182-189.

Upon reconstitution of the virus, RhCMV ΔRh182-189 was isolated and characterized. After confirming that the BAC cassette had been excised using PCR, restriction digest and southern blotting of viral DNA was performed. Southern blot indicated that recombinant virus had been isolated with no wild-type contamination since we were unable to detect Rh182 or Rh189 using probes generated against the each ORF (Fig 2.3C). Since deletion of Rh182-189 may affect transcription of the surrounding ORFs, we tested whether Rh181 and Rh190 RNAs were expressed using RT-PCR. Both Rh181 and Rh190 were expressed by ΔRh182-189 at levels similar to wild type RhCMV infection (data not shown). Lastly, we analyzed the growth properties *in vitro* by viral growth curves. The single-step growth curve performed with an MOI=1 showed that ΔRh182-189 grew similar to wild-type RhCMV, achieving the same viral titer (Fig 2.3D). We concluded that we had successfully isolated a recombinant RhCMV lacking Rh182-189, that deletion of Rh182-189 did not prevent transcription of Rh181 and Rh190, and that the recombinant virus ΔRh182-189 did not have any growth defects in tissue culture.

Deletion of RhUS2-11 only partially restores MHC-I HC synthesis in infected cells

In order to determine if steady state levels of MHC-I were restored in cells infected with ΔRh182-189 compared to wild-type RhCMV, we performed western blot and cell surface labeling. Compared to cells infected with wild-type RhCMV, ΔRh182-

189-infected cells showed only a partial restoration of the amount of MHC-I on the cell surface (Fig 2.4A). Similarly, there was only a partial restoration of the total amount of MHC-I heavy chain (HC) present in ΔRh182-189-infected cells compared to wild type-infected cells as shown by western blot (Fig 2.4B). Furthermore, pulse-chase labeling of ΔRh182-189-infected cells showed that HC synthesis was efficiently blocked during a 10-min metabolic labeling time (Fig 2.4C). Furthermore, the effect seen on the HC was not a general knockdown of cellular protein synthesis, but rather seemed HC specific. There was no decrease in the ER chaperone calreticulin as seen in western blot analysis (Fig 2.4B), nor in MHC-I light chain β2-microglobulin in pulse-chase experiments (Fig 2.4C). Additionally, when infected TRFs were metabolically labeled for 30 minutes very little HC was recovered, whereas we were able to recover similar amounts of transferrin receptor and vimentin as in mock-infected cells (Fig 2.4D). These data indicated that unlike HCMV, RhCMV contained a gene or genes outside the US2-11 homologous region that was able to specifically interfere with MHC-I expression. Because this gene was able to block MHC-I heavy chain synthesis during a short metabolic labeling time, we termed this gene Viral Inhibitor of Heavy Chain Expression, or VIHCE.

VIHCE is encoded within the Rh158-180 genetic region

Thus far, no strain of HCMV has been shown to have a VIHCE. We hypothesized then that VIHCE was likely encoded by a viral ORF that has no homolog in HCMV. Since the viral MHC-I inhibitors in HCMV (and their RhCMV homologs) are grouped together in the same region of the genome, we first tested whether VIHCE was encoded

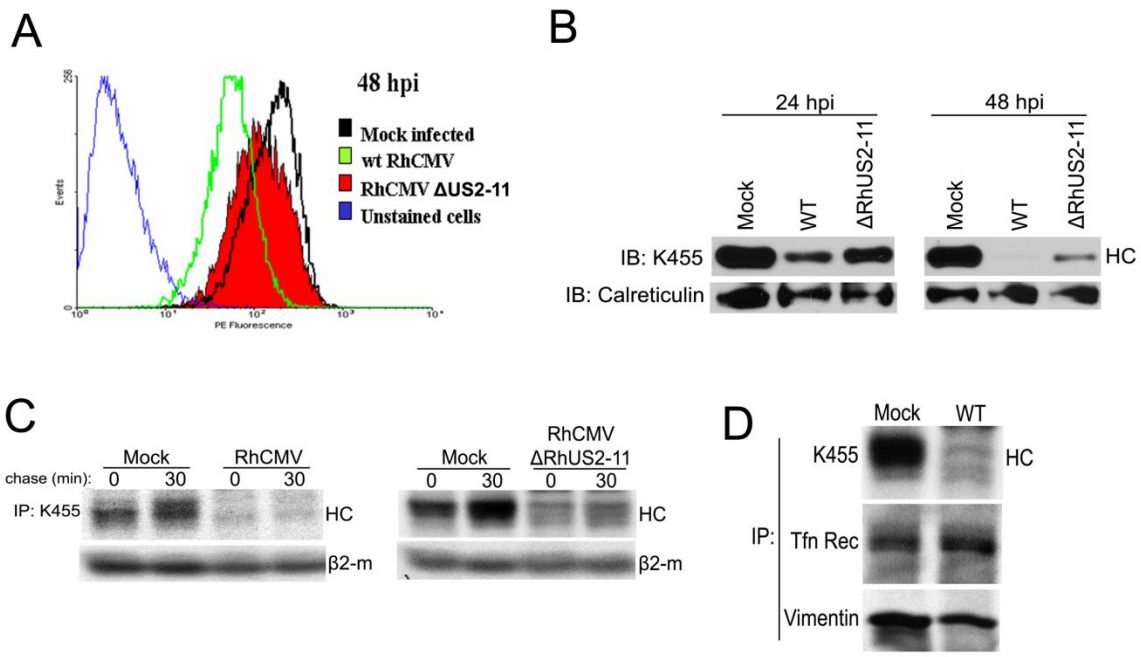


Fig 2.4 RhCMVΔ182-189 retains the ability to block MHC-I synthesis. A) Surface expression of MHC-I during RhCMV infections. Mock-black line. Wild type RhCMV-green line. RhCMVΔ182-189-solid red. B) Western blot analysis of whole cell lysate from TRFs infected with wild type (WT) RhCMV or RhCMVΔ182-189 at 24 and 48 hours post infection (hpi). Calreticulin is shown as a loading control. C) Pulse-chase labeling of 10 min and immunoprecipitation of total MHC-I from Mock-infected or RhCMV-infected TRFs. D) Pulse-labeling of 60 min and IP of MHC-I, Tfn Rec (Transferrin receptor) or Vimentin from Mock-infected or RhCMV-infected TRFs.

by Rh190 or Rh191. These two overlapping ORFs are located immediately upstream of Rh189, the US11 homolog. Using the RhCMV BAC protocol as described, we created the recombinant virus ΔRh182-191. However, MHC-I heavy chain synthesis was still efficiently blocked in fibroblasts infected with ΔRh182-191 (Fig 2.5A). We also tested whether Rh181 encoded VIHCE. Rh181 is a homolog of HCMV US1, which is not known to affect MHC-I, but is located adjacent to US2. We thus constructed a RhCMV lacking Rh181. Again, MHC-I heavy chain synthesis was still efficiently blocked during infection with RhCMVΔ181 (Fig 2.5B). We conclude that VIHCE is not encoded by Rh181, Rh190 or Rh191.

Aside from Rh182-191, the RhCMV genome encodes a predicted 220 ORFs, of which 95 have no significant homology to any HCMV ORF. Certain regions of the genome are concentrated with RhCMV-specific ORFs, such as Rh1-17, Rh27-41, Rh45-53, and Rh158-180 (Fig 1.2). The Rh158-180 region had particular potential for encoding VIHCE for two main reasons. One was the large percentage of RhCMV-specific genes. Only 2 of the 23 predicted ORFs have homologs in HCMV strain AD169. Second was the significant number of potential immunomodulatory genes encoded within this region. These include Rh158, an IL-8 homolog; Rh160.1, an IL-10 homolog; Rh163, a TNF-receptor homolog; and several predicted single transmembrane glycoproteins [66]. We therefore targeted this region for deletion to determine if we could restore MHC-I heavy chain synthesis during infection.

In order to determine if VIHCE was encoded within the Rh158-180 region, we created two recombinant viruses that lacked this region. One recombinant only lacked Rh158-180, while a second recombinant lacked both Rh158-180 and Rh182-189 (Since

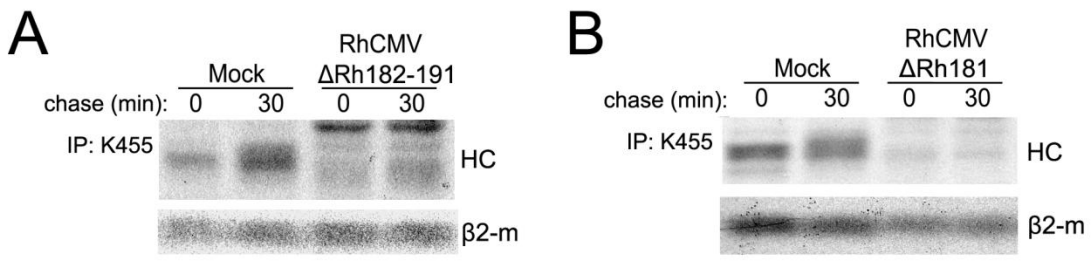


Fig 2.5 VIHCE is not encoded by Rh181, Rh190, or Rh191. Pulse-chase labeling of 10 min and immunoprecipitation of total MHC-I from Mock-infected or RhCMV-infected TRFs.

the BAC cassette is located between the Rh181 and Rh182 ORFs, it was not possible to delete the entire genomic region between Rh158-189). The ΔRh158-180 virus was created using the RhCMV BAC as described. The ΔRh158-180ΔRh182-189 virus was created by using the ΔRh182-189 BAC as a parent, and subsequently deleting Rh158-180 in the same manner (Fig 2.2). In both recombinants, the Kan^r cassette was not excised in order to limit the loss of DNA from the genome. Both recombinant BACs were characterized as described for the ΔRh182-189 BAC. Southern blotting of the BAC DNA indicated the loss of rh158, rh168, and rh180 in both viruses (Fig 2.6A). Rh182 and rh189 were also confirmed deleted from ΔRh158-180ΔRh182-189, while the control Rh207 was present in all three in the expected EcoRI restriction fragment. Interestingly, the reconstituted viruses ΔRh158-180 and ΔRh158-180ΔRh182-189 showed no obvious growth defects despite the large deletions of 18.5Kbp and 25.5Kbp, respectively. Both viruses grew to titers similar to those seen by wild type RhCMV.

To determine if deletion of Rh158-180 eliminates VIHCE, we infected fibroblasts with ΔRh158-180 and ΔRh158-180ΔRh182-189 and analyzed MHC-I synthesis by pulse-chase. During infection with ΔRh158-180ΔRh182-189, MHC-I HC was synthesized to levels similar to those seen in mock infected cells (Fig 2.6B, 0 chase time points). Additionally, the heavy chain appeared stable as there was no decrease of the protein over a 30-min chase (Fig 2.6B, 30-min chase time points). During infection with ΔRh158-180, MHC-I HC was initially synthesized as in mock infected cells, but subsequently degraded over the 30-min chase time by the Rh182-189 genes (Fig 2.6B). This degradation of newly synthesized heavy chain by Rh182-189 could be blocked using proteasomal inhibitors, resulting in the isolation of a deglycosylated cytosolic

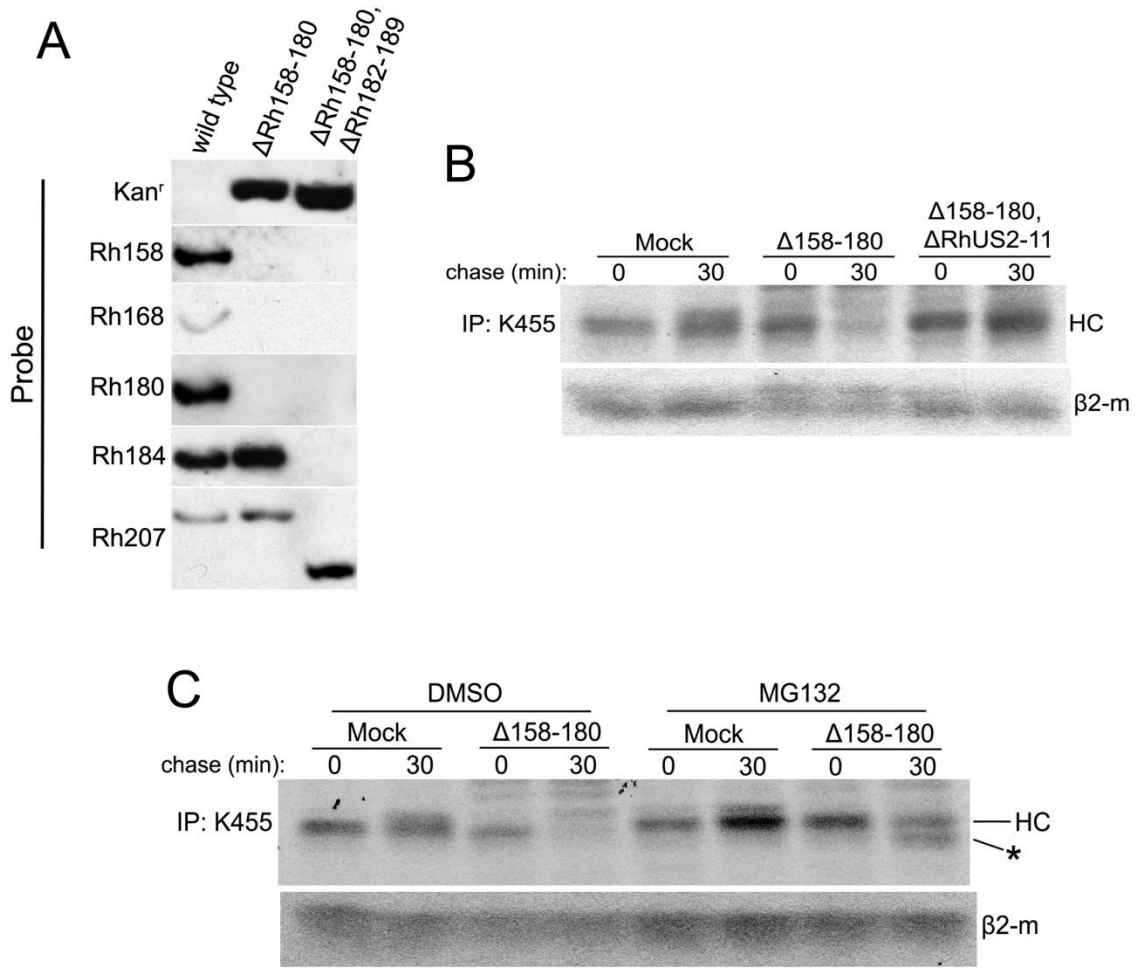


Fig 2.6 VIHCE is encoded within the Rh158-180 region. A) Southern blot analysis of the recombinant BACs RhCMVΔ158-180 and RhCMVΔ158-180,Δ182-189. B) Pulse-chase labeling for 10 min of TRFs infected with WT or recombinant RhCMV followed by IP of total MHC-I. In C) cells were incubated with 50μM MG132 or DMSO for 1 hour prior to and during labeling. (*) indicates a deglycosylated heavy chain cytosolic degradation intermediate stabilized by MG132.

intermediate (Fig 2.6C). This result is consistent with the described mechanism of action of the US2 and US11 homologs. These data indicate that VIHCE is located within the Rh158-180 region, since deletion of these genes results in the loss of VIHCE function (i.e., an initial restoration of the synthesis of MHC-I HCs). The data also suggest that VIHCE acts prior to the US2-11 homologs in the MHC-I antigen presentation pathway.

Rh178 is necessary for VIHCE

To determine which ORF(s) within the Rh158-180 region encoded VIHCE, we took two parallel approaches. All of the predicted ORFs were cloned into the mammalian expression vector pUHD10.1 and tested independently by transfection and staining of MHC-I on the cell surface. However, none of the predicted ORFs had a significant impact on surface expression of MHC-I (data not shown). These results indicated that either one or more of our expression constructs failed, or that VIHCE was likely encoded by a transcript that was not included in the original ORF predictions for RhCMV.

The second approach taken to determine the gene encoding VIHCE was further deletional mapping using the RhCMV BAC. Since viruses with and without VIHCE offered a clear phenotypic difference in pulse-chase experiments, we were able to track the VIHCE gene by subsequent smaller deletions within the Rh158-180 region. Using the wild type RhCMV BAC as the parent, we first created viruses lacking either Rh158-168 or Rh167-180 and analyzed MHC-I HC synthesis during infection with each of these. Whereas MHC-I HC expression was still blocked during infection with ΔRh158-168, HC was initially synthesized during ΔRh167-180 infection (Fig 2.7). This indicated VIHCE was expressed from the Rh167-180 region. Similarly, MHC-I HC was synthesized during

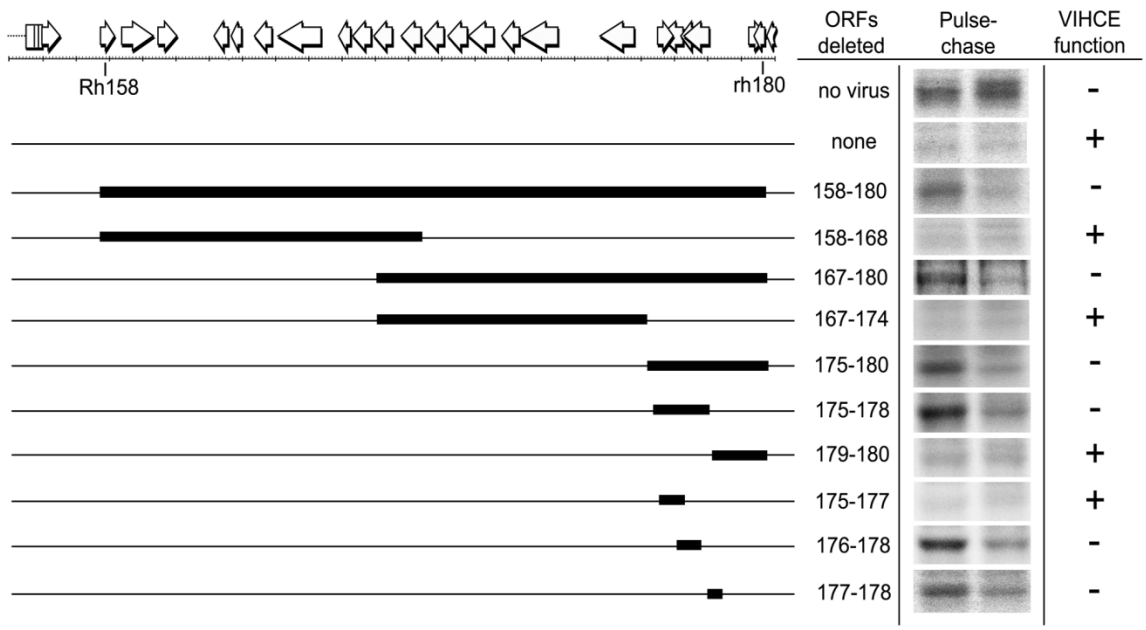


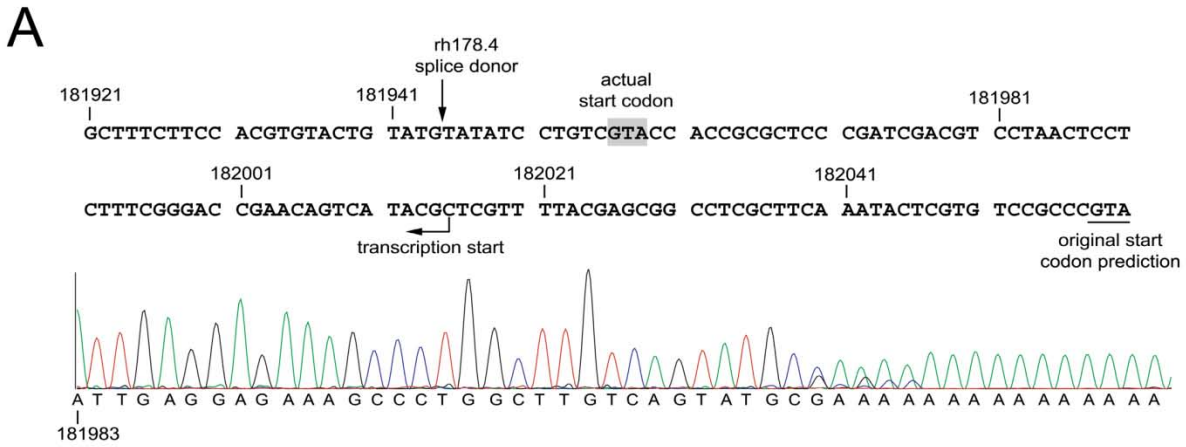
Fig 2.7 rh178 is required for VIHCE. Deletional mapping of VIHCE. Predicted open reading frames between Rh158-180 are shown as open white arrows. Solid black rectangles indicate the region of deletion. Pulse-chase labeling for 10 min with the indicated recombinant virus was performed followed by IP with K455. Lack of VIHCE is readily apparent by the initial synthesis of HC (left column) followed by Rh182-189-mediated destruction (right column).

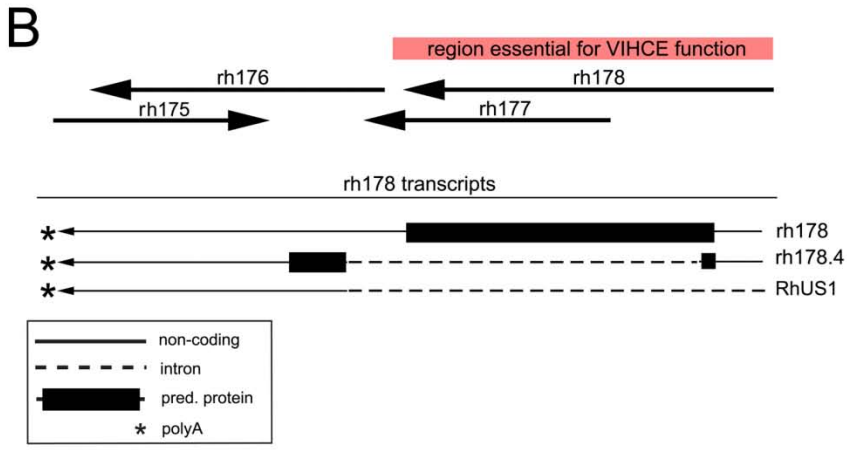
infection with Δ175−180, Δ175-178, Δ176−178, and Δ177-178, but not Δ167-174, Δ179-180, and Δ175-177 (Fig 2.7). These data indicate that the Rh178 ORF is necessary for VIHCE function.

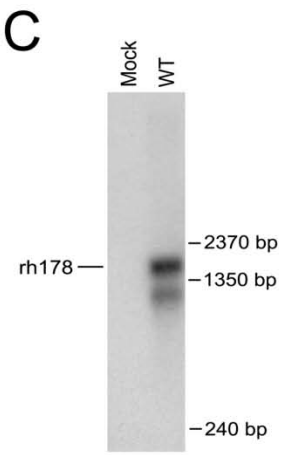
Identification of the Rh178 transcripts expressed during RhCMV infection

Since the predicted Rh178 ORF had no effect on the surface expression of MHC-I when expressed independently, we performed detailed mapping to determine the exact transcripts made from this region during viral infection. This was done using 5' and 3' RACE, northern blot, and PCR and sequencing of cDNA. RACE was performed using cDNA made from viral RNA isolated at 24 hours post infection. Sequencing of the 5' RACE product determined that the transcription start site of Rh178 was at genomic position 182015bp, downstream of the predicted ATG start codon (Fig 2.8A). Thus the originally predicted start codon was incorrect. The first ATG after the transcription start site is in frame with the original ATG prediction, but is located 102bp downstream. The protein predicted to be encoded by the Rh178 transcript is 34 amino acids shorter than what was originally predicted [66]. The 3' end of the transcript was identified by 3' RACE. The Rh178 transcript terminates at a polyadenylation signal located 845bp from the predicted stop codon, giving a total expected transcript size of 1550bp. We also confirmed a previous report indicating that this polyA site is also used by the Rh181 transcript [244] (Gene Bank Accession: AF474179).

In order to determine the possible presence of other transcripts and to confirm Rh178 transcription during infection, we used northern blot and PCR of randomly primed cDNA. PCR indicated the presence of a splice-variant of Rh178 that utilizes the same


A
rh178.4
splice donor
actual
start codon
181921
181941
181981
GCTTTCTTCC ACGTGTACTG TATGTATATC CTGTCGTACC ACCGCGCTCC CGATCGACGT CCTAACTCCT
182001
182021
182041
CTTTCGGGAC CGAACAGTCA TACGCTCGTT TTACGAGCGG CCTCGCTTCA AATACTCGTG TCCGCCCGTA
transcription start
original start
codon prediction
A T T G A G G A G A A A G C C C T G G C T T G T C A G T A T G C G A A A A A A A A A A A A A A
181983


B
region essential for VIHCE function
rh176
rh178
rh175
rh177
rh178 transcripts
rh178
rh178.4
RhUS1
non-coding
intron
pred. protein
polyA


C
Mock
WT
rh178
2370 bp
1350 bp
240 bp

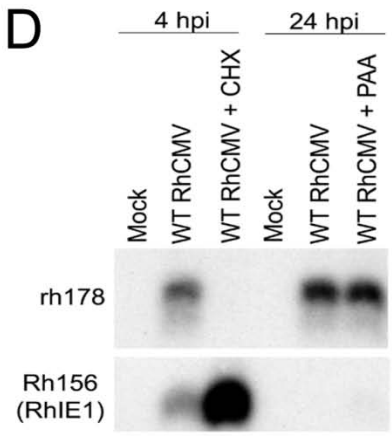

D
4 hpi
24 hpi
Mock
WT RhCMV
WT RhCMV + CHX
Mock
WT RhCMV
WT RhCMV + PAA
rh178
Rh156
(RhIE1)

Fig 2.8 Identification of rh178 transcripts. A) Complementary sequence of the RhCMV genome from 181921-182060bp. Underlined at 182058bp is the original predicted start codon for rh178. Transcription actually begins at 182015bp as determined by 5' RACE (see sequence chromatogram below genomic sequence). Shaded in gray is the first ATG codon of the transcript. Also noted is the splice donor site for *rh178.4* which is spliced at 181944bp. B) Predicted ORFs and experimentally confirmed transcripts in the *rh178* region. The red rectangle indicates the region essential for VIHCE function as determined by deletions in several independent recombinants. Large black arrows indicate positions of ORFs *rh175-178*. C) Northern blot analysis of total RNA isolated from mock or WT RhCMV-infected TRFs at 24 hours post infection. ORF *rh178* was used to generate ^{32}P-dCTP labeled DNA probe. D) Northern blot analysis of rh178 and Rh156 (IE1) at 4 and 24 hours post infection. Cyclohexamide (CHX) and phosphonoacetic acid (PAA) were included where indicated.

transcription start site as Rh178, but splices shortly after the start codon to a splice acceptor site also utilized by Rh181 (Fig 2.8B). This splice variant is denoted Rh178.4 (Note that Rivailler et al. [72] have noted additional ORFs upstream of *rh178* and denoted them *rh178.1*, *rh178.2*, and *rh178.3*). Northern blot analysis using the predicted *rh178* coding region as probe revealed two transcripts (Fig 2.8C). A larger predominant transcript of approximately 1550bp corresponds to the expected size of *rh178*. The smaller transcript likely corresponds to *rh178.4*. We conclude from these data that *rh178* is transcribed during RhCMV infection, creating a transcript of 1550bp encoding a predicted 639aa polypeptide.

We also determined the kinetic class of gene expression to which rh178 belonged. While no rh178 transcript was detected in the presence of the translational inhibitor cyclohexamide, rh178 was detected in the presence of the DNA replication inhibitor phosphonoacetic acid (PAA) (Fig 2.8D). Since rh178 required de novo viral protein synthesis, but not viral DNA replication, it is classified as an early gene.

Rh178 is sufficient for VIHCE

Having identified the correct Rh178 transcript, we next asked if Rh178 was sufficient for VIHCE function. To test this, we cloned Rh178 into a replication defective adenovirus vector [245]. The adenovirus vector lacks the E1 gene of adenovirus, preventing replication in non-complementing cell lines. It also lacks E3, which has been shown to inhibit surface expression of MHC-I [246, 247]. Thus the adenoviral vector itself should not downregulate MHC-I. Upon transduction of fibroblasts with Rh178-expressing adenovirus, we observed a decrease in the cell surface expression of MHC-I

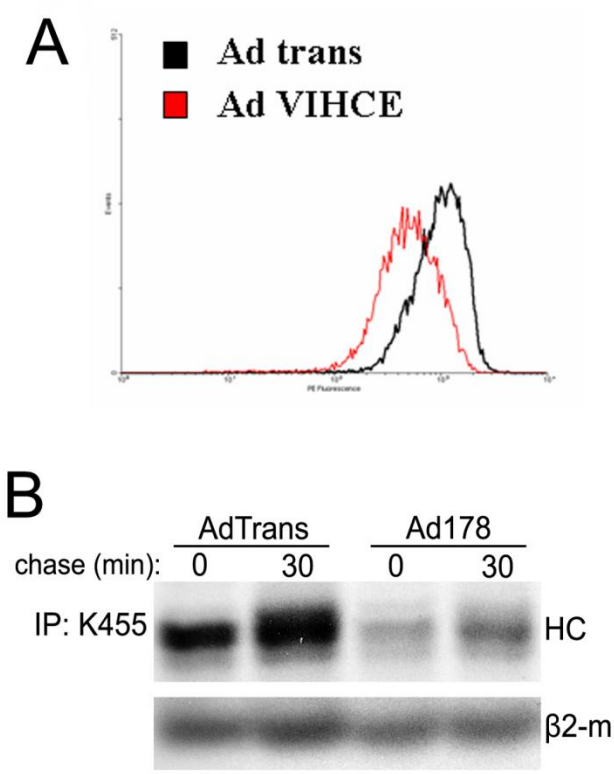


Fig 2.9 rh178 is sufficient for VIHCE. A) Surface expression of MHC-I 48 hours after transduction with either and adenovirus expressing the tetracycline-responsive transactivator (AdTrans, control) or an adenovirus expressing rh178. B) HC expression in TRFs transduced with replication deficient recombinant adenovirus AdTrans (expressing tetracycline responsive transactivator as a control) or AdTrans together with Ad178 (expressing rh178) for 24 hours, followed by a 10-min pulse label and 30-min chase.

compared to the control adenovirus vector expressing the tetracycline-responsive transactivator (Fig 2.9A). In pulse-chase experiments, we observed a phenotype similar to that seen during RhCMV infection. MHC-I HC synthesis was blocked while the light chain β2-m was unaffected (Fig 2.9B). These data suggest that Rh178 is sufficient for VIHCE function.

The Rh178 encoded protein mediates VIHCE

The observed VIHCE phenotype is a knockdown of MHC-I heavy chain expression. Since this is the same phenotype observed for inhibitory RNAs, it was possible that Rh178 expressed a microRNA. In order to determine whether Rh178 encoded a microRNA, we introduced a frameshift mutation in the Rh178 coding region, and we analyzed a codon-optimized Rh178 transcript.

To create the Rh178 frameshift mutant, a single nucleotide was introduced into the ORF directly after the ATG start codon (rh178FS; Fig 2.10A). This mutation leads to the production of a polypeptide of 99aa in length with no sequence homology to Rh178 or any other known protein. The introduction of the Rh178 frameshift into the virus genome required the mutation of the Rh178 5'UTR, where 20bp of viral sequence was replaced with 93bp of sequence from the recombination vector including an FRT site (Fig 2.10A). In order to control for this, we created a virus with the same 5'UTR mutation, but contained an intact Rh178 transcript (rh178FS-Ctrl). Upon infection of TRFs with rh178FS-Ctrl, MHC-I HC synthesis was efficiently blocked as in WT infection (Fig 2.10B). This indicates that the introduced 5'UTR mutation had no effect on VIHCE function. Conversely, during infection of TRFs with rh178FS, MHC-I HC synthesis was

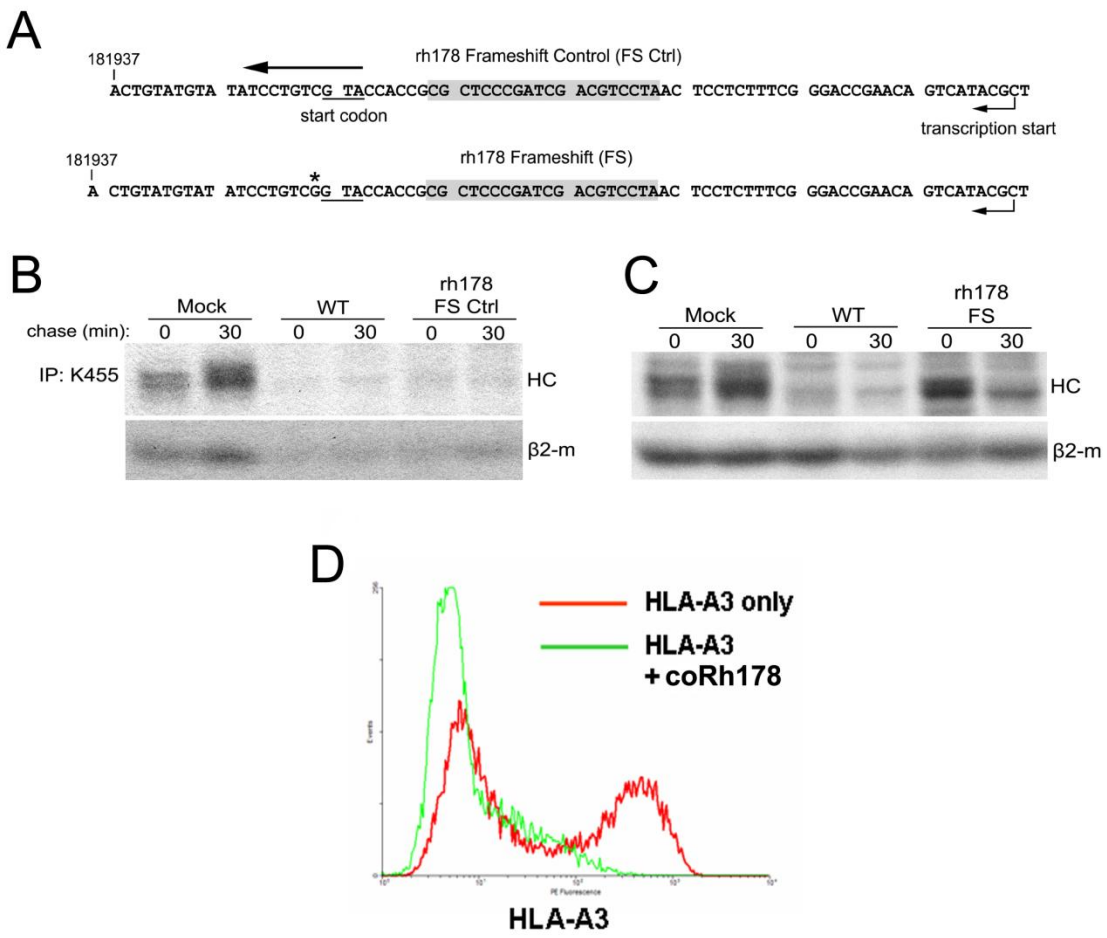


Fig 2.10 rh178 encodes for a protein, not an RNA, that is responsible for VIHCE.

A) Sequence of the rh178 frameshift control and frameshift recombinants. Shown is complementary genomic sequence, with transcripts running from right to left. In each recombinant a 20bp sequence in the 5' UTR of *rh178* (gray boxes) was replaced with 93bp from the recombination vector including the FRT recombination site. (*) indicates the single base insertion causing a frameshift. B) and C) HC expression in TRFs infected with control (part B, right two lanes) or frameshift (part C, right two lanes) viruses as determined by pulse-chase analysis. D) Co-transfection of HeLa cells with codon-optimized rh178 (coRh178) and HLA-A3, followed 48 hours later by cell surface staining of HLA-A3 and FACS.

similar to mock-infected cells after a 10-min metabolic label (Fig 2.10C). HC was subsequently destroyed by Rh182-189. This phenotype indicates that VIHCE has been lost, and that introducing a single nucleotide causing a frameshift in rh178 eliminates VIHCE function. Since a single nucleotide addition would be unlikely to interfere with the production or function of an inhibitory RNA, this is strong evidence that it is the rh178 encoded protein that is responsible for VIHCE.

As an additional confirmation that the rh178 protein is responsible for VIHCE, we analyzed a codon optimized rh178 construct. The codon optimized rh178 (coRh178) was synthesized by GeneArt (Toronto) and was only 74% identical to the wild-type rh178 while still encoding the same polypeptide (Appendix A). Upon co-transfection of coRh178 with the human MHC-I molecule HLA-A3, surface expression of HLA-A3 was reduced compared to transfection of HLA-A3 alone (Fig 2.10D). This indicates that the codon optimized Rh178 is functional, and as the coRh178 mRNA is 26% different than the WT Rh178 mRNA, this is further evidence that it is the Rh178 protein that is responsible for VIHCE.

Rh178 encodes an ER-resident transmembrane protein

To determine the subcellular localization of Rh178, we created a Rh178-HA expression construct with the HA epitope tag at the C-terminus. TRFs were co-transfected with Rh178-HA and K5-FLAG, an epitoped tagged ER-resident expressed by KSHV. Immunofluorescent staining for HA showed a staining pattern indicative of ER localization (Fig 2.11A). Co-staining with FLAG antibody indicated that Rh178 co-localized with K5. These data suggest that Rh178 is an ER-resident protein.

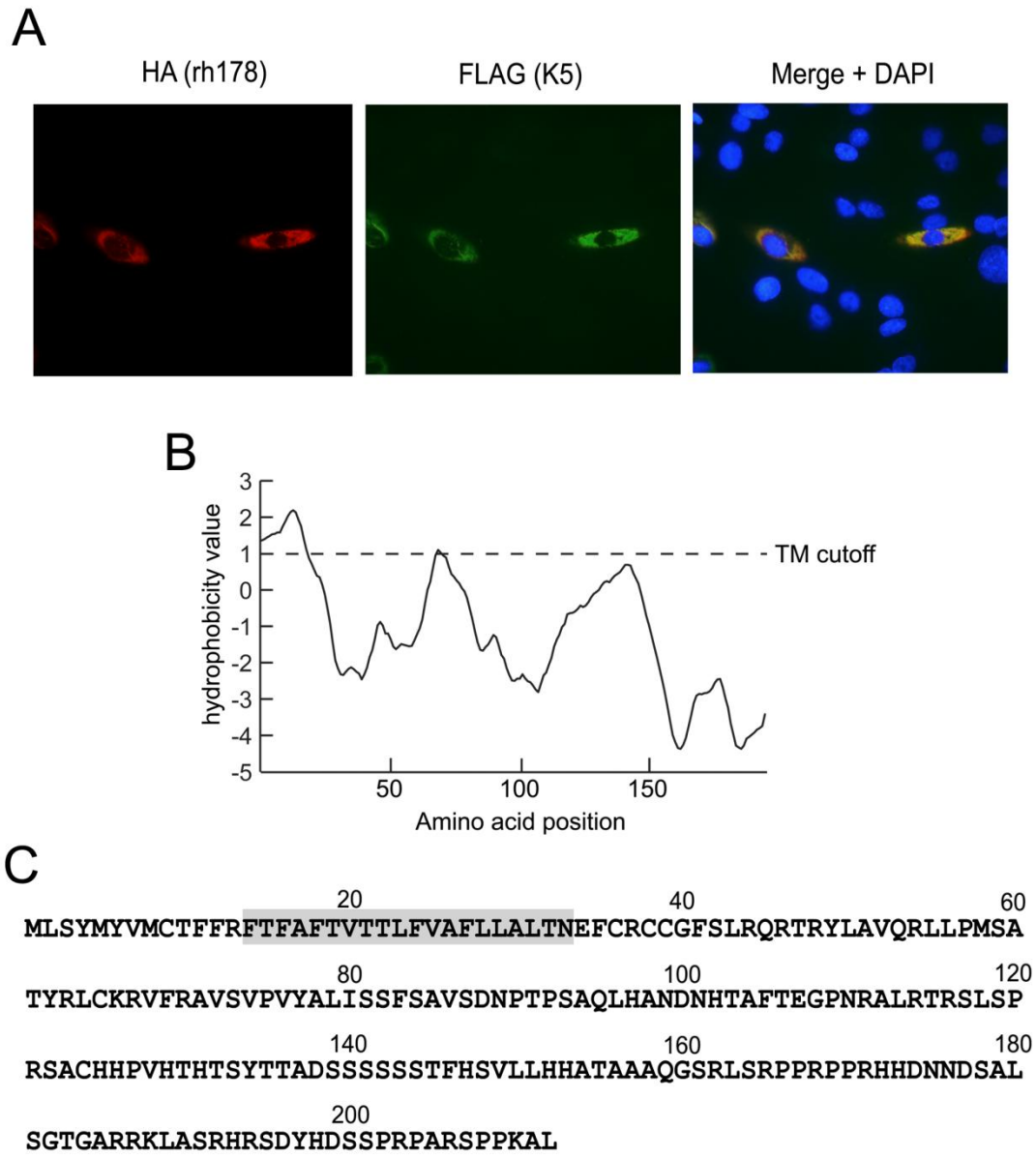


Fig 2.11 rh178 is a 212aa ER localized protein. A) Immunofluorescence analysis of TRFs 24 hours after transfection with HA-tagged rh178 together with FLAG-tagged K5 from KSHV. B) Hydrophobicity graph of rh178 (TopPred, http://bioweb2.pasteur.fr/). TM refers to a predicted transmembrane domain cutoff value. C) Complete polypeptide sequence of rh178. Shaded in gray is the predicted signal anchor sequence.

The predicted topology of Rh178 is that of a type 1b transmembrane protein. Hydrophobicity analysis shows three hydrophobic domains, of which only the first is predicted to span a membrane (Fig 2.11B). This first hydrophobic domain is predicted to encode a signal anchor, a non-cleaved membrane spanning signal sequence. Thus, the most likely topology for Rh178 is a short lumenal tail in the ER with the bulk of the protein residing in the cytosol.

The rh178 protein (Fig 2.11C), with a predicted molecular weight of approximately 24 kDa, does not display significant homology with non-RhCMV sequences in the genomic database. Aside from two possible N-linked glycosylation sites, it has no notable conserved domains or markers in its primary amino acid sequence. Nor do any significant alignments appear when secondary and tertiary structure of the protein are predicted by computer models [248].

Discussion

In this chapter we have described the identification of a novel immunomodulatory protein expressed exclusively by rhesus cytomegalovirus. This is a surprising result since RhCMV also contains the fully functional homologs of the HCMV US6-family of MHC-I inhibitors. We can only speculate as to the evolutionary pressure that caused RhCMV to select yet another inhibitor of antigen presentation. One potential possibility is that as the genetic makeup and expression of MHC genes evolved after the split of humans and old world primates, each species of CMV adapted to its new MHC environment. Interestingly, the rhesus macaque MHC-I locus is much more polygenic than that of the human MHC [249]. It is possible that because of the additional number of MHC-I alleles

that can be expressed in a given rhesus macaque, RhCMV had to evolve an additional protein to overcome this pressure. This will be discussed in more detail in Chapter 5.

The rh178 ORF appears to be unique to rhesus cytomegalovirus. It is conserved between strains of RhCMV, but shares no homology to any sequence found in the other sequenced primate CMVs. This includes no homolog in any of the seven sequenced human CMV strains, nor a homolog in chimpanzee CMV. Additionally, there appears to be no significant homology to any other known mammalian protein.

VIHCE is located within an interesting region of the RhCMV genome. Not only are there a large number of RhCMV-specific ORFs encoded within this region, but there is significant divergence in this region between different strains of RhCMV. For example, when RhCMV strain 180.92 was compared to strain 68.1, it was discovered that 68.1 encoded 10 ORFs not found in strain 180.92 [72]. All of these were encoded within the U_L/b'-like Rh158-180 region. This genomic location is extremely variable between strains of HCMV, and RhCMV seems to follow the same rule. This is somewhat interesting since the U_L/b' region of HCMV can be located near the end of the genome in some isoforms. However since RhCMV does not appear to isomerize, the Rh158-180 region is never located near the end of the genome. Conserved genes between viruses are largely located towards the center of the genome, while the ends can be frequently changed. This particular region in RhCMV, or primate CMVs in general, then must have some other property that allows for the high amount of strain variation despite its more centralized location in the genome.

Lastly, we have found that independent expression of the wild type Rh178 protein is very poor. Upon transfection of Rh178 constructs, very few cells express the Rh178

protein as detected by immunofluorescence. Even when adenovirus transduction was used, ensuring that every cell in culture received Rh178-encoding DNA, expression was generally poor unless a very high multiplicity of infection was used. Conversely, the codon-optimized Rh178 construct allowed for very high expression of Rh178 protein regardless of how it was introduced into cells. It is interesting to note the discrepancy between the wild type and codon optimized Rh178, since one would imagine the virus would have optimized the coding transcript by evolutionary means. While we never successfully identified the reason for the poor protein expression, it is clear that in the context of virus infection Rh178 works quite well, although it is less than 100% efficient. It is likely that the virus has in fact optimized Rh178 for expression in the context of *in vivo* virus replication, which may not directly translate to independent expression in tissue culture cells. Furthermore, it is possible that Rh178 is a relatively newly evolved protein, and that given many more years of virus replication within a rhesus macaque population, further changes in Rh178 may occur that would subsequently lead to better expression in culture.

CHAPTER 3

VIHCE prevents MHC-I HC biosynthesis in a signal peptide-dependent manner

Introduction

Inhibition of MHC-I antigen presentation is a common theme seen across virus families. The mechanisms that viruses have evolved in order to block this cellular pathway vary widely. These include targeting nearly every step of the pathway including MHC-I transcription, peptide transport into the ER, peptide loading, MHC-I assembly, maturation, and surface expression. Thus far, most characterized mechanisms of viral inhibition of MHC-I antigen presentation, including all of those performed by CMVs, block MHC-I after complete translation of the MHC-I heavy chain.

We have shown that Rh178-encoded VIHCE reduces MHC-I HC synthesis both during RhCMV infection and when expressed independently of virus infection (See chapter 2). This reduction is seen as a block in the synthesis of new MHC-I HCs (Fig. 2.4C), which eventually results in a decrease in the steady state levels of MHC-I both in total cell lysate and on the cell surface (Fig 2.4A,B). While the block in new MHC-I HC synthesis caused by VIHCE is not complete, it is a significant knockdown in protein expression. The residual HC that is recovered with Rh178 present indicates that VIHCE was either incomplete or that VIHCE did not equally affect all MHC-I alleles present in TRFs. In this chapter, we explore the molecular mechanism by which VIHCE is able to block MHC-I heavy chain biosynthesis.

Results

VIHCE does not cause rapid degradation of MHC-I heavy chains

To determine how VIHCE was preventing MHC-I HC expression, we began an analysis of newly synthesized HCs by metabolic labeling infected cells for various times and by pulse-chase experiments. Since our initial results showed a decrease in the recovery of HC after a 10-min metabolic label, we examined whether significantly more HC could be recovered after a longer labeling time. TRFs were infected with either WT RhCMV or ΔRh182-189 (ΔRhUS2-11), metabolically labeled for 60 or 120 minutes, and HC immunoprecipitated. Consistent with previous results examining steady state levels of HC, significantly less HC was recovered from cells infected with RhCMV containing VIHCE (ΔRhUS2-11) compared to Mock-infected cells during both labeling periods (Fig 3.1A). Again we observed a slight increase in the amount of HC recovered in ΔRhUS2-11 infected cells compared to WT RhCMV infected cells, indicating that VIHCE is not complete in TRFs.

The observation of a significant reduction in newly synthesized HCs during a 10-min metabolic label is indicative of either extremely rapid degradation of HCs immediately following synthesis or a block in production occurring prior to or during HC translation. Since both HCMV (US2 and US11) and RhCMV (Rh182 and Rh189) already express proteins that cause rapid degradation of HCs, we examined whether VIHCE was causing rapid degradation of HCs by performing very short metabolic labelings. US2 and US11 have been shown to reduce the half-life of MHC-I heavy chain to less than 3 minutes [192], meaning that if VIHCE was acting in a similar fashion much of the HC could be degraded during a 10-min label. However, upon pulse-labeling cells for only 5

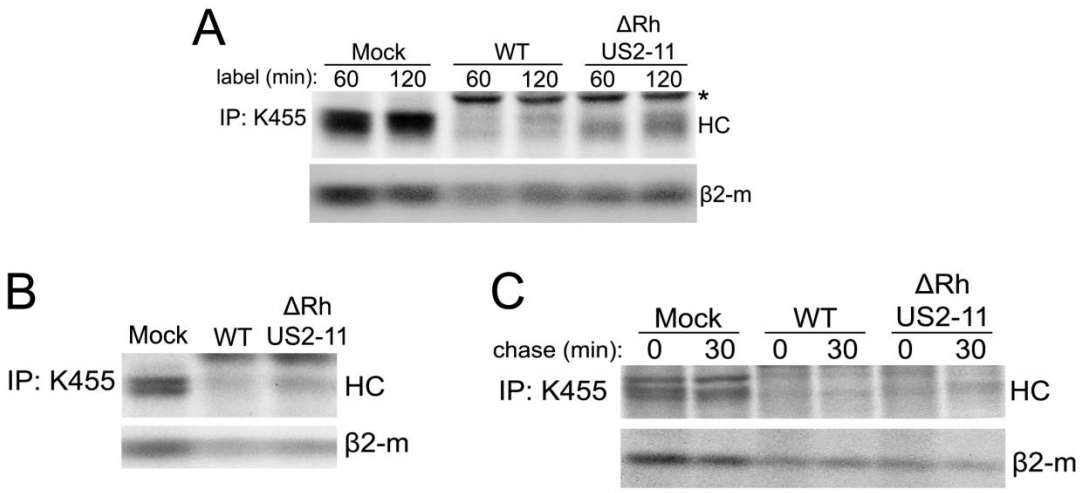


Fig 3.1 VIHCE efficiently blocks synthesis of MHC-I heavy chains. A) IP of total MHC-I upon labeling with ^{35}S-Met/Cys for the indicated time. (*) All IPs from WT and recombinant RhCMV- infected cells contain antibody-binding proteins around 55kDa (see Appendix B) which likely correspond to the RhCMV homologues of the Fc-receptor UL119-118 of HCMV. B) IP for MHC-I following a 5-min metabolic label. C) IP for MHC-I following a 1-min metabolic label with or without a 30-min chase.

minutes, we still recover dramatically less HC in Δ182-189 infected cells compared to Mock (Fig 3.1B). Even during a 1-min label, which is sufficient time to label a small amount of HC, we recover less HC from Δ182-189 infected cells compared to Mock (Fig 3.1C). These data suggest that HC is not rapidly degraded, but likely never completely synthesized.

Despite the likelihood that VIHCE was preventing complete HC synthesis, we examined whether it was merely delaying MHC-I maturation, perhaps initially masking the molecule before it was released. To test this, we performed a metabolically label in Mock- or Δ182-189-infected TRFs for 10-min and chased them from 30 to 90 minutes. We recovered significantly less HC at all time points in the Δ182-189 infected cells (Fig 3.2A). This suggests that VIHCE was not masking MHC-I and delaying its maturation.

To rule out that HC was not recovered due to epitope masking by a viral protein or because HC was in a complex with NP40-insoluble proteins, we lysed cells in SDS to disrupt protein complexes and denature the HCs prior to immunoprecipitation. Using either a monoclonal antibody that recognizes only free HC (HC-10) [250] or K455, we were unable to recover increased amounts of HC under these conditions (Fig 3.2B). Taken together these data suggest that RhCMV either prevents complete HC synthesis or degrades HC prior to complete protein synthesis.

Since co-translational degradation is mediated by proteasomes [251] we wanted to determine whether HC translation could be completed in the presence of proteasome inhibitors. TRFs were infected with ΔRhUS2-11 and treated with the proteasomal inhibitor MG132. However, no significant increase in HC recovery was observed either when total MHC-I was immunoprecipitated with K455 from NP40-lysates or with HC-10

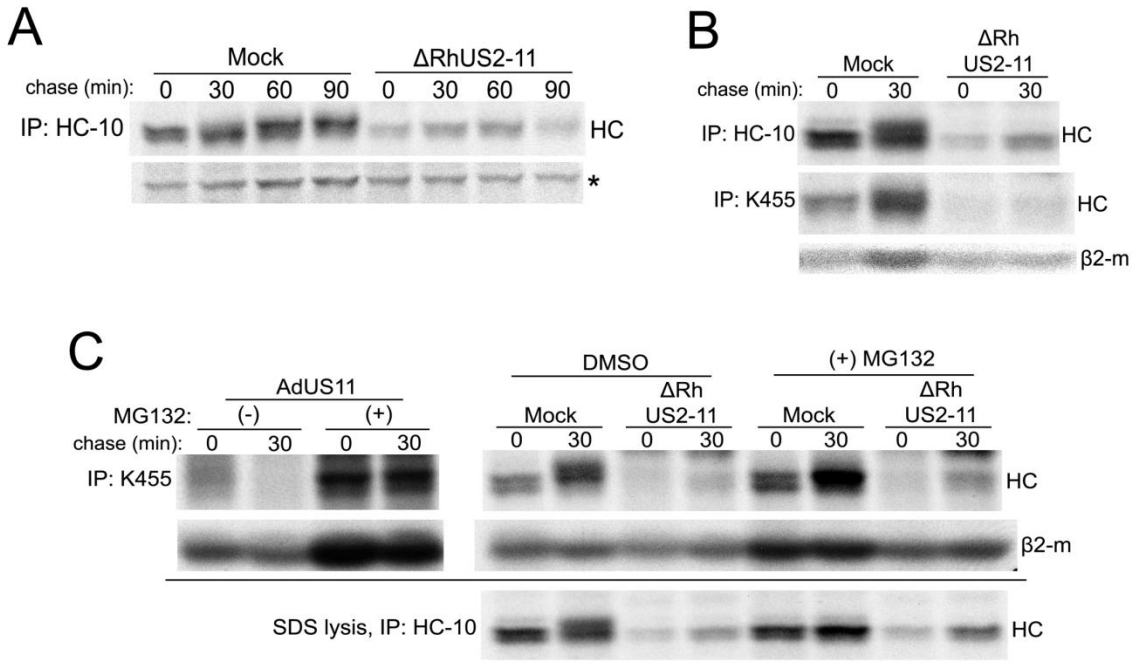


Fig 3.2 rh178 does not mask HC, nor can HC be stabilized with proteasomal inhibitors in the presence of VIHCE. A) HC synthesis is not delayed. Cells were radiolabeled for 10 min followed by chase of indicated times. After SDS lysis, IP was performed using HC-10 antibody, which recognizes free MHC-I HC. (*) A non-MHC-I-specific band indicating protein loading. B) Pulse-chase labeling of 10 min and IP of total MHC-I or HC. Cells were lysed in SDS buffer prior to IP with the indicated antibody. C) Pulse-chase labeling and IP of RhCMV-infected TRFs treated with proteasome inhibitor. Where indicated TRFs were incubated with 50μM MG132 or DMSO during 60-min of Met/Cys starvation, 10-min label, and 30-min chase. For control, TRFs were transduced with AdUS11 (MOI=25), a recombinant adenovirus expressing HCMV US11, for 24 hours followed by NP40-lysis and IP with K455. Shown for RhCMV-infection is both NP-40 lysis (top panel) and SDS-lysis (bottom panel) prior to IP with the noted antibody.

from SDS-lysates (Fig 3.2C). In contrast, HC was stabilized in cells transduced with Adenovirus expressing HCMV US11. The proteasomal inhibitors Lactacystin and ZL_3VS also failed to stabilize HC in ΔRhUS2-11-infected cells (data not shown). Conversely, during RhCMV infection in the absence of VIHCE, RhUS2-US11-mediated HC degradation could be blocked with MG132 (Fig 2.6C). This suggests that VIHCE acts prior to RhUS2-US11 in the MHC-I antigen presentation pathway.

MHC-I HC mRNA is intact and associates with actively translating ribosomes during RhCMV infection.

Our previous data strongly suggest that RhCMV VIHCE inhibits the expression of the MHC-I HC prior to or during polypeptide synthesis. It was therefore possible that VIHCE was affecting the MHC-I HC mRNA, thus preventing protein expression. However, there was no decrease in the amount of HC mRNA during RhCMV infection as shown by northern blot (Fig 3.3A) and quantitative RT-PCR (data not shown). Furthermore, northern blot analysis indicates that the HC mRNA is intact, as no cleavage products or smaller fragments are detected. We next examined the presence of the HC mRNA in various cellular RNA fractions. Compared to GAPDH mRNA, there was no significant difference in the levels of HC mRNA in either the nuclear or cytoplasmic-enriched RNA fractions (Fig 3.3B). Additionally, HC mRNA was equally present in the polyadenylated mRNA fraction isolated from Mock or RhCMV-infected cells. These data indicate that HC mRNA transcription, poly-adenylation, splicing and export to the cytosol are not affected by RhCMV infection.

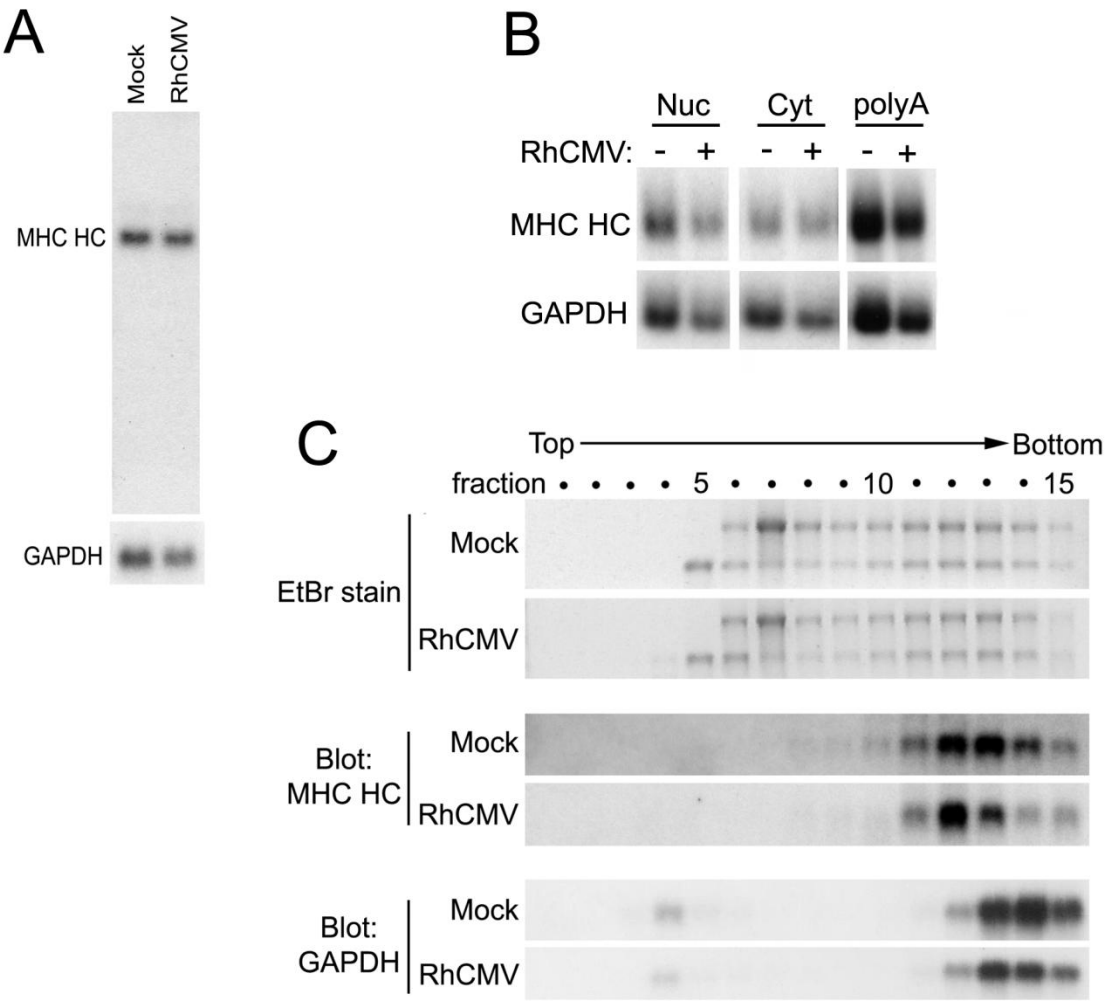

A
Mock
RhCMV
MHC HC
GAPDH
B
Nuc
Cyt
polyA
RhCMV: - + - + - +
MHC HC
GAPDH
C
Top
Bottom
fraction • • • • 5 • • • • 10 • • • • 15
EtBr stain
Mock
RhCMV
Blot: MHC HC
Mock
RhCMV
Blot: GAPDH
Mock
RhCMV

Fig 3.3 VIHCE does not affect HC mRNA stability or ribosome binding. A) Northern blot analysis of HC- or GAPDH-specific mRNA from total RNA isolated at 24 hours after Mock- or RhCMV-infection. The ^{32}P-dCTP labeled probes were generated using rhesus-derived cDNAs for HC or GAPDH as templates. B) Northern blot analysis of enriched nuclear (nuc), cytoplasmic (cyt), and polyadenylated (polyA) RNA fractions from mock or RhCMV-infected cells at 24hpi. C) Polyribosome fractionation and northern blot analysis. TRFs were either mock infected or infected with wild-type (WT) RhCMV at MOI=3 for 24 hours followed by isolation and fractionation of polysomes. Ethidium Bromide (EtBr) staining of a denaturing agarose gel shows the amount and ratio of 18S and 28S rRNA present in each fraction, indicating the presence of ribosomal subunits. Polysomes sediment to higher, denser fractions. Lower panels show northern blots of the gel using the HC and GAPDH-specific probes.

Since the MHC-I HC mRNA is present and seemingly intact in the cytosol, VIHCE is likely blocking HC synthesis at or subsequent to translation. To determine whether the association of HC mRNA with ribosomes is inhibited by VIHCE we analyzed the polyribosome distribution of HC mRNA in RhCMV-infected cells [252]. Total RNA was isolated from sucrose gradient fractions of Mock and RhCMV-infected cell lysates. Ethidium bromide staining of the isolated RNA indicates the ribosome composition of each fraction, e.g. fraction 5 contains mostly small ribosomal subunits, fraction 7 contains mostly large ribosomal subunits, and the higher fractions 10-15 contain multiple complete ribosomes (Fig 3.3C, top panel). Therefore mRNAs with multiple ribosomes bound will sediment to the higher gradient fractions.

When the RNA from the sucrose gradient fractions was analyzed by northern blot, the MHC-I HC mRNA sedimented to the polyribosome fractions 12 and 13 in both Mock- and RhCMV-infected cells (Fig 3.3C). Small shifts in polyribosome density were observed in RhCMV infection for both HC and GAPDH mRNA, suggesting virus infection causes a slight reduction of ribosomal occupancy on cellular transcripts. Thus, it seems that VIHCE does not inhibit the association of polyribosomes with HC mRNA.

As further confirmation that VIHCE was not affecting ribosome association with HC mRNA, we compared the polyribosome distribution in cells infected with RhCMV with and without VIHCE. As expected, the sedimentation profile of the HC mRNA was the same in RhCMV infection regardless of the presence of VIHCE (Fig 3.4A).

Though HC mRNA was associated with polyribosomes, it was possible that the ribosomes were not actively translating but instead stalled on the transcript. To determine if in fact HC mRNA associated ribosomes were active, we incubated the cells with

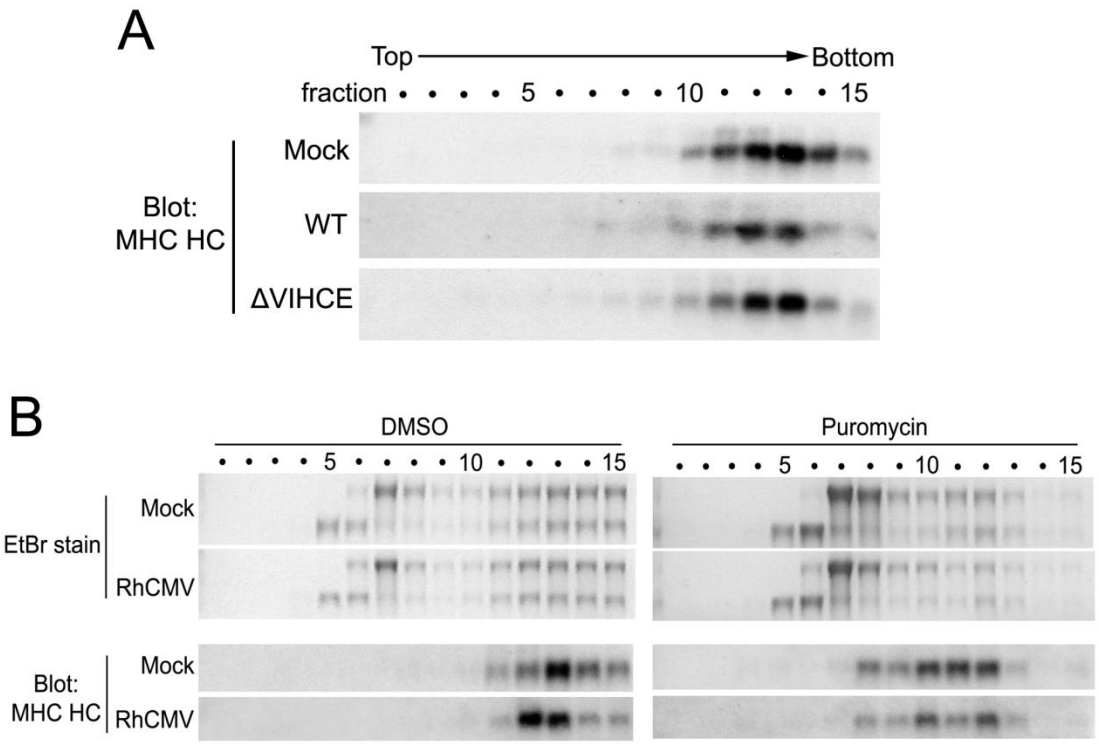


Fig 3.4 VIHCE does not prevent ribosomal activity on HC mRNA. A) Polyribosome fractionation as in Fig 3.3C, after either mock infection or infection with wild type (wt) RhCMV or RhCMV lacking Rh175-180 (ΔVIHCE). B) Polyribosome fraction as in Fig 3.3C, with either DMSO or 100μg/ml puromycin added 4 minutes prior to harvesting.

puromycin for 4 min. This short incubation caused a shift in the HC mRNA polyribosome profile in both Mock and RhCMV-infected cells, indicating the loss of ribosomes from the transcript (Fig 3.4B). Since puromycin is a polypeptide chain terminator incorporated by peptidyl transferase, this result indicates that the ribosomes bound to HC mRNA are actively translating. This result also confirms that the sedimentation of the HC mRNA to those fractions is because of ribosome-association, and not because of an association with some other macromolecular complex.

One method of translational suppression by miRNAs has been suggested to be premature ribosome dissociation from mRNAs [253]. This was shown in ribosome run-off assays by blocking translation initiation and subsequently timing the rate of ribosome falloff. To examine if RhCMV caused premature ribosome dissociation from MHC-I HC mRNA, we incubated cells for brief times with hippuristanol prior to polyribosome sedimentation. Hippuristanol is a compound isolated from coral that blocks translation initiation by inhibiting eIF4a RNA-binding activity [254]. Upon adding hippuristanol to cells, actively translating ribosomes continue translation until normal dissociation, but no new translation begins. Thus you can determine the rate of ribosome fall off (Fig 3.5A). Mock and WT RhCMV-infected cells were incubated with hippuristanol for 0, 3, 5, or 7 min. Ribosome dissociation was determined by graphing the percentage of MHC-I HC mRNA in each polyribosome fraction. We observed no increase in the rate of ribosome fall off in RhCMV-infected cells compared to Mock-infected (Fig 3.5B). In fact, we observed a slight ribosome retention on HC mRNA during RhCMV infection. These data suggest that RhCMV VIHCE does not cause premature ribosome dissociation from HC mRNA.

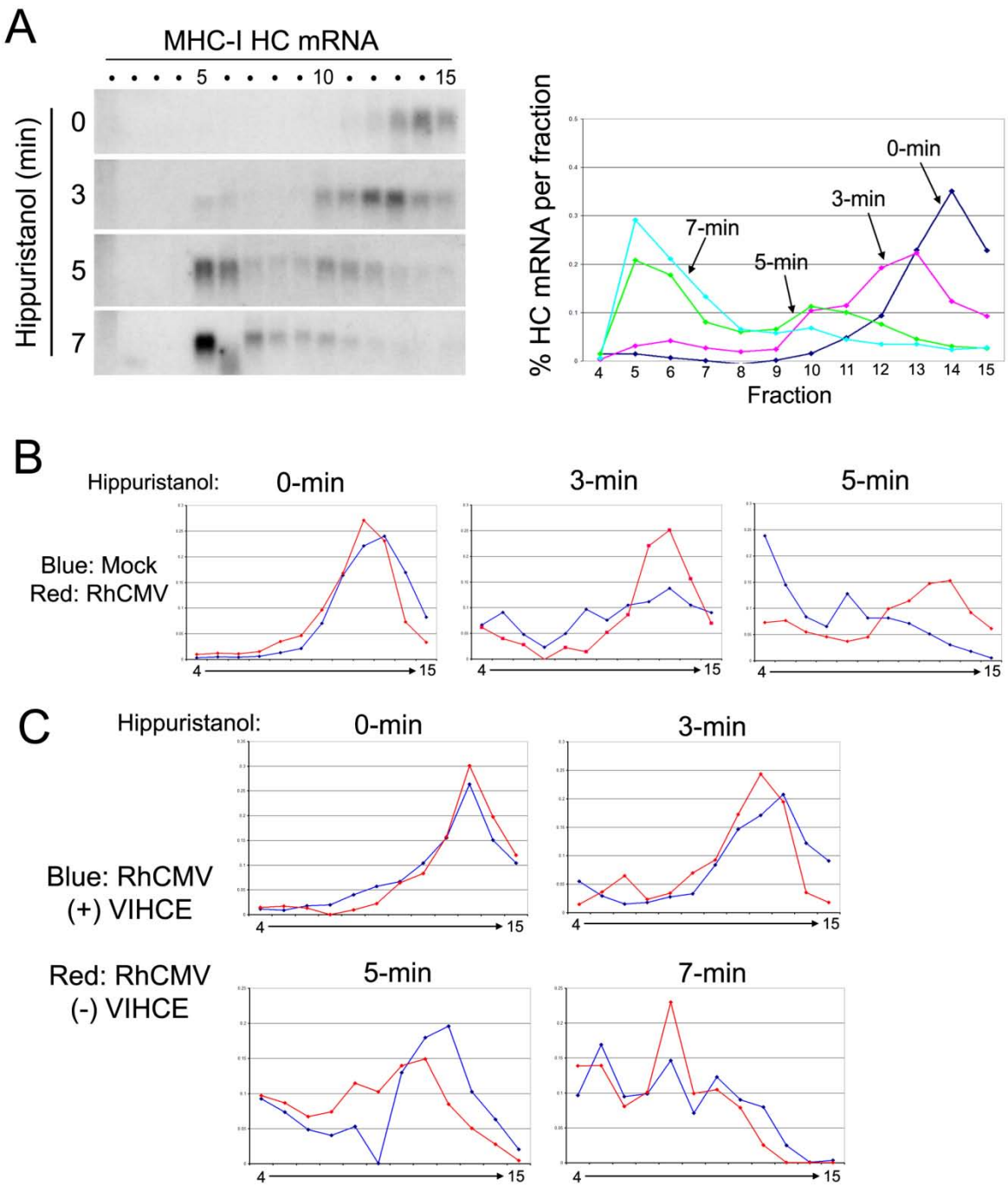

A
MHC-I HC mRNA
5
10
15
Hippuristanol (min)
0
3
5
7
% HC mRNA per fraction
0-min
3-min
7-min
5-min
Fraction
B
Hippuristanol:
0-min
3-min
5-min
Blue: Mock
Red: RhCMV
C
Hippuristanol:
0-min
3-min
Blue: RhCMV
(+) VIHCE
Red: RhCMV
(-) VIHCE
5-min
7-min

Fig 3.5 VIHCE does not cause premature ribosome dissociation from HC mRNA. A) Representative polyribosome fractionation with hippuristanol treatment. Left panel: Northern blot for HC mRNA in polyribosome fractions after treatment for 0, 3, 5, or 7 minutes with the translation initiation inhibitor hippuristanol. Right panel: Graph of the percentage of HC mRNA in each polyribosome fraction at each treatment time point. B) Graphs of HC mRNA as in part A, with RNA from either mock or wild type RhCMV infection treated with hippuristanol for the indicated time. C) HC mRNA graphs as above, after infection with RhCMV either with or without VIHCE present.

The previous result indicated that VIHCE may cause ribosome retention on HC mRNA. In order to examine if this was the case, we infected cells with RhCMV with and without VIHCE and compared HC mRNA ribosome runoff. With VIHCE present, there was a slight delay in ribosome falloff seen after the 5 min hippuristanol treatment (Fig 3.5C). However, by 7 minutes virtually all the ribosomes had dissociated from HC mRNA in both infections. Since it is unlikely that such a slight delay in ribosome dissociation from HC mRNA in the presence of VIHCE could itself account for a dramatic reduction in protein synthesis, we conclude that VIHCE does not significantly affect ribosomal runoff from MHC-I mRNA.

Taken together these data suggested that HC mRNA is transcribed normally in RhCMV-infected cells and that protein translation is not inhibited at the level of initiation or elongation. However, since full-length HC protein cannot be recovered it seems most likely that HC translation is not completed.

VIHCE requires a primate MHC-I heavy chain signal peptide for efficient targeting

Observations similar to VIHCE were reported for translation inhibition by microRNAs that bind to the 3'-UTR of target transcripts. Similar to VIHCE, mRNAs that are targeted by a given microRNA are found in an active polyribosomal complex but a translated polypeptide intermediate can not be recovered even in the presence of proteasome inhibitors [255]. To examine the possibility that VIHCE targets the 3'-UTR of HCs we tested the ability of VIHCE to block synthesis of HC with or without its native 3'-UTR. Since antibodies to rhesus HCs are not available, and VIHCE is able to block expression of human HCs (Fig 3.6A), we chose to examine VIHCE function on HLA-A3.

To determine whether the 3'-UTR was required for this inhibition we transiently expressed HLA-A3 with or without its native 3'-UTR in TRFs. Following transfection we infected cells with either RhCMV containing VIHCE (ΔRhUS2-11) or RhCMV lacking VIHCE (ΔRhUS2-11,Δrh178). Expression of both HLA-A3 carrying the native 3'-UTR or HLA-A3 carrying a heterologous vector-derived 3'-UTR sequence was reduced in the presence of VIHCE (Fig 3.6B). Since the 5'-UTR was vector-derived in both constructs, we conclude that VIHCE does not target the native UTRs of HC mRNA.

Translation of type I transmembrane proteins such as HC is dependent upon an N-terminal signal peptide (SP) that mediates translocation across the ER membrane. Upon translation initiation, the SP is recognized by the signal-recognition particle (SRP) which binds to the SP and arrests translation. This is followed by docking of the translation complex to the SRP-receptor which aids the transfer of the ribosomal/mRNA/nascent polypeptide complex to the SEC61 translocon [256]. Translation then resumes and the nascent polypeptide chain is imported into the lumen of the ER. The fact that VIHCE requires the HC coding sequence suggested that the HC protein might be at least partially translated and that VIHCE acts on the nascent polypeptide. Compared to human HC, we observed that the murine MHC-I molecule H2-K^b was more resistant to VIHCE (data not shown). We hypothesized that this resistance was encoded in the amino-terminus of H2-K^b, specifically the SP. To test this hypothesis we replaced the SP of HLA-A3 with that of H2-K^b. As a further control, we also introduced the SP of CD4 which is more divergent from the HLA-A3 SP (Fig 3.6C). In both instances we observed that expression of the chimeric protein was much less reduced by virus expressing VIHCE compared to native HLA-A3. Remarkably, the SP of K^b is quite similar to that of HLA-A3, yet

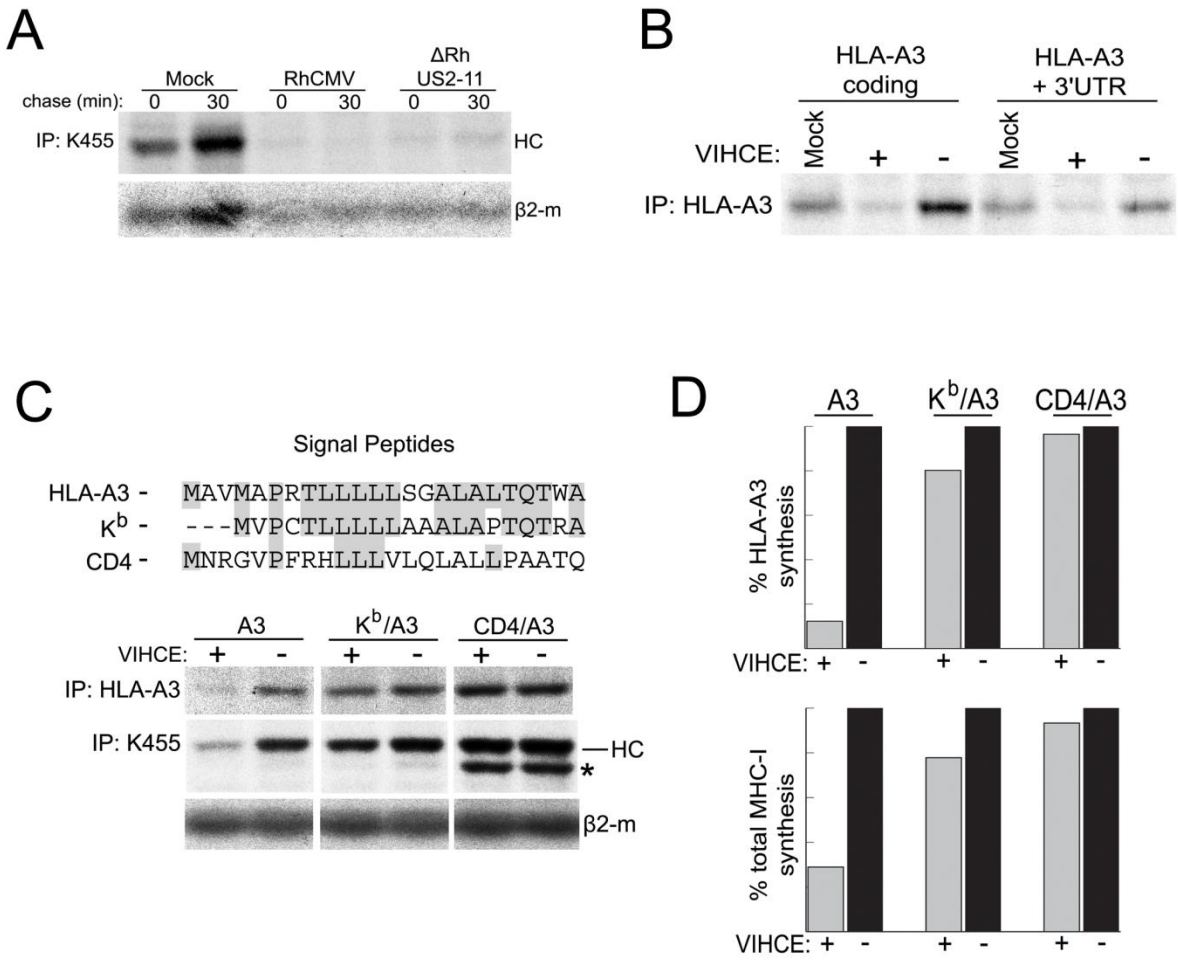

A
Mock
RhCMV
ΔRh US2-11
chase (min): 0 30 0 30 0 30
IP: K455
HC
β2-m
B
HLA-A3 coding
HLA-A3 + 3'UTR
VIHCE: Mock + - Mock + -
IP: HLA-A3
C
Signal Peptides
HLA-A3 - MAVMAPRTLLLLLSGALALTQTWA
Kb - ---MVPCTLLLLLAAALAPTQTRA
CD4 - MNRGVPFRHLLLVLQLALLPAATQ
A3
Kb/A3
CD4/A3
VIHCE: + - + - + -
IP: HLA-A3
IP: K455
HC
*
β2-m
D
A3
Kb/A3
CD4/A3
% HLA-A3 synthesis
VIHCE: + - + - + -
% total MHC-I synthesis
VIHCE: + - + - + -

Fig 3.6 VIHCE requires the primate MHC-I signal peptide for efficient targeting. A) Rh178 inhibits expression of human HC. THFs were infected with the indicated virus at MOI=3 for 24 hours, followed by a 10-min pulse-label, a chase of 30-min and IP with K455. B) UTR-independent inhibition of HLA-A3 expression by rh178. TRFs were electroporated with pEF1α containg the indicated HLA-A3 construct. After 24 hours, cells were either mock infected or infected with recombinant RhCMV (MOI=3) containing VIHCE (+; ΔRh182-189) or lacking VIHCE (-; ΔRh178, ΔRh182-189). After an additional 24 hours, cells were labeled for 30-min, lysed in NP-40, and HLA-A3 was immunoprecipitated. C) Upper panel: Amino acid sequence of the signal peptides used in chimeric HLA-A3 HCs. Gray shading indicates identity with the HLA-A3 signal peptide. Lower panel: TRFs were electroporated with native HLA-A3 (A3) or the indicated SP-chimera (the HLA-A3 signal peptide was replaced with the H2-K^b or the CD4 signal peptide in K^b/A3 or CD4/A3, respectively) prior to infection with RhCMV, metabolic labeling and IP as in 7B. (*) indicates an uncharacterized HC-band that appears prominently in IPs from CD4/A3 transfectants and that could represent a deglycosylated or truncated HC. D) Quantification of HLA-A3 or total HC expression from 7C shown as a percent relative to HC levels in the absence of VIHCE. All experiments are representative of several replicates.

expression of HLA-A3 with the K^b SP was restored to almost the same levels as observed for the CD4 SP (Fig 3.6D). Therefore, we conclude that the SP of primate MHC-I is required for VIHCE to inhibit HC translation. The fact that VIHCE requires the MHC-I SP further suggests that VIHCE interferes with SP-dependent translocation which would lead to translation arrest and rapid, co-translational destruction of the resulting protein fragments.

Discussion

Here we report that VIHCE, the unique RhCMV inhibitor of MHC-I antigen presentation, is able to specifically block the complete translation/translocation of MHC-I heavy chains by targeting the signal peptide sequence of the molecule. This is the first description of a viral protein that is able to specifically block translation of a cellular immune protein. This is also the first description of any protein that blocks translation/translocation by signal sequence recognition. There is, however, a description of a small molecule that can specifically inhibit cotranslational translocation whose specificity is determined by the targets signal peptide (Chapter 5, [257]).

The phenotype caused by VIHCE is somewhat unusual. Generally it is a knockdown in HC protein synthesis, similar to what is seen with inhibitory RNAs. However, a strange aspect of this knockdown is the detection of actively translating ribosomes on the HC mRNA. Furthermore, the polysome analysis suggests a rather high occupancy of ribosomes on the mRNA, similar to levels seen in cells without VIHCE. This implies translation occurring at a near normal rate. Yet very little complete protein is detected, possibly pointing to co-translational degradation as a strong potential

mechanism. A more detailed analysis of possible VIHCE mechanisms is found in Chapter 5.

The peptide binding domain of MHC-I molecules is very polymorphic in order to accommodate a higher number of antigenic peptides. Conversely, the signal peptide is highly conserved not only between MHC-I alleles within a species, but also between related species. One reason for this conservation is that a segment of the cleaved signal peptide is presented on the non-polymorphic HLA-E molecule to the negative signaling receptor CD94/NKG2A or C of NK cells [258]. This relationship within the immune response prevents significant changes from being made within the MHC-I signal peptide and thus makes it an attractive stable target for a virus. Additionally, this particular peptide sequence appears to be limited to MHC-I heavy chains, as searches for other proteins that contain a similar sequence have yet to identify any close matches.

CHAPTER 4

The US2-11 homologous region in RhCMV is necessary for reinfection, but not primary infection of rhesus macaques

Introduction

The species specificity of cytomegaloviruses, and thus the lack of an animal model for HCMV, limits the *in vivo* study of HCMV genes to their homologs in CMVs of other species. Non-human primate (NHP) models provide an excellent model system for studying infectious disease. NHPs are much more closely related to humans than small animal models, plus they are an outbred population. Rhesus macaques are one such NHP model commonly used for many biological questions. They also harbor their own cytomegalovirus, RhCMV. RhCMV is closely related to HCMV and contains homologs to the HCMV US6-family of MHC-I antigen presentation inhibitors as well as a single unique MHC-I modulator.

The HCMV inhibitors of antigen presentation (VIPRs) are able to prevent cytotoxic T lymphocyte (CTL) lysis in cell culture. At least one VIPR has also been shown to prevent viral antigen presentation *in vivo* using the MCMV model [236]. Larger questions remain, however, about the overall role of inhibiting MHC-I antigen presentation for CMV infection *in vivo*. In the mouse model, MCMV lacking all of its VIPRs is still able to infect, replicate and persist in the animal [238]. There is however a reduction in viral titers in the salivary gland [240].

Because of the greater similarity between humans and rhesus macaques, and thus HCMV and RhCMV, we used the RhCMV model to study the role of CMVs inhibitors of

MHC-I antigen presentation *in vivo*. We wanted to address several questions. First was whether RhCMV lacking its MHC-I inhibitors could infect, replicate and persist in naïve rhesus macaques. While this had been demonstrated in the mouse model, it was possible that a different result may be obtained in the NHP model. Additionally, we wanted to compare the immune response to an MHC-I evasion mutant with that generated to WT RhCMV. Since VIPRs prevent T cell recognition, one might expect a better T cell response to a virus that cannot inhibit antigen presentation.

A third question regarding VIPRs *in vivo* is whether they play a role in superinfection. Superinfection, or reinfection, is the phenomenon of a pathogen positive host being infected again by a variant of the same pathogen. CMVs are able to reinfect already CMV positive hosts with additional strains. This has been demonstrated in both MCMV, HCMV, and RhCMV [44, 241, 242]. It is possible that in the presence of existing CMV infection and an existing CMV immune response that VIPRs are required for CMV reinfection. Here we address these questions by comparing infection in rhesus macaques between wild type RhCMV and RhCMV lacking some or all of its VIPRs.

Results

Characterization of recombinant RhCMVs used *in vivo*

In order to evaluate the role of MHC-I antigen presentation inhibitors during CMV infection *in vivo*, we created recombinant RhCMVs that lack the VIPRs. We initially created two viruses: a "double-knockout" RhCMV lacking both the US6-homologous Rh182-189 region and VIHCE; and a "single-knockout" RhCMV that lacks only the US6-homologous Rh182-189 region (Fig 4.1A). Since these viruses would

ultimately be used in animals that already were CMV-infected, we also engineered both mutants to express an exogenous antigen. In this case, we used a codon-optimized SIVgag under control of the constitutively active EF1α promoter. The SIVgag was placed in the deleted Rh182-189 region. This allows for specific tracking of the mutant viruses and the immune response to them compared to WT RhCMV.

The single-knockout virus was created using the RhCMV BAC as described in the materials and methods with the exception that the pCP015 plasmid, and consequent PCR product for recombination, also contained the SIVgag expression cassette. After removal of the Kan^r cassette, the single-knockout was used as a parental strain to make the double-knockout. Rh178 was then deleted by recombination. However, because of the residual FRT recombination sequence left after deletion of Rh182-189, a different Kan^r cassette was used. pOriF5 contains a Kan^r cassette flanked by the mutated F5 FRT sites. These will not recombine with the wild type FRT sites, and this eliminates the possibility of unwanted FRT recombination.

Both recombinant BACs were screened by restriction digest and confirmed to have intact viral genomes (data not shown). PCR analysis and direct sequencing of the region of deletion in the BACs also confirmed that the regions of interest were removed from the genomes, and that the SIVgag expression cassette was in place. After the reconstitution of virus by electroporation into TRFs, we characterized their growth and gene expression profiles. Semi-quantitative RT-PCR confirmed that the ORFs surrounding those deleted were still expressed from the viruses, including Rh181 and Rh190 (Fig 4.1B). Other viral

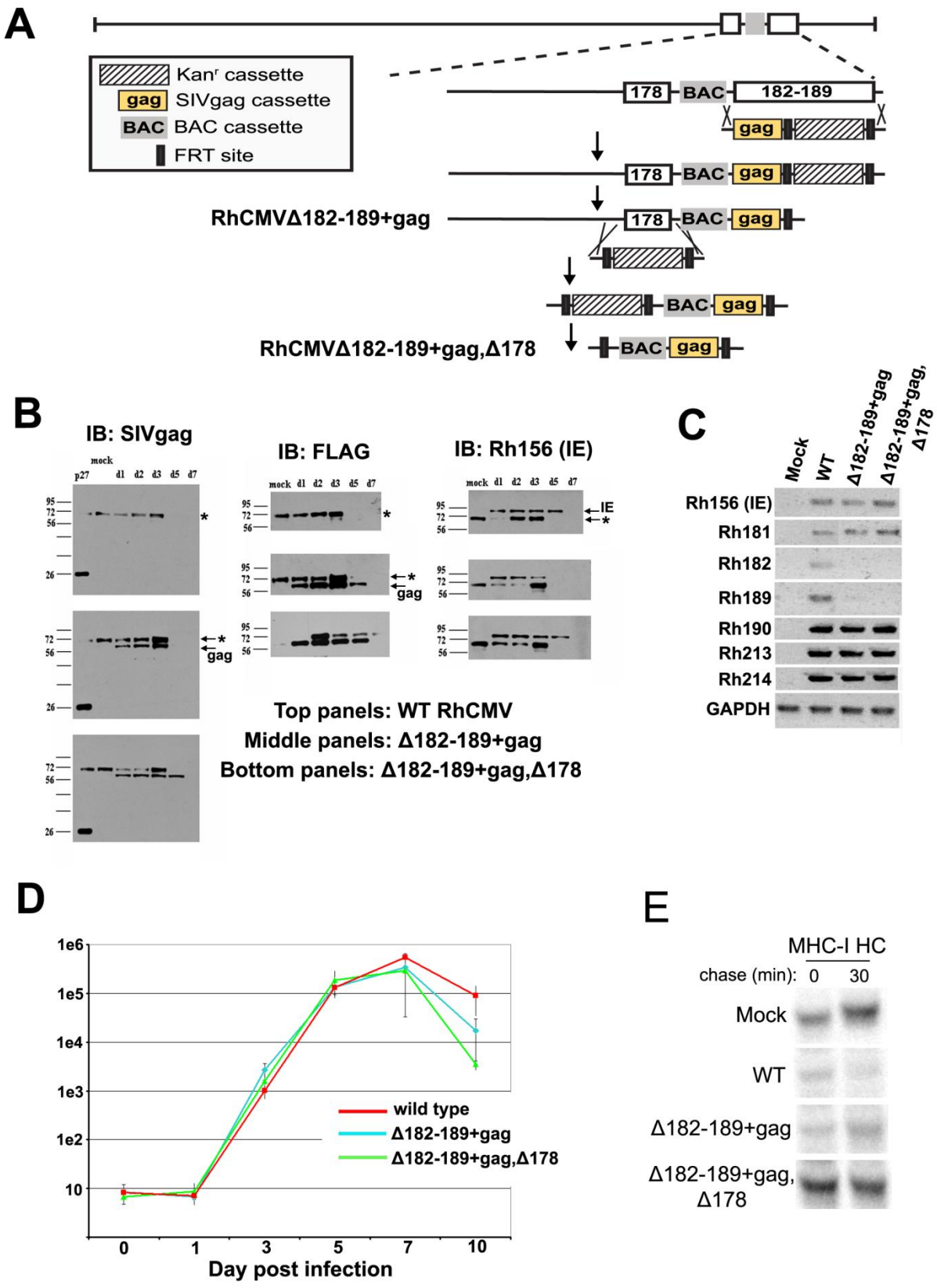

A
Kanr cassette
gag SIVgag cassette
BAC BAC cassette
FRT site
178
BAC
182-189
gag
RhCMVΔ182-189+gag
RhCMVΔ182-189+gag,Δ178
B
IB: SIVgag
IB: FLAG
IB: Rh156 (IE)
mock
p27
d1 d2 d3 d5 d7
gag
IE
Top panels: WT RhCMV
Middle panels: Δ182-189+gag
Bottom panels: Δ182-189+gag,Δ178
C
Mock
WT
Δ182-189+gag
Δ182-189+gag, Δ178
Rh156 (IE)
Rh181
Rh182
Rh189
Rh190
Rh213
Rh214
GAPDH
D
1e6
1e5
1e4
1e3
1e2
10
0 1 3 5 7 10
Day post infection
wild type
Δ182-189+gag
Δ182-189+gag,Δ178
E
MHC-I HC
chase (min): 0 30
Mock
WT
Δ182-189+gag
Δ182-189+gag, Δ178

Fig 4.1 Creation and characterization of RhCMVs lacking VIPRs and expressing SIVgag. A) Schematic of the construction of RhCMVΔ182-189+gag and RhCMVΔ182-189+gag, Δ178. The EF1α promoter-driven SIVgag cassette was inserted where Rh182-189 was removed. For details see materials and methods. B) Western blot analysis of infected cell lysates. TRFs were infected with either wild type RhCMV or recombinant RhCMVs and immunoblotted (IB) for SIVgag, FLAG, or Rh156. The SIVgag construct is FLAG-tagged at its carboxy terminus. (*) indicates an ~70kDa protein that cross-reacts with our secondary antibody. C) Semi-quantitative RT-PCR. cDNA was synthesized from total RNA isolated from infected cells at 24hpi, and PCR performed with the indicated primer sets. D) Multi-step growth curve for recombinant RhCMVs. TRFs were infected at an MOI=0.1 and supernatant harvested and titered at the indicated times. E) Pulse-chase analysis of cells infected with the indicated RhCMV. At 24hpi, cells were labeled for 10 min and chased for the indicated times. HC was immunoprecipitated with the K455 antiserum.

transcripts Rh156(IE1), Rh213, and Rh214 were also expressed, while PCRs for Rh182 and Rh189 were negative (Fig 4.1B).

Western blot of infected cell lysate confirmed that both SIVgag and the IE1 homolog Rh156 proteins were expressed (Fig 4.1C). Since the SIVgag construct includes an epitope tag, it could be detected both by an SIVgag specific antibody and the FLAG epitope antibody. Rh156 was detected by specific antibody. The growth properties of the viruses were characterized in tissue culture by multistep growth curve. TRFs were infected at an MOI=0.1 and viral release into the supernatant measured over 10 days. Growth of both recombinant viruses was comparable to the growth of the WT RhCMV virus (Fig 4.1D). Lastly, each recombinant was characterized with regards to its ability to affect MHC-I expression. As expected, pulse-chase analysis revealed that the single-knockout, lacking only the US6-homologous region, was still able to block expression of MHC-I heavy chains through VIHCE (Fig 4.1E). Conversely, MHC-I heavy chain expression was not affected in cells infected with the double-knockout lacking both the US6-homologous region and VIHCE (Fig 4.1E). We conclude that the recombinant single knockout (RhCMVΔ182-189+SIVgag) and double knockout (RhCMVΔ182-189+SIVgag,Δ178) viruses have no genomic, growth, or gene expression defects, and they each have the expected phenotype with regards to MHC-I expression.

RhCMV VIPRs are not required for primary infection of rhesus macaques

An initial question regarding the RhCMV MHC-I evasion mutants is whether or not they can infect and replicate, at least initially, in animals that have no anti-CMV immune response. We examined whether RhCMV lacking all or most of its MHC-I

inhibitors could infect CMV naïve animals by measuring the CD4+ T cell response, CD8+ T cell response, and antibody response. The presence of an anti-CMV T cell response is indicative of at least some viral replication, as UV-inactivated RhCMV that does not replicate *in vivo* does not induce a measurable T cell response (L. Picker, personal communication).

Six CMV naïve rhesus macaques were used for this study. Two animals received WT RhCMV+SIVgag, two animals received the single-knockout virus RhCMVΔ182-189+SIVgag, and two received the double knockout RhCMVΔ182-189+SIVgag,Δ178. All viruses were given at a dose of 10^7 PFU subcutaneously. There was no indication of any abnormal inflammation at the site of infection.

Probably the best and most sensitive indication of virus replication is the induction of a virus-specific T cell response. Lymphocytes were collected from peripheral blood (PBMC) and by bronchoalveolar lavage (BAL), a technique involving flushing lymphocytes from the lung. Collected cells were stimulated *ex vivo* with RhCMV or gag antigen, and CD4+ and CD8+ lymphocytes were sorted and analyzed for expression of TNF-α and CD69, an activation marker. A CMV-specific CD4+ T cell response (Fig 4.2) was observed in all three infections, indicating that all three viruses were able to infect and replicate in the infected animals. Both anti-SIVgag CD8+ and CD4+ responses were also detectable in both PBMC and BAL (Fig 4.3), indicating successful expression of the exogenous antigen from the recombinant CMVs. Interestingly, the magnitude of the T cell response was similar for both wild type infection and infection with the MHC-I evasion mutants. All the responses were persistent, with a similar percentage of positive lymphocytes out to day 112.

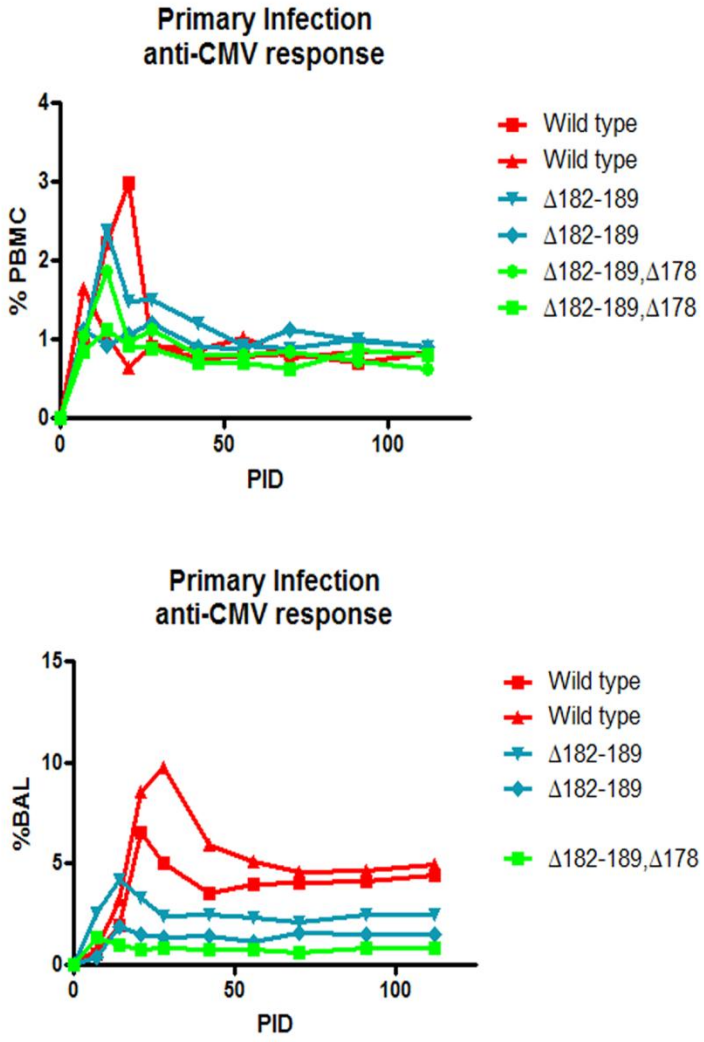


Fig 4.2 Infection of naïve rhesus macaques with RhCMV lacking Rh182-189 induces a CMV-specific CD4+ T cell response. CMV naïve rhesus macaques were infected with 10^7 PFU subcutaneously with the indicated virus (note all viruses also express SIVgag, including the wild type). Lymphocytes were isolated from peripheral blood (PBMC, Top panel) or bronchoalveolar lavage (BAL, Bottom panel) and sorted for CD4+ T cells reactive against total CMV lysate.

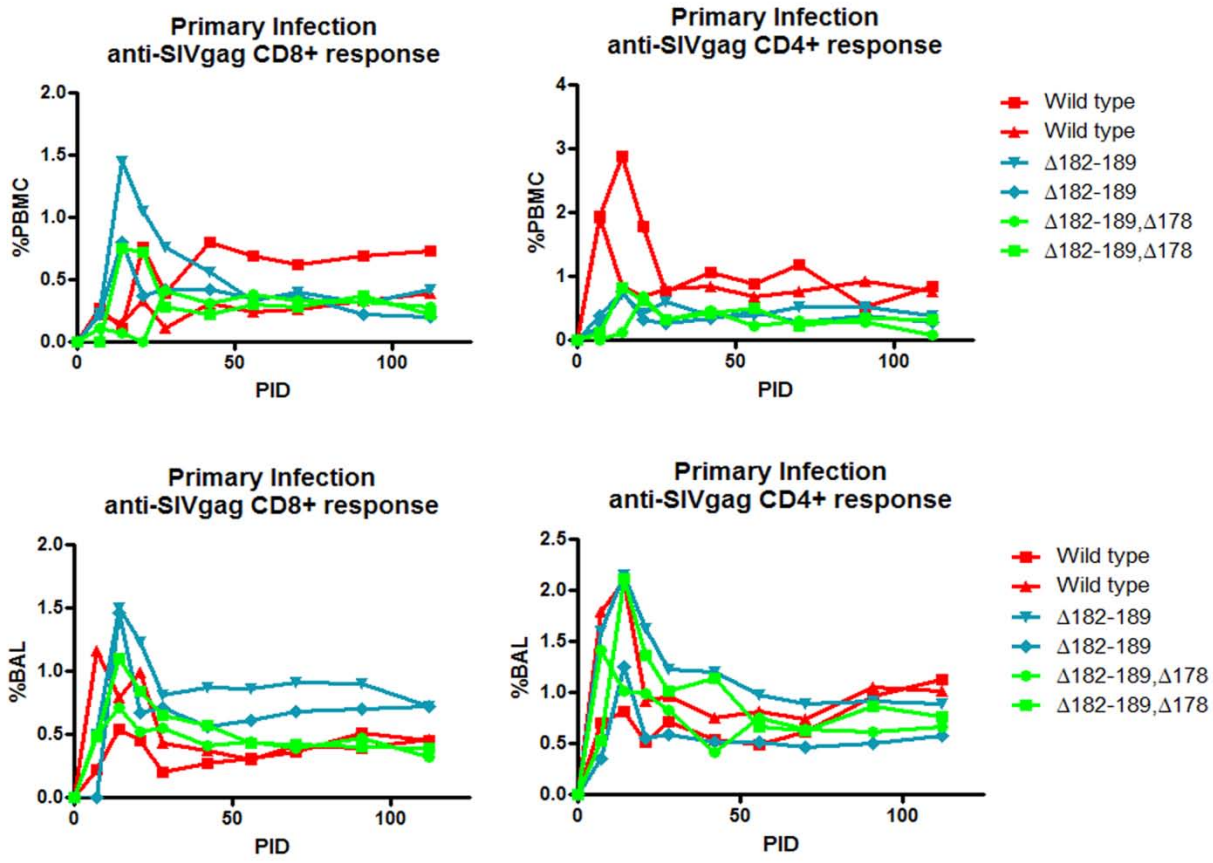


Fig 4.3 Infection of naïve rhesus macaques with RhCMV lacking Rh182-189 induces an SIVgag-specific T cell response. Cells were isolated as in Fig 4.2 and materials an methods. Shown is the percentage of SIVgag reactive CD8+ (left panels) and CD4+ T cells (right panels) in lymphocytes isolated from PBMCs (top panels) and BAL (bottom panels).

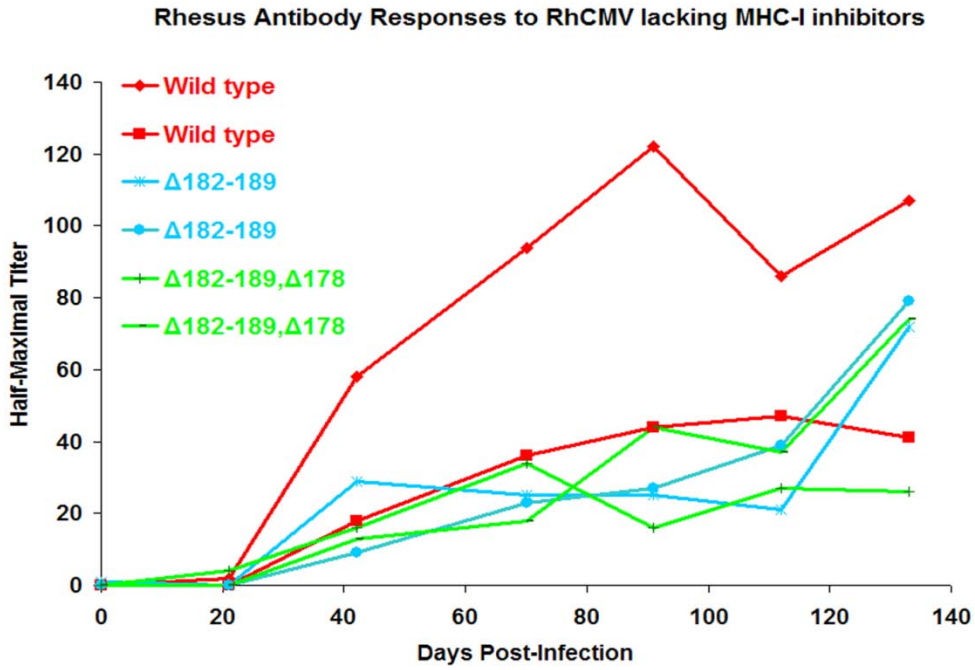


Fig 4.4 Infection of naïve rhesus macaques with RhCMV lacking Rh182-189 induces an antibody response similar to wild type infection. CMV-specific antibodies were titered by ELISA at the indicated times after RhCMV infection of naïve rhesus macaques.

The antigen specific antibody titers in sera were measured by ELISA. The CMV-specific antibodies in all infected animals indicates successful seroconversion (Fig 4.4). The antibody response in the animals infected with the MHC-I evasion mutants was similar to that seen in WT RhCMV infection.

Taken together, these data indicate that the RhCMV inhibitors of MHC-I antigen presentation are not required for primary infection of rhesus macaques. By these measures, the induction of the T cell and antibody response to infection with the MHC-I evasion mutants appears to be like WT RhCMV infection. While the results thus far are similar to those seen in the mouse model, it is not yet clear if the virus is able to persist in the host or if there is a difference in viral titers in the saliva as seen with the MCMV mutants [240].

Reinfection of CMV positive rhesus macaques requires the RhCMV VIPRs

Cytomegaloviruses have the remarkable ability to reinfect a seropositive animal despite the presence of a strong anti-CMV immune response. This reinfection has been demonstrated in rhesus macaques as well [259]. Since there is such a large T cell response generated to viral infection that cannot prevent reinfection of a new strain, we questioned whether the viral inhibitors of MHC-I antigen presentation played a role in CMVs ability to reinfect. This issue had not been examined in other CMV animal models.

This study used six rhesus macaques. All six animals had been naturally infected with WT RhCMV present in the colony. Animals were then experimentally reinfected with 10^7 PFU of RhCMV subcutaneously. Two animals were reinfected with WT

RhCMV+SIVgag. Four animals were initially infected with the double-knockout RhCMVΔ182-189+SIVgag,Δ178.

Animals reinfected with the WT RhCMV+SIVgag developed a strong anti-SIVgag CD8+ and CD4+ (Fig 4.5) T cell response. Reinfection virus was also being shed in urine as shown by the detection of SIVgag expression virus in co-culture assay. In contrast, animals reinfected with RhCMVΔ182-189+SIVgag,Δ178 did not induce a detectable immune response. Neither SIVgag-specific CD8+ or CD4+ cells were recovered from PBMCs or BAL (Fig 4.5). Additionally there was no SIVgag-expressing virus detected in the urine by co-culture assay. To rule out the possibility of an error in protocol, the same four animals were challenged again with the double-knockout RhCMVΔ182-189+SIVgag,Δ178 after 91 days. Again no T cell response was detected. This indicates that RhCMV lacking all of its MHC-I evasion genes is unable to reinfect a rhesus macaque with an existing anti-CMV immune response. The lack of a T cell response is indicative of a lack of any significant virus replication.

In order to determine if the RhCMV-specific MHC-I inhibitor VIHCE was sufficient to rescue RhCMV in reinfection, we next challenged the same four rhesus macaques with 10^7 PFU of the single-knockout RhCMVΔ182-189+SIVgag. However, the single-knockout RhCMVΔ182-189+SIVgag also failed to induce a CD8+ or CD4+ T cell response in either PBMC or BAL. The same four animals were again challenged with the single-knockout RhCMVΔ182-189+SIVgag 70 days later, and again no T cell response was induced. Together these data suggest that VIHCE is not sufficient for RhCMV reinfection, and that the US2-US11 homologous region in RhCMV is necessary

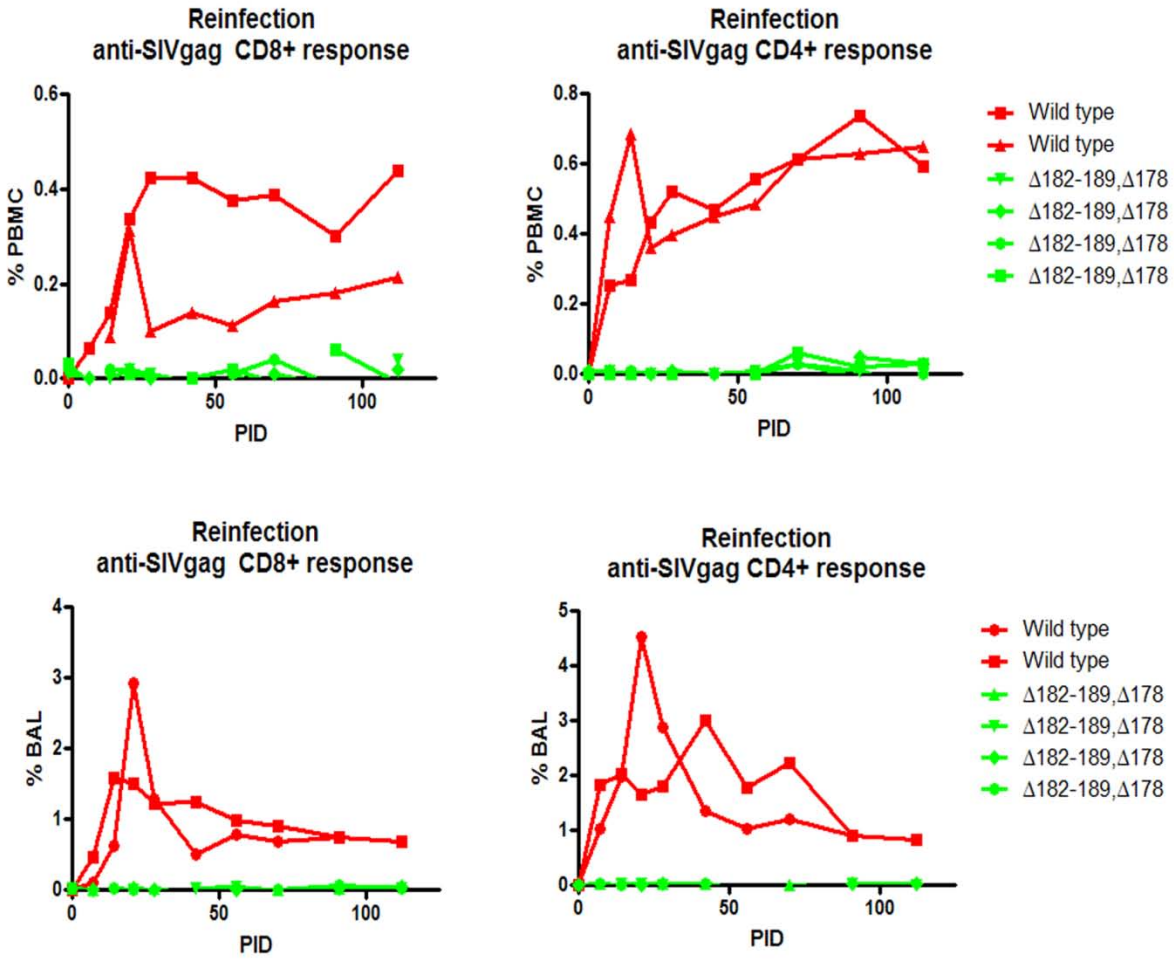


Fig 4.5 Reinfection of CMV-seropositive rhesus macaques with RhCMV lacking Rh182-189 fails to induce an SIVgag-specific T cell response. RhCMV seropositive rhesus macaques were infected with 10^7 PFU subcutaneously with the indicated virus. Lymphocytes were isolated and stimulated as described in materials and methods. Shown is the percentage of SIVgag reactive CD8+ (left panels) and CD4+ (right panels) in lymphocytes isolated from PBMCs (top panels) and BAL (bottom panels).

for RhCMVs ability to reinfect a rhesus macaque with an existing anti-CMV immune response.

Discussion

In this chapter we have analyzed infection of rhesus macaques with RhCMV lacking MHC-I inhibitory proteins. In doing so we have identified a previously unreported role for CMV inhibitors of antigen presentation (VIPRs), in that they are necessary for RhCMV reinfection of seropositive hosts. We have also found that similar to mice infected with MCMV lacking its VIPRs, lack of VIPRs in RhCMV seemed to have no effect on the kinetics or magnitude of the immune response during primary infection. This is the first study analyzing the *in vivo* role of the US6-family of proteins, and has established RhCMV as a viable model for studying CMV inhibition of antigen presentation *in vivo*.

The fact that the immune response to primary CMV infection is similar regardless of the presence of MHC-I inhibitors indicates a greater role for other viral immunomodulators during this time. CMV contains a myriad of innate immune response inhibitors, and these likely play the greater role in suppressing CMV-immunity immediately upon primary infection. The MHC-I inhibitors, our data suggest, are more important in protecting the virus from existing immunity. It will be interesting to observe if the virus persists in these primary infections in the face of the established immunity, and whether memory inflation will occur as during wild type RhCMV infection.

Another suggestion from these results is the limited role of direct presentation in priming the immune response. This would imply that indirect presentation, or cross-

presentation, is the major mechanism for priming anti-CMV immunity. Cross-presentation occurs when phagocytic cells such as macrophages and dendritic cells take up fragments of protein from infected cells that have died from infection. These antigenic fragments can then enter both the MHC-I and MHC-II antigen presentation pathways, and thereby stimulate naïve CD8+ and CD4+ T cells. The phagocytic cells themselves are not infected, but are able to present antigenic peptide from other cells. Since the priming of the T cell responses is the same regardless if CMV is inhibiting direct antigen presentation, cross-presentation is a likely alternative.

We demonstrate that deletion of the Rh182-189 region, regardless of the presence of VIHCE, renders RhCMV unable to reinfect a seropositive animal. This implicates the CMV US6-family in avoiding recognition from preexisting T cells. It is possible that in deleting Rh182-189 we inadvertently deleted a protein involved in some other critical innate immunomodulatory function, and that is the reason for not establishing re-infection. This seems unlikely however, since the virus lacking Rh182-189 was able to infect naïve animals with functional innate responses. It is more likely that the inability to reinfect is a result of the inability to escape the existing T-cell response, and that RhCMV is eliminated before it is able to establish infection.

CHAPTER 5

Discussion and Future directions

In this dissertation we have described the identification and characterization of viral inhibitor of heavy chain expression (VIHCE), a novel immunomodulatory protein encoded by RhCMV. VIHCE represents not only a novel mechanism for viral inhibition of antigen presentation, but also protein translation in general. This is the first description of a protein that prevents the complete translation of a specific substrate by targeting the signal peptide.

Perspectives on VIHCE

Viruses utilize multiple mechanisms to regulate host protein expression. The viral inhibitors of MHC-I alone indicate the myriad of ways even one single virus can manipulate a cellular pathway to block normal host protein expression. However, this viral inhibition is not limited to post-translational effects. Transcriptional shutoff is also a common tactic, as is regulation of translation. Translational regulation itself can occur by multiple means, from inhibiting initiation, 5' cap-snatching, attacking poly-A binding protein, or miRNA mediated translational suppression and mRNA cleavage.

Preventing synthesis of host cell proteins is a common mechanism utilized by many viruses. RNA viruses in particular have long been known to perform host-shut off mechanisms during productive replication. However, these mechanisms have largely been a generalized shut-off of host cell protein synthesis, rather than specifically targeting a protein or subset of proteins. Picornaviruses, for example, encode a proteinase

that cleaves host translation initiation factors so that cap-dependent translation is blocked [260]. Since picornaviral proteins are translated using internal ribosome entry sites (IRES), this limits competition for other cellular metabolic components and favors translation of viral proteins. This type of generalized shut-off has been demonstrated in many other viruses, including rotaviruses, adenoviruses, and alphaviruses [261].

While translation inhibition of a specific protein by a viral protein had previously not been reported, gene silencing by RNAs can lead to the same end result. Inhibitory RNAs have now been identified as important regulators of protein expression in virtually all biological systems, including viruses [262]. MicroRNAs (miRNAs) are short (~22nt) non-coding RNAs that are derived from larger, stem-loop forming precursors. The miRNAs associate with ribonucleoproteins to form a miRNA-induced silencing complex, or RISC, and target mRNAs by complementarity within the 3' UTR of the target. A precise match results in cleavage of the target mRNA, while an imprecise match typically leads to the repression of protein synthesis. The mechanism by which protein synthesis is inhibited is not fully understood. Several different posttranscriptional repression methods have been suggested. These include cotranslational degradation [255] and premature termination [253], among others [263].

In one report, Nottrott et al. [255] demonstrated that miRNAs cause a strong decrease in protein expression despite having no effect on the mRNA. Additionally, the target protein could not be stabilized with proteasomal inhibitors, and the target mRNA was associated with actively translating ribosomes that "ran-off" upon blocking translation initiation. These data exactly parallel what we observe in the presence of VIHCE. The researchers hypothesized that the target protein was rapidly degraded upon

translation and attempted to detect any protein fragments by placing an epitope tag on the amino-terminus. However, they were unable to detect any fragments in the presence of the miRNA. They thus concluded that the miRNA was either inducing co-translational degradation of the protein or masking the protein, even though they presented no direct evidence for this.

In a similar study, Petersen et al. [253] showed small interfering RNAs (siRNAs) repressed protein synthesis without affecting target mRNA, and that the target mRNA was associated with actively translating ribosomes. However, upon performing ribosome run-off assays, they observed a moderately increased rate of ribosome dissociation from target mRNAs in the presence of the siRNA. They concluded that the siRNA was causing premature ribosome dissociation from targeted mRNAs. This is somewhat questionable, however, since the increased fall-off of ribosomes was rather weak. Additionally, the steady state levels of polysome mRNA were identical with or without siRNA, and one would expect that if ribosomes were falling off the target mRNA faster, the steady state polysome distribution would be shifted towards fewer ribosomes. Regardless, both of these inhibitory RNA studies indicate a phenotype similar to VIHCE.

It is clear that VIHCE is acting as a protein and is not an inhibitory RNA. Additionally the observation of similar phenotypes does not guarantee the same mechanism for translational suppression. However it does not rule out the possibility that VIHCE interacts with the same cellular machinery as inhibitory RNA complexes such as the RISC complex. A major question for both VIHCE and inhibitory RNAs is what becomes of the polypeptide that is, at least partially, synthesized. The two scenarios

proposed above are co-translational degradation of the HCs or partial translation resulting in small HC fragments unrecognizable by our antibodies.

Co-translational degradation has been shown to be mediated by the proteasome, and it has been estimated that 50% of nascent proteins are co-translationally degraded in cells [251]. While we are unable to detect HC in the presence of proteasomal inhibitors, it is possible that proteasomes still mediate HC destruction. VIHCE could conceivably directly link the HC to the proteasome, perhaps requiring an active proteasome for HC destruction. In that regard, it would be interesting to examine the polyribosome distribution on MHC-I HCs in the presence of proteasomal inhibitors. Alternatively, co-translational degradation may be mediated by other cellular proteases. Cytosolic proteases have multiple functions, and are known to contribute in processing intracellular antigens that can end up in the MHC-II pathway [264]. VIHCE may redirect the nascent HC to another cellular protease.

A second possibility that would explain the active ribosome association with MHC-I HCs would be incomplete translation. A similar situation was recently observed for non-stop RNA. In these experiments, translational arrest resulted in protein fragments that are rapidly degraded by the proteasome [265]. Therefore, it is possible that HC translation intermediates are degraded by the proteasome despite the fact that we were unable to detect a degradation intermediate in the presence of proteasome inhibitors. Possible reasons why such breakdown products were not identified are their potentially small and heterogenous size and their extremely rapid degradation. HC-derived intermediates might also lack the epitopes recognized by the HC-specific antibodies used in this work. To test if this was the case, an amino-terminus epitope-tagged HC construct

could be designed to identify any small protein fragments that may not otherwise be identifiable. These may not be identifiable, however, since this same strategy failed to detect any protein fragments during miRNA-mediated translational suppression as mentioned above.

How might VIHCE be acting?

Since very little full length MHC-I HC is detectable when VIHCE is present, it is possible that HC is not translocated into the ER. The specific inhibition of ER translocation by a protein has not been described. However, a small molecule has been identified that inhibits the co-translational translocation of vascular cell adhesion molecule 1 (VCAM-1). Cotransin, as well as CAM741, a molecule of similar structure, were identified in screens for inhibitors of cell adhesion molecules [257, 266]. Interestingly, these molecules inhibit VCAM-1 translocation in a signal peptide-dependent manner. Initially it was thought that this inhibition was selective for VCAM-1. Subsequently it was identified that CAM741 can also block translocation of vascular endothelial growth factor (VEGF) [267]. Yet the results from these studies suggest at least an extremely narrow target range for the molecules.

Like VIHCE, cotransin/CAM741 did not affect the mRNA of its target, nor affect ribosome binding, yet very little protein was recoverable. Through *in vitro* translocation assays using pancreatic microsomal membranes, both groups were able to show a block in translocation that was dependent upon the signal peptide. Translocation of a chimeric molecule with an alternate SP was not blocked, and transferring the VCAM-1 SP to another molecule blocked its translocation when cotransin/CAM741 was present [257,

266]. Subsequently it was determined that the VCAM-1 SP was incorrectly associating with Sec61β in the translocon in the presence of the compounds [268]. This resulted in the translation of the VCAM-1 protein into the cytosol. In contrast with VIHCE, however, VCAM-1 could be stabilized with proteasomal inhibitors. This indicates a mechanism that upon blocking translocation, the resultant protein is fully translated into the cytosol where it is subsequently destroyed by the proteasome. While this doesn't seem to be the case for VIHCE, it is possible that VIHCE acts in a similar fashion in preventing correct SP association with the Sec61 translocon, thereby causing MHC-I HC synthesis in the cytosol where it is degraded in a non-proteasome-mediated fashion. To test this it must first be addressed whether the HC ribosome-SP complex interacts with the translocon at all. This could be achieved through co-immunoprecipitation or chemical cross-linking experiments.

While the wild-type HLA-A3 signal peptide is efficiently targeted by VIHCE, the very similar mouse MHC molecule H2-K^b signal peptide is not. This is somewhat surprising since they only differ at 6 of 21 residues. Certainly one or more of those six residues must be critical for targeting. Similarly, CAM741 was much less efficient at blocking the mouse VCAM-1 SP despite it sharing 17 of 24 residues with the human VCAM-1 [268]. It was then shown that substitution of a single amino acid (V21A) in the murine VCAM-1 was sufficient to increase to CAM741 sensitivity by over 5-fold, and substitution of two amino acids (V21A and L18M) increased sensitivity by over 10-fold. Thus, single amino acid differences between SPs can have dramatic effects on the sensitivity to translocational blocking. And while there is the obvious large difference of cotransin/CAM741 being small, chemical compounds and VIHCE being a protein, these

studies at least provide precedent for translocation inhibition of a narrow target range dependent upon a few important residues in the signal peptide sequence.

There are several questions yet to be answered with regards to the function of VIHCE. Since it seems as though at least the signal peptide is being translated, one potential function of VIHCE would be to prevent SRP association with the SP. Normally, if the SRP did not bind to the SP, one would expect that the protein would be translated into the cytosol, improperly folded, and subsequently degraded. This seems unlikely in the case of VIHCE. On one hand, it would be expected that degradation would take place in a proteasome-dependent fashion. Since proteasomal inhibitors fail to stabilize any HC, it seems as though VIHCE does not mediate proteasomal HC degradation. Another reason this seems unlikely is if HC were translated into the cytosol, we would likely be able to detect the complete protein in very short metabolic labeling experiments. Our HC antibodies are able to recognize misfolded HC, thus the conformation of the HC should make no difference for recognition. Since we are unable to detect any HC even in short labeling times, it seems more plausible that the HC protein is either never completely synthesized or degraded co-translationally by other cellular proteases.

Similarly, VIHCE may not only prevent SRP binding, but also directly bind the SP and ribosome and stall translation. The function of the SRP is to recognize the SP and stall translation until the ribosome can dock at the translocon. It is possible that VIHCE is taking the place of the SRP, stalling HC translation and preventing ribosome docking at the translocon. One way to test this would be to determine if the HC polyribosome complex was membrane associated. Normally the HC polyribosome complexes will be associated with the ER membrane, and thus by isolating membrane and cytosolic

fractions you could determine where the HC polysomes were being translated. A second way to test this would be to perform coimmunoprecipitation experiments or chemical crosslinking experiments to determine if the HC polysome is associating with the Sec61 translocon. Evidence that would argue against this model could include the polyribosome fractionations performed as described in Chapter 3. If VIHCE were stalling translation immediately after SP synthesis, one would expect very few ribosomes associated with the mRNA since the SP sequence is only 72 base pairs. However, we observed polysome sedimentation at nearly the same density in cells with or without VIHCE present (Fig 3.4A), suggesting a high number of ribosomes on the mRNA.

Since we have shown VIHCE to be ER-associated, it seems more likely that recognition of the SP occurs at the ER membrane rather than in the cytosol with the SRP. VIHCE could then potentially prevent SRP binding to the SRP-receptor. This would predictably result in the HC polysomes fractioning with the cytosol, rather than membranes. Similarly, VIHCE may prevent SP association with the Sec61 translocon. If either of these was the case, one may expect VIHCE to co-precipitate with either the SRP-receptor or Sec61 components. This would also prevent recognition of HC with Sec61 in chemical cross-linking experiments. Alternatively, VIHCE may prevent the proper association of the SP with the Sec61 translocon, similar to cotransin/CAM741. If this were the case you should observe an association between nascent HC SP and Sec61, and perhaps the cross-linking pattern with and without VIHCE may differ.

These previous possible mechanisms are based on the assumption that the active portion of VIHCE is facing the cytosol. Although this is the predicted topology, it is possible that the portion of VIHCE that recognizes the MHC-I HC is on the lumenal side

of the ER. Topology analysis will help determine which of these is correct. If, however, VIHCE is a lumenal ER protein, it must recognize the SP as it enters the lumen through the translocon. VIHCE might then "cap" the translocon and prevent complete synthesis of the HC, or it could redirect the HC for cotranslational degradation by an ER protease.

It is not uncommon for viruses to target a cellular protein with little room for mutation or a slow evolutionary rate. Since viruses evolve much more rapidly than these cellular "Achilles heels", they make for good targets. The extracellular domains of MHC-I, particularly the peptide-binding regions, are highly polymorphic and evolve rapidly. In contrast, the cleaved SP is highly conserved among different MHC-I alleles including the rhesus macaque MaMu and the human HLA genes [269]. Many signal peptides for MaMu-I, MaMu-3 and MaMu-A show less than 3 amino-acids difference to either HLA-A, B or C alleles and some MaMu-SPs are identical to HLA-SPs [270]. A possible reason for the high conservation of HLA signal peptide sequences is the fact that a conserved nonapeptide (VMAPRTLLL in the HLA-A3 sequence) is presented by the non-polymorphic HLA-E molecule to the negative signaling receptor CD94/NKG2A or C of NK cells [258]. This insures that the immune system can monitor normal MHC-I synthesis in the cell. This system seems to be conserved in RM, although some alleles start at the methionine within the peptide [269]. Interestingly, the SP of the HCMV UL40 glycoprotein contains this peptide which is presented by HLA-E in HCMV-infected cells in a TAP-independent fashion [143, 144]. Thus, HCMV is able to subvert this cellular checkpoint with a decoy peptide and allow for normal expression of HLA-E despite also inhibiting normal MHC-I synthesis and TAP transport. This evasion mechanism seems to

be conserved in RhCMV as well. The RhCMV ORF Rh67 encodes the same nonapeptide within its signal peptide, which otherwise shares only 19 % identity with UL40 [66]. It will be interesting to examine the hypothesis that the SP of ORF Rh67 is VIHCE-resistant despite containing this conserved peptide.

The raises another question regarding the fate of the HC protein, and that is what happens to the HC signal peptide? Since VIHCE recognizes the HC SP, the peptide is presumably made. By affecting the SP, VIHCE may end up reducing HLA-E expression. Since the loading of HLA-E requires HC SP cleavage and subsequent peptide processing [271], normal HLA-E expression may indicate that the SP is cleaved and processed, providing some insight into where and how VIHCE is working.

A particularly interesting question from an evolutionary perspective is why RhCMV selected for yet another MHC-I evasion mechanism. Since both RhCMV and HCMV contain the US6-family homologs, this gene family was present in the CMV infecting the common ancestor of humans and rhesus macaques. Since the split of the two species, HCMV did not add any ORFs to its MHC-I evasion repertoire, or it possibly lost VIHCE. Regardless, RhCMV had evolutionary pressure to evolve (or retain) the additional MHC-I inhibitor. One possible explanation for this may have to do with how the rhesus and human MHC-I loci independently evolved. In order to maintain diversity, MHC-I genes are both polymorphic and polygenic. The human MHC-I loci encodes three HCs: HLA-A, HLA-B, and HLA-C. This means each human can express up to six different MHC-I HC alleles, three from each chromosome. The polymorphism in the classical MHC-I molecules is largely within the peptide-binding cleft. Thus human pathogens that target MHC-I molecules need only worry about targeting the conserved

regions of 3 HLA proteins. Rhesus macaques have evolved a much more polygenic MHC-I repertoire. An individual rhesus macaque can have as many as 22 active MHC-I genes [269]. This number can vary widely between individual animals. Thus, a pathogen infecting rhesus macaques has to face a potentially much wider array of MHC-I molecules. These individual MHC-I HCs also seem to lack allelic polymorphism [249], suggesting that rhesus macaques leaned towards diversity in gene number and gene combination rather than the polymorphic allele approach evolved by humans.

The US2 and US11 encoded proteins are known to selectively degrade certain MHC-I alleles. It is not known if the RhCMV homologs to these are also selective. While it is uncertain at this point if VIHCE targets specific MHC-I alleles, it could be predicted that since VIHCE targets the highly conserved SP that its HC targeting would be rather broad. It then stands to reason that *in vivo*, where the US6-family homologs of RhCMV face a high number of different MHC-I molecules, destruction of certain molecules may be incomplete. Thus RhCMV selected for VIHCE, a protein that targets a highly conserved portion of all MHC-I molecules. In cell culture, VIHCE is typically incomplete when expressed by itself in the context of virus infection (See chapters 2-3). In order to more efficiently eliminate MHC-I, RhCMV requires both VIHCE and the US6-family. This necessity for efficient MHC-I inhibition and the complexity of the rhesus macaque MHC locus may help explain the evolution of VIHCE.

Perspectives on CMV MHC-I inhibition *in vivo*

Our data from primary infection of rhesus macaques indicate that RhCMVs MHC-I inhibitors are not necessary for primary infection. These data, thus far, mirror what has been concluded in the mouse model [238]. The findings bring up interesting points about the induction of the CMV immune response and the important viral components during the initial stages of infection. For one it tends to implicate viral inhibitors of innate immunity as being more important during the initial stages of infection, which is not entirely surprising. Furthermore it suggests that the direct presentation of peptides from virally infected cells may not contribute to priming the CD8+ T cell response in any considerable fashion. Last, the persistence of virus may suggest that even after an adaptive anti-CMV response is mounted, the virus is able to hide somewhere and avoid immune eradication even without MHC-I inhibitors.

Innate immunity is the first response to virus infection. Infected cells can be induced into an anti-viral state within the cell when virus is recognized. The recognition of viral infection by a cell is usually through pattern recognition molecules (PRM) that detect the presence of common components of viral infection such as dsRNA, nonmethylated CpG DNA, or ssRNA in the wrong subcellular compartment. The effects of this PRM activation are broad ranging. Among the effects are the maturation of dendritic cells, which enhances antigen presentation and promotes T cell stimulation, and the induction of type 1 interferons (IFN). The IFNs not only induce anti-viral proteins within the infected and neighboring cells, but also stimulate adaptive immunity and drives it towards the anti-viral T_H1 type response. Thus the innate response plays an important role in helping develop the anti-CMV T cell response.

As mentioned briefly earlier, CMV contains a multitude of inhibitors of innate immunity. These viral inhibitors of the innate immune response are likely playing a more important role than the MHC-I inhibitors in the establishment of infection, since RhCMV lacking its inhibitors of MHC-I seems to have no problem establishing itself, at least initially. Since the size of the anti-CMV T cell response is virtually the same during primary RhCMV infection regardless of the presence of MHC-I inhibitors, one could predict that the innate response is also similar. As the viral innate inhibitors are still active in the virus lacking VIPRs, they are likely contributing to not only establishing infection, but perhaps in dampening the adaptive response. So the CMV immune evasion genes may have roles in a sequential manner, parallel to the type of immune response that dominates at certain times after infection.

A second implication from our data and others is that direct presentation of peptide from infected cells plays little role in priming the anti-CMV immune response. As mentioned earlier, cross-presentation results when a non-infected professional antigen presenting cell (APC) such a macrophage, dendritic cell, or B cell, uptakes exogenous antigen obtained from dead, dying, or compromised cells. This antigen is then processed and used to stimulate naïve T cells. This is an important system that the immune system has evolved, in that it largely avoids direct manipulation by pathogens. While viruses can and do attempt to prevent cell death, or they evolve proteins that resist processing in some cases, it is a difficult task to manipulate a cell that it has not directly infected. CMV is known to infect monocytes, the precursor to dendritic cells and macrophages, and it can infect the differentiated APCs themselves [272]. Therefore it was conceivable that upon virus entering the host, professional APCs were infected by CMV, viral antigen

processed, and enough MHC-I expressed to stimulate naïve CD8+ T cells. If this was the case, one would expect that eliminating the viral VIPRs would allow for better direct presentation in these APCs, and a stronger CD8+ T cell response would be mounted. That we do not see a stronger T cell response upon deletion of the VIPRs indicates that this direct presentation pathway likely plays little role in priming the CD8+ T cell response in normal infection.

One unanswered question from our studies is what happens to CMV lacking it VIPRs in a primary infection once the adaptive immune response is generated? While this role of VIPRs during CMV persistence is still undetermined in the primate model, it has been shown in the mouse model that MCMV lacking VIPRs can persist [238]. Should this persistence also occur in the rhesus macaque, it raises some puzzling questions. Why can a virus lacking VIPRs persist in the face of a "typical" immune response, but cannot infect an animal in the face of the same response? Or are the immune responses truly the same? It is possible that while the magnitude of the responses is the same in wild type and VIPR deleted infections, the functionality is somehow different. One way to test this would be to infect a naïve animal with RhCMV lacking its VIPRs, then attempt to reinfect that animal with another RhCMV lacking its VIPRs. If the immune response to the first virus was able to prevent reinfection with the second virus, as you would expect, you could conclude that the immune response was functionally similar to that derived from wild type RhCMV infection. If the responses were functionally similar, the virus must be able to hide somewhere in the host. Perhaps it gains access to an immunologically privileged site and is successfully able to replicate. Alternatively, the other viral immune evasion mechanisms could continue to thwart the immune response

and allow for persistence. It will be important for these studies to not only determine if RhCMV lacking VIPRs can persist, but also determine the level of viremia. We are attempting to quantitate virus shedding to determine if there is a difference in the amount of virus present during infection without VIPRs. Along these lines, it was noted in the mouse model that MCMV lacking its VIPRs replicated to lower titers than wild type within the salivary gland [240]. This suggests yet another role for MHC-I inhibitors, in that they lead to increased virus in tissue that promotes transmission.

It will be interesting to compare the long term infection of RhCMV lacking VIPRs to the wild type in primary infection. As discussed earlier, infection with wild type CMV leads to memory inflation of some antigen specific T cells. The current model is that persistent viral gene expression or perhaps low level replication continues to stimulate T cells over the life of the host, causing a continuous expansion of certain clones. It is conceivable that VIPRs slow this rate of expansion by blocking T cell stimulation. It is also possible that VIPRs are necessary when the CD8+ T cell inflation reaches some critical mass, and that perhaps a virus lacking its VIPRs would eventually be cleared. Unfortunately these are very long term experiments and could not be readily tested in a short time.

Our results from chapter 4 indicate that in the face of an existing immune response, CMV requires the US6-family of VIPRs in order to reinfect. The ability of CMV to reinfect has been suggested for some time due to the identification of multiple strains of the virus in infected humans. Originally this was thought to only occur in those individuals with altered immune systems such as AIDS patients [273, 274], organ

transplant recipients [275], or pregnant women [44, 276]. But infection with multiple strains of CMV has been detected in organ and blood samples from patient necropsy [277], and in women who regularly attended clinics for sexually transmitted disease [278]. Additionally, infection with multiple strains of MCMV has been detected in wild mice [241, 279] and in laboratory mice [279], suggesting that CMV reinfection is not limited to HCMV. Appropriately, reinfection of RhCMV in rhesus macaques has also now been identified (chapter 4 and [259]). HCMV reinfections can have severe clinical outcomes, as the presence of multiple CMV strains during congenital CMV infection strongly correlated with lethal outcome during gestation [280]. Multiple CMV infections in transplant recipients also correlated with increased viral load [281], delayed clearance [282], and graft rejection [281].

Reinfection has clear evolutionary advantages. It expands the target host range of a particular strain to include those with preexisting immunity to the virus. Additionally, recombination may occur between multiple strains of a virus in a single host, allowing for viruses to share genes and alleles that aid in fitness. Also, coinfection with different MCMVs in mice led to a complementation between the strains and increased viral fitness [283]. Reinfection is also not limited to CMV. Infection with multiple strains of other herpesviruses such as VZV [284], HSV [285, 286], KSHV [287], and EBV [288] has been documented. Nor is it limited to herpesviruses, as it also occurs with infections of HIV [289]. It is unknown whether these natural reinfections occur while the host is completely healthy or if perhaps there is a certain level of opportunism associated with it.

While the precise mechanism for reinfection in these systems is not known, reinfection in general seems to occur because of one of three reasons. One is an

inefficient immune response during primary infection by the pathogen. A second is a high degree of antigenic diversity in the pathogen population, as is the case for influenza or respiratory syncicial virus. Here we document a third, which is sufficient ability of the pathogen to avoid immune recognition. CMV can reinfect a host despite containing nearly identical antigenic make-ups and despite an extremely large pre-existing immune response. However, this is dependent upon the ability of CMV to avoid certain immune recognition. In the absence of inhibitors of the MHC-I pathway, RhCMV presumably gets wiped out by the existing anti-CMV T cells before any significant viral replication takes place. When the VIPRs are present, RhCMV is able to sufficiently avoid T cell killing in order to establish itself in the animal. Whether VIPRs are necessary after the initial steps of reinfection is an interesting question. The persistence of RhCMV lacking VIPRs in the primary infection would give an indication of this. However, one could directly test this in a reinfection model by creating a CMV with inducible MHC-I inhibitors. Induction initially upon reinfection should allow for the virus to establish itself. Subsequently turning off the VIPRs would address the question of their necessity after the establishment of reinfection. One could even perform a time course of induction to determine precisely how long VIPRs were needed for reinfection.

The ability of CMVs to reinfect brings up a significant difficulty in the development of a CMV vaccine. Is a vaccine that prevents infection even possible? Does protective immunity to CMV exist? Obviously the immune response that is induced to natural CMV infection, despite its magnitude, is not sufficient to prevent reinfection. There is evidence, however, that pre-existing CMV immunity has some protective effects. In one study, 40% of CMV-naïve mothers of children in day care became

infected, whereas only 7% of CMV-positive mothers were reinfected [290]. Additionally the transplacental transmission rate of CMV was lower in seropositive women compared to those who had primary infection during pregnancy [291]. In a direct challenge study, it was shown that pre-existing natural immunity was protective to low dose challenge and that subjects who had been given the attenuated Towne strain as a vaccine were partially protected [292]. Passive immunoglobulin immunity has also been shown to be partially protective in transplacental transmission [293]. Thus, while neither natural immunity nor any tested vaccines can entirely prevent infection and subsequent disease, there is evidence it can help reduce incidence, transmission, and disease severity. This also suggests that the VIPRs are not 100% effective at establishing reinfection. The variability may come from genetic diversity, perhaps in the MHC locus, or from a particular antigenic response. While a majority of the HCMV genome can be antigenic (see Chapter 1), perhaps responses to certain ORFs can be more protective than others.

When CMV does cause disease, primary infection is generally more severe than reinfection. For example, reinfection during pregnancy is associated with a reduced state of disease for the neonate compared to primary infection [41-44]. However, reinfection itself can be problematic as mentioned above. Results from this work provides new targets in helping to prevent reinfection. It will first be necessary to define the exact MHC-I evasion requirements for reinfection. Our results simply demonstrate that the US2-11 homologous region is necessary. But are all of these necessary, or just some? Further recombinant viruses lacking some of the US2-11 genes will help address this. Once the critical genes for reinfection are identified, the major difficulty would then become finding specific inhibitors of these. One possibility would be the development of

miRNA-mediated silencing. The second major obstacle would be in delivering these inhibitors in a prophylactic manner. This could help reduce the incidence of reinfection in particularly vulnerable individuals, such as during pregnancy or organ transplantation.

One potential application of this work is in the design of replication-competent vaccines and gene therapy vectors. Viral vaccine vectors are modified viruses designed to deliver exogenous antigens into the host with the intent of creating an immune response to the exogenous antigen. The purpose is to create protective immunity against a pathogen without having to use potentially dangerous attenuated versions of the pathogen. Both replication defective and replication competent viruses are being utilized for gene delivery. However, replication competent vaccine vectors are much better able to induce a T cell response. A major hurdle in vector delivery into humans is the presence of pre-existing immunity to the vector. Most strategies involve multiple administrations of the vector, and the T cell response generated to the vector itself is able to limit the efficacy of subsequent uses. Additionally, some viruses being developed as vectors, such as adenovirus type 5, have a high amount of pre-existing immunity in the population. Our results suggest that one possible way to allow for efficient reinfection would be in modifying the vectors to encode immune evasion molecules. This might allow for more efficient gene expression and immune response to the exogenous antigen.

Lastly, it is likely that VIPRs have other roles in viral infection than simply reinfection. There are viruses, some poxviruses for example, that contain VIPRs yet are unable to reinfect. Although there are exceptions in some animals [294], anti-poxviral immunity is very protective against reinfection. Poxviral vaccines work so well that Variola (Smallpox) was able to be eradicated through a rigorous worldwide vaccination

effort. Since poxviruses such as myxoma virus contain at least one VIPR yet are unable to reinfect, this suggests other potential roles for these genes during virus infection. VIPR-mediated reinfection may require other elements that vary between specific viruses, such as a certain cellular or tissue tropism, a certain rate or level of gene expression, timing of gene expression, or a certain level of effectiveness of VIPRs on MHC-I expression.

Future directions on VIHCE

There are clearly many questions surrounding the function of VIHCE. In general, it needs to be established if VIHCE inhibits some step in HC translation, induces co-translational degradation of HC, or blocks translocation of HC. Probably the best way to determine if VIHCE blocks translocation is to use it in an *in vitro* translocation assay using microsomes. Since it is unknown if VIHCE interacts with cellular components that might be required for its function, this may have to be done with microsomes isolated from VIHCE expressing cells rather than translating VIHCE *in vitro*. However, the *in vitro* translation/translocation assay would allow for the addition of various cellular components independently to determine what is necessary. Co-immunoprecipitations or cross-linking experiments with VIHCE would be useful in determining with which cellular components VIHCE is interacting.

If VIHCE is inhibiting translation at some point after initiation but prior to termination, it should be possible to detect fragments of the HC molecule that get synthesized. As these products would likely be degraded very rapidly, it may be necessary to stabilize them with proteasomal inhibitors. Additionally, in order to detect

these products, it would be necessary to epitope tag the amino-terminus. This itself could prove somewhat tricky, since we know VIHCE needs to recognize the SP itself which lies at the amino terminus. Thus inserting an epitope tag between the SP and the remainder of the protein may interfere with VIHCE recognition of the HC.

Cotranslational degradation may be possible to directly test in this case. Previously, cotranslational degradation has been measured by a ubiquitin (Ub) sandwich technique [251]. In this system, the protein tested for cotranslational degradation (HC in our case) is placed in frame between two stable reporter domains. The three proteins are connected by Ub moieties that are recognized and cleaved cotranslationally by Ub-specific processing proteases. Thus, the first reporter domain is translated and clipped from the peptide at the Ub moiety. This exposes the beginning of the HC polypeptide, the SP. If cotranslational degradation then begins, it will likely proceed through the HC sequence and through the second Ub moiety and second reporter. This means that the ratio of the first reporter to the second reporter in the cells would be greater than 1. If the HC is degraded posttranslationally, the second Ub moiety will be clipped as the first, and translation of the second reporter should occur normally. Thus the ratio of reporters would be near 1. This system could be used to specifically detect cotranslational degradation of HCs in the presence of VIHCE.

Another goal of future research on VIHCE should be in better determining what is necessary for targeting the HC. Thus far our work only implicates the residues in the SP as necessary for efficient targeting. It is not yet clear if other parts of the HC molecule are also necessary, or if the SP is sufficient for VIHCE targeting. Further mutations and chimeric molecules will help determine this.

There is also the opportunity to perform mutagenesis of VIHCE itself in order to determine important functional domains. The VIHCE protein sequence has no homology to any other known protein, and it will be interesting to determine which domains are carrying out its function.

Future directions on MHC-I inhibitors *in vivo*

The most pertinent question regarding the function of MHC-I inhibitors *in vivo* is whether RhCMV lacking its VIPRs can continue to persist during a primary infection. This will require continuous monitoring of virus shedding and it would also be best to quantify the viral load compared to wild type infection. Related to this is the continuing immune response to infection of RhCMV lacking VIPRs. Does it continue to expand at the same rate as in wild type infection?

A question in the reinfection model is whether the US2-11 homologous region is sufficient for reinfection. We have shown that they are necessary, but will need to reinfect animals with a RhCMV lacking only VIHCE to determine if this region is sufficient.

Another critical experiment is a CD8+ T cell depletion study. The implication from our work is that CD8+ T cells are able to immediately kill cells infected with RhCMV lacking VIPRs in a reinfection setting. Thus, if the CD8+ T cells are depleted using antibody, the RhCMV lacking VIPRs should be able to reinfect. If CD8+ depletion fails to restore reinfection, it may suggest that CD4+ T cells play a critical role and that the US2-11 region may be inhibiting CD4+ T cell activation during wild type reinfection.

Lastly, it is important to note that our study only implicates the entire US2-11 region as being necessary for reinfection. It does not specifically implicate VIPRs. While it is likely that reinfection is due to the VIPRs within the region, there are four genes of unknown function that are also lacking during our reinfection attempts. Two of those have limited sequence homology to HCMV US8 and US10, which are known to at least interact with MHC-I. However, it will be important to rule out that these other genes are playing a role by using additional recombinant viruses, and show that reinfection specifically needs the VIPRs of RhCMV.

Conclusions

In this dissertation, we have described the development of RhCMV as a model for HCMV inhibition of MHC-I antigen presentation *in vivo*. We have characterized the ability of RhCMV to inhibit the MHC-I pathway, identifying both conserved ORFs and a novel ORF involved in blocking this process. We have also discovered a previously unreported role for the MHC-I inhibitors *in vivo*, that they are necessary for CMV to reinfect seropositive hosts. From our work we can make several conclusions:

1) RhCMV contains an additional inhibitor of the MHC-I pathway encoded by the rh178 ORF. We have termed this inhibitor VIHCE.

2) VIHCE is an ER-localized protein capable of inhibiting the complete biosynthesis of the MHC-I heavy chain.

3) VIHCE does not affect the MHC-I heavy chain mRNA or affect active ribosome associations with heavy chain mRNA.

4) VIHCE does not mediate rapid degradation of MHC-I heavy chains, but rather acts by blocking translation or translocation.

5) In order to efficiently target the MHC-I heavy chain, VIHCE requires the conserved primate HC signal peptide.

6) Based on the induction of a T cell response, the RhCMV inhibitors of MHC-I antigen presentation are not required for the initial infection of CMV naïve hosts.

7) The induction of the cellular immune response to the exogenous antigen expressed from RhCMV during primary infection is similar regardless of the presence of the viral inhibitors of MHC-I.

8) The Rh182-189 region (homologous to HCMV US2-11) is required for RhCMV reinfection of CMV seropositive hosts.

9) VIHCE alone is not sufficient to rescue RhCMV reinfection when the virus lacks the Rh182-189 region.

Many questions remain to be answered with regards to these projects. Similar to US2 and US11 providing insights into the cellular pathway of retrotranslocation, further characterization of VIHCE may provide new insights into the translation and translocation of MHC-I heavy chain, or membrane proteins in general. Further studies of the role of CMVs MHC-I inhibitors *in vivo* will lead to a better understanding of CMV pathogenesis and immunity, which in turn will lead to improved anti-viral treatments and perhaps a successful vaccine.

Effort statement

In chapter 2, all of the described work was performed by C. Powers, with the exception of the cloning of the Rh182-189 ORFs, which was performed by N. Pande. In chapter 3, all of the described work was performed by C. Powers. In chapter 4, the creation and characterization of all recombinant RhCMVs was performed by C. Powers. Infections of rhesus macaques was performed by A. Legasse. *Ex vivo* T cell stimulations and staining was performed by S. Hansen and J. Edgar. ELISA was performed by D. Siess.

Materials and Methods

Cells and viruses: Telomerized rhesus fibroblasts (TRFs) [295] and telomerized human fibroblasts (THFs) were obtained from Jay Nelson and maintained in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum, 100U/mL penicillin and 100ug/mL streptomycin. HeLa cells and HEK293 cells were obtained from ATCC and maintained in the same media as above. RhCMV strain 68.1 was obtained from Scott Wong [66] and propagated in TRFs. Recombinant RhCMVs were created as described below using the RhCMV BAC obtained from Peter Barry [296]. RhCMV was purified by infection of 10 T-175 tissue culture flasks at an MOI of 0.1 to 1. After 100% CPE was reached, plates were scraped in the existing media and the mix frozen at -80C. Virus was thawed at 37C, followed by a low speed spin (10 min, 3000rpm Sorvall tabletop centrifuge). Supernatant was spun again for 15 min at 7000rpm in the sorvall JLA-16.250 rotor. Supernatant was then spun over 5ml of 20% sorbitol, 0.2M Tris pH 8.0, at 22Krpm in a beckman SW-28 ultracentrifuge rotor. Pelleted virus was resuspended in PBS and stored at -80C. Recombinant rh178 adenovirus was created using the AdEasy vector system according to the manufacturers protocol (Stratagene). Adenoviruses AdTrans and AdUS11 were obtained from David Johnson.

Infections and Growth Curves: Infections were performed in complete DMEM. CMV was thawed at RT, diluted into DMEM and placed on cells for 2 hours with intermittent rocking. Cells were then washed 1x with PBS and complete DMEM added. For growth curves, 6-well dishes of TRFs were infected at the indicated MOI for 2 hours, washed 2x

with PBS, and an equal amount of DMEM added to each well. At each time point, supernatant was removed, cells pelleted, and supernatant stored at -80C until titering.

Plasmids: rh178-HA was cloned by PCR from RhCMV BAC DNA into pUHD10.1. HLA-A3 constructs were expressed from pEF1α, a modified version of pCDNA3.1(-) (Invitrogen, Carlsbad, CA). The cytomegalovirus promoter was excised from pCDNA3.1(-) by digesting with Mlu I and Nhe I, followed by klenow treatment and ligation. The EF1α promoter (obtained from Jay Nelson) was then ligated into the Xho I site to create pEF1α. HLA-A3 was obtained by PCR from Jurkat T-cell cDNA using the forward primer 5'ctggaattcatggccgtcatggcgccccgaac and the reverse primer 5'gtcggatcctcacactttacaagctgtgag to amplify the coding region only or the reverse primer 5'gtcggatccttaggaatcttctcc to include the 3'UTR.

Transfection and Nucleofection: Transfections were performed with Effectene (Qiagen). 50-200ng of expression plasmid was mixed with 25ng reporter plasmid (GFP) into 150ul of buffer EC. 8µl of enhancer was added and the sample briefly mixed and placed at RT for 5 min. 5µl of Effectene was added, vortexed for 5-10 seconds, and let sit at RT for 10 min. 1mL of regular media was added, followed by dropwise addition of the mix onto 6-cm tissue culture dishes. MHC-I expression plasmids were transfected into TRFs using the AMAXA Nucleofector II (AMAXA Biosystems, Gaithersburg, MD). 2.5µg of expression plasmid was mixed with 100µl of cell line solution L. Between 1e6 and 2.5e6 TRFs were resuspended with the DNA/buffer mix and electroporated using the T-030 program. 0.5ml of RPMI with 10% FBS was added immediately and cells placed

at 37C for 45 min to recover. They were then plated into dishes with DMEM. Transfection efficiency was monitored with a GFP reporter and was consistently >90%. Infections with RhCMV were performed 24 hours after electroporation.

Metabolic labeling and immunoprecipitation: Cells were starved for 30-min, except where noted, using DMEM without serum, methionine (Met) or cysteine (Cys). Labeling was performed for indicated times using Pro-mix ^{35}S-Met/Cys (GE Healthcare) at 400 μCi/mL. To chase the label, cells were washed 3x in phosphate buffered saline (PBS) followed by incubation at 37ºC in DMEM with 10% FBS containing 90μg/mL Met and 188μg/mL Cys. For NP-40 lysis, cells were lysed for 30 minutes at 4ºC in 1% NP-40 in PBS with complete protease-inhibitor cocktail (Roche). For SDS lysis, cells were lysed for 10 minutes at 25ºC in 0.6% SDS in PBS with complete protease-inhibitor cocktail, then diluted in 3x volume of 1.2% triton X-100 in PBS prior to immunoprecipitation. For 1-minute pulses, cells were trypsinized, pelleted, washed 1x with PBS, and resuspended in 200μl DMEM (- Met/Cys) in an eppendorf tube. 500μCi/mL ^{35}S-Met/Cys was added and the cells placed in a 37ºC heat block. After 1-min, 1ml of chase media was added to quench, cells were pelleted, washed 2x in chase media, lysed or resuspended in 1ml chase medium for the indicated time.

Antibodies: Polyclonal sera K455 recognizes both chains of the MHC-I heterodimer, assembled and unassembled (obtained from Per Peterson) [247]. HC-10 only recognizes free MHC-I heavy chains [250]. HLA-A3 antibody was purified from the GAP A3 hybridoma, obtained from ATCC (HB-122). Antibodies to Calreticulin, Transferrin,

Vimentin, HA and FLAG were obtained, respectively, from Stressgen (Victoria, BC), Zymed (S. San Francisco, CA), Biomeda (Burlingame, CA), Santa Cruz, and Sigma. Secondary Alexa Fluor-conjugated antibodies 594 goat anti-rabbit and 488 goat anti-mouse were obtained from Invitrogen.

Polyribosome fractionation and northern blots: Approximately $5x10^6$ TRFs were either Mock-infected or RhCMV-infected for 24 hours. Fresh media was placed on the cells for 45-minutes, and cells were placed on ice and washed 2x with cold PBS containing 0.1 mg/ml cycloheximide (Sigma). All subsequent steps were performed at 4ºC. Cells were lysed for 10 min using 600µl of polysome lysis buffer (15mM Tris, pH 7.4, 15mM $MgCl_2$, 0.3M NaCl, 1% Triton x-100, 0.1 mg/mL cycloheximide, 1 mg/mL heparin). Lysates were cleared at 12,000 x g for 10 min. The supernatant was layered onto the top of a 10-50% sucrose gradient composed of sucrose in polysome lysis buffer excluding Triton x-100. The gradients were centrifuged at 35,000 rpm in a Sorvall SW-41 rotor for 3 hours. 750µl fractions were collected from the top of the gradient. After adding 4.25ml of 5.65M guanidine HCl, each fraction was ethanol precipitated (-20ºC overnight). RNA was pelleted at 15,000 x g for 30 min, washed with 70% ethanol, dried at 25ºC, and resuspended in 400µl RNAse-free water. RNA was then re-precipitated by adding 40µl 0.3M sodium acetate and 900µl 100% ethanol, washed with 70% ethanol and resuspended in 50µl RNAse-free water.

For Northern blotting, 10µl of each fraction was separated on a denaturing 1% agarose gel containing 1X MESA (Boston BioProducts, Worcester, MA) and 3.7% formaldehyde and transferred to Immobilon-Ny+ nylon membrane (Millipore) by capillary blotting in

20xSSC. RNA was fixed by air drying at 25ºC for 30 min and baking at 80ºC for 2 hours. Radiolabeled probes were generated by random priming of PCR fragments of the gene of interest with random hexamers, ^{32}P-dCTP, and large DNA polymerase fragment (Klenow, Invitrogen) for 30 min at 37C. Probes were then separated from free nucleotides by exclusion columns (Roche sephadex G-50). After denaturing the probe at 100ºC for 10 min, the probe was chilled on ice and added to 5mL ExpressHyb hybridization solution (Clontech) for hybridization. Membranes were pre-hybridized for 30 min at 68ºC followed by probe hybridization for 2 hours, rinsed and washed twice with 2X SSC, 0.05%SDS followed by two washes in 0.1X SSC, 0.1% SDS, and autoradiography.

RNA and reverse transcription: Total RNA was harvested from cells using the Qiagen RNeasy mini protocol. For RNA fractionation, cells were lysed on ice in buffer RLN (50mM Tris-Cl pH8.0, 140mM NaCl, 1.5mM $MgCl_2$, 0.5% NP-40). Nuclei and membranes were pelleted at 300 x g for 3 min. RNA from the supernatant was purified by RNeasy protocol and considered enriched for cytoplasmic RNA. RNA from the pellet was purified by RNeasy protocol and considered enriched for nuclear RNA. Polyadenylated RNA was purified using oligotex beads and mRNA mini protocol (Qiagen). For reverse transcription, 1µg of total RNA was treated with DNAse, then cDNA synthesized with Superscript III (Invitrogen) according to the manufacturers protocol.

Southern Blotting: Southern blots were performed using nonradioactive DIG-labeled probes according to the manufacturers protocols (DIG starter kit II, Roche). Briefly, probes were generated by random priming and DIG-labeled nucleotides. Digested DNA was run on a standard agarose gel, treated for 15 min with 0.25N HCl, 30 min with 0.5N NaOH 1.5M NaCl, rinsed with deionized water, then 30 min with 1M Tris·Cl, pH 8, 1.5 M NaCl. The DNA was then transferred to immobilon Ny+ (Millipore) via cappillary action with 10XSSC. The blot was air dried, baked for 2 hours at 80C, prehybridized for 30 min, hybridized with denatured probe for 4 hours, washed, probed with anti-DIG antibody, washed, and detected by chemiluminescence.

Immunofluorescence and immunoblot: For IFA, transfected cells were fixed with 3.7% formaldehyde for 40 minutes, washed twice with PBS, quenched with 50mM NH_4Cl for 10 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 7 min prior to staining. For immunoblot, cells were lysed in 1% NP-40 in PBS with protease inhibitor cocktail, mixed 1:1 with Laemmli loading buffer, and SDS-PAGE performed. Transfer was done using a semi-dry transfer apparatus (Owl).

RACE: Total RNA from TRFs infected with WT RhCMV (or RhCMV lacking *rh175-178* as a negative control) for 24 hours was used. For 3' RACE, cDNA was synthesized using an oligo-dT anchor (5'gaccggatccgaattcgtcgactttttttttttttttv). PCR was performed from cDNA using a PCR anchor primer (5'-gaccggatccgaattcgtcgac) and a gene specific primer. For 5' RACE, cDNA was synthesized with a gene specific primer (*rh178* 5'-catttgcatgcagctgtgcg). 10µg cDNA was then treated with terminal deoxynucleotidyl

transferase and 0.5mM dATP at 37ºC for 30 min, followed by purification and PCR using a nested gene specific primer (*rh178* 5'-gcgcgaaacacgcgtttgc) and the oligo-dT anchor.

Recombinant virus

RhCMV BAC was maintained in EL250 bacterial cells which contain heat-inducible λ-recombination (rec) genes and an arabinose-inducible FLP recombinase [297]. All bacterial growth was performed at 30ºC unless otherwise noted. To induce the rec genes, a 200ml culture of EL250s was grown in LB to OD=0.35 at 600nm and placed at 42ºC for 17 min. Bacteria were then placed on ice for 10 min and made electrocompetent by washing 4x with 250ml 10% glycerol and snap freezing in liquid nitrogen.

To make the PCR product for recombination, primers containing 40-50bp of homology to the sequences flanking the RhCMV region to be removed were used to amplify a Kanamycin (Kan) resistance cassette from plasmid pCP015 [298]. The pCP015 forward primer binding site (5'gtaaaacgacggccagt) and reverse primer binding site (5'gaaacagctatgaccatg) were added to the 3' end of the mutagenesis primers.

Competent EL250s containing WT RhCMV BAC were then electroporated with the PCR product for recombination using a MicroPulser (Biorad, Hercules, CA) and selected for Kan and Chloramphenicol (Cm) resistance at 30ºC on LB agar. Clones were first screened to insure no plasmid contamination. To induce the FLP recombinase to excise the Kan^r cassette, clones were grown in LB with Cm only to OD=0.5 at 600nm and incubated with 1mg/mL arabinose for 1 hr, diluted 1:10 in LB with Cm, and plated on LB agar with Cm. Following replica plating on LB agar with Kan and Cm, selected colonies that had lost Kan^r were characterized by restriction digest, southern blot, and partial

sequencing. Virus was reconstituted by electroporation of TRFs with 5-10μg of BAC DNA. The Kan^r cassette was not removed from the deletion viruses shown in Fig 3A in order to reduce the total amount of DNA removed from the genome. Sequences for the primers used for all recombinant BACs can be found in Appendix C.

For the double deletion virus Δ158-180,ΔRhUS2-11, we used the ΔRhUS2-11 BAC as a parent and deleted Rh158-180 as detailed above. We also did not remove the Kan resistance cassette in this BAC due to the presence of the FRT recombination site from the RhUS2-11 deletion and to lessen the amount of total DNA removed from the genome.

For the SIVgag-expressing mutants, a modified version of pCP015 with a SIVgag expression cassette inserted was used to generate the PCR product for recombination. For the double deletion virus containing SIVgag, the Δ182-189+gag BAC was used as a parent. However, for the second recombination to remove Rh178, a different Kan^r plasmid was used. pOri5K contains Kan^r cassette flanked by mutant FRT sites that do not recombine with the wild type FRT sites.

BAC DNA preps: BAC DNA was prepared via modified miniprep protocol. 10mL of BAC culture was grown overnight. Cells were resuspended in 300μl buffer P1 (50mM Tris-HCl pH 8, 10mM EDTA, 100μg/mL RNAseA), lysed in 300μl buffer P2 (0.2M NaOH, 1% SDS), and protein precipitated with 350μl P3 (3M KOAc pH5.5). 0.5mL phenol-chloroform-isoamyl alcohol was added and samples rocked at RT for 10 min. After spinning for 10min at 13Krpm, DNA was precipitated in 0.3M NaOAc and 2 volumes ethanol.

***Ex vivo* T cell stimulation and sorting:** Total leukocytes were collected from rhesus macaque whole blood or BAL fluid by spinning at 3000rpm for 20 minutes on a ficoll cushion. Cells were washed twice with Hanks buffered salt solution (HBSS), resuspended in RPMI with 10% FBS, and lymphocytes counted using a Coulter counter. Approximately 1.2e6 lymphocytes were stimulated with the co-stimulatory anti-CD28 and anti-CD49d antibodies and one of the following at 37ºC for 1 hour: antibodies only, staphylococcal enterotoxin B (SEB) superantigen as a positive control, overlapping 15-mer SIVgag peptides, or clarified lysate from RhCMV-infected cultured fibroblasts. After 1 hour, 10μg/mL brefeldin A was added to prevent cytokine secretion. The cells were then incubated at 37ºC for 6 hours. Following the antigen stimulation, cells were washed in cold PBS. Cells were then stained with the following antibodies: anti-CD3, anti-CD4, and anti-CD8. They were then permabilized and stained with anti-TNFα and anti-CD69, both indicators of activation. The percentage of CD4 or CD8 T cells expressing TNFα and CD69 was then normalized to the memory subset. Memory correction was done by dividing the total percentage of responders by the percentage of CD4 or CD8 T cells with a memory phenotype. Memory phenotype is defined as CCR7(-), CD45RO(+), CD28(+), and CCR5(+). These T cell stimulations have been previously described [259, 299].

References

1. Pellett, P.E. and B. Roizman, *The Family Herpesviridae: A Brief Introduction*, in *Fields Virology; Fifth Edition*, D.M. Knipe and P.M. Howley, Editors. 2007, Lippincott, Williams & Wilkins: Philadelphia. p. 2479-2499.
2. McGeoch, D.J., F.J. Rixon, and A.J. Davison, *Topics in herpesvirus genomics and evolution.* Virus Res, 2006. **117**(1): p. 90-104.
3. Roizman, B., et al., *Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses.* Intervirology, 1981. **16**(4): p. 201-17.
4. Roizman, B., Knipe D.M., and Whitley, R.J., *Herpes Simplex Viruses*, in *Fields Virology*, D.M. Knipe, Howley, P.M., Editor. 2007, Lippincott, Williams, and Wilkins: Philadelphia.
5. Arduino, P.G. and S.R. Porter, *Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features.* J Oral Pathol Med, 2008. **37**(2): p. 107-21.
6. Pugh, R.N., R.I. Omar, and M.M. Hossain, *Varicella infection and pneumonia among adults.* Int J Infect Dis, 1998. **2**(4): p. 205-10.
7. Echevarria, J.M., I. Casas, and P. Martinez-Martin, *Infections of the nervous system caused by varicella-zoster virus: a review.* Intervirology, 1997. **40**(2-3): p. 72-84.

8. Patel, R.A., H.J. Binns, and S.T. Shulman, *Reduction in pediatric hospitalizations for varicella-related invasive group A streptococcal infections in the varicella vaccine era.* J Pediatr, 2004. **144**(1): p. 68-74.

9. Jackson, M.A., V.F. Burry, and L.C. Olson, *Complications of varicella requiring hospitalization in previously healthy children.* Pediatr Infect Dis J, 1992. **11**(6): p. 441-5.

10. Brogan, T.V., et al., *Group A streptococcal necrotizing fasciitis complicating primary varicella: a series of fourteen patients.* Pediatr Infect Dis J, 1995. **14**(7): p. 588-94.

11. Krauss, H., *Zoonoses : infectious diseases transmissible from animals to humans*. 3rd ed. 2003, Washington, D.C.: ASM Press. xvii, 456.

12. Ward, K.N., *The natural history and laboratory diagnosis of human herpesviruses-6 and -7 infections in the immunocompetent.* J Clin Virol, 2005. **32**(3): p. 183-93.

13. Ganem, D., *Kaposi's Sarcoma-associated Herpesvirus*, in *Fields Virology*, D.M. Knipe, Howley, P.M., Editor. 2007, Lippincott, Williams and Wilkins: Philadelphia.

14. Soulier, J., et al., *Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease.* Blood, 1995. **86**(4): p. 1276-80.

15. Cesarman, E., et al., *Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas.* N Engl J Med, 1995. **332**(18): p. 1186-91.

16. Evans, A.S., J.C. Niederman, and R.W. McCollum, *Seroepidemiologic studies of infectious mononucleosis with EB virus.* N Engl J Med, 1968. **279**(21): p. 1121-7.

17. Rickinson, A.B., Kieff, E., *Epstein-Barr Virus*, in *Fields Virology*, D.M. Knipe, Howley, P.M., Editor. 2007, Lippincott, Williams and Wilkins: Philadelphia.

18. Deiss, L.P., J. Chou, and N. Frenkel, *Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA.* J Virol, 1986. **59**(3): p. 605-18.

19. Staras, S.A., et al., *Seroprevalence of cytomegalovirus infection in the United States, 1988-1994.* Clin Infect Dis, 2006. **43**(9): p. 1143-51.

20. Nichols, W.G. and M. Boeckh, *Recent advances in the therapy and prevention of CMV infections.* J Clin Virol, 2000. **16**(1): p. 25-40.

21. Alford, C.A., et al., *Congenital and perinatal cutomegalovirus infection.* Rev Infect Dis, 1990. **12**(7): p. 745-53.

22. Stratton, K.R., J.S. Durch, and R.S. Lawrence, in *Vaccines for the 21st century*. 2000, National Academy Press: Washington, DC.

23. Ho, M., *The history of cytomegalovirus and its diseases.* Med Microbiol Immunol, 2008. **197**(2): p. 65-73.

24. Minder, W., *Die Aetiologie der Cytomegalia infantum.* Schweiz Med Wochenschr, 1953. **83**: p. 1180–1182.

25. Craig, J.M., et al., *Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease.* Proc Soc Exp Biol Med, 1957. **94**(1): p. 4-12.

26. Rafailidis, P.I., et al., *Severe cytomegalovirus infection in apparently immunocompetent patients: a systematic review.* Virol J, 2008. **5**: p. 47.

27. Sorlie, P.D., et al., *A prospective study of cytomegalovirus, herpes simplex virus 1, and coronary heart disease: the atherosclerosis risk in communities (ARIC) study.* Arch Intern Med, 2000. **160**(13): p. 2027-32.

28. Streblow, D.N., S.L. Orloff, and J.A. Nelson, *Do pathogens accelerate atherosclerosis?* J Nutr, 2001. **131**(10): p. 2798S-2804S.

29. Zhou, Y.F., et al., *Cytomegalovirus infection of rats increases the neointimal response to vascular injury without consistent evidence of direct infection of the vascular wall.* Circulation, 1999. **100**(14): p. 1569-75.

30. Bruning, J.H., et al., *Enhancement of transplantation-associated atherosclerosis by CMV, which can be prevented by antiviral therapy in the form of HPMPC.* Transpl Int, 1994. **7 Suppl 1**: p. S365-70.

31. Lemstrom, K., et al., *Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection.* Circulation, 1995. **92**(9): p. 2594-604.

32. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. **202**(5): p. 673-85.

33. Akbar, A.N. and J.M. Fletcher, *Memory T cell homeostasis and senescence during aging.* Curr Opin Immunol, 2005. **17**(5): p. 480-5.

34. Simon, C.O., et al., *CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation.* J Virol, 2006. **80**(21): p. 10436-56.

35. Grattan, M.T., et al., *Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis.* Jama, 1989. **261**(24): p. 3561-6.

36. Mocarski, E.S.J., T. Shenk, and R.F. Pass, *Cytomegaloviruses*, in *Fields Virology; Fifth Edition*, D.M. Knipe and P.M. Howley, Editors. 2007, Lippincott, Williams & Wilkins: Philadelphia. p. 2702-2772.

37. Deayton, J., et al., *Loss of cytomegalovirus (CMV) viraemia following highly active antiretroviral therapy in the absence of specific anti-CMV therapy.* Aids, 1999. **13**(10): p. 1203-6.

38. O'Sullivan, C.E., et al., *Decrease of cytomegalovirus replication in human immunodeficiency virus infected-patients after treatment with highly active antiretroviral therapy.* J Infect Dis, 1999. **180**(3): p. 847-9.

39. Hassan, J. and J. Connell, *Translational mini-review series on infectious disease: congenital cytomegalovirus infection: 50 years on.* Clin Exp Immunol, 2007. **149**(2): p. 205-10.

40. CDC, *Cytomegalovirus during pregnancy*. 2008: http://www.cdc.gov/cmv/pregnancy.htm.

41. Fowler, K.B., S. Stagno, and R.F. Pass, *Maternal immunity and prevention of congenital cytomegalovirus infection.* Jama, 2003. **289**(8): p. 1008-11.

42. Stagno, S., et al., *Congenital cytomegalovirus infection: The relative importance of primary and recurrent maternal infection.* N Engl J Med, 1982. **306**(16): p. 945-9.

43. Fowler, K.B., et al., *The outcome of congenital cytomegalovirus infection in relation to maternal antibody status.* N Engl J Med, 1992. **326**(10): p. 663-7.

44. Boppana, S.B., et al., *Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity.* N Engl J Med, 2001. **344**(18): p. 1366-71.

45. Pass, R.F., et al., *Congenital cytomegalovirus infection following first trimester maternal infection: symptoms at birth and outcome.* J Clin Virol, 2006. **35**(2): p. 216-20.

46. Ross, S.A., et al., *Hearing loss in children with congenital cytomegalovirus infection born to mothers with preexisting immunity.* J Pediatr, 2006. **148**(3): p. 332-6.

47. Williamson, W.D., et al., *Progressive hearing loss in infants with asymptomatic congenital cytomegalovirus infection.* Pediatrics, 1992. **90**(6): p. 862-6.

48. Fowler, K.B., et al., *Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection.* J Pediatr, 1997. **130**(4): p. 624-30.

49. Boppana, S.B., et al., *Congenital cytomegalovirus infection: association between virus burden in infancy and hearing loss.* J Pediatr, 2005. **146**(6): p. 817-23.

50. Noda, S., et al., *Cytomegalovirus MCK-2 controls mobilization and recruitment of myeloid progenitor cells to facilitate dissemination.* Blood, 2006. **107**(1): p. 30-8.

51. Sinclair, J. and P. Sissons, *Latency and reactivation of human cytomegalovirus.* J Gen Virol, 2006. **87**(Pt 7): p. 1763-79.

52. Sinzger, C., et al., *Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues.* J Gen Virol, 1995. **76 (Pt 4)**: p. 741-50.

53. Toorkey, C.B. and D.R. Carrigan, *Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues.* J Infect Dis, 1989. **160**(5): p. 741-51.

54. Patrone, M., et al., *Cytomegalovirus UL131-128 products promote gB conformational transition and gB-gH interaction during entry into endothelial cells.* J Virol, 2007. **81**(20): p. 11479-88.

55. Ryckman, B.J., et al., *Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells.* J Virol, 2008. **82**(1): p. 60-70.

56. Camozzi, D., et al., *Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53.* J Gen Virol, 2008. **89**(Pt 3): p. 731-40.

57. Muranyi, W., et al., *Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina.* Science, 2002. **297**(5582): p. 854-7.

58. Marschall, M., et al., *Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina.* J Biol Chem, 2005. **280**(39): p. 33357-67.

59. Varnum, S.M., et al., *Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome.* J Virol, 2004. **78**(20): p. 10960-6.

60. Chee, M.S., et al., *Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169.* Curr Top Microbiol Immunol, 1990. **154**: p. 125-69.

61. Murphy, E., et al., *Reevaluation of human cytomegalovirus coding potential.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13585-90.

62. Elek, S.D. and H. Stern, *Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero.* Lancet, 1974. **1**(7845): p. 1-5.

63. Cha, T.A., et al., *Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains.* J Virol, 1996. **70**(1): p. 78-83.

64. McGeoch, D.J., et al., *Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses.* J Mol Biol, 1995. **247**(3): p. 443-58.

65. Davison, A.J., et al., *The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome.* J Gen Virol, 2003. **84**(Pt 1): p. 17-28.

66. Hansen, S.G., et al., *Complete sequence and genomic analysis of rhesus cytomegalovirus.* J Virol, 2003. **77**(12): p. 6620-36.

67. Rawlinson, W.D., H.E. Farrell, and B.G. Barrell, *Analysis of the complete DNA sequence of murine cytomegalovirus.* J Virol, 1996. **70**(12): p. 8833-49.

68. Vink, C., E. Beuken, and C.A. Bruggeman, *Complete DNA sequence of the rat cytomegalovirus genome.* J Virol, 2000. **74**(16): p. 7656-65.

69. McGregor, A., F. Liu, and M.R. Schleiss, *Identification of essential and non-essential genes of the guinea pig cytomegalovirus (GPCMV) genome via*

transposome mutagenesis of an infectious BAC clone. Virus Res, 2004. **101**(2): p. 101-8.

70. Powers, C. and K. Fruh, *Rhesus CMV: an emerging animal model for human CMV.* Med Microbiol Immunol, 2008. **197**(2): p. 109-15.

71. Asher, D.M., et al., *Persistent shedding of cytomegalovirus in the urine of healthy Rhesus monkeys.* Proc Soc Exp Biol Med, 1974. **145**(3): p. 794-801.

72. Rivailler, P., et al., *Genomic sequence of rhesus cytomegalovirus 180.92: insights into the coding potential of rhesus cytomegalovirus.* J Virol, 2006. **80**(8): p. 4179-82.

73. Lockridge, K.M., et al., *Primate cytomegaloviruses encode and express an IL-10-like protein.* Virology, 2000. **268**(2): p. 272-80.

74. Pande, N.T., et al., *Rhesus cytomegalovirus contains functional homologues of US2, US3, US6, and US11.* J Virol, 2005. **79**(9): p. 5786-98.

75. Alcendor, D.J., et al., *Analysis of the rhesus cytomegalovirus immediate-early gene promoter.* Virology, 1993. **194**(2): p. 815-21.

76. Barry, P.A., et al., *Nucleotide sequence and molecular analysis of the rhesus cytomegalovirus immediate-early gene and the UL121-117 open reading frames.* Virology, 1996. **215**(1): p. 61-72.

77. Kravitz, R.H., et al., *Cloning and characterization of rhesus cytomegalovirus glycoprotein B.* J Gen Virol, 1997. **78 (Pt 8)**: p. 2009-13.

78. Kropff, B. and M. Mach, *Identification of the gene coding for rhesus cytomegalovirus glycoprotein B and immunological analysis of the protein.* J Gen Virol, 1997. **78 (Pt 8)**: p. 1999-2007.

79. Yue, Y., et al., *Characterization and immunological analysis of the rhesus cytomegalovirus homologue (Rh112) of the human cytomegalovirus UL83 lower matrix phosphoprotein (pp65).* J Gen Virol, 2006. **87**(Pt 4): p. 777-87.

80. McCormick, A.L., et al., *Differential function and expression of the viral inhibitor of caspase 8-induced apoptosis (vICA) and the viral mitochondria-localized inhibitor of apoptosis (vMIA) cell death suppressors conserved in primate and rodent cytomegaloviruses.* Virology, 2003. **316**(2): p. 221-33.

81. Spencer, J.V., et al., *Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10.* J Virol, 2002. **76**(3): p. 1285-92.

82. Penfold, M.E., et al., *Characterization of the rhesus cytomegalovirus US28 locus.* J Virol, 2003. **77**(19): p. 10404-13.

83. Jones-Engel, L., et al., *Temple monkeys and health implications of commensalism, Kathmandu, Nepal.* Emerg Infect Dis, 2006. **12**(6): p. 900-6.

84. Swack, N.S. and G.D. Hsiung, *Natural and experimental simian cytomegalovirus infections at a primate center.* J Med Primatol, 1982. **11**(3): p. 169-77.

85. Vogel, P., et al., *Seroepidemiologic studies of cytomegalovirus infection in a breeding population of rhesus macaques.* Lab Anim Sci, 1994. **44**(1): p. 25-30.

86. Huff, J.L., et al., *Differential detection of B virus and rhesus cytomegalovirus in rhesus macaques.* J Gen Virol, 2003. **84**(Pt 1): p. 83-92.

87. Lockridge, K.M., et al., *Pathogenesis of experimental rhesus cytomegalovirus infection.* J Virol, 1999. **73**(11): p. 9576-83.

88. Macchia, I., et al., *Expression of CD8alpha identifies a distinct subset of effector memory CD4+ T lymphocytes.* Immunology, 2006. **119**(2): p. 232-42.

89. Chan, K.S. and A. Kaur, *Flow cytometric detection of degranulation reveals phenotypic heterogeneity of degranulating CMV-specific CD8+ T lymphocytes in rhesus macaques.* J Immunol Methods, 2007. **325**(1-2): p. 20-34.

90. Pitcher, C.J., et al., *Development and homeostasis of T cell memory in rhesus macaque.* J Immunol, 2002. **168**(1): p. 29-43.

91. Walker, J.M., et al., *Multicolor flow cytometric analysis in SIV-infected rhesus macaque.* Methods Cell Biol, 2004. **75**: p. 535-57.

92. Kaur, A., et al., *Direct relationship between suppression of virus-specific immunity and emergence of cytomegalovirus disease in simian AIDS.* J Virol, 2003. **77**(10): p. 5749-58.

93. Kaur, A., et al., *Cytotoxic T-lymphocyte responses to cytomegalovirus in normal and simian immunodeficiency virus-infected rhesus macaques.* J Virol, 1996. **70**(11): p. 7725-33.

94. Kaur, A., et al., *Decreased frequency of cytomegalovirus (CMV)-specific CD4+ T lymphocytes in simian immunodeficiency virus-infected rhesus macaques: inverse relationship with CMV viremia.* J Virol, 2002. **76**(8): p. 3646-58.

95. Estes, J.D., et al., *Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection.* J Infect Dis, 2006. **193**(5): p. 703-12.

96. Janeway, C., *Immunobiology : the immune system in health and disease*. 5th ed. 2001, New York: Garland Pub. xviii, 732.

97. Park, B., et al., *Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing.* Cell, 2006. **127**(2): p. 369-82.

98. Koszinowski, U.H., M.J. Reddehase, and S. Jonjic, *The role of CD4 and CD8 T cells in viral infections.* Curr Opin Immunol, 1991. **3**(4): p. 471-5.

99. Krmpotic, A., et al., *Pathogenesis of murine cytomegalovirus infection.* Microbes Infect, 2003. **5**(13): p. 1263-77.

100. Polic, B., et al., *Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection.* J Exp Med, 1998. **188**(6): p. 1047-54.

101. Jonjic, S., et al., *A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection.* J Virol, 1988. **62**(5): p. 1653-8.

102. Klenerman, P. and A. Hill, *T cells and viral persistence: lessons from diverse infections.* Nat Immunol, 2005. **6**(9): p. 873-9.

103. Koszinowski, U.H., M. Del Val, and M.J. Reddehase, *Cellular and molecular basis of the protective immune response to cytomegalovirus infection.* Curr Top Microbiol Immunol, 1990. **154**: p. 189-220.

104. Reddehase, M.J., et al., *Stalemating a clever opportunist: lessons from murine cytomegalovirus.* Hum Immunol, 2004. **65**(5): p. 446-55.

105. Steffens, H.P., et al., *Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence.* J Virol, 1998. **72**(3): p. 1797-804.

106. Walter, E.A., et al., *Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor.* N Engl J Med, 1995. **333**(16): p. 1038-44.

107. Einsele, H., et al., *Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy.* Blood, 2002. **99**(11): p. 3916-22.

108. Boeckh, M., et al., *Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity.* Blood, 2003. **101**(2): p. 407-14.

109. Munks, M.W., et al., *Genome-wide analysis reveals a highly diverse CD8 T cell response to murine cytomegalovirus.* J Immunol, 2006. **176**(6): p. 3760-6.

110. Munks, M.W., et al., *Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection.* J Immunol, 2006. **177**(1): p. 450-8.

111. Moss, P. and N. Khan, *CD8(+) T-cell immunity to cytomegalovirus.* Hum Immunol, 2004. **65**(5): p. 456-64.

112. Khan, N., et al., *Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals.* J Immunol, 2002. **169**(4): p. 1984-92.

113. Ouyang, Q., et al., *Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old.* J Clin Immunol, 2003. **23**(4): p. 247-57.

114. Ouyang, Q., et al., *Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly.* Exp Gerontol, 2004. **39**(4): p. 607-13.

115. Voehringer, D., M. Koschella, and H. Pircher, *Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1).* Blood, 2002. **100**(10): p. 3698-702.

116. Northfield, J., et al., *Does memory improve with age? CD85j (ILT-2/LIR-1) expression on CD8 T cells correlates with 'memory inflation' in human cytomegalovirus infection.* Immunol Cell Biol, 2005. **83**(2): p. 182-8.

117. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell.* Nature, 1998. **393**(6684): p. 474-8.

118. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-3.

119. Gamadia, L.E., et al., *Properties of CD4(+) T cells in human cytomegalovirus infection.* Hum Immunol, 2004. **65**(5): p. 486-92.

120. Gamadia, L.E., et al., *Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease.* Blood, 2003. **101**(7): p. 2686-92.

121. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.

122. Tu, W., et al., *Persistent and selective deficiency of CD4+ T cell immunity to cytomegalovirus in immunocompetent young children.* J Immunol, 2004. **172**(5): p. 3260-7.

123. Dunn, H.S., et al., *Dynamics of CD4 and CD8 T cell responses to cytomegalovirus in healthy human donors.* J Infect Dis, 2002. **186**(1): p. 15-22.

124. Sester, M., et al., *Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus.* J Virol, 2002. **76**(8): p. 3748-55.

125. Tomazin, R., et al., *Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells.* Nat Med, 1999. **5**(9): p. 1039-43.

126. Hegde, N.R., et al., *Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation.* J Virol, 2002. **76**(21): p. 10929-41.

127. Odeberg, J., et al., *Human cytomegalovirus protein pp65 mediates accumulation of HLA-DR in lysosomes and destruction of the HLA-DR alpha-chain.* Blood, 2003. **101**(12): p. 4870-7.

128. Lee, A.W., et al., *Human cytomegalovirus alters localization of MHC class II and dendrite morphology in mature Langerhans cells.* J Immunol, 2006. **177**(6): p. 3960-71.

129. Redpath, S., et al., *Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10.* J Immunol, 1999. **162**(11): p. 6701-7.

130. Baca-Jones, C., *RCMV downregulates surface expression of MHC class II*. 2007.

131. Tay, C.H., E. Szomolanyi-Tsuda, and R.M. Welsh, *Control of infections by NK cells.* Curr Top Microbiol Immunol, 1998. **230**: p. 193-220.

132. Biron, C.A., K.S. Byron, and J.L. Sullivan, *Severe herpesvirus infections in an adolescent without natural killer cells.* N Engl J Med, 1989. **320**(26): p. 1731-5.

133. Vilches, C. and P. Parham, *KIR: diverse, rapidly evolving receptors of innate and adaptive immunity.* Annu Rev Immunol, 2002. **20**: p. 217-51.

134. Beck, S. and B.G. Barrell, *Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens.* Nature, 1988. **331**(6153): p. 269-72.

135. Browne, H., et al., *A complex between the MHC class I homologue encoded by human cytomegalovirus and beta 2 microglobulin.* Nature, 1990. **347**(6295): p. 770-2.

136. Fahnestock, M.L., et al., *The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides.* Immunity, 1995. **3**(5): p. 583-90.

137. Colonna, M., *Immunology. Unmasking the killer's accomplice.* Nature, 1998. **391**(6668): p. 642-3.

138. Borges, L. and D. Cosman, *LIRs/ILTs/MIRs, inhibitory and stimulatory Ig-superfamily receptors expressed in myeloid and lymphoid cells.* Cytokine Growth Factor Rev, 2000. **11**(3): p. 209-17.

139. Cosman, D., et al., *A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules.* Immunity, 1997. **7**(2): p. 273-82.

140. Chalupny, N.J., et al., *Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142.* Biochem Biophys Res Commun, 2006. **346**(1): p. 175-81.

141. Wills, M.R., et al., *Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis.* J Immunol, 2005. **175**(11): p. 7457-65.

142. Lee, N., et al., *HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5199-204.

143. Tomasec, P., et al., *Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40.* Science, 2000. **287**(5455): p. 1031.

144. Ulbrecht, M., et al., *Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis.* J Immunol, 2000. **164**(10): p. 5019-22.

145. Tomasec, P., et al., *Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141.* Nat Immunol, 2005. **6**(2): p. 181-8.

146. Armstrong, M., et al., *The UL14 encodes an EndoH-sensitive glycoprotein that is retained in the ER.* . 2006.

147. Cosman, D., et al., *ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor.* Immunity, 2001. **14**(2): p. 123-33.

148. Kubin, M., et al., *ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells.* Eur J Immunol, 2001. **31**(5): p. 1428-37.

149. Sutherland, C.L., et al., *UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells.* J Immunol, 2002. **168**(2): p. 671-9.

150. Welte, S.A., et al., *Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein.* Eur J Immunol, 2003. **33**(1): p. 194-203.

151. Wu, J., et al., *Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein.* J Immunol, 2003. **170**(8): p. 4196-200.

152. Dunn, C., et al., *Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity.* J Exp Med, 2003. **197**(11): p. 1427-39.

153. Rolle, A., et al., *Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein.* J Immunol, 2003. **171**(2): p. 902-8.

154. Sainz, B., Jr., et al., *Synergistic inhibition of human cytomegalovirus replication by interferon-alpha/beta and interferon-gamma.* Virol J, 2005. **2**(1): p. 14.

155. Torigoe, S., et al., *Cytofluorographic analysis of effects of interferons on expression of human cytomegalovirus proteins.* J Virol Methods, 1993. **45**(2): p. 219-28.

156. Gribaudo, G., et al., *Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription.* Virology, 1993. **197**(1): p. 303-11.

157. Chin, K.C. and P. Cresswell, *Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus.* Proc Natl Acad Sci U S A, 2001. **98**(26): p. 15125-30.

158. DeFilippis, V.R., et al., *Interferon regulatory factor 3 is necessary for induction of antiviral genes during human cytomegalovirus infection.* J Virol, 2006. **80**(2): p. 1032-7.

159. Navarro, L., et al., *Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon- stimulated response element-binding complex.* Mol Cell Biol, 1998. **18**(7): p. 3796-802.

160. Boehme, K.W., et al., *Human cytomegalovirus elicits a coordinated cellular antiviral response via envelope glycoprotein B.* J Virol, 2004. **78**(3): p. 1202-11.

161. Netterwald, J.R., et al., *Postattachment events associated with viral entry are necessary for induction of interferon-stimulated genes by human cytomegalovirus.* J Virol, 2004. **78**(12): p. 6688-91.

162. Browne, E.P., et al., *Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs.* J Virol, 2001. **75**(24): p. 12319-30.

163. Taylor, R.T. and W.A. Bresnahan, *Human cytomegalovirus immediate-early 2 gene expression blocks virus-induced beta interferon production.* J Virol, 2005. **79**(6): p. 3873-7.

164. Taylor, R.T. and W.A. Bresnahan, *Human cytomegalovirus IE86 attenuates virus- and tumor necrosis factor alpha-induced NFkappaB-dependent gene expression.* J Virol, 2006. **80**(21): p. 10763-71.

165. DeFilippis, V. and K. Früh, *Rhesus cytomegalovirus particles prevent activation of interferon regulatory factor 3.* J Virol, 2005. **79**(10): p. 6419-31.

166. Skaletskaya, A., et al., *A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7829-34.

167. Poncet, D., et al., *An anti-apoptotic viral protein that recruits Bax to mitochondria.* J Biol Chem, 2004. **279**(21): p. 22605-14.

168. Arnoult, D., et al., *Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria.* Proc Natl Acad Sci U S A, 2004. **101**(21): p. 7988-93.

169. Saederup, N., et al., *Cytomegalovirus-encoded beta chemokine promotes monocyte-associated viremia in the host.* Proc Natl Acad Sci U S A, 1999. **96**(19): p. 10881-6.

170. Kaptein, S.J., et al., *The r131 gene of rat cytomegalovirus encodes a proinflammatory CC chemokine homolog which is essential for the production of infectious virus in the salivary glands.* Virus Genes, 2004. **29**(1): p. 43-61.

171. Hahn, G., et al., *Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes.* J Virol, 2004. **78**(18): p. 10023-33.

172. Wang, D. and T. Shenk, *Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18153-8.

173. Beisser, P.S., et al., *Chemokines and chemokine receptors encoded by cytomegaloviruses.* Curr Top Microbiol Immunol, 2008. **325**: p. 221-42.

174. Heo, J., et al., *Polymorphisms within human cytomegalovirus chemokine (UL146/UL147) and cytokine receptor genes (UL144) are not predictive of sequelae in congenitally infected children.* Virology, 2008. **378**(1): p. 86-96.

175. Penfold, M.E., et al., *Cytomegalovirus encodes a potent alpha chemokine.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9839-44.

176. Miller-Kittrell, M., et al., *Functional characterization of chimpanzee cytomegalovirus chemokine, vCXCL-1(CCMV).* Virology, 2007. **364**(2): p. 454-65.

177. Friedman, D.J. and R.P. Ricciardi, *Adenovirus type 12 E1A gene represses accumulation of MHC class I mRNAs at the level of transcription.* Virology, 1988. **165**(1): p. 303-5.

178. Weissman, J.D., et al., *HIV-1 tat binds TAFII250 and represses TAFII250-dependent transcription of major histocompatibility class I genes.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11601-6.

179. Guerreiro-Cacais, A.O., et al., *Inhibition of heavy chain and beta2-microglobulin synthesis as a mechanism of major histocompatibility complex class I downregulation during Epstein-Barr virus replication.* J Virol, 2007. **81**(3): p. 1390-400.

180. Levitskaya, J., et al., *Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1.* Nature, 1995. **375**(6533): p. 685-8.

181. Gilbert, M.J., et al., *Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product.* Nature, 1996. **383**(6602): p. 720-2.

182. Ahn, K., et al., *Human cytomegalovirus inhibits antigen presentation by a sequential multistep process.* Proc Natl Acad Sci U S A, 1996. **93**(20): p. 10990-5.

183. Lehner, P.J., et al., *The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6904-9.

184. Hill, A., et al., *Herpes simplex virus turns off the TAP to evade host immunity.* Nature, 1995. **375**(6530): p. 411-5.

185. Koppers-Lalic, D., et al., *Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing.* Proc Natl Acad Sci U S A, 2005. **102**(14): p. 5144-9.

186. Fruh, K., et al., *A viral inhibitor of peptide transporters for antigen presentation.* Nature, 1995. **375**(6530): p. 415-8.

187. Boname, J.M., et al., *Viral degradation of the MHC class I peptide loading complex.* Immunity, 2004. **20**(3): p. 305-17.

188. Bennett, E.M., et al., *Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression.* J Immunol, 1999. **162**(9): p. 5049-52.

189. Jones, T.R., et al., *Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11327-33.

190. Kavanagh, D.G., U.H. Koszinowski, and A.B. Hill, *The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment.* J Immunol, 2001. **167**(7): p. 3894-902.

191. Dasgupta, A., et al., *Cowpox virus evades CTL recognition and inhibits the intracellular transport of MHC class I molecules.* J Immunol, 2007. **178**(3): p. 1654-61.

192. Wiertz, E.J., et al., *The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol.* Cell, 1996. **84**(5): p. 769-79.

193. Boname, J.M. and P.G. Stevenson, *MHC class I ubiquitination by a viral PHD/LAP finger protein.* Immunity, 2001. **15**(4): p. 627-36.

194. Kerkau, T., et al., *The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules.* J Exp Med, 1997. **185**(7): p. 1295-305.

195. Ziegler, H., et al., *A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments.* Immunity, 1997. **6**(1): p. 57-66.

196. Reusch, U., et al., *A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation.* Embo J, 1999. **18**(4): p. 1081-91.

197. Piguet, V., et al., *HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes.* Nat Cell Biol, 2000. **2**(3): p. 163-7.

198. Hewitt, E.W., et al., *Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation.* Embo J, 2002. **21**(10): p. 2418-29.

199. Gewurz, B.E., et al., *Antigen presentation subverted: Structure of the human cytomegalovirus protein US2 bound to the class I molecule HLA-A2.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6794-9.

200. Furman, M.H., et al., *The human cytomegalovirus US10 gene product delays trafficking of major histocompatibility complex class I molecules.* J Virol, 2002. **76**(22): p. 11753-6.

201. Tirabassi, R.S. and H.L. Ploegh, *The human cytomegalovirus US8 glycoprotein binds to major histocompatibility complex class I products.* J Virol, 2002. **76**(13): p. 6832-5.

202. Huber, M.T., et al., *Human cytomegalovirus US7, US8, US9, and US10 are cytoplasmic glycoproteins, not found at cell surfaces, and US9 does not mediate cell-to-cell spread.* J Virol, 2002. **76**(11): p. 5748-58.

203. Llano, M., et al., *Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets.* Eur J Immunol, 2003. **33**(10): p. 2744-54.

204. Beersma, M.F., M.J. Bijlmakers, and H.L. Ploegh, *Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains.* J Immunol, 1993. **151**(9): p. 4455-64.

205. Jones, T.R., et al., *Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains.* J Virol, 1995. **69**(8): p. 4830-41.

206. Schust, D.J., et al., *Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11.* J Exp Med, 1998. **188**(3): p. 497-503.

207. Barel, M.T., et al., *Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation.* J Immunol, 2003. **171**(12): p. 6757-65.

208. Barel, M.T., et al., *Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2- and US11-mediated degradation.* Int Immunol, 2006. **18**(1): p. 173-82.

209. Machold, R.P., et al., *The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complex (MHC) class I heavy chains.* J Exp Med, 1997. **185**(2): p. 363-6.

210. Story, C.M., M.H. Furman, and H.L. Ploegh, *The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products.* Proc Natl Acad Sci U S A, 1999. **96**(15): p. 8516-21.

211. Thilo, C., et al., *Dissection of the interaction of the human cytomegalovirus-derived US2 protein with major histocompatibility complex class I molecules: prominent role of a single arginine residue in human leukocyte antigen-A2.* J Biol Chem, 2006. **281**(13): p. 8950-7.

212. Furman, M.H., H.L. Ploegh, and D. Tortorella, *Membrane-specific, host-derived factors are required for US2- and US11-mediated degradation of major histocompatibility complex class I molecules.* J Biol Chem, 2002. **277**(5): p. 3258-67.

213. Lilley, B.N., D. Tortorella, and H.L. Ploegh, *Dislocation of a type I membrane protein requires interactions between membrane-spanning segments within the lipid bilayer.* Mol Biol Cell, 2003. **14**(9): p. 3690-8.

214. Lilley, B.N. and H.L. Ploegh, *A membrane protein required for dislocation of misfolded proteins from the ER.* Nature, 2004. **429**(6994): p. 834-40.

215. Ye, Y., et al., *A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol.* Nature, 2004. **429**(6994): p. 841-7.

216. Loureiro, J., et al., *Signal peptide peptidase is required for dislocation from the endoplasmic reticulum.* Nature, 2006. **441**(7095): p. 894-7.

217. Lee, S., B. Park, and K. Ahn, *Determinant for endoplasmic reticulum retention in the luminal domain of the human cytomegalovirus US3 glycoprotein.* J Virol, 2003. **77**(3): p. 2147-56.

218. Lee, S., et al., *Structural and functional dissection of human cytomegalovirus US3 in binding major histocompatibility complex class I molecules.* J Virol, 2000. **74**(23): p. 11262-9.

219. Park, B., et al., *Human cytomegalovirus inhibits tapasin-dependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion.* Immunity, 2004. **20**(1): p. 71-85.

220. Cresswell, P., et al., *Mechanisms of MHC class I-restricted antigen processing and cross-presentation.* Immunol Rev, 2005. **207**: p. 145-57.

221. Grandea, A.G., 3rd and L. Van Kaer, *Tapasin: an ER chaperone that controls MHC class I assembly with peptide.* Trends Immunol, 2001. **22**(4): p. 194-9.

222. Purcell, A.W., et al., *Quantitative and qualitative influences of tapasin on the class I peptide repertoire.* J Immunol, 2001. **166**(2): p. 1016-27.

223. Williams, A.P., et al., *Optimization of the MHC class I peptide cargo is dependent on tapasin.* Immunity, 2002. **16**(4): p. 509-20.

224. Schoenhals, G.J., et al., *Retention of empty MHC class I molecules by tapasin is essential to reconstitute antigen presentation in invertebrate cells.* Embo J, 1999. **18**(3): p. 743-53.

225. Thammavongsa, V., et al., *HLA-B44 polymorphisms at position 116 of the heavy chain influence TAP complex binding via an effect on peptide occupancy.* J Immunol, 2006. **177**(5): p. 3150-61.

226. Ahn, K., et al., *The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP.* Immunity, 1997. **6**(5): p. 613-621.

227. Hengel, H., et al., *A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter.* Immunity, 1997. **6**(5): p. 623-32.

228. Arora, S., P.E. Lapinski, and M. Raghavan, *Use of chimeric proteins to investigate the role of transporter associated with antigen processing (TAP) structural domains in peptide binding and translocation.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7241-6.

229. Gaudet, R. and D.C. Wiley, *Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing.* Embo J, 2001. **20**(17): p. 4964-72.

230. Hewitt, E.W., S.S. Gupta, and P.J. Lehner, *The human cytomegalovirus gene product US6 inhibits ATP binding by TAP.* Embo J, 2001. **20**(3): p. 387-96.

231. Ahn, K., et al., *Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47.* Embo J, 1996. **15**(13): p. 3247-55.

232. Tomazin, R., et al., *Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP.* Embo J, 1996. **15**(13): p. 3256-66.

233. Pinto, A.K. and A.B. Hill, *Viral interference with antigen presentation to CD8+ T cells: lessons from cytomegalovirus.* Viral Immunol, 2005. **18**(3): p. 434-44.

234. Kleijnen, M.F., et al., *A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface.* Embo J, 1997. **16**(4): p. 685-94.

235. Krmpotic, A., et al., *The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo.* J Exp Med, 1999. **190**(9): p. 1285-96.

236. Holtappels, R., et al., *Cytomegalovirus misleads its host by priming of CD8 T cells specific for an epitope not presented in infected tissues.* J Exp Med, 2004. **199**(1): p. 131-6.

237. Gold, M.C., et al., *The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by M45-specific CTL but does not alter the*

immunodominance of the M45-specific CD8 T cell response in vivo. J Immunol, 2002. **169**(1): p. 359-65.

238. Gold, M.C., et al., *Murine cytomegalovirus interference with antigen presentation has little effect on the size or the effector memory phenotype of the CD8 T cell response.* J Immunol, 2004. **172**(11): p. 6944-53.

239. Munks, M.W., et al., *Viral interference with antigen presentation does not alter acute or chronic CD8 T cell immunodominance in murine cytomegalovirus infection.* J Immunol, 2007. **178**(11): p. 7235-41.

240. Lu, X., et al., *Murine cytomegalovirus interference with antigen presentation contributes to the inability of CD8 T cells to control virus in the salivary gland.* J Virol, 2006. **80**(8): p. 4200-2.

241. Booth, T.W., et al., *Molecular and biological characterization of new strains of murine cytomegalovirus isolated from wild mice.* Arch Virol, 1993. **132**(1-2): p. 209-20.

242. Rizvanov, A.A., et al., *Generation of a recombinant cytomegalovirus for expression of a hantavirus glycoprotein.* J Virol, 2003. **77**(22): p. 12203-10.

243. Gossen, M. and H. Bujard, *Tight control of gene expression in mammalian cells by tetracycline-responsive promoters.* Proc Natl Acad Sci U S A, 1992. **89**(12): p. 5547-51.

244. Chang, W.L., et al., *A recombinant rhesus cytomegalovirus expressing enhanced green fluorescent protein retains the wild-type phenotype and pathogenicity in fetal macaques.* J Virol, 2002. **76**(18): p. 9493-504.

245. He, T.C., et al., *A simplified system for generating recombinant adenoviruses.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2509-14.

246. Burgert, H.G. and S. Kvist, *An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens.* Cell, 1985. **41**(3): p. 987-97.

247. Andersson, M., et al., *Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance.* Cell, 1985. **43**(1): p. 215-22.

248. Kelley, L.A., R.M. MacCallum, and M.J. Sternberg, *Enhanced genome annotation using structural profiles in the program 3D-PSSM.* J Mol Biol, 2000. **299**(2): p. 499-520.

249. Otting, N., et al., *Unparalleled complexity of the MHC class I region in rhesus macaques.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1626-31.

250. Stam, N.J., et al., *HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy.* Int Immunol, 1990. **2**(2): p. 113-25.

251. Turner, G.C. and A. Varshavsky, *Detecting and measuring cotranslational protein degradation in vivo.* Science, 2000. **289**(5487): p. 2117-20.

252. Lodish, H.F., *Alpha and beta globin messenger ribonucleic acid. Different amounts and rates of initiation of translation.* J Biol Chem, 1971. **246**(23): p. 7131-8.

253. Petersen, C.P., et al., *Short RNAs repress translation after initiation in mammalian cells.* Mol Cell, 2006. **21**(4): p. 533-42.

254. Bordeleau, M.E., et al., *Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A.* Nat Chem Biol, 2006. **2**(4): p. 213-20.

255. Nottrott, S., M.J. Simard, and J.D. Richter, *Human let-7a miRNA blocks protein production on actively translating polyribosomes.* Nat Struct Mol Biol, 2006. **13**(12): p. 1108-14.

256. Song, W., et al., *Role of Sec61alpha in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocation channel.* Cell, 2000. **100**(3): p. 333-43.

257. Besemer, J., et al., *Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1.* Nature, 2005. **436**(7048): p. 290-3.

258. Lopez-Botet, M., et al., *NK cell recognition of non-classical HLA class I molecules.* Semin Immunol, 2000. **12**(2): p. 109-19.

259. Price, D.A., et al., *Induction and evolution of cytomegalovirus-specific CD4+ T cell clonotypes in rhesus macaques.* J Immunol, 2008. **180**(1): p. 269-80.

260. Etchison, D., et al., *Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex.* J Biol Chem, 1982. **257**(24): p. 14806-10.

261. Schneider, R.J. and I. Mohr, *Translation initiation and viral tricks.* Trends Biochem Sci, 2003. **28**(3): p. 130-6.

262. Pfeffer, S., et al., *Identification of virus-encoded microRNAs.* Science, 2004. **304**(5671): p. 734-6.

263. Eulalio, A., E. Huntzinger, and E. Izaurralde, *Getting to the root of miRNA-mediated gene silencing.* Cell, 2008. **132**(1): p. 9-14.

264. Strawbridge, A.B. and J.S. Blum, *Autophagy in MHC class II antigen processing.* Curr Opin Immunol, 2007. **19**(1): p. 87-92.

265. Ito-Harashima, S., et al., *Translation of the poly(A) tail plays crucial roles in nonstop mRNA surveillance via translation repression and protein destabilization by proteasome in yeast.* Genes Dev, 2007. **21**(5): p. 519-24.

266. Garrison, J.L., et al., *A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum.* Nature, 2005. **436**(7048): p. 285-9.

267. Harant, H., et al., *Inhibition of vascular endothelial growth factor cotranslational translocation by the cyclopeptolide CAM741.* Mol Pharmacol, 2007. **71**(6): p. 1657-65.

268. Harant, H., et al., *The translocation inhibitor CAM741 interferes with vascular cell adhesion molecule 1 signal peptide insertion at the translocon.* J Biol Chem, 2006. **281**(41): p. 30492-502.

269. Daza-Vamenta, R., et al., *Genetic divergence of the rhesus macaque major histocompatibility complex.* Genome Res, 2004. **14**(8): p. 1501-15.

270. Urvater, J.A., et al., *Mamu-I: a novel primate MHC class I B-related locus with unusually low variability.* J Immunol, 2000. **164**(3): p. 1386-98.

271. Bland, F.A., et al., *Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E.* J Biol Chem, 2003. **278**(36): p. 33747-52.

272. Jahn, G., et al., *Human cytomegalovirus infection of immature dendritic cells and macrophages.* Intervirology, 1999. **42**(5-6): p. 365-72.

273. Drew, W.L., et al., *Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: documentation by Southern blot hybridization.* J Infect Dis, 1984. **150**(6): p. 952-3.

274. Spector, S.A., K.K. Hirata, and T.R. Newman, *Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome.* J Infect Dis, 1984. **150**(6): p. 953-6.

275. Chou, S.W., *Acquisition of donor strains of cytomegalovirus by renal-transplant recipients.* N Engl J Med, 1986. **314**(22): p. 1418-23.

276. Shen, C.Y., et al., *Cytomegalovirus recurrence in seropositive pregnant women attending obstetric clinics.* J Med Virol, 1993. **41**(1): p. 24-9.

277. Meyer-Konig, U., et al., *Simultaneous infection of healthy people with multiple human cytomegalovirus strains.* Lancet, 1998. **352**(9136): p. 1280-1.

278. Chandler, S.H., H.H. Handsfield, and J.K. McDougall, *Isolation of multiple strains of cytomegalovirus from women attending a clinic for sexually transmitted disease.* J Infect Dis, 1987. **155**(4): p. 655-60.

279. Gorman, S., et al., *Mixed infection with multiple strains of murine cytomegalovirus occurs following simultaneous or sequential infection of immunocompetent mice.* J Gen Virol, 2006. **87**(Pt 5): p. 1123-32.

280. Arav-Boger, R., et al., *Polymorphisms of the cytomegalovirus (CMV)-encoded tumor necrosis factor-alpha and beta-chemokine receptors in congenital CMV disease.* J Infect Dis, 2002. **186**(8): p. 1057-64.

281. Coaquette, A., et al., *Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients.* Clin Infect Dis, 2004. **39**(2): p. 155-61.

282. Humar, A., et al., *Cytomegalovirus (CMV) glycoprotein B genotypes and response to antiviral therapy, in solid-organ-transplant recipients with CMV disease.* J Infect Dis, 2003. **188**(4): p. 581-4.

283. Cicin-Sain, L., et al., *Frequent coinfection of cells explains functional in vivo complementation between cytomegalovirus variants in the multiply infected host.* J Virol, 2005. **79**(15): p. 9492-502.

284. Taha, Y., et al., *Reactivation of 2 genetically distinct varicella-zoster viruses in the same individual.* Clin Infect Dis, 2006. **43**(10): p. 1301-3.

285. Klein, R.J., *Reinfections and site-specific immunity in herpes simplex virus infections.* Vaccine, 1989. **7**(5): p. 380-1.

286. Roest, R.W., et al., *Genotypic analysis of sequential genital herpes simplex virus type 1 (HSV-1) isolates of patients with recurrent HSV-1 associated genital herpes.* J Med Virol, 2004. **73**(4): p. 601-4.

287. Beyari, M.M., et al., *Multiple human herpesvirus-8 infection.* J Infect Dis, 2003. **188**(5): p. 678-89.

288. Gratama, J.W., et al., *Detection of multiple Epstein-Barr viral strains in allogeneic bone marrow transplant recipients.* J Med Virol, 1992. **37**(1): p. 39-47.

289. Allen, T.M. and M. Altfeld, *HIV-1 superinfection.* J Allergy Clin Immunol, 2003. **112**(5): p. 829-35; quiz 836.

290. Adler, S.P., et al., *Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age.* J Infect Dis, 1995. **171**(1): p. 26-32.

291. Plotkin, S.A., *Is there a formula for an effective CMV vaccine?* J Clin Virol, 2002. **25 Suppl 2**: p. S13-21.

292. Plotkin, S.A., et al., *Protective effects of Towne cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge.* J Infect Dis, 1989. **159**(5): p. 860-5.

293. Nigro, G., et al., *Passive immunization during pregnancy for congenital cytomegalovirus infection.* N Engl J Med, 2005. **353**(13): p. 1350-62.

294. McKeever, D.J., et al., *Studies of the pathogenesis of orf virus infection in sheep.* J Comp Pathol, 1988. **99**(3): p. 317-28.

295. Chang, W.L., et al., *Replication of rhesus cytomegalovirus in life-expanded rhesus fibroblasts expressing human telomerase.* J Virol Methods, 2002. **104**(2): p. 135-46.

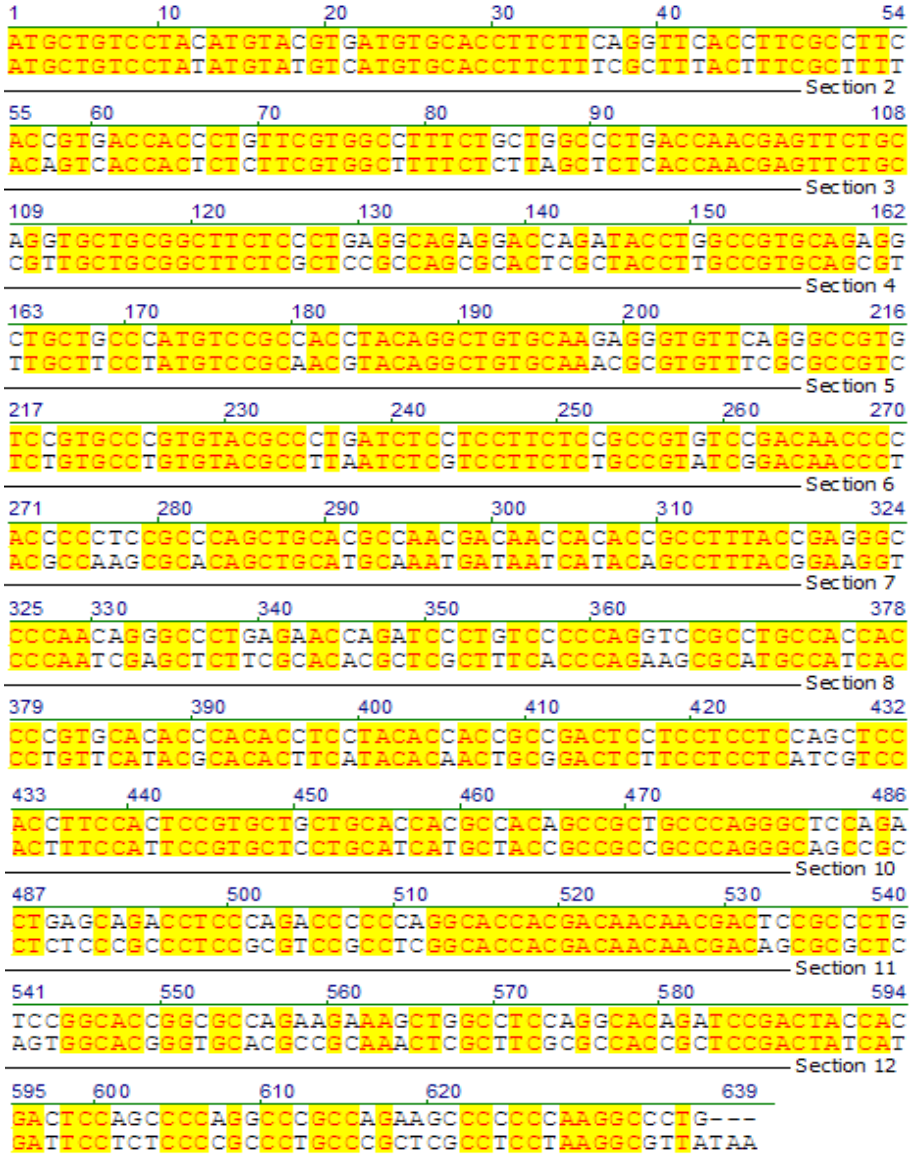
296. Chang, W.L. and P.A. Barry, *Cloning of the full-length rhesus cytomegalovirus genome as an infectious and self-excisable bacterial artificial chromosome for analysis of viral pathogenesis.* J Virol, 2003. **77**(9): p. 5073-83.

297. Lee, E.C., et al., *A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA.* Genomics, 2001. **73**(1): p. 56-65.

298. Cherepanov, P.P. and W. Wackernagel, *Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant.* Gene, 1995. **158**(1): p. 9-14.

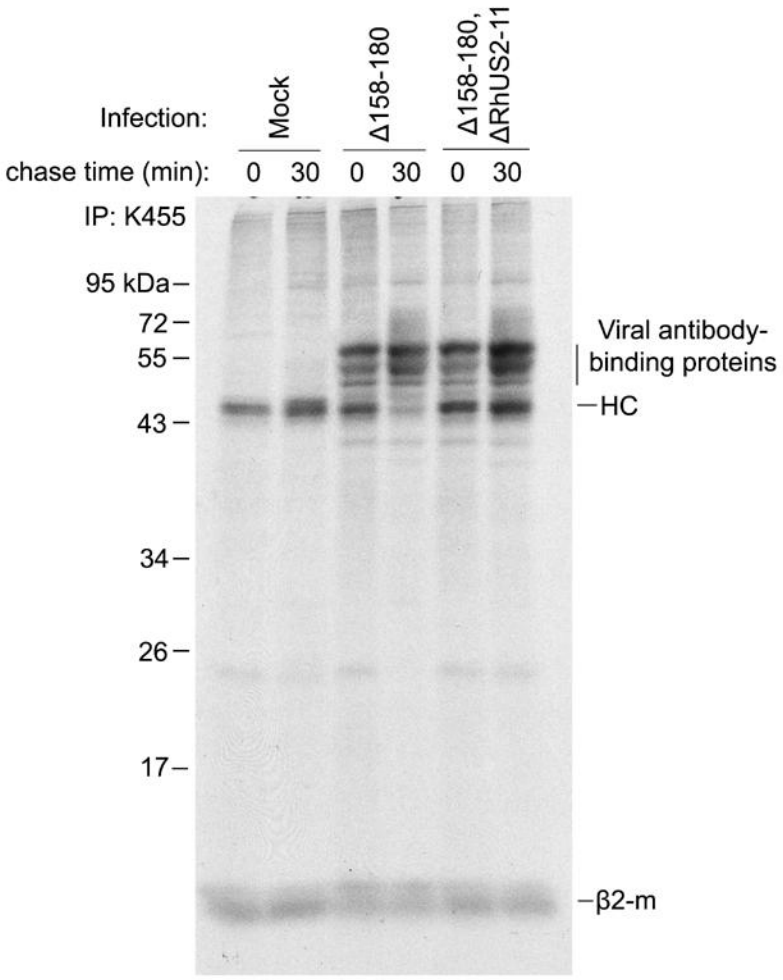
299. Pitcher, C.J., et al., *HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression.* Nat Med, 1999. **5**(5): p. 518-25.

Appendix A



Appendix A: Comparison of wild type rh178 and codon optimized rh178 nucleotide sequence. Codon optimized rh178 shares only 73.9% nucleotide identity with wild type rh178. Top sequence: codon optimized rh178. Bottom sequence: wild type rh178 sequence. Conserved nucleotides are highlited in yellow.

Appendix B



Appendix B: RhCMV contains viral antibody binding proteins that are not specific to the immunoprecipitated antigen. Complete autoradiograph from Fig 2.6B showing pulse-chase and IP during infection with RhCMV Δ158-180 and Δ158-180, ΔRhUS2-11. Indicated on the left side are molecular weight estimates. This indicates the viral antibody binding proteins that are not shown in IPs from other figures since they are non-specific to the immunoprecipitated antigen.

Appendix C

Forward mutagenesis primer (5'-3')	Reverse mutagenesis primer (5'-3')	ORFs deleted
agaaatcgcaaagacattctggtttctgttttgtttcatt	aagcccttcacatgaatcccataaatcataaatgacaggttac	RhUS2-US11
gcaagattacagagtgtaaaagacggcatgttatctacggta	cagggtacgagccgaattaacaggatagtcataggtaggt	Rh158-180
gcaagattacagagtgtaaaagacggcatgttatctacggta	tctgctcacgagctgccctgaagacaactaaccaccacaacccgcgaat	Rh158-168
ccgacggtgttttttatctgccattcttgctcgcgtcagtccatgcg	cagggtacgagccgaattaacaggatagtcataggtaggt	Rh167-180
ccgacggtgttttttatctgccattcttgctcgcgtcagtccatgcg	ttattttaaattatgggctgggtacttacttgaaggtcaagagcacggcc	Rh167-174
gtggtggctgctggccaagtaacgatccacaccatgaattgtcgcactag	cagggtacgagccgaattaacaggatagtcataggtaggt	Rh175-180
gtggtggctgctggccaagtaacgatccacaccatgaattgtcgcactag	cgctccctcggcctgactgatgactagtcatcgcacgcctcttcccgcccgt	Rh175-178
gggcgggaagaggcgtgcgatgactagtcatcagtcaggccgagggagcg	cagggtacgagccgaattaacaggatagtcataggtaggt	Rh179-180
gtggtggctgctggccaagtaacgatccacaccatgaattgtcgcactag	cacattcatccgacacttttatacgaacaaactcgccagt	Rh175-177
ggccatgaaaagtcctaggagaaacacaacgcaaagagca	tctgccgtatcggacaaccctacgccaagcgcacagctgc	Rh176-178
ggtgatggcatgcgcttctgggtgaaagcgagcgtgtgcg	cgctccctcggcctgactgatgactagtcatcgcacgcctcttcccgcccgt	Rh177-178

Appendix C: Primer recombination sequences for BAC recombinant. Note: These sequences are only the primer portion containing homology to RhCMV and do not contain the pCP015 primer binding sites.