Natural Killer Cell Evasion By Rhesus Cytomegalovirus

by

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Chapter 1 INTRODUCTION

2.1 HERPESVIRUSES

1.1.1 Herpesviridae

Viruses within the *Herpesviridae* family are encapsulated, enveloped, double-stranded DNA viruses, with large coding potentials that have co-evolved with their hosts for the past 200 million years. The *Herpesviridae* family is further divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*, which is based upon duration of replication cycle, cell tropism, sites of latency, and species restriction. A total of eight viruses comprising all three subfamily members of the *Herpesviridae* family are known to infect humans and can establish persistent infections that cannot be completely cleared by the host. Viruses within the *Alphaherpesvirinae* subfamily include human pathogens: Herpes simplex virus 1 and 2 (HSV-1, HSV-2) and Varicella-Zoster virus (VZV), which replicate their genomes comparatively quick (hours), infect a wide range of tissues types (mucosal epithelium, neuronal), establish latency in sensory ganglia, and display broad host tropism. The *Betaherpesvirinae* subfamily members have a slower replication cycle (days), a more restricted host tropism, and include Human Cytomegalovirus (HCMV) and Human herpesvirus 6 and 7 (HHV-6/7). Initial infection can occur in a broad spectrum of tissue types, but can be predominantly

found in the salivary gland, bone marrow, and spleen, which are also common sites of latency. Members of the *Gammaherpesvirinae* subfamily, which includes Epstein-Barr virus (EBV) and Kaposi's sarcoma associated virus (KSHV), have the most restricted host tropism and tissue tropism (lymphotropic) of the *Herpesviridae* family members, but vary in length of replication cycle. EBV and KSHV maintain latency in B cells and are associated with lymphoproliferative disorders such as Burkitt's lymphoma, a disease caused by EBV. In addition, KSHV is the causative agent of Kaposi's sarcoma, a vascular tumor of endothelial cell origin (1).

Despite their differences, a number of biological features tie these family members together. For instance, during a lytic infection, all herpesviruses go through a temporal cascade of viral gene expression: immediate early, early, and late. The final result of this productive gene expression program is the lysis of the host cell and release of infectious viral progeny. Another unifying feature of this virus family is the latency gene expression program. While differences between subfamilies dictate cell tropism and conditions leading to reactivation, the viral genomes are maintained as closed circular episomes within the latently infected cells. These infections do not result in viral progeny, but instead express transcripts needed to maintain latent viral genomes (2). Immune evasion is also instrumental to herpesviruses' ability to maintain lifelong infections. Multiple immune evasion strategies employed by all three subfamilies have been identified including: evasion of T cells and NK cells, inhibition of apoptosis, and interference with the interferon response. All herpesviruses encode DNA polymerases, which have adapted to be quite adroit in herpes viral DNA replication due, in part, to multiple associated viral gene products aiding in the replication process. The comparative

massive size of their genomes relative to other virus families makes it necessary for these viruses to facilitate expedited DNA metabolism, and therefore hijack or subvert normal host cell DNA synthesis (1). However, within their enormous coding capacity still lie many questions as to how these viral genes cooperate to produce viral progeny and promote their coexistence with their host.

1.1.2 Cytomegalovirus

1.1.2.1 HCMV Epidemiology and Pathogenesis

HCMV, also known as HHV-5, is the prototypic virus of the Beta-herpesvirus family (3). In the U.S., it is estimated that over 50% of the population is seropositive for HCMV. In developing regions of the world, the percentage of the population infected can reach 100% (4). HCMV is the number one cause of congenital birth defects due to opportunistic infections. It results in loss of hearing and mental retardation, affecting 1 in 750 children born each year (5, 6). However, whether CMV seropositive mothers have natural immunity that protects neonates from symptomatic disease is unclear. A recent meta-analysis of congenital HCMV infections compared the number of infants diagnosed with hearing loss due to vertical transmission of CMV from either primary or non-primary infections in the mother. Both groups of infants had approximately a 12% incidence rate of deafness (7). Similarly, another study found that incidence rates of hearing loss due congenital CMV infections in populations with near 100% CMV seroprevalence were similar to other populations with much lower CMV seroprevalence (8). Of the infants that do contract CMV *in utero*, only 10% to 15% will be symptomatic at birth (9-11). Combined with the fact that CMV is not screened for at birth, it is difficult to determine whether treatments may lower disease related sequelae. However, antivirals

such as ganciclovir are recommended for infants with severe disease especially of the central nervous system (12).

In immunocompromised populations (i.e.—infants and patients on immunosuppressing drugs), HCMV infection causes high morbidity and mortality (13). The mechanisms of pathogenesis in pediatric asymptomatic CMV infections resulting in hearing loss and neurologic sequelae are poorly understood; however, there are common clinical findings found amongst infants with symptomatic congenital CMV infections. For example, 50% of infants born with symptomatic congenital CMV infections are underweight for their gestational age, have an enlarged liver and spleen, and diagnosed with microcephaly. In addition, they commonly display petechial rash and jaundice (11). HCMV has been shown to both infect and prevent proliferation of neural precursor cells (14, 15), which possibly leads to the neurological sequelae found in congenital CMV infections. Likewise, infection of the placenta has also been implicated to play a role in pathogenesis, as CMV infection may alter its development and lead to placenta insufficiency (16, 17).

1.1.2.2 CMV Biology

1.1.2.2.5 Genome Organization

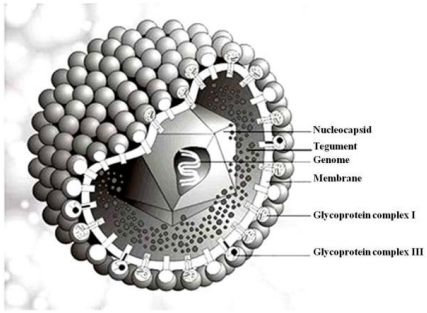
All herpesvirus family members have genomes that exist as monopartite, linear, dsDNA; which contain both repeated sequences (of either direct or inverted orientation) and non-repeated sequences—referred to as unique regions. The CMV genome has two terminal repeats that flank the unique long (U_L) and unique short (U_S) regions, and two inverted internal repeat sequences that divide the U_L and U_S sequences. The repeated sequences facilitate the inversion of the U_L and U_S sequences, thus CMV can exist as one

of four different isoforms—all of which are infectious and found in equimolar concentrations. The ability of CMV to isomerize can lead to large deletions of its genome. One such example is exemplified by the lab-adapted HCMV strain AD169, which contains a large deletion of 15kB and is denoted as U_Lb'. The U_Lb' region is not necessary for growth in cultured fibroblasts. However, the U_Lb' region is maintained in clinical isolates, likely because it encodes multiple immune evasion genes, as well as for glycoproteins that facilitate entry into endothelial and epithelial cells. This gene region also encodes products necessary for maintaining latency, which are necessary to maintain the virus in its natural host *in vivo*. Open reading frames (ORFs) contained in the HCMV U_L and U_S regions are prefaced with either UL or US respectively, and have been previously reported to total over 220 predicted ORFs (18, 19). However, (20) Stern-Ginossar and colleagues found 751 ORFs using ribosomal profiling, albeit many of these transcripts encode proteins less then 20 AA or result from start sites downstream to previously annotated ORFs. HCMV has 13 gene families, which arose due to gene duplications and allow for rapid evolution of the gene family members (21). The complexities of the CMV genome and their functional relevance are still being unraveled.

1.1.2.2.5 Virion Structure

Although larger than other herpesvirus virions (200-300 nm diameter), the CMV virion has a similar spherical structure containing an icosahedral capsid consisting of 162 capsomeres that encloses the dsDNA genome. The virion tegument layer surrounds the capsid and this is enclosed in a lipid bilayer envelope containing viral glycoproteins. The increased size of CMV virions is due to the increased amount of tegument. In HCMV,

this amorphous protein layer makes up 50% of the total virion protein composition, where pp65 is the most abundant tegument protein (18). In addition to viral protein, viral RNA is also found within the tegument layer as well as cellular proteins and RNA (22). The viral envelope glycoproteins can be divided into three types of complexes: gCI, gCII and gCIII (23). Cell attachment is mediated by the gCI (a homodimer of gB) and gCII (a heterodimer of gM and gN). The interactions between gCI and gCIII facilitate HCMV entry into host cells via the gCIII heterotrimer, gH/gL/gO, or gCIII pentameric complex, gH/gL/UL128/UL130/UL131A. The pentameric complex is associated with increased infectivity of epithelial/endothelial cells (24, 25).



(With permission from The Big Picture Book of Viruses: Herpesviridae)

Figure 1.1 HCMV Virion

1.1.2.2.5 Cellular tropism

The previous section describes the viral envelope glycoproteins present in the virion in order to gain host cell entry. Indeed, the current paradigm in the field has been that trimer (gH/gL/gO) was needed for entry into fibroblasts, whereas pentamer (gH/gL/UL128/UL130/UL131A) was necessary for the virus to enter epithelial/endothelial cells; however, this dogma has recently been put into question by Zhou et al. (25) who show that trimer is needed to facilitate fusion-mediated entry into all

cell types, and that pentamer is necessary to broaden tropism through yet undetermined mechanisms. Interestingly, HCMV protein UL148 was recently described as affecting the ratio of trimer to pentamer by binding gO and retaining it in the ER as evidenced by UL148-mediated increased gO endoglygosidase H (endoH) sensitivity (26). Despite the focus on entry of endothelial and epithelial cells, CMV actually has quite a broad cellular tropism that includes: monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, hepatocytes, and fibroblasts (27).

1.1.2.2.5 Replication Cycle

The lytic replication cycle of CMV starts with attachment of the virus and is completed with release of viral progeny by lysing the host cell (Fig 1.2). Most herpesviruses including CMV have initial attachment to host cells via glycosaminoglycans, usually heparan sulfate (28). The attachment step brings the virion in closer proximity to the host cell and allows for the binding step. All herpesviruses contain glycoproteins that reside in the lipid bilayer envelope. Three of these have been shown to be essential for entry and are conserved across the subfamilies: gB, gH, and gL. Multiple receptors have been identified as binding and entry receptors and vary somewhat depending on the virus (28). The binding of host receptors by the virus leads to membrane fusion.

Following entry, the virion is uncoated, whereby the capsid and associated tegument proteins are released into the cytoplasm and travel along microtubules and are directed to to the nuclear pore. Mediated by capsid and tegument viral proteins, the linear viral DNA translocates through nuclear pores and becomes circularized. The viral DNA

genome is immediately silenced by host cell intrinsic factors, which are subsequently degraded by CMV tegument protein, pp71, allowing viral transcription to proceed. The first genes to be transcribed are the immediate early genes. This class of viral gene is defined experimentally as a gene that can be detected when protein synthesis is inhibited by drugs like cyclohexamide. The translated proteins, IE1 and IE2 are transcription factors responsible for expression of early and late genes. They are also negative regulators of immediate early transcription by binding to the viral major immediate early promoter (MIEP) as repressors. The early genes are responsible for making viral genomes, and include DNA replication machinery. The late genes are largely composed of structural genes involved in viral assembly. Capsids, encoded by late genes accumulate in the nucleus and package viral genomes. Once assembled, the encapsulated genomes bud from the nucleus. Viral glycoproteins mature through the ER-golgi and a process of secondary envelopment occurs as the particles undergo another round of envelopment into the virion assembly compartment composed of membranes derived from trans-Golgi and early endosomes. The viral particles are released through exocytosis or cell lysis (1).

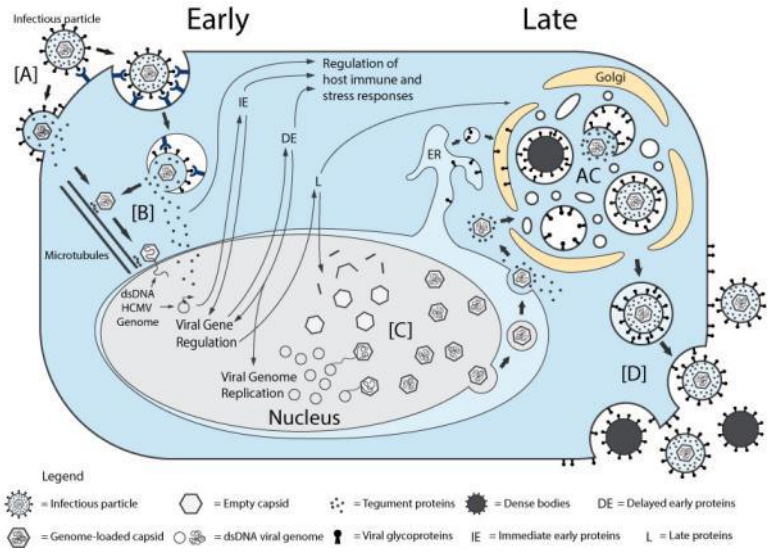


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Figure 1.2 Overview of the HCMV life cycle

1.1.2.2.5 Latency, Persistence & Reactivation

The terms acute and persistent are commonly used to describe the pattern of viral infections within a host. Broadly defined, acute infections are rapid and subsequently cleared by the host adaptive immune response. Persistent infections are long lasting and occur due to the virus evading multiple lines of host immune defenses. However, most viruses that give rise to infections described as persistent do go through an initial acute infection period during primary infection.. The acute phase can be followed by a subsequent chronic stage, where the virus is ultimately cleared or a latent stage ensues, which lasts the lifetime of the host. Latency is characterized by three general features: 1) low to nonexistent viral gene expression 2) poor recognition of virally infected cells by host immune system, and 3) retention of an intact viral genome allowing future viral progeny to be generated during reactivation (1). CMV is categorized as a persistent virus that maintains a life long infection within its host. However, this persistence is not solely dependent upon latently infected cells. Indeed, CMV is known to establish persistent infections within endothelial and epithelial cells that continue to shed virus, but CMV also maintains its genome in latently infected CD34+ hematopoietic cells (33). CMV is capable of infecting these cells, but cannot undergo full lytic replication. Instead, the CMV genome is maintained as an episome and latency associated gene products are expressed (34, 35). One gene product associated with the latency gene expression profile is latent undefined nuclear antigen, LUNA, which is encoded within transcripts that are antisense to the UL81-UL82 (38, 39). LUNA transcripts can be found in CMV-infected $CD34^{+}$ cells and are reduced upon their differentiation and CMV reactivation (40). Another latency-associated transcript, UL111a (41), encodes the viral homologue of

interleukin-10 and is thought is promote immune evasion of latently infected cells (42). Interestingly, the UL133-UL138 locus, encoded within the U_Lb' region that is dispensable for replication in fibroblasts, is also necessary to restrict replication in hematopoietic cells (43). Recently, the G protein-coupled receptor, US28, was also reported to promote latency in hematopoietic cells (518). Additionally, multiple microRNAs are expressed during latency that restrict viral DNA replication. For example, mirUL-112-1 targets the IE1 transcript (44), and has been postulated that this translational repression along with chromatin remodeling at the MIEP within CMV-infected $CD34^+$ cells is needed to restrict IE expression to enter and maintain latency (36, 45). Changes in the cellular environment can reactivate of HCMV from latency is associated with the maturation of latently infected cells into differentiated phenotypes, and is dependent on viral IE expression. The exact changes in the host cellular and extracellular factors required for reactivation are currently unknown. Future research is needed to elucidate which cell types can be latently infected and under what conditions, as well as the requirements of reactivation.

1.1.2.3 Host Immune Response to CMV Infection

CMV is able to setup a life long persistent infection within its host; however, this does not preclude the fact that the immune system mounts a multifactorial attack against the virus. Normal immune control of CMV prevents overt disease by keeping the virus in check. The immune response is comprised of the following three branches: intrinsic, innate, and adaptive. The role each type of immunity has on detection and clearance of CMV will be discussed below.

1.1.2.3.1 Intrinsic Immunity

Intrinsic immune components are constitutively expressed, and ever-present within the cell. One of the first intrinsic immune molecules described was APOBEC3G, a cytidine deaminase responsible for hypermutation in ssDNA during reverse transcription leading to the production of nonviable retroviruses (46). Intrinsic factors also limit the replication and spread of CMV. Within the nucleus, large protein complexes, known as ND10 or PML bodies, function to regulate a wide variety of cellular functions related to cellular fitness and stability (47). Utilizing sumolyation domains, the Death-associated protein 6 (DAXX) and ATRX are recruited to the ND10 bodies localized at histones and are responsible for transcriptional repression (48), including repression of the CMV MIEP (49, 50). It is no surprise then that CMV employs multiple strategies to avoid the intrinsic antiviral activity of ND10 bodies (51, 52). The tegument protein, pp71, is delivered to the nucleus after entry and degrades DAXX, leading to the expression of IE proteins that further degrade or disrupt additional proteins within ND10 bodies and allow for further transcriptional activation of downstream viral genes (52).

1.1.2.3.2 Innate Immunity

The innate immune response encompasses both molecular and cellular components defined by their ability to rapidly respond to a broad array of pathogens and features commonly associated with incoming pathogens without prior exposure. These features (i.e. —LPS, CpG, and dsRNA) referred to as pathogen-associated-molecular-patterns (PAMPs), are detected by a diverse set of pattern recognition receptors (PRRs),

which exist both in the cytosol and on the cell's surface. An example of PRRs are the Toll-like receptors (TLRs), which are intracellular and cell surface resident receptors. TLR2 has been reported to be triggered by HCMV (53). Additional intracellular dsDNA PRR sensors have been described that detect CMV including: IFI16, ZBP1, and AIM2 (54-56). The activation of PRRs leads to the activation of downstream signaling cascades via pathways such as the NFκB pathway and the IRF-3 pathway, which converge into the JAK/STAT pathway and result in the expression of pro-inflammatory cytokines such as type I/II interferon and interferon stimulated genes (ISGs). These pathways are responsible for inducing inflammation and eliciting an antiviral state within the host including the activation of caspases leading to apoptosis of virally infected cells, inhibition of protein synthesis, upregulation of factors involved in the antigen presentation pathway including Major Histocompatibility complex Class I (MHCI), maturation and recruitment of effector cells to the site of infection, changes in the vasculature leading increased blood flow, and activation of the adaptive immune response (57).

PRRs can be triggered in cells through infection; however, sentinel cells like macrophages and dendritic cells (DCs), also express PRRs that can be triggered while scavenging dead and dying cells. Once mature, DCs in particular, upregulate chemokine receptors necessary to migrate to the lymph nodes and prime naïve T cells. However, since DCs infected with CMV are hindered from differentiation and maturation, they are also inhibited in their ability to stimulate T cell activation and proliferation (58, 59)

Another important innate immune cell type controlling CMV infection is the Natural Killer (NK) cell. Their importance is elegantly demonstrated by the findings of

Biron et al. (60), which describes a patient with a rare SCID disorder resulting in the complete lack of NK cells. Over the course of the patient's lifetime, the patient succumbed to severe disease resulting from herpesvirus infections, even though they had a normal repertoire of B and T cells (60). Another team studied a patient suffering from CMV infection that had normal NK and B cell development, but was deficient for T cells. After 6 weeks, the virus was undetectable, despite the fact that they had no specific T cell response against the infection (61). The unique features of NK cell biology and the role of CMV NK cell evasion will be discussed at length in the forthcoming sections of this dissertation.

1.1.2.3.3 Adaptive Immunity

The adaptive immune response is comprised of cellular and humoral arms (T cells and antibodies, respectively) that work to rid the host of pathogen. CMV targets both arms in a variety of ways. During a cellular immune response, three signals are required by primary T cells in order to induce proliferation and activate signaling cascades leading to effector functions such as release of cytokines and cytolysis of infected cells. Signal one requires the recognition of MHC, of which there are two different classes that interact directly with T cells. MHCI is expressed on the surface of most host cells, whereas MHC class II is expressed primarily on antigen presenting cells. Both molecules present peptides, derived from the proteasome, loaded into their peptide binding groove during protein maturation in the ER. These peptides can be non-self peptides derived from intracellular pathogens or they can be self-antigens but self-reactive T cells are typically deleted in the thymus during development. During signal one, this non-self

peptide/ MHC complex is recognized and bound by a specific T cell receptor (TCR). T cells also express a co-receptor that is needed to transduce the signal and is involved in binding peptide/MHC along with the TCR. Traditionally, CD8 associates with TCRs that bind MHC I and CD4 associates with TCRs that bind MHCII; however, this paradigm has been shifted due to recent work involving RhCMV, and will described in further detail below (62). Signal two, costimulation, is achieved through binding members of the Ig and TNFR families, (eg. CD28 with B7). Signal three, provided by cytokines, are needed to perpetuate T cell differentiation and survival.

CMV has a dramatic effect on the CTL compartment of the host, where 10-20 % of the total CD8 T cell population can be comprised of CMV-specific CD8 T cells. This increase in CTLs known as memory inflation, an effect seen in RhCMV, MCMV and HCMV (63-66). The inflationary CTLs have an effector memory phenotype characterized as having low levels of CD62L and CCR7 (lymph node honing markers) allowing them to be in the periphery, where they can rapidly respond as they are cytolytic and produce high levels of the cytokines, TNFα and INFγ. They also lack exhaustion markers such as PD-1 (67).

Antibodies are also produced due to CMV infection. B cells are activated when their B cell receptors recognize CMV peptide presented on MHC by antigen presenting cells (APC)s. This stimulates the B cells to produce antibodies. The most common antibodies are specific CMV proteins like IE1, tegument proteins, envelope glycoproteins (68). However, while both of these responses are important for controlling the virus, neither antibodies nor CTLs are capable of completely clearing CMV from the host.

1.1.2.4. CMV Models

Since CMV has strict species specificity, animal models cannot be used to directly study HCMV *in vivo.* Therefore, animal models have been developed that study orthologous CMVs and the infection of their natural mammalian host to study CMV replication, pathogenesis and pathology, host immune responses and CMV immune evasion. The mouse CMV (MCMV): mouse infection model is the most established animal model. This model first developed in 1954 with the isolation of the Smith strain of MCMV from a mouse salivary gland (69). Advantages of the MCMV model include lower cost, increased statistical power through larger group size, availability of genetic knockout and isogenic inbred mouse strains, and mouse specific reagents that have been well-characterized (i.e.—tetramers, antibodies, etc.). MCMV was cloned into a bacterial artificial chromosome, which allowed for mutagenesis of the viral genome to further study specific mechanisms of virally encoded gene products (70). However, there are limitations to the knowledge we can gain using MCMV as a model of HCMV infections of humans due to evolutionary divergence between both host and virus. For instance, the murine NK cell receptor and ligand repertoire varies considerably compared to human NK cells (71). Further, the MCMV virus encodes a protein that itself is a NK cell activating ligand, a fact that is not recapitulated in the HCMV genome (72). In addition to MCMV several other animal models have been developed including rat (RCMV), guinea pig (GPCMV), and Rhesus macaque (RhCMV). GPCMV has been instrumental in understanding congenital CMV sequelae due to relatedness of human and guinea pig placentae (RR 35,36). Advances in our understanding and treatment of CMV-mediated transplant-associated vascular disease have been made using the RCMV model (73-75).

However, due to the fact that CMV co-evoles with its the host, significant evolutionary divergence has occurred between rodent and human CMV genomes (76), especially with respect to non-essential genes (77). Due to the relatedness of RhCMV to HCMV and the relatedness of their respective hosts, RhCMV infection of Rhesus macaques (RM) has emerged as an important animal, which will be explored in more detail below.

1.1.2.4.1 Rhesus CMV

1.1.2.4.1.1 Overview

RhCMV was first isolated from the urine of a healthy RM in 1974 and the strain was named 68-1 (78). This was later cloned into a BAC, which has allowed for greater ease of viral genome mutagenesis (79). The BAC derived 68-1 strain has undergone extensive sequencing and proteomics. The genome is over 220,000 Kbps and encodes over 260 ORFs (77, 80). Interestingly, similar mutations found in lab adapted strains of HCMV in the ULb' region (81) have also been identified in BAC derived RhCMV 68-1, where both viruses were extensively passaged in fibroblasts and have the same commensurate deletion of UL128 important for epithelial cell tropism (80). Only two ORFs have been characterized as RhCMV-specific and not found in the HCMV genome; Rh178, responsible for the inhibition of MHC-I translation (82) and Rh10, a viral homologue to the host gene for the cyclooxygenase-2 enzyme that enhances growth in endothelial cells (83). Importantly, most gene families within the HCMV genome are conserved in RhCMV (77).

1.1.2.4.1.2 RhCMV as a model for HCMV

In order for a scientific finding obtained using an animal model to add insight to the biology of human disease, the pathology of the disease needs to be similar between what is seen in humans and the particular animal model you are using. The commonalties of RhCMV infection in RMs with HCMV infection in humans are a major advantage of using the RhCMV model. In immunocompetent RMs, similar to humans, CMV infections are asymptomatic. Additionally, CMV infection is pervasive throughout the population, where in nearly 100% of RMs in primate centers are CMV positive and populations in the wild have been reported as 95% CMV positive (84, 85). Seroconversion occurs during the first year of life and then the RMs continue to shed virus in both the saliva and urine (78, 86, 87). Another important feature is that like HCMV, RhCMV is able to superinfect a RhCMV-seropositve. The genes responsible for superinfection, those that inhibit antigen presentation, are also conserved between RhCMV and HCMV (88, 89). In addition, the unique immune responses seen in HCMV infections such as high numbers of $CD4^+$ and $CD8^+$ CMV-specific T cells (64, 65) and gB-specific antibodies are also found during RhCMV infection (68).

CMV infection is often concomitant in AIDS patients who have succumbed to disease due to their HIV infection. RM that are infected with related simian immunodeficiency virus (SIV) also develop an AIDS-like phenotype, conferring vulnerability to CMV-related disease (90, 91). The co-infection of RhCMV and SIV has become an important model for the study of CMV-related disease in an immunocomprised host (92). Built on the shoulders of SIV animal models (93-96), use of CMV as vaccine vector to immunize against HIV is currently being investigated (62, 89,

97, 98). The details of these efforts will be discussed below in greater detail, but is mentioned here to illustrate the relevance and importance of using RhCMV as a model for HCMV.

1.2. NK CELL BIOLOGY

1.2.1. Overview

NK cells are an important subset of the innate immune system that are rapid responders in the recognition of both cellular transformation and infection. Individuals with genetic mutations that result in an absence of NK cells suffer from severe herpesvirus infections and ultimately succumb to the disease (60), NK cells express inhibiting and activating receptors that regulate their behavior. NK cell inhibitory receptors bind MHCI, which is present on the surface of healthy, non-infected cells. Receptor:MHCI engagement transduces an inhibitory signal and prevents NK cell lysis of the target cell. NK cell activating receptors recognize stress inducible ligands, which are only expressed upon viral infection and cellular transformation. The combination of activating signals and a lack of inhibitory signals transduced through the NK cell receptors elicit a variety of effector functions. In general, activated NK cells lyse target cells and produce a variety of cytokines, notably interferon-γ (IFN-γ), which is important in the activation of T cells. The following section will review the important features of NK cell biology including development, regulation of inhibition and activation, and recent advances describing NK cell memory.

1.2.2. Lineage, Development & Phenotype

NK cells make up between 2-20% of lymphocytes depending on the tissue type (99, 100). As with all lymphocytes, including B and T cells, their development begins in the bone marrow as pluripotent HSCs. Signaling through the tyrosine kinase, FLT3, drives differentiation and gives rise to the CLP (101, 102). Although the factors required for further differentiation into progenitor NK cells are not clearly established, cells that are committed to the NK cell lineage are defined by the expression of interleukin-2 (IL)-2 and IL-15Rβ (103). The lineage committed NK cells are able to bind additional IL-15, which drives their differentiation into a mature phenotype (101, 104).

Mature NK cell populations differ in distribution and effector function, as well as the amounts and types of inhibitory and activating receptors they express. Human NK cells were initially grouped into 2 major subsets—either $CD3^-/CD56^{dim}$ or $CD3^-/CD56^{bright}$ (100, 102, 105) . CD56, also known as Neural Cell Adhesion Molecule (NCAM), is a glycoprotein in the immunoglobulin (Ig) superfamily that binds heparin sulfates (106-110). The majority (90%) of NK Cells are $CD3^-/CD56^{dim}$, primarily reside in the blood and spleen, and upon activation are cytolytic and secrete interferon-λ (111, 112). Consistent with an effector phenotype, $CD3^-/CD56^{dim}$ NK cells also express the activating receptor, CD16 (113), responsible for antibody (Ab) dependent cellular cytotoxicity (ADCC) (114-117). Conversely, the minority population (10%) of $CD3^-/CD56^{bright}$ NK Cells do not express factors associated with cytoxicity, but upon stimulation secrete high amounts of cytokine—namely tumor necrosis factor (TNF)-α and interferon-λ, and are largely found in the lymph nodes (LN) (118-125). Evidence suggests that the CD56bright NK cell population are the precursors to CD56dim subset,

(104, 117, 126-128) and can be phenotypically distinguished by a wide differential in surface marker expression, which confer their functional differences (Fig 1.3 (129, 130)).

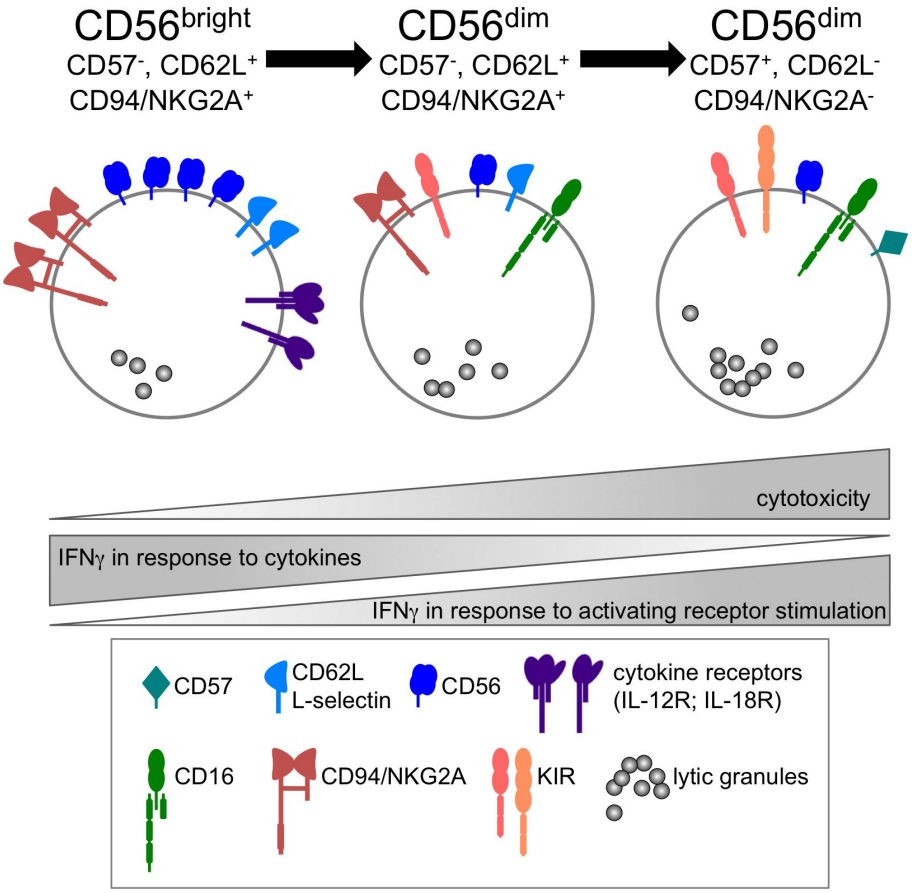

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Figure 1.3: Human NK cell differentiation and maturation

CD56^{bright} NK cells express CCR7^{+}, which is likely necessary for homing to the lymph nodes and other secondary lymphoid tissue (131, 132), whereas CD56^{dim} NK cells express CXCR1 and ChemR allowing for homing to the peripheral tissue during inflammation (133, 134). CD56^{bright} NK cells also express higher amounts of adhesion molecules compared to CD56^{dim} NK cells, thus allowing for the differences in observed tissue distribution (135, 136).

As discussed below, the expression of inhibitory receptors is essential to transduce signals necessary for NK cell activation. Therefore, not surprisingly, CD56^{bright}

and $CD56^{dim}$ NK cells express different inhibitory receptors. $CD56^{bright}$ NK cells express the inhibitory receptor heterodimer CD94/NKG2A, whereas the $CD56^{dim}$ NK cells express inhibitory killer cell Ig-like receptors (KIR)s (101, 137-140).

CD57 has been reported as a marker of senescence in $CD8^{+}$ T cells (106, 108-110, 141-144). However, data also supports CD57 expression as a marker of $CD8^{+}$ T cell terminal differentiation (111, 145), a finding that was recapitulated in the $CD56^{dim}$ NK subset by the Lanier lab showing that $CD57^{+}$/ $CD56^{dim}$ NK cells were more cytolytic and secreted more cytokine then their $CD57^{-}$ counterparts when activated via CD16 (113, 146-148).

Despite close evolutionary ties, RM NK cells do not express CD56 (126, 149, 150). Instead, the inhibitory receptor NKG2A is used as a specific marker for RM NK cells (151, 152). Another important distinction is that RM NK cells highly express CD8α, whereas human NKs do not (129, 147).

1.2.3. NK Cell Receptors

1.2.3.1. NK Cell Inhibition

NK cells survey the environment in response to signals transduced through their receptors. One important molecule in this environment is MHCI. In humans, the human leukocyte antigen (HLA) complex encodes 6 different MHCI genes. HLA-A, B, and C are classical MHCI proteins that bind β2 microglobulin (β2m) and are highly polymorphic –with the current counts of documented alleles reaching over ten thousand (131). HLA-E, F, G are non-classical MHCI molecules, which are less polymorphic. Not only is classical MHCI important in its role of presenting antigen to T cells, it also has the major function of engaging inhibitory receptors on NK cells, thus preventing activation

and downstream effector functions. Classical MHCI is present on the surface of all nucleated cells, and serves as a indicator for the health of an individual cell. MHCI cell surface expression is often downregulated due to viral infections and cellular transformation. Therefore, even though these cells are able to evade T cell recognition, NK cells can detect the lack of MHCI via inhibitory NK cell receptors. NK cells express a wide variety of inhibitory receptors, some of which are type I glycoproteins within the immunoglobulin (Ig) superfamily, such as killer cell Ig-like receptors (KIRs) and leukocyte Ig-like receptors (LILRs) and bind classical MHCI. Other inhibitory receptors, like CD94-NKG2A, fall into the type II C-type lectin binding protein family.

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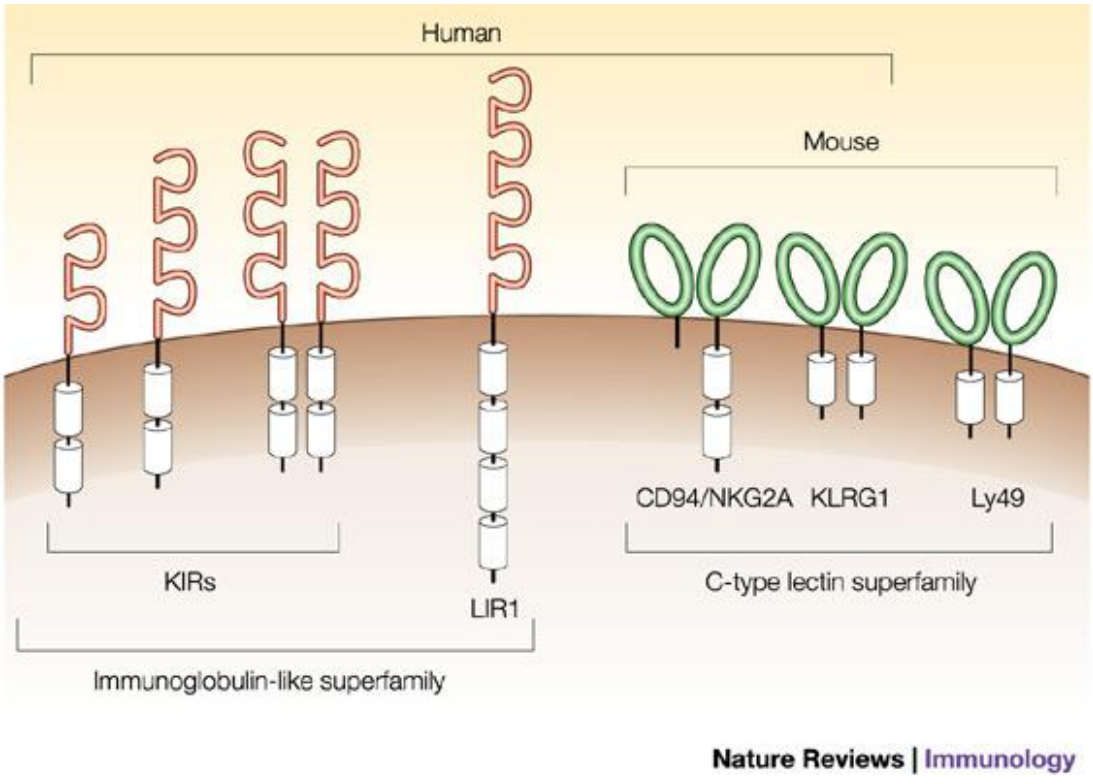


Figure 1.4: Inhibitory NK cell receptors

However, all inhibitory receptors have the common motif, I/L/V/SxYxxL/V (in single-letter amino acid code, slashes separate alternative amino acids that may occupy a given position) within their cytoplasmic tail known as the immunoreceptor tyrosine-based inhibitory motif (ITIM). Within this motif is a tyrosine residue that becomes phosphorylated by syk family kinases after engagement with ligand and recruits phosphatases, SHP-1 and SHP-2 (133), eliciting a signaling cascade which results in actin remodeling (135) and the prevention of immune synapse formation (153-155). In addition, the inhibitory receptors form microclusters upon stimulation that suppress activating receptors from forming clusters also needed for actin remodeling (137, 138, 140). Thus, ITIMs mediate a long list of interactions that converge to prevent the initiation of NK cell activation. This dominance of inhibition over activation ensures that NK cells do not attack the host to cause immunopathology.

In addition to the cytosolic ITIM domains, the inhibitory KIRs contain either two or three Ig domains and are designated as either KIR2D or KIR3D, respectively. The KIR2D receptors commonly bind HLA-C alleles, whereas KIR3D binds HLA-B or HLA-A. Following the “2D” or “3D” designation is either “L” for long cytoplasmic tail, which contains either one or two ITIMS or “S” for short cytoplasmic tail without ITIMs, and are thus activating receptors, which will be discussed in more detail below (141-144). Different isoforms of KIRs recognize different HLA alleles, and only slight changes in amino acid sequence are necessary to have an effect on specificity. For instance, two common HLA-C polymorphisms at positions 77 and 80 are recognized by two different isoforms of KIR2DL (145). This highly polymorphic region of the genome is encoded within the leukocyte receptor complex (LRC) in both humans and old world monkeys

(146-148), 22 *Mamu-KIR* genes, both activating and inhibitory, have been discovered in RM thus far (156).

The mouse genome does not encode the KIR family, but instead has its own analogous family of receptors—Ly49. These receptors also bind MHCI; however, they are type 2 C-type lectin binding proteins. Although, they have divergent structures from KIRs, these molecules also have ITIMs and recruit tyrosine phosphatases – thereby blocking activation. Another commonality with KIRs, a subset of Ly49 receptors are activating receptors that lack ITIMs. Interestingly, one inhibitory Ly49 receptor, Ly49I, binds an MCMV protein, m157, in addition to MHCI (72). Much work has been done studying the interactions of MCMV and NK cells, with great focus on the interactions with the Ly49 receptor family in particular. The details of MCMV interactions with the Ly49 receptor family will be discussed in greater detail in the section review of CMV NK cell evasion.

The LRC gene region encodes additional inhibitory receptors. Leukocyte immunoglobulin (Ig)-like receptors (LILR) in humans (157, 158) and orthologous Paired Ig-like receptors (PIR) genes in mouse can both can bind MHCI, although these orthologs differ in expression patterns as PIRs are not expressed on mouse NK cells (159-162). The human LILRB1 binds with low affinity to nearly all HLA proteins including non-classical MHCI. LILRs are highly expressed on monocytes and B cells, and seem to play a minor role in regulating NK function compared to other inhibitory receptors (157, 158, 163).

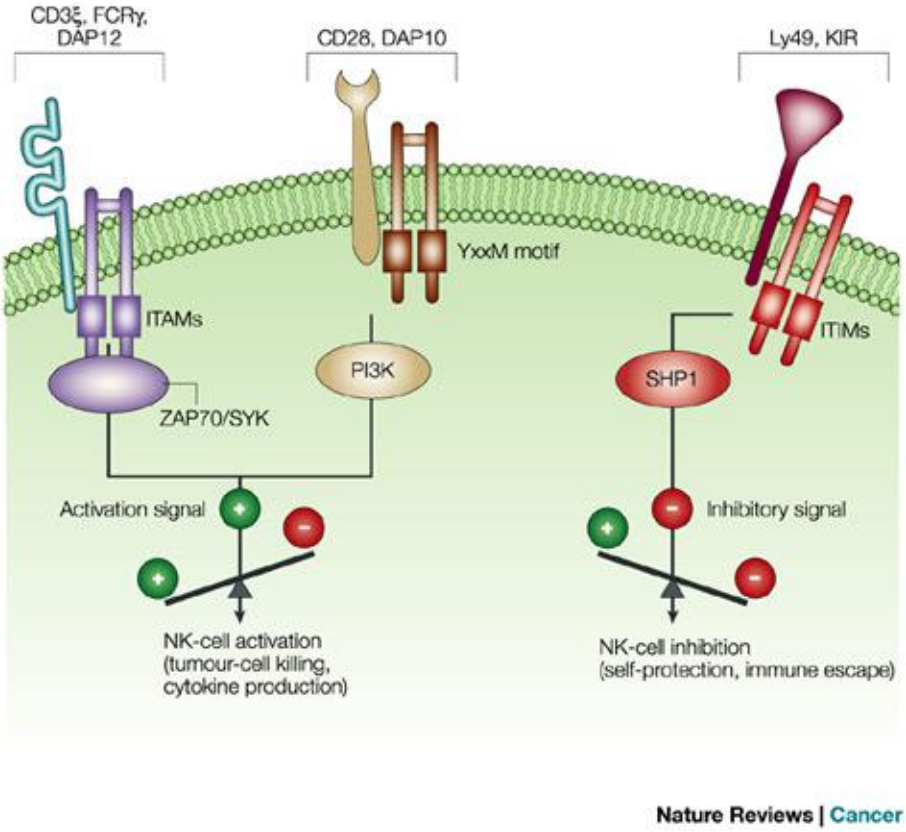
Located in the NK complex of human, RM, and mouse are the *CD94* and *NKG2* gene families that encode receptors that bind non-classical MHCI: HLA-E, mamu-E, and

Qa-1^b, respectively (164-167). HLA-E differs from classical MHC-I in that peptides, that bind the groove are not derived from the sampling of all actively translated proteins. Instead the leader sequence from MHCI, which is cleaved during co-translation, binds to the peptide cleft (165, 166, 168, 169). Interestingly, HLA-E cannot bind its own signal peptide. CD94 can dimerize with several different members of the NKG2 family (in humans, NKG2A, -C, -E, and -F) (170); however, only NKG2A leads to inhibition due an ITIM in its cytoplasmic tail (171-175). The *CD94/NKG2* genes are far less polymorphic compared to the *Ly49* and *KIR* genes (176, 177). Other proteins in this family that heterodimerize with CD94 are activating receptors and will be discussed below; however, it should be noted that NK cells can be double positive for both the inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimers. However, the CD94/NKG2A receptor has higher affinity for MHCI 209. The CD94/NKG2 receptors are not constitutively expressed, and expression can be modulated by cytokines IL-15 (178-180), TGF-β (181), and IL-12 (182).

1.2.3.2. NK Cell Activation

The ability of NK cells to lyse target cells deficient of MHCI was first described by Karre et al. (183). They elegantly show that MHC1 deficient tumors where more effectively lysed by natural killer cells compared to tumor cells that were $MHCI^+$ (183, 184). Raulet et al. (185) demonstrated that bone marrow derived from β2m (-/-) donors (MHC null), could not graft into MHC matched recipient mice either heterozygous or wt for β2m. These data helped to shape the “missing self” hypothesis, stating that NK cells will kill target cells that do not express MHCI–a marker for “self” (186). The past 25

years of work has expounded upon and clarified the roles of MHCI interactions with NK cells and will be described in further detail in the section describing NK cell education. Simplistically, if inhibitory receptors are not engaged then the breaks are released and cannot block signal transduction via activating receptors—thus allowing the NK cell to perform effector functions upon ligand engagement (187-192).



(With permission from Smyth et al, Nature Reviews Cancer, 2002.

Figure 1.5: Control of NK-cell functions by balance of activation/inhibitory signals

NK cell activating receptors vary in both structure and adaptor molecules needed to transduce a signal. Indeed, many activating receptors do not possess domains to signal from directly; instead they associate with adaptor proteins that contain immunoreceptor tyrosine-based activation motif (ITAM) domains with the sequence Asp/Glu-x-x-Tyr-x-x-Leu/Ile x6−8 Tyr xx Leu/Ile, where x denotes any amino acid with 6 to 8 amino acids between the two Tyr xx Leu/Ile elements (193-196). When ITAMs become

phosphorylated a signaling cascade occurs starting with the recruitment tyrosine kinases Syk and ZAP70 (197), resulting in the downstream effects of activation including Ca^{2+} influx, degranulation, and expression of cytokines. Alternatively, NK activating receptors can also signal through other adaptor molecules like DAP10, which signals through the phosphatidylinositol-3 kinase (PI3K) signaling pathway (191, 198, 199).

Some of the earliest research involving NK cells focused on their ability to perform Antibody (Ab) -dependent cellular cytotoxicity (ADCC), which is initiated by a process known as opsonization, where ab bind and coat the surface of the cell. NK cells 8activates the NK cell. The activated NK cell then lyses the cell by forming a immunological synapse with the target cell. Human CD16 can associate with either ITAM containing adaptors CD3ζ or FcεRIγ (105, 193, 200, 201). However, mouse CD16 only associates with FcεRIγ (202-204). Unlike other activating receptors, triggering through CD16 alone is enough to induce cytolysis because it can associate with multiple adaptors, all of which are needed to fully activate the pathway. Other major NK activating receptors need pairwise combinations of receptors that use differing adaptors in order to have the same effect (155, 205).

The NKG2D receptor is a type 2 C-type lectin binding protein that is conserved amongst mammals with very few polymorphisms (157, 170, 177). It belongs to the NKG2 family, but has very little homology to other family members (170). NKG2D forms homodimers (191) that are expressed on NK cells, γδTCR+ T cells, and CD8+ T cells (198, 206-215). In humans, all CD8+ T cells, including naïve T cells express NKG2D (206, 210); however, in mouse only activated T cells express NKG2D (208, 216-218). Thus, naïve mouse T cell cannot receive costimulatory signals through

NKG2D. Another important distinction is their use of adaptor proteins. Human NKG2D solely associates with DAP10, which contains a YxxM tyrosine-based signaling motif (187, 191, 198, 210, 215, 219, 220); however, mice can express two different NKG2D isoforms: NKG2D-L, associating with DAP10, and NKG2D-S, associating with either DAP12 or DAP10 (190, 214, 221). DAP12 recruits Syk and ZAP70 tyrosine kinases, whereas DAP 10 recruits (PI3K) (191, 198). In order for NKG2D to activate the NK cell, another coactivating receptor must also be engaged (205, 217, 222-228).

NKG2D binds ligands containing homology to MHC. One such family of ligands is the MHC class I chain-related (MIC) family, which is comprised of MICA and MICB in humans. This family of genes are encoded within the MHC locus and are highly polymorphic, 103 and 42 alleles, respectively for MICA and MICB (131, 217, 227). RM also encode the *MIC* family. *MIC1* is orthologous to *MICA* and *MIC2* is orthologous to *MICB*. The RM genome also encodes *MIC3*, which is a gene fusion of *MIC1* and *MIC2* (224, 229, 230). All of the MIC proteins have the α1, -2, and -3 domains of MHC but do not bind β2m (224, 227, 229, 231).

The UL16-binding protein (ULBP) family is also conserved amongst mammals. Humans encode for ULBP1-6, which are also MHC-like proteins – containing the α1 and α2 domains, and can be either transmembrane (ULBP1-3, 6) or GPI-anchored (ULBP4, 5) (232, 233). RM encode four *ULBP* homologs including *ULBP1-4* (234, 235). Mice encode the *RAE-1* genes, which are orthologous to the *ULBP* gene family. The *RAE-1* family is comprised of *RAE-1α, RAE-1β, RAE- 1γ, RAE-1δ, RAE-1ε* (236, 237). The genes *MULT1* and *H60* are two more additional MHC-like ligands (α1 and α2 domains) for NKG2D encoded within the mouse genome (234, 238, 239).

NKG2D ligands are induced upon viral infection and following cellular transformation; however, our currnet knowledge of all pathways leading to induction is incomplete. The MIC promoters contain a heat shock binding domain, and can respond to cellular stress (240, 241). It has been shown that they can be induced upon DNA damage response, and are sensitive to chromatin remodeling. HDAC inhibitors can also induce MIC expression (113, 240, 242-251). There are some general trends that are found in cancer cell NKG2DL expression, which will be discussed in the section describing the role of NK cells in cancer. CMV on the other hand upregulates all known NKG2DLs (206, 251-253). This upregulation is CMV specific, as IE1 upregulates ULBP2 transcription and IE2 upregulates MICA/B (254-257)

The other two activating members of NKG2 family are NKG2C and NKG2E. Both form heterodimers with CD94, but have a lower affinity for HLA-E than their inhibitory counterpart, NKG2A (258, 259). CD94/NKG2C associates with DAP12 to transmit downstream signals and elicit NK cell effector functions (258, 260). Of particular interest, NKG2C has been correlated with a memory-like phenotype in natural killer cells and will be discussed in further detail in the section on NK cell memory.

The natural cytotoxicity receptors (NCR) including NKp30, NKp44, and NKp46 are activating receptors within the Ig superfamily. Lacking close homology, these receptors were originally grouped for their ability to induce cytotoxicity without antibody opposed to ADCC (261, 262). Both NKp46 and NKp30 can signal through either CD3ζ or FcεRIγ, whereas NKp44 signals through DAP10 (262-264). They can bind terminal sialic acids and hemgglutinins residing on molecules from incoming pathogens and thus resemble PAMP receptors (264-267). NKp46 is the most ubiquitously expressed NCR,

and the only NCR known to also be expressed in mice (268, 269). In addition to pathogenic ligands, NKp30 binds HLA-B-associated transcript 3 (BAT3), which is exosomally released from tumor cells upon cellular stress (270). The membrane protein, B7-H6, which is upregulated in tumor cell lines is also bound by NKp30 (271, 272). New cancer induced activated ligands continue to be discovered. Recently, the mixed-lineage leukemia-5 protein (MLL-5) was discovered to bind NKp44. This ligand is absent on healthy cells, but detectable on a wide variety of tumor cells (273-279).

As mentioned above, both the KIR and Ly49 receptor families contain members that serve as activating receptors due to their short cytoplasmic tails and associate with ITAM containing adaptor, DAP12 (280-284). The existence of these activating isoforms are likely due to the expansion of gene clusters and CMV has been proposed to place immune pressure on the locus thus driving evolution (285-289), and is exemplified by the example MCMV activating Ly49H evolving from the inhibitory Ly49I (290).

The signaling lymphocytic activation molecule (SLAM) family of receptors contain the immunoreceptor tyrosine-based switch motifs (ITSM) in their cytoplasmic tail, which recruits the Src kinase Fyn (291, 292). The family includes 2B4, NTB-A, and CRACC (293). 2B4 is the best characterized member and expressed on mouse and human NK cells (293). 2B4 binds CD48, which is expressed on all peripheral blood cells and contributes to immune synapse formation during T cell activation (292, 294). The co-activator receptor, 2B4, is only efficiently stimulated when coupled with engaged receptors that signal through ITAMs and/or YINM motifs (205).

The activating receptors DNAM-1, NKp80 and NKp65 contain signaling motifs within their cytoplasmic tail, thus do not associate with any of the signaling adaptors

mentioned thus far. Both NKp80 and NKp65 bind the c-type lectins AICL (295) and KACL (296), respectively; and signal through a hemi-ITAM in their cytoplasmic tail (172, 297). DNAM-1 binds nectins CD112 and CD155 (176, 298, 299), and can signal through tyrosine based motifs that recruit the Src kinase Fyn and the Ser/Thr kinase PKC (178, 180, 300).

1.2.4. NK Cell Education

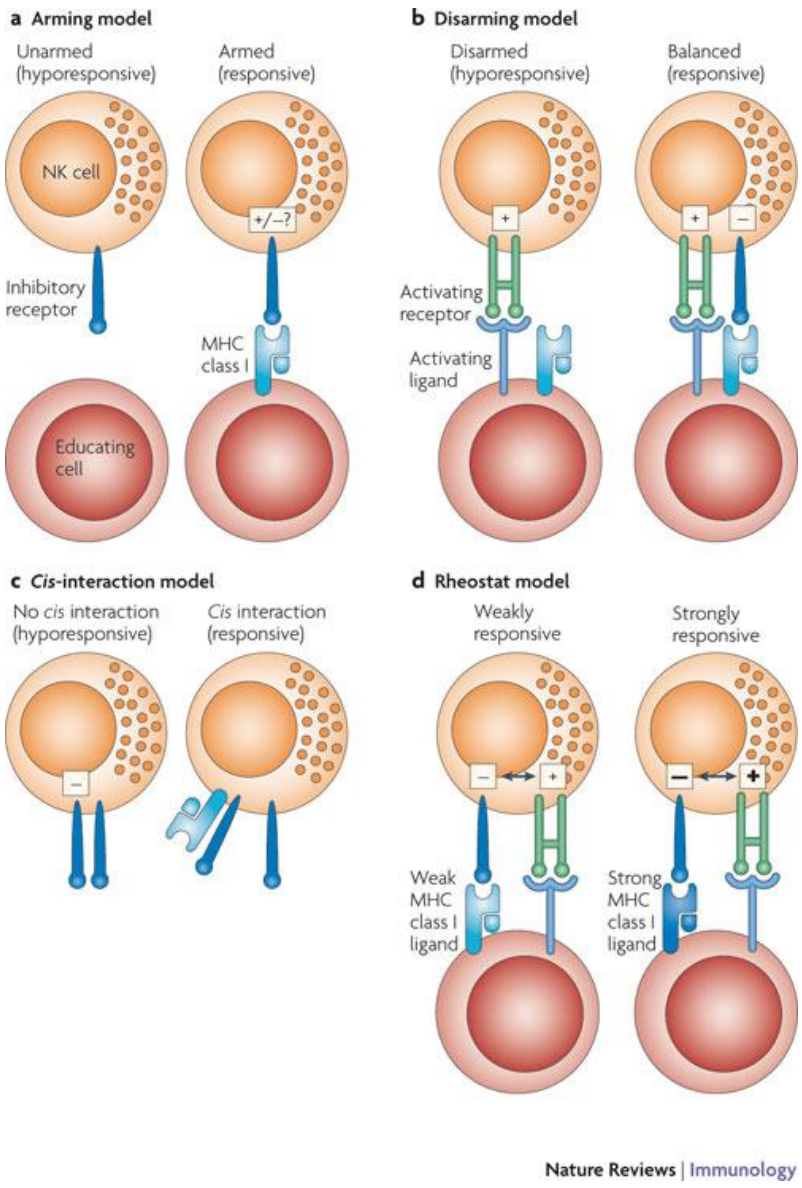
1.2.4.1. Missing self

NK cells must induce self-tolerance in order to protect the host from auotreactivity. Just like T cells, NK cells must learn not to kill healthy cells. T cells do this by negative selection in the thymus. NK cells use their receptors to regulate their gene expression and control effector function. The term NK education refers to the process NK cells use to prevent autoreactivity when in contact with healthy cells and to become effectors when in contact with target cells. The inhibitory receptors provide a means to enact a hyporesponsive state within the NK cell. However, this theory did not explain multiple reports in mice and humans that some subsets of NK cells do not express inhibitory receptors, and yet still maintain a mature phenotype (72, 301-309). The concept of NK education was expanded to describe the MHC-dependent and MHC-independent affects on NK cell development and responsiveness. The process of NK education is complex and dynamic. The amount of MHC in the environment determines NK cell responsiveness. NK cell behavior can be regulated through the expression and sequestration of both its activating and inhibitory receptors. Several models have been used to describe these phenomena, which will be described in further detail below.

1.2.4.2. Models of NK cell education

Licensing

The concept of licensing was first described by Kim et al. (192). Whereby, they showed that signaling through the ITIM on the inhibitory receptor after MHC binding sensitizes the NK cell to become more effector like, as evidenced by increased cytokine release and cytolytic killing (192). There is no clonal editing for NK cells like there exists for T cells. Thus, NK cells that do not receive signaling through ITIMs are said to be unlicensed, and as such have decreased functionality due to this lack of signal and do not become self reactive. The NK cells that have been licensed are, in effect, educated. While the term licensed was first used to describe MHC dependent NK cell education, the term is now used ubiquitously for NK cell education in general.



With permission from Höglund et al. Nature Reviews Immunology 10, 724-734 (October 2010)

Figure 1.6 NK cell education models

Arming/disarming model

The Raulet lab proposed the arming and disarming model (303, 310) to explain the observation that some NK cells do not express inhibitory receptors; however, these cells did display a mature phenotype and achieved self-tolerance. They propose that NK

cells initially have a high basal level of responsiveness. However, without inhibitory receptors to bind MHC and with continued activation through the activation receptors, the NK cells effectively become anergic and without the ability to perform effector functions are “disarmed.” This model seemed to have biologically support since both T and B cells can be induced into anergy after continuous activation. The flip side to the “disarming” model is the “arming model.” The two models are similar in that both theories describe NK cell responsiveness as a MHC-dependent process. The “armed” model differs because it suggests that NK cells start with a low basal level of responsiveness and that MHC binding to inhibitory NK cell receptors transduces a signal that propels it into an “armed” and responsive state (301, 303).

Rheostat concept

NK cells are exposed to varying amounts of inhibitory signals in their environment, and in addition can express varying amounts of inhibitory NK cell receptors. The Rheostat model was proposed by both Raulet and Höglund (311-313), and takes into account this spectrum in intensity of inhibitory signals. In this Rheostat model, the most responsive state is due to an increased amount of inhibitory NK cell receptors on the surface and high affinity MHC/inhibitory receptor binding (311-313). Loss of inhibitory NK cell receptors decreases responsiveness (312). Reports have shown that human NK cells possess varying amounts of inhibitory NK cell receptors and these receptors differ in strength of signal transduced due to the number of inhibitory receptors and subset of MHC they bind (202, 204, 259).

Cis interacting model

The aforementioned models involve binding MHC in trans. The work of Chalifour et al. (314) show that Ly49 receptors can bind MHC in cis and that this interaction can sequester the inhibitory NK cell receptors and prevent them from binding MHC in trans resulting in NK cell activation. No evidence of human inhibitory NK cell receptor binding of MHC in cis was reported at the time of this dissertation. Therefore, it is difficult to say whether this phenomenon is specific to the Ly49 family and murine NK biology or whether it also occurs for humans.

All encompassing Tuning model

The field now commonly refers to NK cells as being either educated or uneducated depending on their expression of inhibitory receptors capable of binding ligand and their resulting responsiveness. NK cell education may also be driven by MHC-dependent or –independent processes (292, 315). The term educated takes into account the rheostat model and the idea that increased overall interactions with inhibitory receptors actually makes the NK cell more responsive when it eventually comes into contact with target cells low in MHCI/ inhibitory receptor ligand, and increased surface expression of activating ligands. Interestingly, hyporesponsiveness achieved due to stimulation without proper initial inhibition is not a fixed endpoint for the NK cell. Indeed, multiple reports have shown that a change in cytokine or MHCI in the environment can restore responsiveness, rendering the uneducated NK cell newly educated (316).

1.2.5. NK-DC crosstalk

As discussed above, NK cells need IL-15 to drive their differentiation, proliferation, and expression of effector functions. One of the main avenues of IL-15 delivery to NK cells is from dendritic cells (DC). In addition to IL-15, other cell-cell interactions between DC and NK cells have been characterized that facilitate NK cell proliferation (210, 212) and secretion of IFNγ (209-211, 214, 215). For example, co-stimulatory molecules such as B7 and CD40 present on DCs interact with CD28 and CD40L, respectively, found on NK cells to stimulate their proliferation (210). Activated monocyte-derived DCs can upregulate activating CD69 expression on NK cells (216-218). In addition, the secretion of IL-12 by DCs has also been reported to be an important player in NK cell activation and stimulator of cytolysis (210, 215). Indeed, immune synapse formation between DCs and NK cells polarizes the DCs, and they secrete IL-12 from preassembled stores (214). Interestingly, the initial mode of stimulus to DC determines the necessary downstream DC-NK signaling interactions to trigger NK cell activation. If DCs are stimulated with LPS, IL-12 is required for subsequent release of INFγ. However, if the DCs are stimulated with IFNα, then DC expression of NKG2DLs is necessary to elicit IFNγ secretion (198).

As NK cells become activated, they release IFNγ, which is one of the major cytokines that in turn facilitates the maturation and differentiation of DCs (217, 222, 224-228). In addition, NK cells induce DCs to produce TNFα (217, 227)as well as IL-12 {Piccioli:2002da, (224, 229). The stimulation of DCs by NK cells allows for the induction of the cellular arm of the adaptive immune response as evidenced by the detection of Th1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells (224, 227, 229).

1.2.6. NK cell memory

The first experimental evidence of memory-like functions in NK cells was described using a classic model of hapten-induced contact hypersensitivity (CHS) in mice (232). In this model, haptens were applied to the skin of Rag2-null mice (i.e., sensitized), which are B and T cell deficient but possesses NK cells. The mice were rechallenged with haptens and a CHS response was detected. However, mice lacking B, T, and NK cells did not produce a CHS response under the same conditions. Further, the NK cells from the Rag2-null mouse livers could be transferred to Rag2-null naïve mice and a CHS response was observed after challenge with haptens. However, this response was not observed with cells isolated from the spleen of the same mouse eliciting the CHS response leading to the term "Liver-restricted memory NK cells". The memory liver NK cells subset are phenotypically CXCR6+ (234) and DX5- (236) and have also shown to respond to viral antigen (234).

Another area of research in NK cell memory focuses on CMV-specific memory NK cells. A striking similarity *in vivo*, between mouse and human CMV models is the detection of an inflationary NK cell population. The first report was made by Sun et al. (240), which demonstrates that Ly49H+ NK cells expand and proliferate due to the MCMV m157 protein. These NK cells were later isolated and stimulated either through the Ly49H+ receptor or a different activating receptor, NK1.1, and found to have increased effector functions. In addition, they transferred the memory NK cells into immunodeficient newborn mice and found that they were able to protect against MCMV infection (240). In addition, activation of DNAM-1 is required for the Ly49H+ NK cell

effector and memory phenotype, which is consequently upregulated on monocytes and DCs due to MCMV infection (244). Data also supports the existence of human memory NK cells due to HCMV infection. NK cells expressing CD94/NKG2C are expanded from less than 10% of the total NK cell population to over 70% and show an activated phenotype in patients following primary HCMV infection or reactivation (246, 248, 249). These expanded cells show increased amounts of IFNγ production and also express a marker of terminal differentiation called CD57 (113, 317). Further proof of their memory-like response was demonstrated by hematopoietic cell transplantation of NKG2C+ NK cells from either CMV+ or CMV- donors into CMV+ recipients. Only the NKG2C+ NK cells from CMV+ donors expanded in recipients–providing strong proof of a secondary response to CMV antigen (249). Multiple studies report an enrichment of inhibitory KIR2DL2/3 expression on NKG2C+ NK cells (251-253), therefore particular MHCI alleles in the environment might have a role in shaping these responses.

A third group of NK cells have showed memory-like phenotypes in response to IL-12, IL-15, and IL-18, referred to as cytokine-induced memory-like NK cells (254, 256, 257). Indeed, Cui et al. (258) demonstrated that adoptive transfer of DCs and NKs, as opposed to NKs alone, evoked increased tumor infiltration, due to cytokine exposure encountered during DC-NK cross talk. Rölle et al. (261) report that IL-12 secreted by CD14+ monocytes drive expansion of NKG2C+ cells in an HLA-E dependent manner.

Two reports from 2015 provide evidence that epigenetic modifications are responsible for the memory phenotype in FcRγ-deficient human NK cells isolated from HCMV infected individuals (263, 264). Interestingly, Schlums et al. (264) found that the memory-like NK cell subset had DNA methylation patterns similar to cytotoxic T cells.

1.3 CMV IMMUNE MODULATION

CMV is a persistent virus, which is not cleared by the host immune system. In order to achieve lifelong infections, the virus has developed multiple mechanisms of evading host defenses. CMV dedicates a large amount of its coding potential exclusively to immune evasion. In the previous section, focusing on NK cell biology, the role of MHCI in shaping the NK cell response was highlighted. This molecule is important not only for NK cell function, but also for T cell recognition. The next section will focus on how CMV evades cellular immunity—both adaptive and innate via T cell and NK cell evasion.

1.3.1 T cell evasion

During CMV infection, MHCI is actively retained and degraded by a group of proteins in the US6 family (HCMV US2-11) (271). US2 and US11 are both responsible for retrotranslocation of MHCI from the ER resulting in proteasomal degradation (273, 275-279). US3 in cooperation with tapasin, binds MHCI in the ER and prevents maturation (280, 282, 283). Lastly, US6 inhibits antigen presentation by blocking the transporter associated with antigen processing (TAP) (285, 287, 289, 318). The HCMV homologues of US2, US3, US6, and US11 in RhCMV are Rh182, Rh183, Rh184, and Rh189, respectively. Although they only share between 33 and 43% sequence identity (80), the gene family is positioned similarly in the genomes and, most importantly, found to be functional homologues (88). RhCMV encodes an additional inhibitor of antigen presentation, Rh178, which arrests translation of MHC (82, 319). The deletion of Rh182-

189+Rh178 lead to complete restoration of MHCI on cell surface levels (319). CMV seropositive hosts can be co-infected with a secondary persistent infection following primary infection in a process called superinfection. However, the Rh182-Rh189 RhCMV deletion mutant was unable to superinfect CMV seropositive RM since infected cells could no longer down modulate MHCI surface expression. Therefore, antigen presentation is restored along with subsequent activation of CMV-specific CD8+ T cells (89). Indeed, the capacity to superinfect was demonstrated to be CD8+ dependent, as the Rh182-Rh189 deletion mutant regained the ability to superinfect following CD8+ T cell depletion (89).

1.3.2 NK cell evasion

The role of NK cell evasion by CMV was first examined experimentally using the MCMV mouse model in which different strains of mice had varying susceptibilities to CMV (265, 267, 320-323). This susceptibility was eventually mapped to the Cmv-1 locus. A resistant isoform in C57BL/6 mouse was able to control MCMV infection; however, the *beige* (eg. BALB/c strain) mouse succumbed to MCMV infection due expression of the susceptible isoform (298, 299, 324). The responsible gene was later mapped to the Ly49 gene family and identified as Ly49H (activating receptor on NK cells), which is absent in susceptible mouse strains (272, 300). The basis of these differences in susceptibility were further elucidated when it was discovered that an MCMV viral protein, m157, bound to Ly49H in resistant mice. This receptor had a high affinity for m157 and interestingly did not bind MHCI, but was an activating receptor that associates with DAP10 (72, 325). In addition, m157 can bind the inhibitory Ly49I receptor in susceptible mice; however, this receptor was only found expressed on 10% of the NK cells (72, 274).

Common to both MCMV and HCMV is the avoidance of detection by the NK cell activating receptor, NKG2D (301, 302, 304, 305, 307-309, 326-335). As described in detail above, this promiscuous receptor has a myriad of ligands, in both mouse and human, all of which are targeted by the virus. The first CMV immunoevasin targeting NKG2DLs to be identified was HCMV UL16 (301, 336-338). Cosman et al. (301) found that an UL16-Fc fusion protein bound both a human T cell line and a Burkitts' lymphoma cell line. They created cDNA expression libraries and screened for UL16 binding partners. The first UL16 ligand identified was MICB; however, the related receptor MICA was not bound by UL16 (243, 247, 250, 301, 339). A later study by the Steinle group (310, 340), showed that differnetial binding of UL16 to MICB and not MICA involved only five key amino acid changes between MICA and MICB. Cosman et al. (301) additionally identified ULBP1, a previously uncharacterized protein, thus discovering a new gene family encoding ligands that bound NKG2D. By searching for similar sequences they also identified ULBP2 and 3; however, only ULBP2 bound to UL16 (301). UL16 prevented cell surface trafficking of these molecules by retention in the ER by means of a tyrosine-based motif in the C-terminus, evidenced by endoH sensitivity in both MICB and UL16 and co-localization of the proteins in the ER/cis-Golgi (333, 341-345). In addition, UL16 was shown to co-immunoprecipatate with MICB suggesting direct interaction (342, 346). Three additional ULBP protein family members were identified as ULBP4-6 (233, 347-349); however, only ULBP6 was shown to also be targeted by UL16 (336, 349). HCMV does indeed target MICA and ULBP3 via UL142, which also retains these particular NKG2DLs in the ER (304, 350-352). However, the interaction between UL142 and MICA was mediated by the transmembrane domain of

UL142 and not a YQRL retrieval motif like that found in the cytoplasmic tail of UL16 (342, 351, 353). Of note, a particular MICA allele, MICA*008, is truncated and lacks the cytoplasmic tail, and part of the transmembrane domain. MICA*008 is not sequestered in the ER by UL142, and interestingly it represents the most prevalent allele in the human population (351, 354, 355). However, this escape variant likely applies selective pressure on the virus to evolve, producing yet another locus adapted to target MICA*008 in particular. Indeed, recent work describes the function of US9, which was previously unknown, showing that it is able to target the MICA*008 allele and redirect it to the lysosome (356). It is worth mentioning that US9 was named as one of the HCMV genes with a high variance in mutation rate, thus allowing the virus to adapt to it hosts (357). UL142 is expressed with late kinetics, and at early time points during infection, MICA was already being downregulated from the surface (328, 341, 351, 358-364). Fielding et al. (255) created a UL142 deletion virus, which was not associated with increased MICA cell surface expression after infection. These results led them to hypothesize that another HCMV gene was also targeting MICA. Indeed, these observations led to the discovery of US18 and US20, which redirect MICA to the lysosome (255). In addition to regulation of NKG2DLs at the post-transcriptional level, HCMV also targets the MICB for degradation through the expression of viral microRNA Mir-UL112-1. The 5' UTRs of the MICs are well conserved, but a single nucleotide difference between the transcripts of MICA and MICB provides specificity for MICB alone (359, 360, 365, 366).

The effects of HCMV infection on the human NK cell activating and inhibitory ligands are striking; however, ethical considerations prevent experimental examination of these phenomena. Thus, much of the functional relevance of CMV NK cell evasion has

been researched using the mouse model. Although MCMV does not encode homologs to the HCMV NKG2D immunoevasins, through other analogous mechanisms, it also targets NKG2DLs—including multiple members in the RAE-1 family, the H-60 family and Mult-1 (308, 362, 367). The gene product of m152, gp40, downregulates MHCI by means of ER retention (368, 369); however, this protein is not homologous with any members of the HCMV US6 family that inhibit MHCI surface expression. Along with MHCI, gp40 also retains *RAE-1α, RAE-1β, RAE- 1γ, RAE-1δ, RAE-1ε* within the ER targeting the PLWF motif within the cytoplasmic tail (367, 370-373). The gene product of m145 targets MULT-1, by ER dislocation (308, 370, 374). MCMV encodes an additional non-homologous protein encoded by ORF m155 that acts post-transit through the Golgi, and has specificity for murine NKG2DL, H60 (371, 375). The MCMV protein m138 targets the largest breadth of NKG2DL family members and acts by redirecting them to lysosome (308, 372, 373).

In addition to NKG2D, HCMV also subverts surveillance of other activating NK cell receptors. The NCR, NKp30, directly binds HCMV tegument protein pp65. The binding of pp65 does not activate the NK cell, but instead causes the dissociation of the CD3ζ chain, effectively blocking signal transduction (320).

The function of HCMV protein, UL141, was first described by Tomasec et al. (376). They demonstrated that UL141 evades the NK activation receptor, DNAM-1, by retention of CD155 (376). It was later determined that UL141 reduced cell surface expression of CD112, also a ligand of DNAM-1 (377). However, this reduction in cell surface expression is mediated along with the assistance of US2 via proteasomal degradation aided by the initial retention of CD112 by UL141 (378). Evasion of

activating NK cell receptors is not the only function of this protein. UL141 is also able to thwart TRAIL-dependent apoptosis through the sequestration of TRAIL receptors death domain 1 and 2 (379).

CMV has also developed ways to prevent NK attack using strategies independent of targeting activating receptor-ligand interactions. CMV has developed multiple strategies to mimic or induce "missing self" in infected cells. HCMV encodes a decoy MHCI-like molecule, UL18, which has been shown to bind the less frequently expressed NK cell inhibitory receptor, LIR-1, with a 1000x fold binding affinity over MHCI (157, 380). Prod'homme et al. (381) showed that UL18 was able to inhibit LIR-1+ cells; however, LIR-1- NK cells became activated when target cells were transduced using adenovirus expressing UL18. MCMV also encodes and expresses a MHC1 decoy named m144; however, current research with m144 has not recapitulated results observed for UL18 (382). Sequencing of HCMV clinical strains reveal this gene to be polymorphic and effects of UL18 to be strain dependent due to varying affinity of the UL18 variants (383). HLA-E is normally induced due to binding of the signal peptide from MHCI. HCMV encodes UL40, which has a leader sequence that also binds to the HLA-E binding groove and upregulates HLA-E to the surface, which can then bind the NK inhibitory receptor NKG2A/CD94. Interestingly, an alternative downstream start site was discovered in the UL40 transcript, and when this truncated protein is translated, the cleaved signal peptide preferentially binds and upregulates UL18 but not HLA-E (384-390). Multiple MCMV mechanisms have been described involving MHCI decoy molecules including m144, m157, and m04 (382, 391-403). The MCMV protein, m144, has MHCI homology and infection with MCMV containing a m144 deletion results in

NK cell mediated viral clearance (391-395). The MHCI homologue, m157, although first described to bind the activating NK receptor Ly49H, was later found to have a strong binding affinity for Ly49I, an inhibitory receptor. An MCMV m157 deletion mutant was attenuated *in vivo* evidenced by a reduction in viral titer (396, 397). Furthermore, WT MCMV isolated from wild outbred mice had m157 variants that could bind to a wide number of inhibitory Ly49 receptors, though a only few of the variants had specificity for the activating Ly49H. The activating Ly49P binds m157 in MA/My (H2 k) mice and confers MCMV resistance (398, 399). This resistance was shown to be mediated through m04, which acts by binding MHC and upregulating it to the surface (382, 400-403). In addition, m04 was the first evasion molecule that was experimentally shown to be necessary to avoid activation of NK cells due to "missing self" (404-406). Although, binding to an activating receptor may seem counterintuitive to employ for NK cell evasion, m04 also allows for binding of MHC to inhibitory receptors, which can override signaling sensed through activating ligands. Thus, the evolution of MCMV viral evasins and the Ly49 locus is a perfect paradigm of a host:pathogen co-evolutionary arms race.

The newest NK cell immnuoevasin identified to date is UL135, which disrupts actin polymerization necessary for immune synapse formation, thus blocking downstream degranualization for both cytolyitc T cells and NK cells. The effects on actin remodeling are mediated through the independent binding of both talin and abl interactor 1 (ABI1) and ABI2 (407). This impressive list of immunoevasins all work in concert to avoid NK cells, suggesting that NK cell evasion is crucial to CMV fitness *in vivo*.

1.3.3. CMV as a vaccine vector

The goal in vaccine development is to elicit a strong immune response that protects the host from a particular pathogen-induced disease. Ideally, vaccines should generate long-lasting immunity, be relatively low cost to make and distribute, and demonstrate a good safety profile. The unique features of CMV induced immune responses make it an excellent candidate for the generation of long-term immunity. One such feature is the ability of CMV vectors to elicit a strong effector memory CTL response. The effector memory CTL phenotype after CMV inoculation is different from other types of vaccines that elicit mostly central memory T cells. It normally takes 1-2 weeks before memory T cells proliferate and induce a CTL response. Whereas the effector memory T cells are armed and ready, reside in the periphery, and able to lyse newly infected cells. Indeed, RM immunized with a RhCMV based vector expressing SIV antigens were able to protect 50% of RM after challenge with SIV (98, 408). In addition to eliciting effector memory T cells, the success of the CMV-based vectors is also likely due to the unique responses due to the particular strain used as the vector backbone, RhCMV 68-1, which does not express two components of the pentameric receptor complex, UL128 and UL130. CMV-based vectors elicit T cells responses capable of recognizing a broader number of epitopes than generated by conventional vaccine approaches or infection with SIV. Interestingly, 2/3 of the epitopes recognized by the CD8 T cells were MHCII restricted. In addition, some epitopes were recognized by T cells in all animals in the study and have been termed “supertopes” (62). Lastly, CMV can superinfect a seropositive host, allowing for the repeated use of CMV vectors without compromising their immunogenicity. Therefore, CMV vectors have the ability to elicit

effector memory T cells that recognize highly diverse and unique epitopes compared to the canonical epitopes normally recognized in other vaccine designs (62).

1.4. NK CELLS IN OTHER DISEASE MODELS

1.4.1. Viral NKG2D evasion

Interestingly, multiple viruses from a varying array of classifications evade NK detection via the NKG2D receptor. For example, the HIV-1 Nef protein was first characterized for its ability to downmodulate expression of HLA (409), and was subsequently found to downmodulate the cell surface expression of MICA, ULBP1, and ULBP2 (410). Also initially described for its MHCI modulating function, E3/19K was the first adenoviral protein described to evade NK cells by targeting MICA and MICB and preventing cell surface expression through ER retention (411). The human herpesvirus-7 (HHV-7) U21 protein, also first described for its MHCI targeting ability (318), was later found to target NKG2DLs, MICA, MICB, and ULBP1. However, U21 redirects these molecules to the lysosome (412). Members of the Orthopoxvirus family, which in includes cowpox, encode for the secreted protein Orthopoxvirus MHC class I–like protein (OMCP) that antagonizes that function of both mouse and human NKG2D, thereby preventing killing of infected cells (413). Some viral proteins like Kaposi's sarcoma-associated herpesvirus (KSHVZ) K3 and K5 are able to evade multiple avenues of immune detection and target a broad spectrum of molecules containing domains within the Ig superfamily. These proteins are E3 ligases that ubiquitylate their targets and redirect them to the lysosome. Both K3 and K5 target MHCI (414, 415), and in addition, K5 downregulates NKG2DLs (MICA and MICB), the ligand for NKp80 [activation

induced C-type lectin (AICL)] (416-418), and B7-and ICAM-1 (414, 418). Similar to HCMV, KSHV and EBV both encode microRNAs that reduce expression of MICB, miRK-12-7 and miR-BART2-5p, respectively (419-427). Multiple reports demonstrate that detection of HA from influenza and Sendai virus by NCRs (NKp46 and NKp44) leads to killing of infected cells (265, 267, 428-432). This finding was instrumental in leading to the discovery that HA from influenza was able to cause the dislocation of the CD3ζ chain from both NKp46 and NKp30 and disrupt downstream signaling (322, 433, 434). This decoupling of the NK cell signaling pathway reduces NK cell cytotoxicity much in the same way as described for HCMV pp65 mediated inhibition of NKp30 (320, 435). Many viruses evade NK cells through targeting of activating ligands or influencing inhibitory signal transduction; however, Influenza also employs a more “direct” method in addition to evasion of NCR mediated activation. Influenza infects NK cells inducing apoptosis, thus evading NK cells through direct killing (324, 436).

1.4.2. Cancer

The study of viral interactions with the host not only provide insights into immune regulation under diseased conditions, it also highlights the similarities between pathogenic and tumorigenic immune evasion. Indeed, the discoveries of viral evasion mechanisms have lead to parallel developments within the cancer field and vice versa. This has been especially true for the field of NK immunology. Understanding the mechanisms and overall NK biology during cancer formation can lead to new therapeutics. The following section will describe current research describing common NK evasion strategies that are observed in cancer, followed by a look at new therapies that target cancer cells by harnessing the effector functions of NK cells.

1.4.2.1. NK cell ligand modulation

Viruses have evolved the ability to down regulate MHCI as an effective mechanism of immune evasion, as well as the interference with NK activating ligands interacting with their cognate receptor. Both of these strategies have been well documented in cancer immune evasion. For example, activating ligands are commonly induced upon cellular transformation. Indeed, the first known cellular ligand discovered for NKp30, B7-H6, was upregulated in cancer cells but not normal human cells (272, 436). Not surprisingly, based upon prior knowledge of viral immune escape, it was discovered that in patients with melanoma, the ectodomain of B7-H6 was shed into the plasma. Cell surface proteases within multiple types of cancer cells termed "a disintegrin and metalloproteases" (ADAM)-10 and ADAM-17 were responsible for cell surface cleavage of B7-H6 (325, 437). New cancer induced activated ligands continue to be discovered. Recently, the mixed-lineage leukemia-5 protein (MLL-5) was discovered to bind NKp44. The ligand was absent on healthy cells, but detected on a wide variety of tumor cells (274, 438, 439).

It has been widely documented that many cancer types actively induce NKG2DLs (326-335, 440). The exact mechanisms leading to their expression still requires much investigation; however, many advances have been made and current data suggest that NKG2DL expression can be induced due to disregulation within cell signaling cascades. The DNA damage repair pathway mediated by ATM/ATR (ataxia telangiectasia mutated/ATM- and Rad3-related) can be constitutively active in advanced tumors as well as precancerous lesions and is associated with increased expression of NKG2DLs (336-338, 441). These clinical data are experimentally corroborated in reports of using HDAC

inhibitors to turn on the DNA damage response pharmacologically (245, 441), which induces NKG2DL expression and increases NK cell mediated killing (243, 247, 250, 339, 442). After DNA damage, the tumor suppressor, p53, can induce cell cycle arrest as well as activate expression of downstream genes necessary for cell cycle regulation. The microRNAs, miR-34a and miR-34c, induced upon p53 activation were found to target ULBP2 transcripts (347) –a finding that is in disagreement with Gasser et al. (336), who report that p53 was not involved in ULBP2 upregulation in experiments using ovarian epithelial cells from p53-/- mice. An additional report found that the induction of ULBP1 and ULBP2 occurred upon p53 overexpression using non–small cell lung cancer (NSCLC) cell line, H1299 (350). Other signaling pathways involved in the induction of NKG2DLs have also been reported. Deregulation in Her2/Her3 signaling resulting from Her3 overexpression leads to MICA/MICB expression via the downstream PI3K/AKT signaling pathway (447-449). These data highlight the complexities of NKG2DL expression and mechanisms of expression are likely to be cell type and condition dependent.

NKG2DL expression has been shown to be downregulated in cancer cells after exposure to a variety of cytokines. For instance, INFγ was reported to downregulate MICA and ULBP2 on melanoma cells (341, 437). The role of IFNγ may be in part due to the induction of miR-520b, which was able to target the MICA transcript (346, 450). Another cytokine reported to affect NKG2DL expression is TGF-beta, which specifically reduced cell surface expression of MICA, ULBP2, and ULBP4 in glioma cells (353). Even hormones have been cited to affect cell surface expression of NKG2DLs. Indeed, estradiol was reported to induce MICA expression within endometrial cancers (354)

which authors suggested may be due to an Estrogen Receptor Element (ERE) (451) found within the MICA promoter. In a recent report studying the effects of estradiol on human non-small cell lung cancer, estradiol induced the expression of ADAM17, a metalloproteinase, resulting in MICA/MICB shedding (452). In fact, NKG2DL shedding has been documented numerously through out the literature (328, 341, 358-364) and was first described by Groh et al. (453), who found that the shedding of soluble MICs (sMIC) was a mechanism of tumor immune escape by blocking NKG2D receptors and thus preventing engagement with NKG2DL on target cells. In addition, they found that the binding of sMIC led to receptor mediated endocytosis, which led to a global reduction of the amount of NKG2D expressed on NK cells in patients with cancers expressing MICs (360). The blocking of MIC shedding as a possible therapeutic was also shown to be functionally relevant in mice, where expression of a shed resistant MICB prevented tumor growth (362). Interestingly, it has also been reported that MICs are also shed during the release of exosomes (368).

1.4.2.2. NK cell based therapeutics

The discovery of NK shedding has led to a novel target, metalloproteinase inhibitors. In addition to *in vitro* work discussed above, the *in vivo* evidence for metalloproteinase involvement in NKG2DL shedding has been reported (370-373). For example, epirubicin, a drug used in the treatment of hepatocellular carcinoma (HCC) has been shown to reduce the amount of MICA shedding due to reduced levels of ADAM10 in patients (370). Another drug, Triptolide, with known anti-tumor activity was also shown to inhibit ADAM10 *in vitro* (371). A number of clinical trials have tested drugs

blocking metalloproteinases; however, none have been successful as of yet (372, 373). In stark contrast to reports reviewed above, a paper just published from the Raulet lab (454) finds that the mouse NKG2DL, Mult-1, when given intravenously is able to not only prevent tumor growth, but are able to reverse desensitization of NK cells. It is difficult to say whether these results would translate into human based therapeutics, especially since the affinity of NKG2D for Mult-1 is 100 fold higher then other NKG2DLs--human or mouse (237, 455).

In addition to the blocking of NKG2DL shedding, researchers have also investigated the cancer killing effects of current drugs mediated by NKG2DL induction, as many therapeutics already in use to treat cancer, including HDAC inhibitors, upregulate them (247, 250). However, it has also been reported that HDAC inhibitors can decrease cell surface expression of NKp30 activating ligands resulting in decreased NK cell effector functions (456). The first licensed protease inhibitor, bortezomib, is FDA approved for multiple myeloma and was shown to cause cell cycle arrest and apoptosis (457). In addition, bortezomib was reported to induce NKG2DLs resulting in increased NK mediated cytolysis of hepatoma cells (458). Bortezomib used to treat donor acute lymphoblastic leukemia (ALL) cells *ex vivo* in combination with HDAC inhibitor, valproate, and another cytostatic drug, troglitazone, enhanced NKG2D dependent NK cell activation (459).

Much attention has been garnered by ADCC with the successful use of targeted monoclonal antibody treatments. The first monoclonal antibody approved for cancer therapy was Rituximab (460), which binds CD20—a marker expressed on nearly all B cells before differentiating into a plasma cell (461). The role of ADCC is exemplified by

numerous reports showing a correlation between treatment outcome and polymorphisms in CD16, some of which are less effective in binding the Fc region of the mAB (384, 386-390). Indeed, this led to the development of next generation anti-CD20 mABs that were engineered to have either increased CD20 affinity (ofatumumab, (396)) or enhanced FcγRIIIa (obinutuzumab, (398)) receptor binding due to an afucosylated Fc domain.

In addition, the release of cytokine and cross-talk with other immune cells by NK cells has been shown to be important for the overall Th1 skewed, anti-tumor CTL immune response (126, 400-403). However, the overall importance of NK cells in mAB-mediated killing relative to other effector cells is still under debate (404, 406), and which effector cell is most therapeutically important may be dependent upon the specific mAB used and the type of modifications they possess (462). Additionally, NK-DC crosstalk was been reported to induce tumor antigen cross-presentation on DCs, important for priming anti-tumor CTLs (463).

NK cell effector functions are determined by overall signaling transduced through activating and inhibitory receptors, whereby increased MHCI in the environment can have an overall dampening effect on NK cell activation (464). Therefore, it is not surprising that multiple reports have found that MHCI expression can reduce the amount of NK dependent ADCC (465-472). These findings have led to the development of anti-KIR mAbs, IPH2101 and lirilumab, which are currently being tested in combination with other B cell targeting mABs for efficacy in varying hematologic and solid tumor patients in current clinical trials (473-475). ADCC can be enhanced when NKG2D is engaged in addition to CD16 (395, 476, 477). Since NKG2DLs are known to be upregulated in coordination with current cancer treatments that induce DNA damage (245, 459, 478),

the combinatorial effects of targeting cancers using mABs and conventional therapies, now include harnessing NK cells (478), (clinical trials: NCT02252263, NCT01714739, NCT02481297, NCT01592370, & NCT02399917). However, it will be important to take into account reports of NK cell hyporesponsiveness (416, 418), for example CD16 ligand mediated endocytosis can lead to reduced NK cytolytic activity due NK cell exhaustion (416). Some of these challenges are being address by stimulating NK cells through the concomitant administration of cytokine along with other treatments (419, 421-427). The co-stimulatory molecule, CD137, is reported to be upregulated upon CD16 engagement, and an anti-CD137 mAB in coordination with anti-CD20 mABs are currently being investigated (428-430, 432, 473). It will be interesting to follow how these targeted immunotherapies progress with the additional focus on harnessing the role of NK cells.

One form of treatment for haematogical malignancies is allogeneic cellular transplantation (ACT). Unfortunately, one of the major side effects of ACT is graft-versus-host disease (GVHD). The allogeneic T cells recognize the recipient host as foreign and subsequent T cell activation results in severe tissue damage. It was later demonstrated that one could ameliorate the GVHD by depleting T cells from the graft prior to transplant, and current strategies are being developed to completely and/or selectively deplete T cells resulting better patient outcome. One method currently being developed is based on the concept of NK alloreactivity, where the donor NK KIRs are mismatched for the recipient MHCI, resulting in an overall improvement in graft as well as lessening of GVHD (433, 434, 465). One recent study reports a significant decrease in infection rate and increased event free survival in patients when activating KIRs, KIR2DL2 and/or KIR2DL3, were present in addition to inhibitory KIR/MHCI mismatch

(435). Another novel technique currently in trials and reported by Sakamoto et al. (436), describes the expansion of autologous NK cells with an activated phenotype, followed by subsequent infusion. Initial results utilizing this technique show no adverse effects due to expanded autologous NK cell transfusion. These results build upon previous studies using the NK cell line, NK-92, in ACT. This cell line was isolated from a lymphoma patient and has been described as being void of most inhibitory KIRs and expressing a large number of activating KIRs (437). Initial results cite that the infusions of NK-92s into patients were well tolerated and included minor and mixed responses (438, 439). The *ex vivo* manipulations of immune cells have been another area of intense research. Recent successes include the development of chimeric antigen receptor (CAR) T cells. CARs are developed to have single-chain variable fragment (scFv) recognition domains that can bind to antigens unique to or overexpressed by malignant cells, comparable to the variable domains contained within the Fab fragment of an antibody. The scFv domain is linked to a cytosolic signaling domain in order to elicit T cell activation after antigen is bound. For instance, the first generation of CAR receptors were linked to the CD3ζ chain. Subsequent generations also link to a variety of co-stimulatory protein activating domains (440). A clinical trial in patients with acute lymphoblastic leukemia (ALL) (441), generated CARs to target CD19, a major determinate on B cells. The CD19-targeting CAR constructs were transduced into autologous patient T cells, which were then transfused back into the patient. A remarkable 67% (14/21) of patients had a complete response rate and 60% (12/20) were measured as minimal residual disease negative, meaning no leukemia cells could be detected (441). One possible concern with these treatments involves the use of T cells in particular, since it is conceivable that these T

cells can persist in the patient and cause B cell aplasia. Additionally, autologous T cells may not always be an option, and the use of allogeneic T cells can cause GVHD. On the contrary, allogeneic NK cells may be used without causing GVHD, but can still kill target cell via cytolysis. Therefore, current research is in progress to implement the use of CAR NK cells. The first CAR NK cells were designed based upon the NK-92 cell line. Interestingly, Boissel et al. (442), measured ADCC using engineered NK-92 cells expressing CD16 (normally absent) in conjunction with anti-CD20 mABs and compared these results with the cytolytic ability of a CD20 targeting CAR NK-92 cell line. Interestingly, the authors found that the CAR expressing NKs were more effective at killing primary chronic lymphocytic leukemia (CLL) cells (442). Solid tumors are also being successfully targeted using CAR NKs cells. For instance, NK expressing CARs targeting ErbB2 and HER2 selectively kill patient primary cancer cells expressing ErbB2/HER2 and inhibit tumor cell growth using mouse models (443, 445, 446). In a similar fashion, several recent reports show enhanced NK cell killing and tumor growth suppression of glioblastoma cells using NK cells expressing CARs directed against commonly overexpressed tumor-proteins, EGFR and EGFR variant III mutant (EGFRvIII) (447, 449). NK-92 cell lines could be engineered to express any variety of CARs, and thus viable patient treatments could be immediately obtainable in contrast to T cells, which must be isolated from the patient, before they can be genetically engineered to express CARs. A significant disadvantage owes itself to the NK-92 lineage—NK lymphoma (367, 437); therefore, for safety concerns, NK-92 cells must be irradiated before transfusion allowing for effector function but not proliferation (450, 479). To address this issue, research is currently being done to examine CAR expression

in NKs isolated from donor peripheral blood (PB-NK) cells. PB-NK cells can expand *in vivo* and express a wider variety of activating receptors. Along with their longer life span compared to T cells, CAR PB-NK cells are an attractive cancer therapeutic.

1.5. HYPOTHESIS

Currently, six HCMV-encoded genes (UL16, UL142, US9, US18 and US20 and miR-UL112) (255, 301, 304, 356, 365, 480) have been demonstrated to suppress NKG2D activation through the downmodulation of NKG2DLs. However, we are lacking a thorough understanding of how these evasive mechanisms contribute to disease pathogenesis *in vivo*. Since CMVs are highly species specific (HCMV only infects humans), we have chosen to use RhCMV infection in RM as our model system. To date no publications have reported RhCMV downmodulation of NKG2DLs, highlighting the need to discover whether RhCMV also modulates NKG2D signaling and, if so, identify which viral ORFs are responsible and determine their mechanism of action.

We hypothesize that RhCMV encodes one or more genes responsible for the downmodulation of NKG2DLs, enabling RhCMV-infected cells to evade NK cell effector functions.

2. Natural Killer Cell Evasion Is Essential For Primary Infection By Cytomegalovirus

By

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Running Title: Cytomegalovirus infection requires NK cell evasion

2.1 Abstract

The natural killer cell receptor NKG2D activates NK cells by engaging one of several ligands (NKG2DLs). Human cytomegalovirus UL16 and UL142 counteract this activation by retaining NKG2DLs but the importance of NK cell evasion for infection is unknown. Since NKG2DLs are highly conserved in rhesus macaques we characterized how NKG2DL interception by rhesus cytomegalovirus (RhCMV) impacts infection *in vivo*. Interestingly, RhCMV lacks homologs of UL16 and UL142 but instead employs Rh159, the homolog of UL148, to interfere with NKG2DL expression. Rh159 resides in the endoplasmic reticulum and retains most, but not all, NKG2DLs. Deletion of Rh159 increases NKG2DL surface expression and NK cell stimulation by infected cells. Importantly, RhCMV lacking Rh159 cannot infect CMV-naïve animals unless CD8+ cells, including NK cells, are depleted. Since CD8+ T cell evasion is dispensable for primary infection, these results reveal that cytomegaloviral interference with NK cell activation is essential to establish but not to maintain chronic infection.

2.1 Author Summary

Natural killer (NK) cells are an important subset of the innate immune system that rapidly responds to cellular transformation and infection. The importance of NK cell control of viral infection is dramatically illustrated by our results revealing that cytomegalovirus (CMV) is unable to establish infections in healthy individuals unless

NK cell responses are subverted. By studying infection of rhesus macaques with rhesus CMV, a highly representative animal model for human CMV, we identified a key viral factor that allows RhCMV to limit NK cell activation by preventing NK cell activating ligands from trafficking to the cell surface. Importantly, we observed that this avoidance of NK cell activation is essential to establish infection *in vivo* because RhCMV lacking the NK cell evasion factor was unable to infect animals unless NK cells were depleted. By unmasking such viral stealth strategies it might be possible to harness innate immunity to prevent viral infection, the primary goal of CMV vaccine development.

2.2 Introduction

NK cells are a significant component of innate immunity against viruses and NK cell-deficient individuals are highly susceptible to herpesvirus infections (60, 481, 482). Herpesviruses, particularly cytomegalovirus (CMV), encode proteins that either inhibit or activate NK cells and elegant studies with murine CMV (MCMV) revealed a complex relationship between NK cell stimulation and MCMV evasion during infection (481-483). NK cell activation is controlled by inhibitory and activating receptors with inhibitory receptors, such as the KIR and CD94/NKG2 that recognize MHC-I, generally overriding positive signals (464, 484). Destruction of MHC-I by CMVs generates a “missing self” situation that reduces inhibitory signals (420, 485). A major activating receptor on NK cells is NKG2D, which is also expressed on γδ T cells, memory αβ CD8+ T cells and some CD4+ T cells (26, 486). NKG2D interacts with multiple ligands: MHC-I related molecules (MICA and MICB) and the UL16-binding proteins (ULBP1-6) in humans, and the H60, MULT-1 and RAE-1 proteins in mice (reviewed in (342-344, 486, 487)); all of which are induced upon cell stress including viral infection. Both human CMV (HCMV)

and MCMV devote multiple gene products to prevent the surface expression of NKG2DL, presumably because the induction of any one can activate NKG2D (488, 489). In HCMV, UL16 retains ULBP1, 2, 6 and MICB in the endoplasmic reticulum (ER) (255, 343, 344, 349)and MICB is additionally targeted by micro-RNA UL112 (240, 365). In addition, ULBP3 is retained by UL142, whereas MICA is downregulated by UL142, US18 and US20 (255, 351, 352, 490). The fact that both virus and host devote multiple gene products to modulating NKG2D activation suggests an evolutionary arms race (251, 491) that is exemplified by the observation that a recently evolved truncated allele of MICA (MICA*008) is counteracted by HCMV US9 (263, 264, 356).

The impact of NKG2DL interception by HCMV on primary infection and persistence, as well as on reinfection of seropositive individuals is not known. Studies in mice indicate that NKG2DL-inhibitory MCMV gene products are not required for infection but reduced viremia is observed in their absence (489, 492). Interestingly, replacing the NKG2DL-inhibitor m152 with RAE-1γ increased CD8+ T cell responses, both short and long term, despite viral attenuation (232, 234, 493). Thus, increased NKG2D activation might improve the immunogenicity of CMV-based vectors while increasing safety. CMV-based vectors are currently being developed for HIV/AIDS based on findings obtained with RhCMV-vectors in rhesus macaque (RM) models of HIV (26, 62, 494). These studies revealed an unprecedented level of protection by RhCMV-based vaccines against Simian immunodeficiency virus (SIV) along with an unexpected ability of RhCMV to control T cell epitope targeting (62). The close evolutionary ties between the human and RM host also extends to the CMVs with the vast majority of HCMV genes conserved in RhCMV (80, 412). Interestingly however,

while gene products involved in T cell control are largely conserved between RhCMV and HCMV (79, 88), both major NKG2DL-inhibitory HCMV gene products, UL16 and UL142, are notably absent from RhCMV. In contrast, MIC genes are highly conserved in RM encoding three proteins: MIC1 and MIC2 are closely related to MICA and MICB, respectively, whereas MIC3 is a chimera (230, 495). The RM genome encodes ULBP1-4, which are also highly conserved compared to humans (496, 497). Given the conservation of the ligands but not of the viral NKG2DL-inhibitors, we examined whether RhCMV evolved unique NKG2DL-inhibitors. Here, we demonstrate that RhCMV inhibits surface expression of all NKG2DLs tested and we identify Rh159, the homologue of HCMV UL148, as the major gene product responsible for retention of NKG2DLs. Similar to UL16, Rh159 prevents surface expression of multiple NKG2DLs, including MIC-proteins and some, but not all, ULBPs. In contrast, UL148 did not retain NKG2DLs consistent with divergent evolution of protein function from a common ancestor. *In vitro*, deletion of Rh159 increased NKG2DL expression and NK cell stimulation by RhCMV-infected cells. *In vivo*, RhCMV was unable to establish infection in either sero-positive or sero-negative animals when Rh159 was deleted. However, primary infection occurred when the CD8+ cell population, that includes NK cells, was depleted. Our results suggest that, unlike MCMV, NK cell evasion is essential for infection by RhCMV and, given the conservation of host NKG2DL, most likely HCMV as well.

2.4 Results

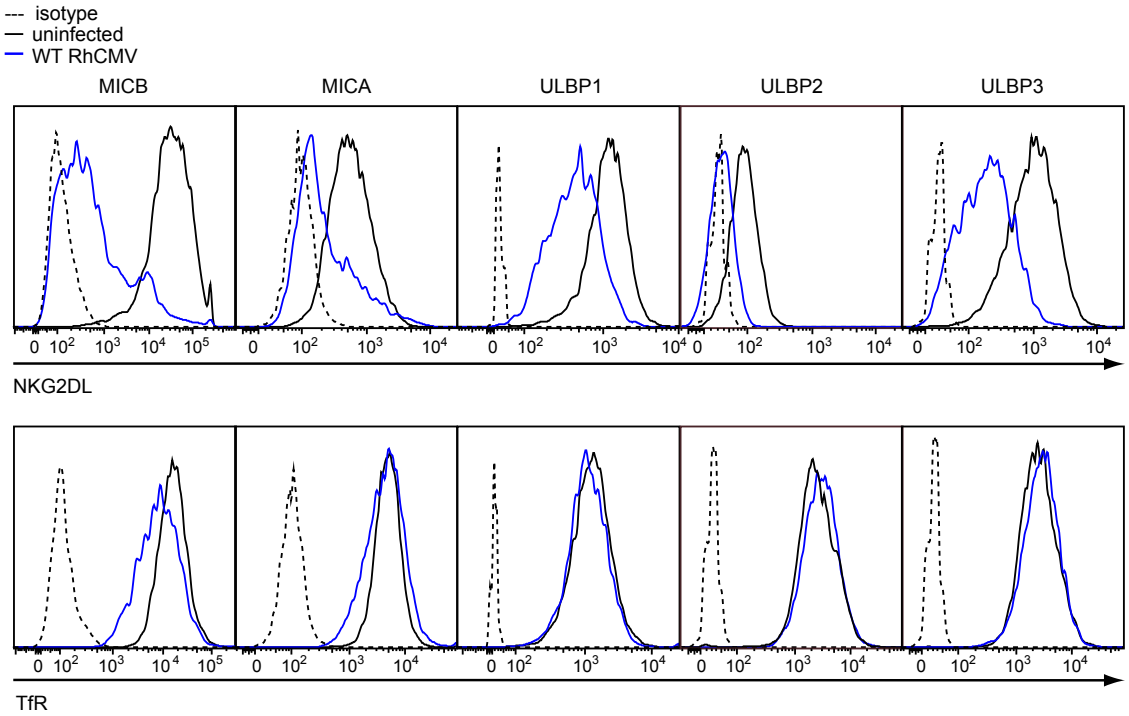
2.4.1. RhCMV inhibits cell surface expression of NKG2DLs

Since both MCMV and HCMV interfere with expression and intracellular transport of NKG2DLs, we hypothesized that RhCMV would similarly affect NKG2DL expression. Given the high degree of homology between human and RM NKG2DLs, we took advantage of a panel of established U373 cell lines stably expressing human ULBP1-3, MICA, or MICB [U373-NKG2DL] (412, 498). To determine whether RhCMV interferes with expression of NKG2DLs we infected U373-NKG2DLs with RhCMV and monitored the cell surface expression levels of each NKG2DL by flow cytometry ("RhCMV" refers to BAC-cloned RhCMV 68-1 (80) unless otherwise noted). Cell surface expression of NKG2DLs was assessed on infected cells by co-staining for IE2+ cells. At 48 hours post-infection (hpi), a substantial decrease in cell surface levels of each of the five human NKG2DL was observed in RhCMV-infected cells (Fig.2.1). In contrast, RhCMV infection did not impact surface expression of transferrin receptor (TfR) suggesting that RhCMV specifically targets NKG2DLs. Since RhCMV interfered with expression of each of the NKG2DLs examined these data suggest that RhCMV targets the full panel of NKG2D-activating ligands presumably to prevent NKG2D-dependent activation of NK cells.

Fig 2.1: RhCMV reduces surface expression of NKG2DLs

U373-NKG2DL were infected with RhCMV (MOI=3) (blue) or left uninfected (black). At 48 hpi surface levels of NKG2DL (upper panel) or TfR (lower panel) were determined by flow cytometry using specific antibodies or isotype control (dotted). Shown is

NKG2DL or TfR cell surface expression on RhCMV-infected cells gated for $IE2^{+}$. The results shown are representative of at least three independent experiments.



2.4.2. MICB is retained in the ER/cis-golgi and associates with Rh159 in RhCMV-infected cells

We hypothesized that RhCMV targeted NKG2DL by a post-translational mechanism since expression of these transfected proteins is controlled by heterologous transcriptional and translational control elements (412). Therefore, we monitored glycosylation of MICB over 24 h of infection by collecting cells at various hpi and digesting cell lysates with Endoglycosidase H (EndoH) or Peptide N-Glycosidase F (PNGaseF) prior to electrophoretic separation and immunoblot with MICB-specific antibodies. Over time, increasing amounts of EndoH-sensitive compared to EndoH-

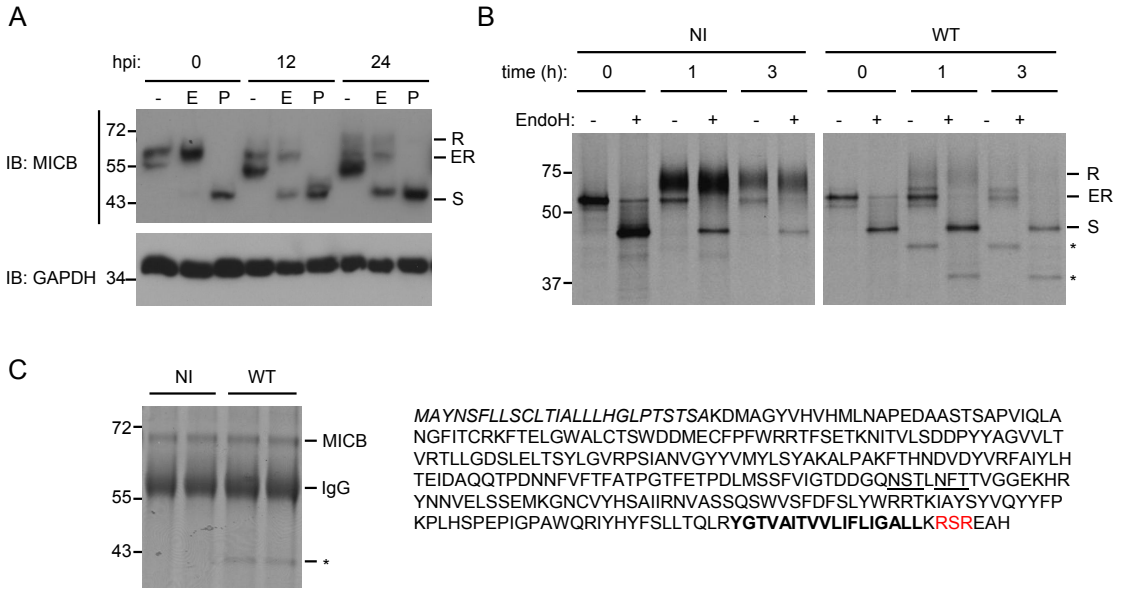
resistant MICB protein accumulated in RhCMV-infected cells (Fig. 2A). Since EndoH specifically removes high mannose oligosaccharides generated in the ER, EndoH sensitivity suggests that RhCMV retained MICB in the ER/cis-Golgi. The decrease of EndoH-resistant MICB over time mirrors the turnover of MICB seen in uninfected cells, thus was likely due to natural turnover (499). To determine whether RhCMV specifically targeted newly synthesized MICB, we immunoprecipitated MICB from metabolically pulse/chase labeled U373-MICB cells at 24 hpi. In uninfected cells the majority of MICB was EndoH resistant at 1 h post-chase (Fig. 2B, left panel). In contrast, the majority of MICB did not attain EndoH-resistance in RhCMV-infected cells (Fig. 2B, right panel), which was consistent with RhCMV preventing NKG2DL maturation. Interestingly, an additional protein species (indicated by *) was observed in MICB-immunoprecipitations in RhCMV-infected, but not in uninfected samples at the 1 h and 3 h time point (Fig. 2B). We hypothesized that the co-precipitating protein, which seemed to be ER-resident since it remained EndoH-sensitive throughout the chase period, was of viral origin. To identify this glycoprotein, we performed preparative MICB-immunoprecipitations from RhCMV-infected U373-MICB cells for analysis by mass-spectrometry (MS). When the immunoprecipitated proteins were separated by SDS-PAGE and visualized by Coomassie-staining, only MICB was observed in samples from uninfected cells whereas approximately equal amounts of MICB and the unknown ~40kDa species were co-precipitated by MICB-specific antibodies from RhCMV-infected cells (Fig. 2C). The ~40kDa protein was excised from the gel, digested with trypsin and analyzed by liquid-chromatography tandem MS (LC-MS/MS). Multiple peptide-species were identified that corresponded in mass to predicted peptides of Rh159, a putative RhCMV glycoprotein

with a predicted MW of 36.8 kDa (34.1 kDa without the putative signal peptide) that is similar to the observed MW of the (de-glycosylated) viral protein co-immunoprecipitating with MICB (Fig. 2B). Rh159 is predicted to contain two N-linked glycosylation sites and to display a type1b transmembrane protein topology with a cleavable signal sequence. A very short cytoplasmic tail of seven amino acids encompasses an RXR ER retrieval motif (Fig.2C). Rh159 was thus a strong candidate for an ER-resident viral glycoprotein that associates with and retains newly synthesized NKG2DL by hijacking the cellular ER-retrieval mechanism.

Fig 2.2: MICB is retained in the ER and associates with Rh159 in RhCMV infected cells

A) Immature MICB is enriched in RhCMV-infected cells. U373-MICB cells were infected with RhCMV (MOI=3) for 12 or 24 h or left uninfected prior to lysis in 1% NP40. Cell lysates were treated with Endoglycosidase H (E), PNGase F (P), or digestion buffer alone (-), separated by SDS-PAGE and immunoblotted with monoclonal antibodies (mAbs) to MICB or GAPDH. Mature glycosylated MICB (67kDa) is EndoH resistant (R), whereas immature MICB retained in the ER/cis-Golgi remains EndoH sensitive (S) (43kDa upon deglycosylation). ER refers to immature, glycosylated MICB that has not been deglycosylated by EndoH (55kDa). **B)** RhCMV inhibits intracellular transport of MICB. U373-MICBs were infected with wild type RhCMV (MOI=3) for 24 h (WT) (visualization by light microscopy confirmed 100% cytopathic effect (CPE)) or left uninfected (NI) followed by metabolically labeling with [35S]cysteine and [35S]methionine for 30 min. The label was then chased for the indicated times (h), cells

were lysed and MICB was immunoprecipitated from cell lysates using a MICB specific antibody. Precipitates were either digested with EndoH (+) or mock treated (-) followed by SDS-PAGE and autoradiography. Stars (*) indicate an EndoH-sensitive protein co-precipitating with MICB in infected cells. **C)** Isolation and identification of Rh159 co-immunoprecipitating with MICB. U373-MICB cells were infected with RhCMV (WT) or left non-infected (NI) as above and cells were lysed at 48 hpi. MICB was immunoprecipitated with anti–MICB mAb, the immunoprecipitates were separated by SDS-PAGE, and proteins visualized by Coomassie Blue staining. The IgG heavy chain (55kDa) is indicated. The 43kDa protein (*) was excised from the gel and digested with trypsin. Mass-spectrometric analysis by LC-MS/MS identified tryptic peptides corresponding to Rh159. The predicted amino acid sequence of Rh159 contains a signal sequence (italics), N-linked glycosylation sites (underlined), a C-terminal transmembrane anchor (bold), and an RXR ER retrieval motif (red). The results shown in A and B are representative of two or more independent experiments.



2.4.3. Rh159 retains MICB in the ER/cis-golgi

To determine whether Rh159 was sufficient to retain MICB, we inserted codon-optimized Rh159 (including a C-terminal FLAG-epitope tag) into a replication-deficient adenovector under control of the tetracycline-regulated promoter. U373-MICB cells were co-transduced with the corresponding construct (Ad159FL) and an adenovector expressing the tetracycline-regulated transactivator (AdtTa). For control we used an adenovector expressing GFP (AdGFP). At 24 hpi, MICB was immunoprecipitated from Ad-transduced U373-MICB cells at 0, 1, and 3 h post- -chase. In U373-MICB cells transduced with AdGFP, the majority of newly synthesized MICB attained EndoH resistance within 1h post-chase (Fig. 3A). In contrast, the majority of MICB remained EndoH-sensitive throughout the chase period in Ad159FL-transduced cells (Fig.3A). We further observed that MICB-specific antibodies co-immunoprecipitated an EndoH-sensitive protein of ~40KDa from Ad159FL-transduced cells but not from Ad-GFP-transduced cells (Fig. 3A). The ~40kDa band was identified as Rh159 by immunoblot

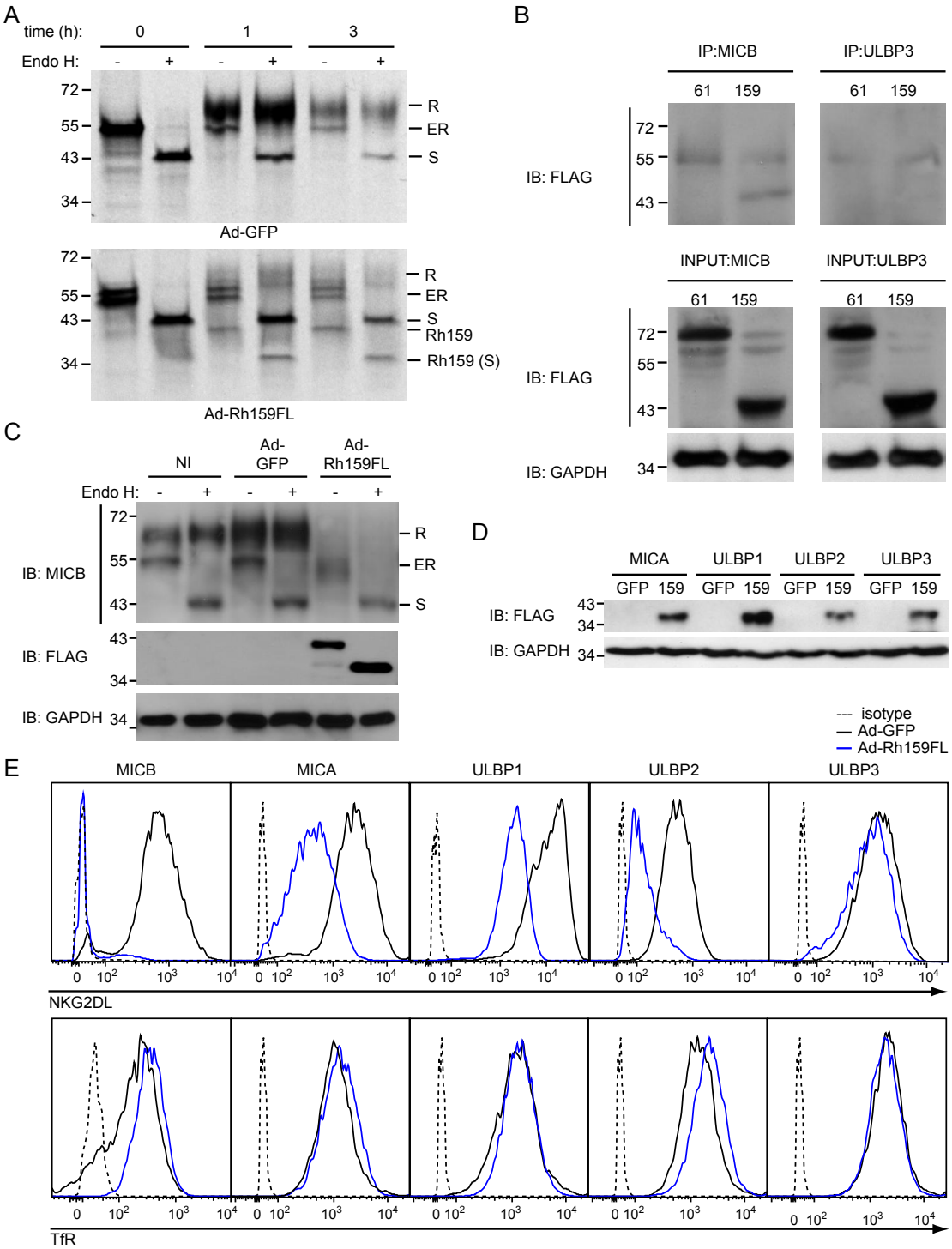
with anti-FLAG antibodies (Fig. 3B) whereas the band was not observed in U373-ULBP3 cells transduced with Ad-159FL or when an adenovector expressing C-terminal FLAG tagged Simian varicella virus (SVV) open reading frame (ORF) 61 (500) (Fig. 3B). Interestingly, steady state protein levels of MICB were strongly reduced and mostly EndoH sensitive at 48 hpi with Ad159FL, suggesting that prolonged retention ultimately leads to degradation of MICB (Fig.3C). Taken together, these data demonstrate that Rh159 associates with and retains MICB in the ER.

To determine whether Rh159 also targets other NKG2DLs, we co-transduced the U373-NKG2DL panel with AdtTA and Ad159FL or AdGFP. Expression of Rh159 was confirmed by immunoblot (Fig. 3C and D) and NKG2DL-surface expression was monitored by flow cytometry. Mean fluorescence intensity (MFI) of MICB was reduced by more than 2 log upon transduction with Ad159FL compared to AdGFP whereas cell surface levels of TfR were not affected (Fig. 3E). Similarly, Rh159-expression reduced surface levels of MICA, ULBP1, and ULBP2 whereas expression of ULBP3 was not affected (Fig. 3E) corroborating the data observed in previous immunoprecipitation experiments (Fig. 3B). Thus, Rh159 impairs the surface expression of most, but not all, NKG2DLs.

Fig 2.3: Rh159 interferes with intracellular transport of NKG2DL

A) Association with Rh159 prevents intracellular transport of MICB. U373-MICB cells were transduced with adenovectors (MOI=80) expressing either GFP (Ad-GFP) or FLAG-tagged Rh159 (Ad-Rh159FL) under control of tetracycline-dependent transactivator provided by co-transduced Ad-tTA (MOI=20). At 24 hpi cells were

metabolically labeled for 30 min with [35S]cysteine + [35S]methionine. Upon chasing the label for the indicated times (h), cells were lysed and MICB was immunoprecipitated with anti–MICB mAb. Precipitates were either digested with EndoH (+) or mock treated (-) followed by SDS-PAGE and autoradiography. **B)** Rh159 co-immunoprecipitates with MICB. Either U373-MICB (MICB, left panel) or U373-ULBP3 (ULBP3, right panel) cells were lysed at 48 h post-transduction with Ad-Rh159FL (159) or an adenovector expressing FLAG-tagged SVV ORF 61 (61) used as a negative control. MICB and ULBP3 were immunoprecipitated with anti–MICB and anti-ULBP3 mAbs, respectively, then immunoblotted with FLAG mAb. Input lanes were loaded with 10% total lysate used in immunoprecipitation and immunoblotted with mABs for FLAG and GAPDH. The results shown are representative of two independent experiments. **C)** Rh159 reduces steady state levels of MICB. U373-MICB cells were lysed at 48 h post-transduction with the indicated Ad-vectors. Lysates were digested with EndoH (+) or mock treated (-) then immunoblotted with mAbs for MICB, FLAG or GAPDH. The results shown are representative of two independent experiments. **D-E)** Rh159 reduces surface expression of MICA, MICB, ULBP1 and ULBP2 but not ULBP3. U373-NKG2DL cells were transduced with Ad-Rh159FL or Ad-GFP as in A) but for 48 h, cells were split into a fractions and either **D)** lysed and immunoblotted with mAbs for FLAG and GAPDH, or **E)** cells were transduced with either Ad-Rh159FL (blue) or Ad-GFP (black) and stained with antibodies specific for the indicated proteins, or isotype control (dotted) and analyzed by flow cytometry. The results shown are representative of three or more independent experiments.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Ad-Rh159FL
E
MICB
MICA
ULBP1
ULBP2
ULBP3
NKG2DL
TfR

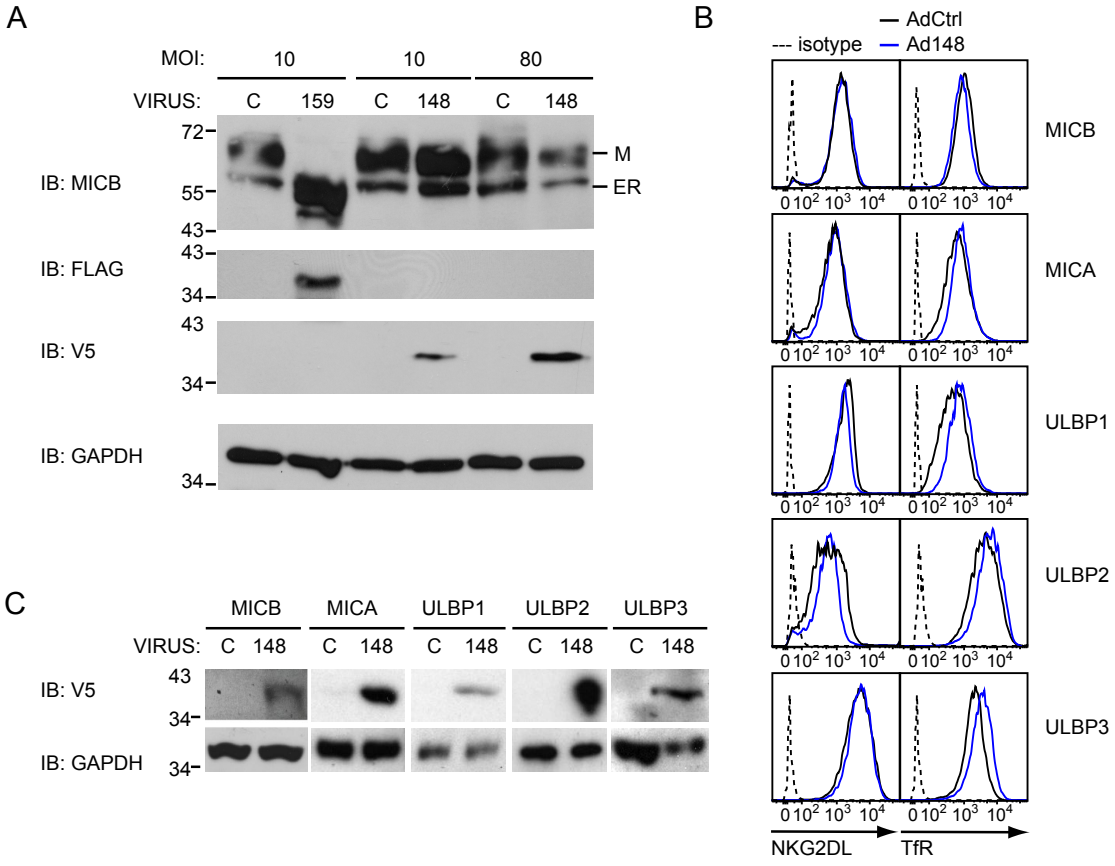
2.4.4. HCMV UL148 does not downregulate NKG2DLs

RhCMV Rh159 shares 30% sequence identity with HCMV UL148, comparable to other RhCMV proteins demonstrated to be functional HCMV protein homologues (80, 88). To determine whether HCMV UL148 would target NKG2DL expression, we transduced each of U373-NKG2DLs with a previously described adenovector expressing UL148 including a C-terminal V5-epitope tag (255). However, in contrast to Rh159, MICB maturation was not affected by UL148 even at high MOI (Fig. 4A) and MICB surface expression remained unchanged after transduction with Ad148 (Fig. 4B). Similarly, cell surface levels of MICA, ULBP1, ULBP2 and ULBP3 were not affected by UL148 (Fig. 4B). Since UL148 expression was verified by immunoblot (Fig. 4A&C), we conclude that despite sequence and positional homology, UL148 and Rh159 diverge in their ability to target NKG2DL. While we cannot rule out that UL148 targets NKG2DL not tested here, previous reports showed increased NKG2DL surface expression upon infection with HCMV lacking UL16 but containing functional UL148 (255). In addition, no decrease in NK cytotoxicity was found when cells transduced with Ad148 were co-incubated with human NK cells when compared to control (407) consistent with our conclusion that HCMV UL148 is not an NK cell evasion factor despite common ancestry with Rh159.

Fig 2.4: Cell surface expression of NKG2DLs is not affected by HCMV UL148

A) MICB maturation is not affected by UL148. U373-MICB cells were transduced with Ad-Ctrl (empty vector) (C), or Ad-UL148 containing a C-terminal V5 tag (148) at MOI of 10 or 80, or Ad-Rh159FL (159) (MOI=10) together with Ad-tTA (MOI=2.5) for 48 h. UL148 and Rh159 expression was verified by immunoblot using anti-V5 antibody or

anti-FLAG antibody, respectively, with antibodies to GAPDH providing a loading control. The mature MICB protein of 67 kDA is designated M. ER refers to immature MICB retained in the ER by Rh159. Results shown are representative of two experiments. **B)** UL148 does not affect cell surface expression of NKG2DL. U373-NKG2DL cells were transduced with Ad-UL148 (blue) or Ad-CTRL (black) (MOI=80) and at 48 h cells were harvested and analyzed for NKG2DL surface expression compared to isotype controls (dotted) by flow-cytometry. Surface expression of TfR was also analyzed for each condition in the U373-NKG2DL panel. **C)** UL148 expression in B) was verified by immunoblot in each of the samples using anti-V5 antibody. GAPDH served as a loading control.



2.4.5. Deletion of Rh159 restores intracellular maturation of MICB in RhCMV-infected cells

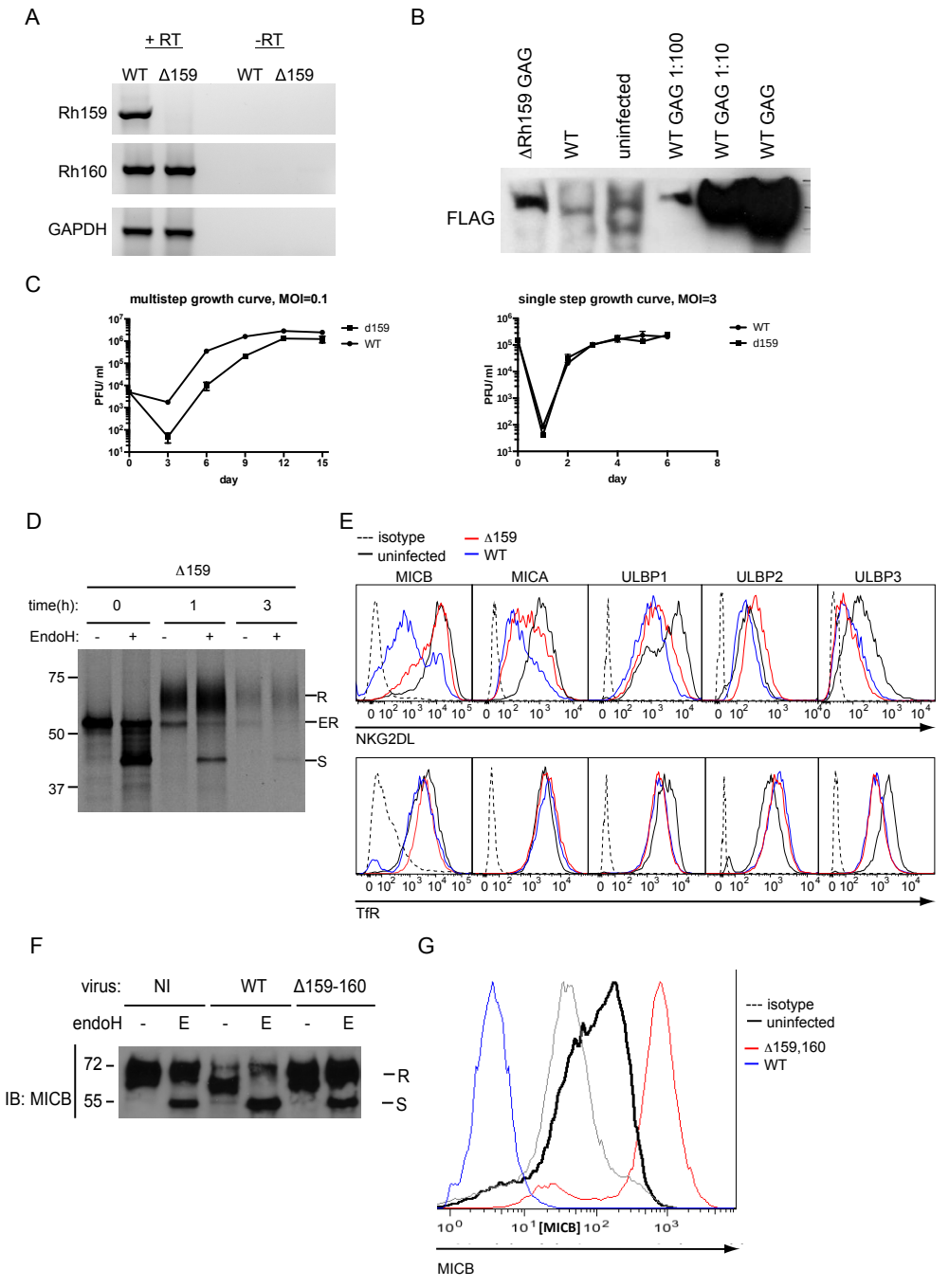
To further demonstrate that Rh159 is responsible for ER-retention of MICB, we created a Rh159 knock out virus (ΔRh159). When MICB maturation was examined by pulse-chase labeling and immunoprecipitation, MICB was found to acquire EndoH resistance at the same rate as observed for uninfected cells (compare Fig. 5A with 2B) suggesting that intracellular transport of MICB was not inhibited. Moreover, MICB surface levels were increased on cells infected with ΔRh159 comparable to uninfected cells (Fig. 5B). Similarly, expression of MICA, ULBP1, and ULBP2 was markedly increased upon infection with ΔRh159 compared to RhCMV, although surface levels were still decreased compared to uninfected cells (Fig. 5B). As expected, deletion of Rh159 did not increase ULBP3-expression. Taken together these data are consistent with Rh159 being predominantly, but not exclusively, responsible for the reduction of MICA, MICB, ULBP1, and ULBP2 surface expression whereas additional RhCMV proteins seem to target ULBP3.

Fig 2.5: Deletion of Rh159 rescues intracellular transport of MICB and surface expression of NKG2DL upon RhCMV infection

A) RT-PCR analysis using RNA isolated from rhesus fibroblasts infected with either (ΔRh159) or (WT) and specific primers to detect Rh159, Rh160, and GAPDH. B) Rhesus fibroblasts were infected with WT RhCMV expressing SIV GAG containing a FLAG epitope tag (WTGAG), (ΔRh159GAG), or WT RhCMV not expressing any exogenous

antigens (MOI=3) for 24 h or left uninfected prior to lysis in 1% NP40. WTGAG samples were additionally loaded at the following dilutions (1:10) and (1:100) treated separated by SDS-PAGE and immunoblotted with monoclonal antibodies (mAbs) to FLAG or GAPDH**. C)** Multiple step (left) and single step (right) growth curves were performed at on telomerized rhesus fibroblasts MOI=0.01 and 3, respectively using either WT ( ) or ΔRh159 (n **). D)** U373-MICBs were infected with ΔRh159 (MOI=3) for 24 h, visualized by light microscopy and were identified as having 100% CPE, then metabolically labeled with [35S]cysteine and [35S]methionine for 30 min prior to chasing the label for the indicated times (data shown in Fig. 2B was done concurrently and used as a control). MICB was immunoprecipitated from cell lysates with anti–MICB mAb. Immunoprecipitates were split and digested with EndoH (+) or mock treated (-) then analyzed by SDS-PAGE and autoradiography. **E)** U373-NKG2DL cells were infected with RhCMV (blue), ΔRh159 (red) (MOI=3) or left uninfected (black) for 48 h. Cell surface levels of NKG2DL or TfR were determined by flow cytometry, using specific antibodies and compared to isotype control (dotted). Depicted is NKG2DL or TfR surface expression on infected cells gated for RhCMV $IE2^{+}$ expression. The results shown are representative of three or more independent experiments. F) U373-MICB cells were infected with RhCMV (WT) or (ΔRh159). Lysates were split and digested with EndoH (+) or mock treated (-) then analyzed by SDS-PAGE and immunoblotted with monoclonal antibodies (mAbs) to MICB or GAPDH G) U373-MICB cells were infected with RhCMV (blue), ΔRh159,160 (red) (MOI=3) or left uninfected (black) for 48 h. Cell surface levels of MICB were determined by flow cytometry, using specific antibodies and compared to isotype control (dotted). Depicted is MICB surface expression on

infected cells gated using CMV specific Ab. The results shown are representative of three or more independent experiments.

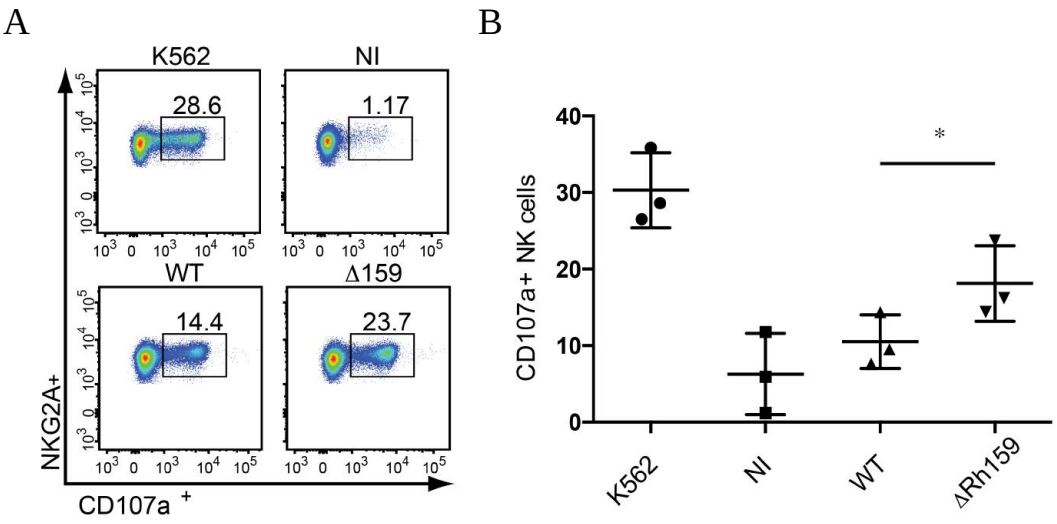


2.4.6. Deletion of Rh159 increases NK cell stimulation by RhCMV-infected cells

To determine whether increased NKG2DL expression by ΔRh159 impacted NK cell stimulation, we infected primary fibroblasts derived from three individual macaques with RhCMV or ΔRh159 and monitored stimulation of purified, autologous NK cells at 48 hpi. Macaque NK cells were magnetically sorted from PBMC using antibodies to NKG2A with the majority of NKG2A+ NK cells expected to co-express NKG2D (152). Following co-incubation with autologous fibroblasts for 4h, NK cell activation was measured by CD107a (lysosomal-associated membrane protein 1), a surrogate marker for degranulation (126). NK cells efficiently recognized the MHC-I-deficient human cell line K562, which was used as a positive control (Fig. 2.6 B). In contrast, NK cells responded only weakly to autologous fibroblasts infected with RhCMV (Fig. 2.6A, B). However, ΔRh159 significantly increased NK cell stimulation by autologous fibroblasts compared to RhCMV or uninfected cells (Fig. 2.6A, B). These observations demonstrate that Rh159 significantly limits NK cell stimulation by RhCMV-infected cells.

Fig 2.6: Deletion of Rh159 increases NK cell stimulation by RhCMV-infected cells *in vitro*.

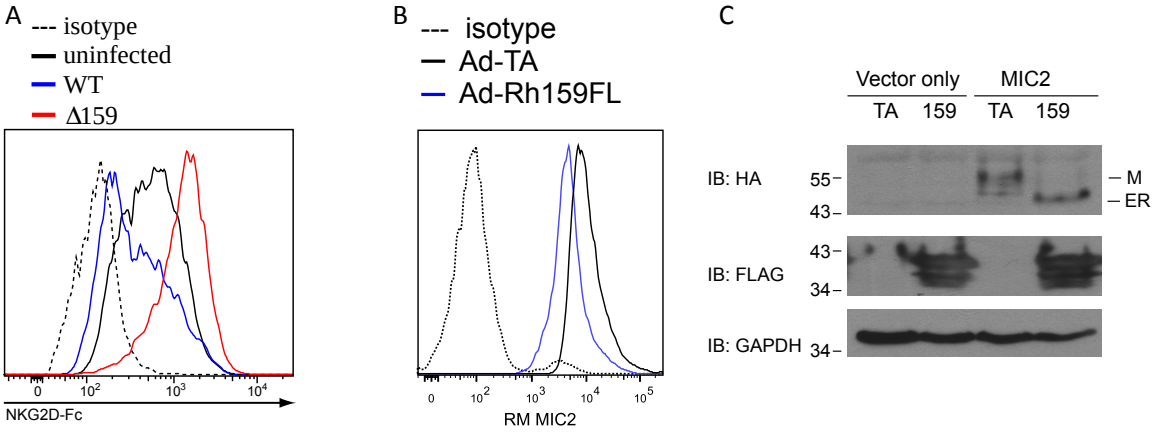
B) and **C)** Increased NK cell stimulation by ΔRh159. PBMCs were isolated from Cynomologus Macaques via Ficoll density separation, followed by sorting for NK cells ($CD3^-/CD8^+/NKG2A^+$). NK cells were stimulated with IL-15 and IL-2 overnight followed by 4 h incubation with autologous fibroblasts infected with either RhCMV or ΔRh159 (MOI=5) or left uninfected for 48 h. MHCI negative K562 cells were used as a positive control. Staining for CD107a, a marker for degranulation, was used to monitor NK cell activation. B shows results from one representative animal. C shows average results from three individual animals pooled from two individual experiments. Statistics performed using repeated measures ANOVA with Bonferroni's correction (*CI>95%), bars indicate SD.



2.4.7. Rh159 targets RM NKG2DLS

In addition to increasing expression of human NKG2DLs in stably transfected U373 cells, ΔRh159 also increased expression of rhesus NKG2DL. As shown in Fig. 2.7A, when rhesus fibroblasts were infected with ΔRh159, increased binding of soluble NKG2D receptor was observed compared to uninfected or RhCMV-infected cells. In addition, the RM homologue for human MICB, MIC2, was ectopically expressed in U373 cells. We then infected the U373 cells expressing MIC2 with Ad159FL. As expected, Rh159 specifically downregulated MIC2 from the cell surface (Fig 2.7B) MIC2 remained endoH sensitive due to infection with Ad159FL compared to control suggesting that Rh159 retains MIC2 in the ER/cis-Golgi (Fig2.7B).

Fig 2.7: Rh159 targets RM NKG2DLs



A) NKG2DL are upregulated by ΔRh159 compared to RhCMV. TRFs were infected with RhCMV (blue), ΔRh159 (red) (MOI=3) or left uninfected (black) for 48 h. Cell surface levels of NKG2DL were determined by flow cytometry using a recombinant NKG2D receptor Fc chimera and compared to isotype control (dotted). Cell surface expression of NKG2DL or TfR on infected cells was determined by gating for RhCMV

$IE2^+$ cells. The results shown are representative of two independent experiments. B) U373 cells were transfected with pCDNA3.1 plasmids expressing MIC2 containing an N-terminal HA tag inserted after the signal sequence. 24 h post transfection, U373-MIC2 cells were transduced with Ad-TA (TA), or Ad-Rh159FL (159) (MOI=10) together with Ad-tTA (MOI=2.5) for 48 h. MIC2 and Rh159 expression was verified by immunoblot using anti-HA antibody or anti-FLAG antibody, respectively, with antibodies to GAPDH providing a loading control. The mature MIC2 protein of 55 kDA is designated M. ER refers to immature MIC2 retained in the ER by Rh159. Results shown are representative of two experiments.

2.4.8. Rh159 is essential for infection

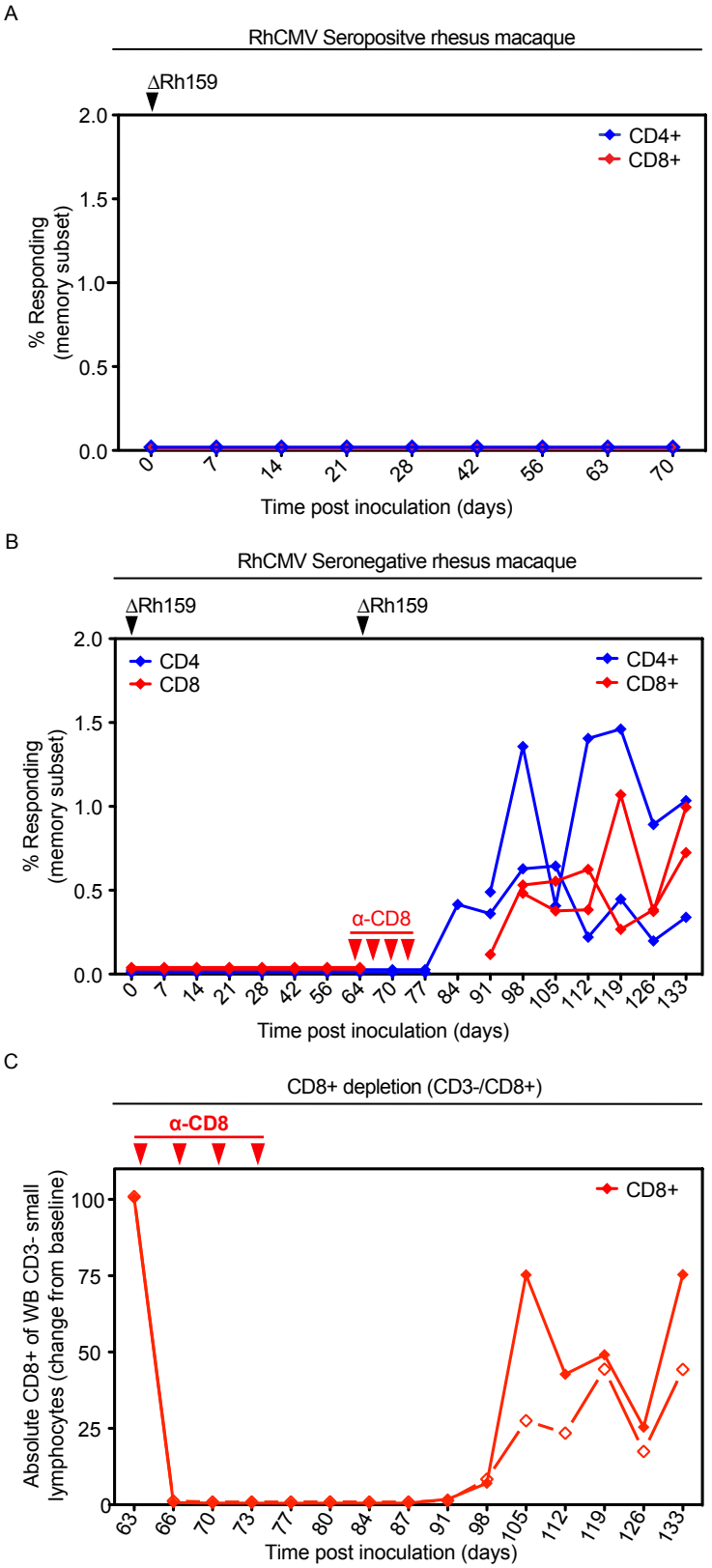
To determine whether decreased NK cell evasion impedes the ability of DRh159 to overcome pre-existing immunity during super-infection, as we reported for T cell evasion (89), we inoculated a CMV-positive animal with DRh159. Since the SIV gag protein was used to replace Rh159 in this construct, we monitored the T cell responses to gag as a surrogate marker for infection. As shown previously, as little as 100 PFU of RhCMV encoding gag are sufficient to elicit gag-specific T cell responses in RhCMV-positive animals (89). Surprisingly; however,, even at a dose of $5x10^6$ PFU, ΔRh159 did not induce gag-specific T cell responses (Fig. 2.8A). Since T cell responses are a sensitive measure of infection, these results suggested that Rh159 is either required to overcome pre-existing immunity (as shown previously for the MHC-I downregulating genes US2-11 (89)) or Rh159 is required for infection regardless of the immune status of the recipient. To determine whether Rh159 was required for primary infection we inoculated two CMV-naïve RM with the same dose of DRh159 and monitored the T cell

response to gag. Similar to the CMV+ animal however, gag-specific T cells were not detected in either of the two CMV-naive RM (Fig. 2.8B). In contrast, wild type RhCMV never failed to induce T cell responses at comparable doses in more than one hundred RM inoculated to date ((62, 89, 494, 501) and data not shown). To determine whether increased clearance by NK cells prevented infection by DRh159, we wanted to monitor infection under conditions that temporarily eliminate NK cells. Since in RM all NK cells express CD8, depletion of CD8+ cells eliminates both CD8+ T cells and NK cells (465). During the first days of infection, CMV-naïve animals lack CMV-specific $CD8^+$ T cells and for this reason evasion of CD8+ T cells is not required for primary infection (89). Therefore, CD8-depletion was used to temporarily eliminate NK cells and T cells on day 63 after the initial inoculation with ΔRh159 (Fig. 2.8C). Re-inoculation of both RM with ΔRh159 resulted in gag-specific $CD4^+$ and (with some delay) $CD8^+$ T cell responses (Fig. 2.8B). Since depletion of non-specific $CD8^+$ T cells was unlikely to impact infection by ΔRh159 we conclude that NK cell evasion by Rh159 is essential for primary infection.

Fig 2.8: Primary infection of rhesus macaques requires evasion of NK cells by Rh159.

A) Rh159 is required for superinfection. At day 0, a RhCMV+ RM was infected subcutaneously (s.c.) with $5x10^6$ PFU of ΔRh159 and the gag-specific T cell responses in PBMC was monitored by ICCS for CD69, TNFα and IFNγ using overlapping SIVgag 15mer peptide mixes. Results are shown as a percentage of total memory $CD4^+$ or $CD8^+$ T cells. No responses above background were measured. **B)** Rh159 is required for primary infection. Two RhCMV-naïve animals were inoculated s.c. with $5x10^6$ PFU

ΔRh159 and SIVgag-specific T cells were monitored as in A). Starting on day 63, RM were treated with anti-CD8 antibody CM-T807 to deplete $CD8^+$ cells and RM were re-inoculated with $5x10^6$ PFU of ΔRh159 on day 64. **C)** The relative frequencies of $CD8^+$ small CD3- lymphocytes in whole blood (WB) of each animal were monitored during CM-T807 treatment.


A
RhCMV Seropositve rhesus macaque
ΔRh159
CD4+
CD8+
% Responding (memory subset)
Time post inoculation (days)
B
RhCMV Seronegative rhesus macaque
ΔRh159
ΔRh159
CD4
CD8
CD4+
CD8+
α-CD8
% Responding (memory subset)
Time post inoculation (days)
C
CD8+ depletion (CD3-/CD8+)
α-CD8
CD8+
Absolute CD8+ of WB CD3- small lymphocytes (change from baseline)
Time post inoculation (days)

2.4 Discussion

Our data indicate that limiting NKG2DL expression on infected cells is essential for RhCMV infection. The conservation of most NKG2DLs between human and RM renders it highly likely that HCMV will similarly depend on NK cell evasion to establish infection. Since multiple NKG2DLs can each engage NKG2D such stringent NK cell control creates an enormous selective pressure to counteract each activating ligand. Consequently, multiple NKG2DL-inhibitory gene products are expressed by HCMV, with UL16 and UL142 being the quintessential NKG2DL-inhibitors acting on multiple NKG2DLs. In contrast to their host targets however, the NKG2DL-inhibitors UL16 and UL142 are not conserved between HCMV and RhCMV. Although RhCMV encodes about 20 ORFs not conserved in HCMV (80) we identified Rh159, the homologue of HCMV UL148, as a broadly acting NKG2DL inhibitor. Similar to UL16, Rh159 is an ER-resident type I glycoprotein that retains MICA, MICB, ULBP1, and ULBP2 in the ER but not ULBP3. A tyrosine-based motif in the cytoplasmic domain of UL16 was implicated in ER-retrieval (342) whereas the luminal domain is thought to interact with NKG2DLs (344). However, so far it has not been possible to demonstrate the formation of a stable complex between NKG2DL and UL16, or any other cytomegaloviral NKG2DL-inhibitor. In contrast, Rh159 was identified in co-immunoprecipitations with MICB consistent with a remarkably stable complex. The predicted type I topology of Rh159 suggests that its ectodomain interacts with NKG2DLs whereas the short, carboxyterminus (KRSREAH) is predicted to expose an RXR ER-retrieval motif (488) responsible for ER-retention of Rh159 and consequently NKG2DLs. However, the role of individual protein domains in Rh159 function has yet to be confirmed experimentally.

Interestingly, HCMV UL148 does not seem to interfere with NKG2DL expression despite clear homology to Rh159 suggesting that the two genes diverge functionally despite common ancestry. Homologues of UL148 are found in all primate CMVs but not in rodent CMVs, whereas UL16 and UL142 are only found in human and chimpanzee CMVs. Since the NKG2DL system predates primate evolution we speculate that the UL148 ancestor most likely evolved to counteract NKG2DL, a function that is conserved in non-human primate CMVs, but substituted with UL16 in human and ape CMVs. In HCMV, UL142 complements UL16 by targeting MICA and ULBP3, which are resistant to UL16. While Rh159 also downregulated MICA, it similarly did not interfere with ULBP3 expression. Since ULBP3 was downregulated in RhCMV-infected cells, it is likely that RhCMV encodes a ULBP3-targeting gene product despite lacking a UL142 homologue. This additional viral NKG2DL inhibitor might also be responsible for the residual reduction of NKG2DL expression in ΔRh159-infected cells. Further work will be required to identify these additional NKG2DL inhibitors and it will be interesting to determine whether upregulation of ULBP3 in their absence would lead to activation of NK cells *in vitro* and stringent control of CMV infection by NK cells *in vivo* as observed for ΔRh159.

The absolute requirement for Rh159 to establish infection was unexpected because deletion of the NKG2DL-inhibitors m138, m145, m152 or m155 from MCMV reduces viral titers, but does not prevent infection of CMV-naïve mice (483). In fact, replacement of m152 with the murine NKG2DL Rae-1 increased $CD8^+$ T cell responses to MCMV (489, 493). This is in stark contrast to the complete absence of T cell responses observed in CMV-naïve animals inoculated with ΔRh159. We consider it

unlikely that ΔRh159 established a low level infection that would not have elicited a T cell responses since CMV-specific T cell responses do not require viral dissemination or spreading from initially infected cells (66, 502). Thus, the most likely explanation for the lack of gag-specific T cell responses in RM infected with ΔRh159 is that NK cells rapidly eliminated infected cells. This conclusion is supported by the induction of gag-specific T cells by ΔRh159 upon treatment with CD8-specific antibodies. Treatment with anti-CD8 monoclonal antibody cM-T807 effectively removes both $CD8^+$ T cells and NK cells from circulation (465). It seems unlikely that T cell depletion permitted infection with ΔRh159, since CMV-naïve animals lack CMV-specific $CD8^+$ T cells at the time of cM-T807 treatment, it seems unlikely that T cell depletion permitted infection with ΔRh159. Moreover, we reported previously that evasion of $CD8^+$ T cells is only required to establish secondary infections in CMV-immune animals, but not for primary infection (89). Thus, although cM-T807 eliminates both T cells and NK cells, $CD8^+$ NK cells were most likely responsible for the suppression of ΔRh159 infection. Interestingly, the magnitude and duration of the T cell response elicited by ΔRh159 did not diminish upon recovery of $CD8^+$ cells suggesting that neither NK cells nor T cells were able to eliminate ΔRh159 once a persistent infection has been established. Similarly, we previously reported that RhCMV lacking MHC-I evasion genes can infect CMV-positive animals upon $CD8^+$ depletion and maintain a persistent infection (89). Taken together these data suggest that both NK and T cells prevent viral spreading from the initial sites of infection, either during primary or secondary infection, respectively, but neither innate nor adaptive cellular responses can eliminate latent viral reservoirs and clear persistent infection, even in the absence of NK or T cell immune evasion mechanisms. We thus expect to observe

long term effector memory T cell responses in the anti-CD8 treated animals despite full recovery of NK and T cell responses. A possible explanation is that latent virus escapes NK and T cell clearance either by being immunologically silent or by using immune evasion mechanisms that differ from the ones used during lytic infection and viral dissemination.

In addition to inhibiting NKG2DL expression, Rh159 was previously shown to enhance infection of epithelial cells (EC) (503). Specifically, it was demonstrated that disruption of the Rh159 gene by transposon insertion reduced growth of RhCMV (strain 68-1) in rhesus retinal ECs by an unknown mechanism. Thus, Rh159 seemed to mediate EC growth independently of the major determinant of EC entry, the pentameric complex gH/gL/UL128/130/131, because 68-1 lacks the UL128 and UL130 subunits (503). Interestingly, it was recently reported that HCMV UL148 affects the ratio of the trimeric (gH/gL/gO) and the pentameric complex in viral particles (26). In the absence of UL148, maturation of gH/gL/gO was impaired thus enhancing the amount of pentameric complexes and infection of ECs. Whether Rh159 similarly affects viral glycoprotein maturation is currently unknown, but it is conceivable that Rh159 might impact the maturation not only of host glycoproteins but also of viral glycoproteins. We recently reported that deletion of the homologs of UL128 and UL130 in RhCMV results in the induction of unconventional $CD8^+$ T cells including MHC-II-restricted $CD8^+$ T cells and responses to supertopes recognized by MHC-disparate animals (62). This unexpected function of pentameric complex components in modulating $CD8^+$ T cell responses could thus potentially be further modified by Rh159. However, ΔRh159 seemed to retain the T cell targeting phenotype of its parental strain 68-1 since unconventional T cell responses

to both MHC-I and MHC-II supertopes were observed in infected animals (data not shown). However, we have not yet determined whether deletion of Rh159 would impact conventional $CD8^+$ T cell responses by UL128-130-intact RhCMV. Thus, Rh159 might play multiple roles *in vivo* that range from NK cell inhibition during primary infection to affecting cell tropism to modulating adaptive T cell responses during persistent infection.

3. RhCMV GENOME ENCODES MULTIPLE GENES RESPONSIBLE FOR NK CELL EVASION

3.1. Introduction

NK cells are an important innate immune cell type that bridges the gap between initial detection of virus and subsequent stimulation of the adaptive immune response. Their importance in controlling CMV is demonstrated not only by the fact that their absence leads to severe herpesvirus disease (60), but also by the astounding number of genes encoded by the virus that specifically have a role in NK cell evasion. The total number of HCMV genes responsible for NK cell evasion stands at eleven, orchestrating their effects using a variety of mechanisms. UL141 inhibits NK activation through DNAM-1 and CD96 by the retention of activation ligands, CD112 and CD155 (376, 377), as well as by inhibiting TRAIL-mediated apoptosis through the intracellular sequestration of TRAIL receptor 2 (379). The tegument protein, pp65, inhibits activation via the NCR activating receptor, NKp30 (320). In an effort to maintain the inhibitory state of NK cells that can be lost due to CMV-mediated-MHCI downregulation (485), the signal peptide of UL40 binds and subsequently upregulates cell surface expression of HLA-E. CMV also encodes a potent MHCI homolog called UL18. Together the upregulation of HLA-E and UL18 block the NK cell recognition of infected cells by engaging NK inhibitory receptors, CD94/NKG2A and LIR-1, respectively (385, 504). UL135 prevents immune synapse formation and downstream NK cell effector functions through actin remodeling (407). However, the majority of the HCMV NK cell evasion genes focus their efforts to prevent cell surface expression of NKG2D activating ligands, MICA/B and ULBP1-6, either by intracellular retention via UL16 and UL142 (301, 304, 342-344, 505), lysosomal

degradation by US18 and US20 (255), proteasomal degradation by US9 (356), or translational repression by miR-UL112 ((365).

However, we are lacking a thorough understanding of how these evasive mechanisms contribute to disease pathogenesis *in vivo*. Since CMVs are highly species specific, we use RhCMV infection in RM as our model system. In Chapter 2, we report on the identification of Rh159 as a novel RhCMV NK cell evasion gene responsible for the downregulation of NKG2DLs by means of ER retention. Importantly, we find that inoculation of RM with RhCMV deleted of Rh159 is unable to establish a primary infection, and that depletion of NK cells restores the ability of the Rh159 deletion virus to infect RM, demonstrating that the inability of the virus to infect is NK cell dependent (Fig 2.8). We also present results that indict that the RhCMV genome encodes additional NK cell evasion genes (Fig 2.3 and 2.5) using a panel of human U373 cells expressing the human NKG2DLs: MICA, MICB, and ULBP1-3. Here, we further examine RhCMV infection in our *in vitro* model, and investigate what additional RhCMV genes may downregulate NKG2DLs. We find that U373 cells are permissive to RhCMV entry, demonstrated by the intranuclear detection of RhCMV IE2. We also demonstrate that viral IE2 expression has a negligible effect on the expression of NKG2DLs from the lentiviral constructs used in our experiments. Importantly, we identified two additional RhCMV gene regions, Rh158-180 and Rh191-202, responsible for the downregulation MICA. RhCMV lacking the gene region Rh158-180 fully restores cell surface MICA expression and cells infected with RhCMV lacking Rh158-180 stimulates NK cell more then cells infected with RhCMV lacking Rh159 alone. In addition, we demonstrate that RhCMV lacking the gene region, Rh191-202, partially restores cell surface MICA

expression; and our data suggests that the RhCMV genes, Rh199 and Rh201, may be the functional homologues of HCMV genes, US18 and US20.

3.2. Results

3.2.1. RhCMV entry of U373 cells

Our lab obtained the human glioblastoma cell line, U373, which had been transduced with lentivirus to stably express human NKG2DLs. In order to test the ability of RhCMV to downregulate NKG2DL surface expression, we first assessed the ability of RhCMV to infect U373 cells. We infected U373s with WT RhCMV at varying MOIs and then stained for IE2 expression, which was detected using immunofluorescence. Even at low MOI (0.1) IE2 was detected in the nucleus of U373 cells, and nearly all cells were positive for IE2 at high MOI (Fig 3.1A). In addition, we obtained a WT RhCMV virus that expresses GFP (RhCMV-GFP). U373s were infected at high MOI with RhCMV-GFP. Using flow cytometry, we show that the majority of infected U373s were positive for GFP (Fig 3.1B, left). In addition, the U373s infected with RhCMV-GFP were also stained with a CMV-specific antibody, which was generated at the VGTI, which was used in subsequent experiments to gate on CMV infected cells.

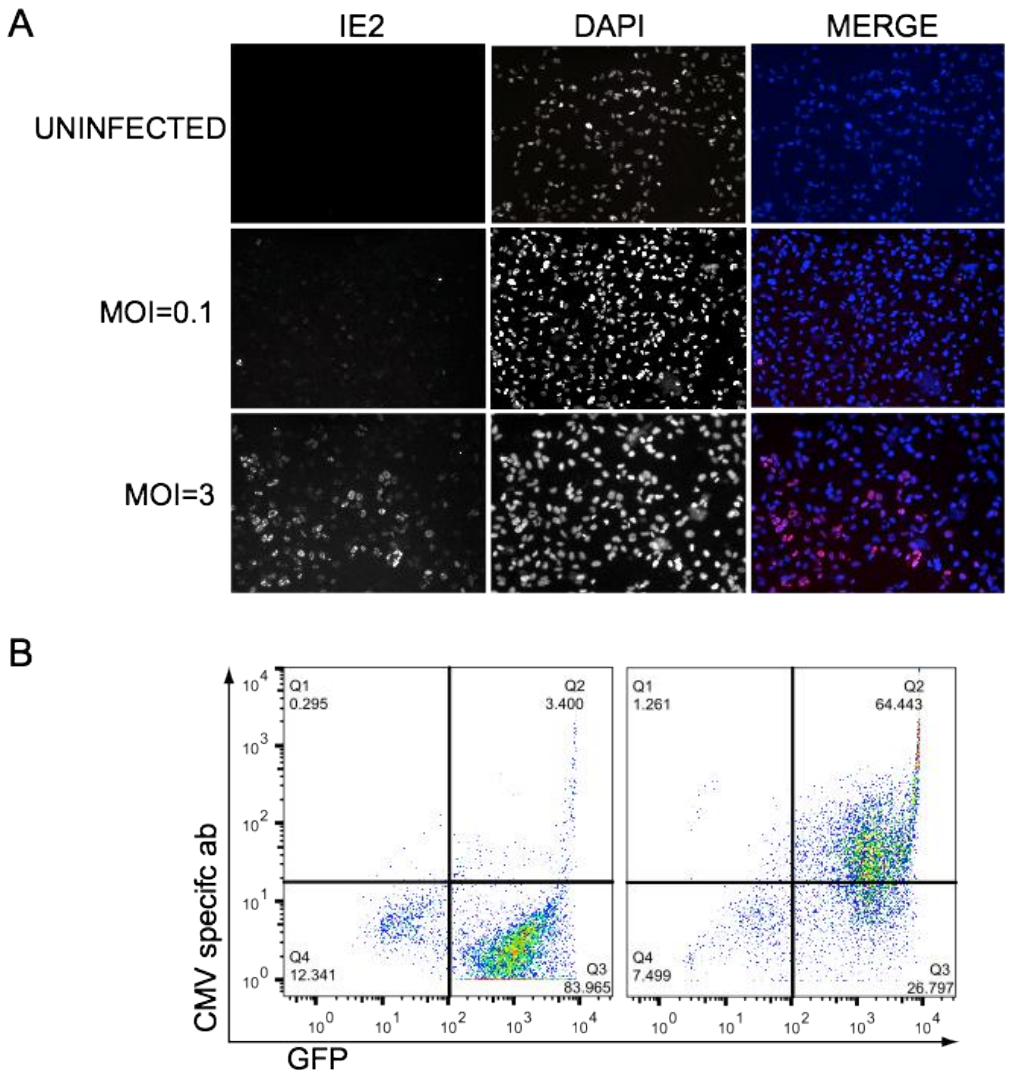


Figure 3.1: U373 cells are permissive to RhCMV infection

A) U373s were infected with RhCMV (MOI=3 or 0.1) or left uninfected. At 24 hpi cells were fixed with 4% paraformaldehyde, permeabilized and then stained with DAPI (blue) and antibodies specific for RhCMV IE2^{+} (red) and visualized by immunofluorescence microscopy. The results shown are representative of two independent experiments. B) U373s were infected with RhCMV-GFP (MOI=3). At 48 hpi, cells were quantified for GFP alone (left panel) or additionally stained with a CMV-specific antibody (right panel) then analyzed using flow cytometry.

3.2.2. RhCMV IE2 does not significantly affect NKG2DL expression

The lentivirus used to stably express the human NKG2DLs are under the HCMV major IE promoter (MIEP) to drive expression. The RhCMV IE2 homolog in HCMV is known to bind and repress the MIEP promoter (51). To address this possible complication, we infected the panel of U373-NKG2DLs with either WT or ΔRh159 RhCMV then measured the amount of corresponding NKG2DL transcript using qRT-PCR. There was a negligible decrease in the levels of MICA, MICB, ULBP2, and ULBP3 transcripts (Fig. 3.2). ULBP1 mRNA was reduced by approximately 50% after infection of either WT or ΔRh159 RhCMV, therefore it could be possible that the effects seen in Fig 2.1&2.5 were due to shut down of the CMV promoter used in our constructs. However, this does not negate the fact that Rh159 plays an important role in NK cell evasion *in vivo* (Fig 2.8).

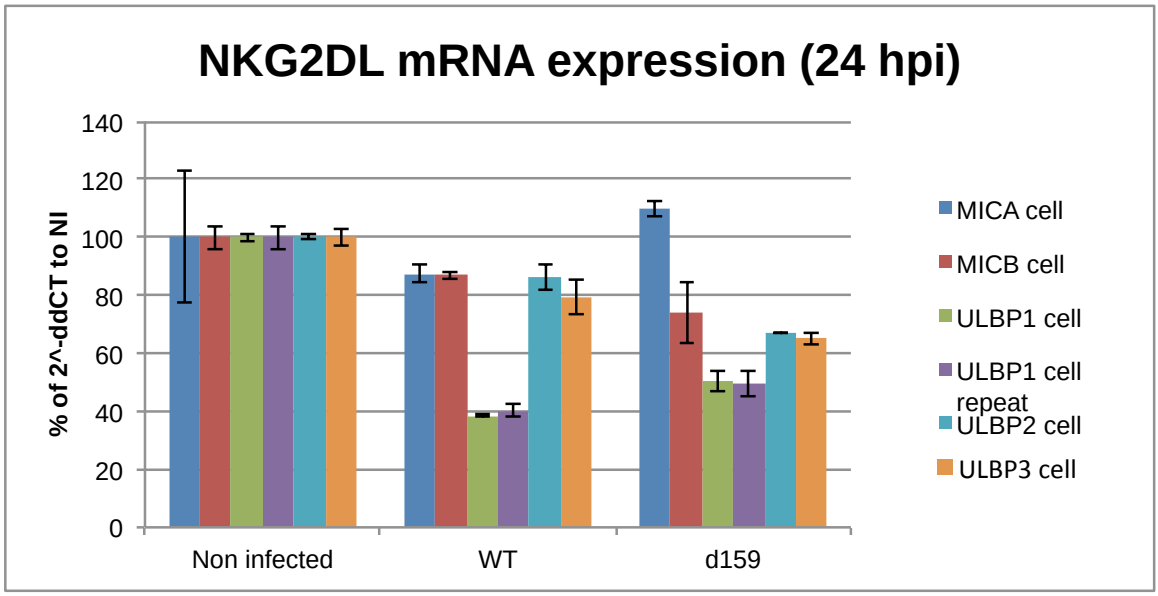


Figure 3.2: NKG2DL mRNA expression by qPCR after infection with RhCMV

U373-NKG2DLs were infected with WT RhCMV or ΔRh159 (MOI=3) for 24 h, followed by RNA extraction to determine NKG2DL mRNA expression by qPCR. Data were normalized to the level of GAPDH mRNA expression in each sample and are shown as percent reduction relative to uninfected control cells. Shown are the means ± standard errors of one independent experiment with three replicates/sample in each experiment.

3.2.3. Rh158-180 encodes additional gene products responsible for downregulation of MICA surface expression

We previously demonstrated that RhCMV is able to retain newly synthesized MICB in the ER (Fig 2.2B, right). Similarly, we immunoprecipitated MICA from metabolically pulse/chase labeled U373-MICA cells at 24 hpi. In uninfected cells the majority of MICA was EndoH resistant at 1 h post-chase (Fig. 3.3A, left). Although not as striking as the lack of MICB maturation due to RhCMV infection (Fig 2.2B, right), a proportion of MICA did remain EndoH-sensitive in RhCMV-infected cells (Fig. 3.3A, right). It is also noteworthy that an additional protein species of the same molecular weight (indicated by *) that was detected in MICB-immunoprecipitations from RhCMV-infected samples (Fig 2.2B) was also detected in MICA-immunoprecipitations from RhCMV-infected samples (Fig. 3.3A, right). Additional analysis needs to be performed in order to confirm the identity of this band.

We have previously shown that infection of U373-MICAs with ΔRh159 compared to WT RhCMV leads to the increased cell surface expression of MICA (Fig. 2.5E). However, deletion of Rh159 did not fully restore MICA surface levels to amounts observed in uninfected cells. (Fig 2.5E). Additionally, comparative levels of MICA

mRNA were observed before and after infection with RhCMV regardless of Rh159 expression (Fig 3.2). Therefore, one or more additional genes responsible for the downregulation MICA cell surface expression are likely to exist. To test this hypothesis, we infected U373-MICAs with either WT or a mutant RhCMV virus containing a large deletion encompassing 22 ORFs—Rh158-180 (ΔRh158-180) and analyzed MICA surface expression by FACS analysis. We found that infection with ΔRh158-180 compared to WT RhCMV fully restored MICA cell surface expression compared to uninfected levels (Fig 3.3B). In addition, MICA detected in lysates from U373-MICAs infected with ΔRh158-180 remained fully endoH-resistant (Fig 3.3C). To determine whether increased MICA expression by ΔRh158-180 led to an increased amount of NK cell activation compared to ΔRh159, we infected primary fibroblasts derived from two individual macaques with WT RhCMV, ΔRh159, ΔRh158-180, ΔRh182-189 or left uninfected, and then monitored stimulation of purified, autologous NK cells at 48 hpi as described previously in Fig 2.7. NK cells were effectively stimulated by positive control cell line K562, (Fig. 3.3D). Similar amounts of NK stimulation were observed in fibroblasts infected with either WT or ΔRh182-189 (lacking 4/5 MHCI immunomodulatory genes) (319) or uninfected. However, NK cells from both individuals had an increase in CD107a staining after co-incubation with fibroblasts infected with ΔRh159. We also observed one individual with a substantial increase in NK stimulation due co-incubation with fibroblasts infected with ΔRh158-180 compared to all other experimental samples (Fig. 3.3D). These results strongly suggest that one or more additional NK cell evasion genes are encoded within the region Rh158-180 and are

responsible for preventing maturation of MICA through the cis-Golgi/ER and are functionally important for the virus to evade NK cells.

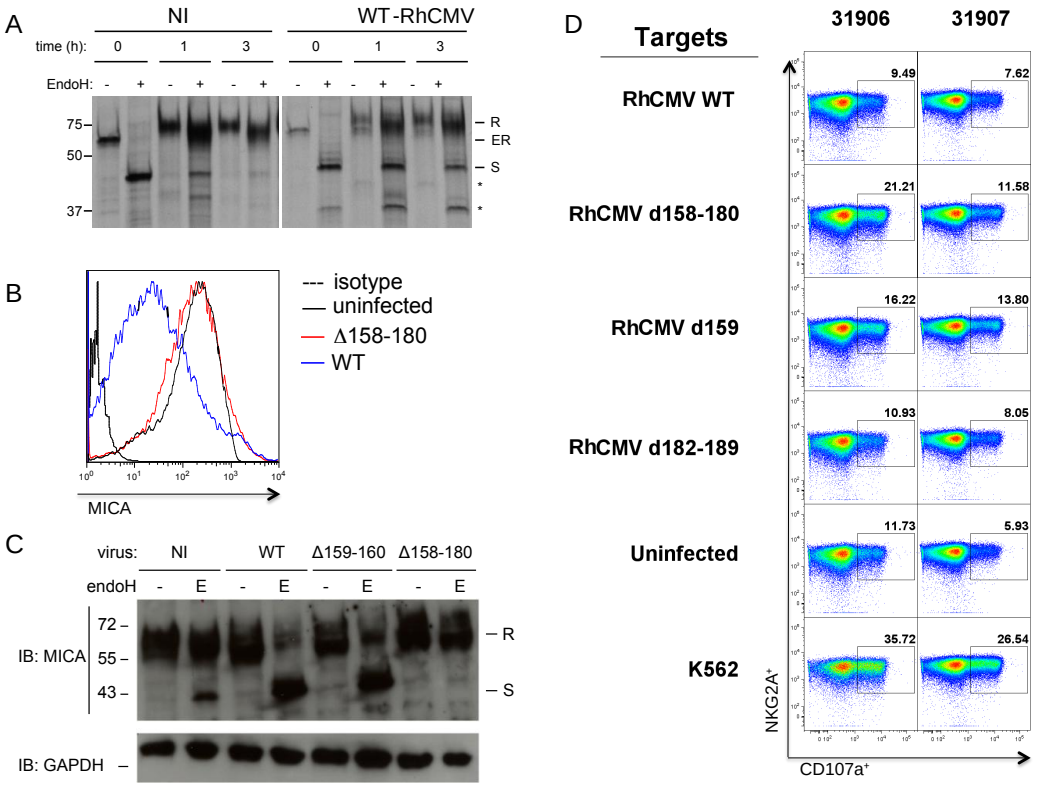


Figure 3.3: Deletion of Rh158-180 rescues maturation and surface expression of MICA upon RhCMV infection

A) U373-MICAs were infected with (WT) RhCMV (MOI=3) for 24 h (visualization by light microscopy confirmed 100% CPE) or left uninfected (NI) followed by metabolically labeling with [35S]cysteine and [35S]methionine for 30 min. The label was then chased for the indicated times (h), cells were lysed and MICA was immunoprecipitated from cell lysates using a MICA specific antibody. Precipitates were either digested with EndoH (+) or mock treated (-) followed by SDS-PAGE and autoradiography. Stars (*) indicate an EndoH-sensitive protein co-precipitating with MICA in infected cells. B) U373-MICAs

were infected with WT RhCMV (blue), ΔRh158-180 (red) (MOI=3) or left uninfected (black) for 48 h. Cell surface levels of MICA were determined by flow cytometry, using MICA specific antibodies and compared to isotype control (dotted). Depicted is MICA surface expression, gated on cells positive for staining with a CMV-specific antibody to control for infection. The results shown are representative of two independent experiments. C) U373-MICAs were lysed at 48 hpi with either WT, ΔRh159, ΔRh158-180 or left uninfected. Lysates were digested with EndoH (+) or mock treated (-) then immunoblotted with mAbs for MICA or GAPDH. The results shown are representative of two independent experiments. D) PBMCs were isolated from Cynomologus Macaques via Ficoll density separation, followed by sorting for NK cells ($CD3^-/CD8^+/NKG2A^+$). NK cells were stimulated with IL-15 and IL-2 overnight followed by 4 h incubation with autologous fibroblasts infected with either WT RhCMV, ΔRh159, ΔRh158-180, ΔRh182-189 (MOI=5) or left uninfected for 48 h. MHCI negative K562 cells were used as a positive control. Staining for CD107a, a marker for degranulation, was used to monitor NK cell activation. Results are from two representative animals.

3.2.4. Rh191-202 encodes additional gene products responsible for downregulation of MICA surface expression

It has been reported that HCMV proteins, US18 and US20, are responsible for the lysosomal degradation of MICA (255). The RhCMV genome encodes the homologs to these proteins—Rh199 and Rh201. We sought out to determine whether Rh199 and Rh201 were indeed the functional homologues of US18 and US20. To test this, we made use of a RhCMV mutant containing a deletion of the gene region Rh191-Rh201 (ΔRh191-202). As with previous deletion RhCMV viruses, we infected U373-MICAs

with ΔRh191-202 and assessed MICA surface expression levels compared to WT RhCMV infection using FACS analysis. We observed an increased amount of MICA surface expression in cells infected with ΔRh191-202 compared to WT RhCMV, although deletion of the gene region Rh191-202 did not restore MICA to uninfected levels (Fig. 3.4).

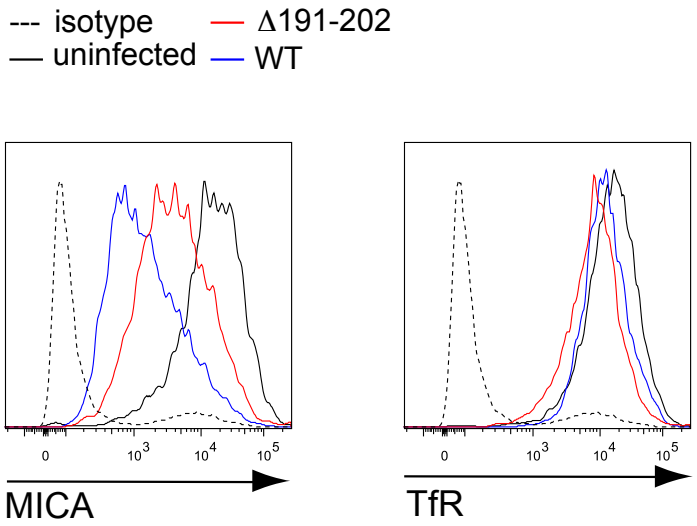


Figure 3.4: Deletion of Rh191-202 increases MICA surface expression

U373-MICAs were infected with RhCMV (blue), ΔRh159 (red) (MOI=3) or left uninfected (black) for 48 h. Cell surface levels of NKG2DL or TfR were determined by flow cytometry, using specific antibodies and compared to isotype control (dotted). Depicted is MICA or TfR surface expression on infected cells gated for RhCMV $IE2^+$ expression. The results shown are of one independent experiment.

3.3. Discussion

Our data indicate that U373 cells are permissive to the entry RhCMV. It is not surprising that IE2 was detected in U373, a glioblastoma cell line, as numerous studies

have reported detection of a wide variety of HCMV proteins including IE1 (506-509), pp65 (506, 510), and gB (506, 507) in samples of patients with glioblastoma. Single-step growth curves of WT and Δ159 RhCMV on U373s are currently underway and will determine whether infectious progeny as also being produced.

It is known that the CMV protein, IE2, can bind and repress the MIEP promoter (511-513). RhCMV and HCMV IE2 AA sequences are 48% identical (514), and therefore it is possible that RhCMV IE2 could bind and repress the HCMV MIEP. Additionally, our *in vitro* system is comprised of a panel of U373s that were transduced with lentivirus containing an HCMV IE promoter to express the NKG2DLs, which we infect with RhCMV that does express IE2 (412). However, we report that the expression of NKG2DLs from the panel of U373-NKG2DLs is not significantly affected by RhCMV IE2 expression with a minor exception of U373-ULBP1. Future experiments will use intracellular FACS to show that RhCMV infection does not affect steady state levels of human NKG2DLs, in contrast to cell surface levels. We will also measure the effects of RhCMVΔRh159 versus WT infection on the cell surface expression of RM ULBP1, as well as the other RM NKG2DLs, driven by the EF1α promoter.

We have generated preliminary data implicating the additional genes regions, Rh158-180 and Rh191-202, as responsible for the reduction of MICA cell surface expression. The co-evolution of both virus and host has led HCMV to evolve multiple genes responsible for downregulating NKG2DLs in an attempt to evade NK cells (286). Therefore, we also expected the RhCMV genome to encode for more NK cells evasion genes then the recently identified, Rh159 (Chapter 2). It is interesting to note that these data suggest no less then three individual genes downregulate MICA cell surface

expression, but most likely employ different mechanisms to enact their common goal. We observed full restoration of MICA on the cell surface due to the deletion of Rh158-180. Further, only the endoH resistant form of MICA was detected by immunoblotting, indicating that MICA had completely matured through the Golgi. However, we also found that the gene region, Rh191-202, targets MICA and is likely expressed in the Rh158-180 deletion mutant. One might then question how complete restoration of MICA cell surface expression is achieved when other gene products are expressed that also target MICA. However, it is conceivable that since MICA is no longer being retained in the ER by Rh159 and assisted by gene products within Rh158-180, that the additional gene/s encoded within Rh191-202 are unable to interact with MICA due to the deletion of the other RhCMV genes, and therefore MICA traffics to the surface unimpeded. Although HCMV proteins, US18 and US20, (homologs to Rh199 and Rh201) target MICA for lysosomal degradation when ectopically expressed (255), the Rh191-202 gene locus may require expression of additional RhCMV genes in order to target MICA. Indeed, other examples of synergistic actions mediated by HCMV viral proteins to hijack cell function have been discovered. A concerted process of protein retention followed by proteasome degradation has recently been reported as the mechanism of CMV-mediated CD112 downregulation (378). UL141 first retains CD112 in the ER, followed by interaction with US2, whereby CD112 is retrotranslocated from the ER and is subsequently degraded by the proteasome (377, 378). It will be interesting to discover what HCMV mechanisms are shared by RhCMV in its battle to evade NK cells, and to elucidate the roles they play *in vivo*.

4 DISCUSSION AND FUTURE DIRECTIONS

This dissertation describes the novel effects of NK cell evasion by RhCMV *in vivo* using the RM model. Our group has used this model to discover the intricate ways that RhCMV mediates immune evasion. Indeed, this dissertation stems from our groups discovery that the downregulation of MHCI from the cell surface by RhCMV leads to the evasion of CD8+ T cells due to the subsequent reduction in antigen presentation. The overall reduction in cell surface MHCI may be instrumental to T cell evasion, but downregulation of MHCI also can be detected by NK killer cells. HCMV is known is modulate NK cell evasion through multiple mechanisms; however, the *in vivo* relevance was not known. Therefore, we set forth to discover: 1) whether RhCMV encodes its own NK cell evasion genes; 2) by what mechanisms these genes exert their effects; and 3) the functional outcome of RhCMV NK cell evasion. In Chapter Two, we present data that identifies Rh159 as a crucial molecule required for RhCMV NK cell evasion and is necessary to establish primary infection *in vivo*. We find that Rh159 is able downregulate NKG2DLs: MICA, MICB, ULBP1, and ULBP2; and that it downregulates these ligands from the cell surface by means of intracellular retention within the ER/cis-Golgi. A mutant virus constructed with a deletion of Rh159 restores surface expression of NKG2DLs, which is sufficient to fully activate NK cells. Importantly, infection of RM with RhCMVΔ159 can prevent RhCMV from establishing its hallmark persistent infection. This project has given great insight into role of RhCMV NK cell evasion, and will have significant impact upon the wider field of NK cell biology and CMV vaccinology, both as a vaccine vector and on the development of a CMV vaccine.

4.1. Perspectives on NK cell evasion by Rh159

CMV is a master of disguise. The virus encodes a multitude of immune evasion genes in order to coexist with its host. This coexistence extends not only throughout the lifetime of one particular host, but CMV has coexisted with its host over a 100 million years, during which time the virus has evolved incredible adaptions to ensure its ability to produce progeny. Indeed, our own genomes bare the effects of this coevolution as evidenced in our expanded repertoire of MHC and KIR gene loci. Meanwhile, CMV has continuously adapted its own genome to evade elimination by our immune system. Indeed, HCMV encodes four genes, US2-11, that each prevent MHCI surface expression that are needed to evade T cell activation. The RhCMV homologues, Rh182-189, were shown by our group to be the functional homologues of US2-11 and through the utilization of the *in vivo* model of RhCMV infection in RM, we demonstrated that these genes were necessary for superinfection. Another important advance generated by this data was progression in the knowledge of MHCI inhibitors in the US6 family, in a particular. Since HCMV cannot be studied in humans due to obvious ethical concerns, RhCMV infection in RM is instrumental to our understanding of CMV evasion. MCMV encodes MHCI inhibitors, m04, m06, and m152; but they are not homologues to the HCMV US6 family. The murine model of MCMV infection demonstrated that modulation of MHCI surface expression due viral MHCI inhibitors were responsible for evasion of T cell activation, but infection of mice with MCMVΔm04+Δm06+Δm152 did not prevent the detection of a strong, prolonged T cell response in immunocompetent mice (515, 516), much like we saw in our own work studying infection of RM with Rh182-189 (89). However, the role of specific HLA allele targeting by MHCI inhibitor

genes cannot be recapitulated in the murine model due to the nature of using isogenic mouse strains. Indeed, in the past five years since the discovery that the US6 family is essential for superinfection, much of the unique biology discovered has been focused on the type and breadth of specific CD8+ cells elicited by RhCMV. These data were only possible due to the RM model of RhCMV infection. Demonstrating to the CMV field, the importance of the RM model.

Likewise, the work of NK cell evasion by RhCMV has provided new information that has not been reported using the murine model. As discussed in Chapter One, MCMV encodes four NK cell evasion genes responsible for downregulating NKG2DLs in mice: m152, m145, m155, and m138. Mutant viruses have been made, a separate deletion virus for each NK cell evasion gene, and tested *in vivo* for their role in downmodulation of NKG2DLs, viral attenuation and effects on specific CD8+ T cell responses. The deletion of any one single gene, restores some NKG2DL to the cell surface; however, all of the deletion viruses were able to establish infection, albeit the viruses were extremely attenuated, which was shown to be mediated by NK cells (367, 374, 375, 482, 517). In an effort to increase NK cell detection and viral attenuation, Jonic and colleges (489) engineered a virus with a deletion of m152 and replaced it with RAE-1γ (RAE-1γMCMV), one of the NKG2DLs targeted by m152. The virus was attenuated in vivo; however, mice infected with RAE-1γMCMV had the surprising effect of inducing a strong, enduring CD8+ T cell response (489). So even with increased amount of NKG2DL on the infected cell surface, the murine NK cells were unable to prevent virus from escaping the site of inoculation as evidenced by a specific CD8+ T cell response. The main conclusion drawn from Chapter 2 was that Rh159 was necessary to establish

primary infection. Similarly to the MCMV NKG2D immunoevasion single deletion mutants, infection with RhΔ159 the did not restore all NKG2DL back to steady state levels; however, the increased expression of NKG2DLs was still sufficient to elicit NK cell activation by RhΔ159-infected cells *ex vivo* and prevent RhΔ159 from establishing infection in either sero-positive or sero-negative RM in *vivo*. What may be the rationale for the discrepancies between these two models? One possible explanation could be the site or route of infection. In murine models, MCMV was delivered intraperitoneally, whereas in our RM model, the virus was administered subcutaneously. It is conceivable that NK cells residing in tissues at the different sites of infection could be functionally and phenotypically different and thus account for the different findings. Another answer may be due to the differences in which the NKG2DL immunoevasins target their cognate ligands. The m152 protein, like Rh159, retains NKG2DLs in the ER; however m152 only retains the RAE-1ligands (367). The MCMV immunoevasin, m138, on the other hand primarily targets members of the H60 family and MULT-1, albeit m138 has specificity for RAE-1ε (479). However, RAE-1δ has the longest residence on the cell surface and mediates the strongest NKG2D-medaited resistance to MCMV infection compared to other RAE-1 ligands (480). Therefore, no one MCMV immunoevasin effectively targets members across all three murine NKG2DL families. Due the complexities and differences leading to the induction of NKG2DLs, it may be necessary to broadly target NKG2DLs from all families. In contrast, Rh159 was able to inhibit NKG2DL cell surface expression from both MIC and ULBP families. Another answer, although certainly not mutually exclusive, may lie in the differences in NKG2DLs between these two species. Both m138 and m145 target MULT-1 (481, 482). Although the deletion of either gene

increases the amount of MULT-1 on the cell surface, a deletion mutant encompassing both of these genes has not been described. Thus it is difficult to determine the relevance of MULT-1 surface expression in cells infected with virus lacking only *m145 or m138* because cell surface expression is only compared to uninfected levels (481, 482), where MULT-1 is actively ubiquitinated and subsequently degraded (484). The maximal amount of MULT-1 on the cell surface could only be determined by deletion of both genes. Therefore, due to the affinity of MULT-1 for NKG2D, both *m145* and *m138* may need to be deleted to fully restore the MULT-1 cell surface levels needed to exact the most potent NK cell response. However, increased surface expression of MICB may be sufficient to stimulate NK cells. Using phylogenetic analysis, a recent report finds that present day MIC genes are derived from a single MICB-like ancestral gene. In addition, multiple herepesviruses express miRNAs that target MICB (420). These data support the importance of targeting MICB in particular to facilitate NK cell evasion.

When work for this dissertation began, the only HCMV proteins reported to downregulate surface expression of NKG2DLs were UL16 and UL142, and unlike the conservation of the MHCI inhibitor genes between HCMV and RhCMV, the RhCMV genome did not encode homologues to UL16 or UL142. Therefore it was quite surprising that when we discovered Rh159 was able to downregulate expression of NKG2DLs, as we expected the gene to be RhCMV specific. However, the HCMV sequence homologue, UL148, although recently shown to be an ER resident protein (26), does not modulate NKG2DL surface expression. We therefore propose that Rh159 can be considered a functional analog of UL16 that can be used as a model for *in vivo* function of UL16. Both UL16 and Rh159 bind MICB with high affinity, as each protein has been demonstrated to

co-immunoprecipitate with MICB. UL16 retains MICB in the ER, and this retention is due to a retrieval motif, YQRL, in the C-terminal tail (342-344). Rh159 also retains MICB in the ER, and although Rh159 does not contain an YQRL motif, it is predicted to have an RXR ER-retrieval motif within the ectodomain (488).

As stated above RhCMV does not encode a UL142 homologue. However, since the beginning of this project several other HCMV proteins have been identified that target NKG2DLs. US18 and US20 were discovered using the Merlin HCMV strain background that contained UL16 and UL18 deletions. They then created deletion mutant viruses that encompassed 56 other nonessential HCMV proteins, and assayed for viruses that showed increased MICA expression by flow cytometry then overexpressed candidate genes in adenovectors in order to identify US18 and US20 (255). In a similar method, our lab has RhCMV deletion mutants that encompass approximately 70 nonessential ORFs, including the US12 family encoded in region Rh191-202. Newly generated data suggest that the RhCMV homologues (Rh199 and Rh201) of US18 and US20 (Fig 3.4). HCMV UL135 uses the novel mechanism of disruption of actin remodeling to prevent immune synapse formation, and thus avoiding effector function. In RhCMV, the sequence homologue is Rh177, and displays only a weak homology to HCMV merlin strain UL135 (33% identity at the amino acid level); however, the HMCV proteins US2-11 display weak homology to Rh182-189 and are indeed functional homologues. Rh177 therefore warrants further investigation, as does Rh164, the sequence homologue to UL141 responsible for TRAIL and DNAM-1 mediated evasion of NK cells. However, all of these genes were present in the RM ΔRh159 infections, and thus whatever effects they have on NK cell evasion was not sufficient to prevent NK cell detection and clearance.

The report of CMV-induced memory NK cells by Sun et al. (240) has opened the door to the real possibilities of NK cell based therapeutics. Memory NK cells are described to undergo clonal expansion, that is IL-12 driven, and then proceed through a contraction phase. These NK cells can be challenged again with specific antigen (ie., m157) and display a greater effector function phenotype and can protect the host against subsequent infection. The field was further advanced by the discovery that m157, a MCMV viral protein that binds Ly49H+ mouse NK cells, was the interaction responsible for the memory NK cell phenotype, showing that a CMV specific viral protein mediated this expansion and that these NK cells were maintained (490). In kind, evidence was provided that HCMV also induces a memory NK cell population that is NKG2C+ (251). A specific CMV viral ligand has not yet been identified as a requirement for this expansion. However, CD94/NKG2C binds to HLA-E, which is upregulated due to CMV infection. It has been hypothesized that this interaction may be the link to the HCMV induced memory NK cell pool. What is not known is whether the expansion of NKG2C+ NK cells is due to the overall upregulation of MHC-E or whether it is due to the presence of specific peptides bound within the groove of MHC-E needed to induce the response?

Another important question in the field is whether specific CMV induced ligand:receptor interactions (eg. m157:Ly49H+) between CMV-infected cells and NK cells is sufficient to induce the memory-like NK cell phenotype or whether the features of CMV persistence is necessary to continually stimulate NK cells in order to maintain a memory-like state. RM infection with the RhΔ159 virus may be one way to determine this important question. The RhΔ159 virus still encodes both Rh67, and therefore can induce the observed HLA-E expression with broad peptide binding capabilities. However,

as described in Chapter Two, this virus is unable to persist. It should be possible to infect RM with the RhΔ159 virus, which encodes SIV. Various timepoints after infection, RM NK cells could be isolated and the co-incubated with DCs that are pulsed with GAG. NK cell could then be tested for activation using a various number of activation assays including the CD107a activation assay used in this dissertation. One could then determine whether a NKG2C+ memory pool occurs only under conditions of WT viral infection, which would support the theory that persistent infection is necessary; however, if a NKG2C+ memory pool could be obtained after infection with the RhΔ159 virus, it would suggest that stimulation through the CD94/NKG2C receptor alone is sufficient to generate CMV specific NK cell memory. Current reports demonstrating that NK cells isolated from HCMV infected individuals have epigenetic modifications supports the later (263, 264). Of course, this work would be dependent on demonstrating that RhCMV infection does indeed induce a memory cell population within RM. New evidence for this was reported during the writing of this dissertation. RM were infected with adenovectors expressing either SIV-Env or SIV-Gag, then waited an impressive 5 years to see if the NK cells were still able to lyse DCs pulsed with either matched or mismatched peptide, and found that both splenic and liver derived NK cells specifically lysed antigen-matched target cells (492). This work suggests that antigen alone is sufficient to generate memory NK cells and that this phenomena is not specific to liver derived NK cells as suggested by data generated in mouse models (232, 234). In addition, they also were able to block DC lysis with the addition of either NKG2A or NKG2C antibody. They did not determine the role of MHC-E presentation. These data suggest that CMV is not necessary to induce

MHC-E in order to generate NK cell memory, but does not rule out the importance of MHC-E presentation to elicit NK cell memory.

4.2. RhCMV NK cell evasion future directions

This dissertation describes the role of Rh159 in RhCMV NK cell evasion. However, RhCMV likely encodes additional NK cell evasion genes. We demonstrate that infection with ΔRh159 did not fully restore surface expression of MICA, MICB, ULBP1 or ULBP2 suggesting that additional genes are responsible for downregulating these ligands. In addition, we found that Rh159 did not target ULBP3. Future work will entail the discovery and characterization of these additional NK cell evasion genes. In our recent preliminary data we show that, MICA was partially restored after infection of cells with RhCMV containing a deletion of gene region Rh191-202. Whether the RhCMV proteins Rh199 and Rh201, are indeed the functional homologues of HCMV proteins, US18 and US20 will be tested. It will also be interesting to determine if the RhCMV proteins have the same specificity for MICA as US18 and US20, or whether they able to target additional NKG2DLs. We also have data supporting the hypothesis that another MICA targeting gene is encoded with the gene region of Rh158-180. Viruses with sub-deletions of the regions Rh158-168 and Rh168-180 have already been constructed in our lab, which can be used to further map the location of the MICA targeting gene. In addition, viruses with further sub-deletions within gene region 168-180 could also be tested if found to be responsible for the MICA targeting phenotype. Since Rh159 did not target ULBP3, our library of RhCMV deletion mutants, including the Rh158-180 deletion mutant, can be used to narrow down the list of possible gene candidates.

Another future direction that is currently ongoing is to determine if Rh159 is the functional analog of HCMV-UL16. To test this, we are engineering a RhCMV vector containing a swap of UL16 for Rh159. RM will be infected the ΔRh159/UL16 replacement virus to answer the question of whether UL16 expression in lieu of Rh159 is sufficient to restore infection of RhCMV. Similarly, an HCMV vector is being constructed with a UL16 deletion. Despite the fact the CMVs displays species-specific tropism, we have shown that HCMV can infect RM and elicit T cell responses (data not shown), and we can therefore infect RM with the HCMVΔUL16 virus and determine if this mutant can establish infection. If the results recapitulate those observed in the Δ159 infection of RM, then we can also determine whether NK cells prevent infection of HCMVΔUL16 by NK cell depletion of RM followed by re-inoculation with HCMVΔUL16. In addition, UL148 (HCMV homologue to Rh159) has been implicated to play a role in tropism and affects the ratio of trimer to pentamer (26). These genes were reported by us to affect the induction of unconventional CD8+T cell responses (62). Therefore, a virus is being constructed to delete Rh159 from the RhCMV vector 68-1.2, which is repaired for UL128-UL131A and to determine whether or not Rh159, alone, could alter the CD8+ T cell restriction. This experiment would also solidify the conclusion that the lack of primary infection by ΔRh159 was indeed purely NK cell dependent and not influenced by differences in tropism.

The role that CMV plays in eliciting and maintaining memory NK cells is another avenue that needs further exploration. Although, it has been shown that antigen alone is sufficient to generate a durable RM memory NK cell population, demonstration that RhCMV can also generate this response is needed. The hypothesis that peptide presented

on MHC-E mediates the expansion of NKG2C+ NK cells needs investigation. The fact that both SIV antigens Env and Gag induce memory RM NK cells is an important new finding that strongly suggests that NK activation via the CD94/NKG2C receptor is required for eliciting NK memory. However, it could be possible that CMV could induce a stronger effector phenotype due to it ability to upregulate MHC-E. In addition to the induction MHC-E restricted CD8+ T cells, it should be tested whether Rh189 and Rh67, are required for CMV specific NK cells. Our group has also regenerated data describing which SIV-Gag peptides that are MHC-E restricted. This information could be used to determine if any of the MHC-E:peptide complexes are more efficient at stimulating memory NK cells, and decipher what determinants are preferred for NKG2C binding in order to elicit the strongest NK cell effector functions.

4.3. Conclusions

In this dissertation, we have shown that RhCMV can downmodulate the expression of NKG2DL from the cell surface. We have additionally discovered a RhCMV gene responsible for this action – Rh159, and characterized its function both *in vitro* and *in vivo*. Rh159 targets MICA, MICB, ULBP1, and ULBP2 and prevents their surface expression. MICB is able to co-immunoprecipitate Rh159, evidence of their direct interaction. In addition, we have determined that Rh159 retains MICB in the ER. We have also determined that the HCMV homologue UL148 does not recapitulate the phenotype of Rh159. Importantly, we have shown that infection of NHP fibroblasts with ΔRh159 can stimulate autologous NHP NK cells *ex vivo*. We have also made the surprising discovery that Rh159 is essential for the primary infection of RM and that this

result is NK cell dependent. The future developments in the field of NK cell biology promise exciting new results and the study of CMV is instrumental to those discoveries.

5 MATERIAL AND METHODS

Cell lines and antibodies

All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU of penicillin/ml and 100 µg of streptomycin/ml and incubated at 37C and 5% CO_2 except where noted. K562 cells were obtained from ATCC. 293A cells were obtained from Invitrogen. 293-CRE expressing cells were a kind gift from Ashley Moses (Oregon Health and Science University). Telomerized Rhesus fibroblasts (TRFs), primary cynomologus fibroblasts and NK cells were obtained from animals housed at the Oregon National Primate Research Center. U373-NKG2DLs were previously described (412). The following primary antibodies were used for immunoprecipitation, flow cytometric and/or immunoblot analysis of U373-NKG2DLs: mouse anti-MICA (AMO1), -MICB (BMO2), -ULBP1 (AUMO2), -ULBP2 (BUMO1), and -ULBP3 (CUMO3) (Axxora), -TfR (M-A712, BD Pharmingen), -GAPDH (6C5) and -V5 (E10) (Santa Cruz), -FLAG (M2) and polyclonal rabbit anti-FLAG (Sigma). For isotype controls mouse $IgG2_a$ (02-6200) or IgG1 (MG100) (Life Technologies) were used. For secondary antibodies, chicken anti-mouse Alexa Fluor 647 (Life Technologies) and streptavidin PE-Cy7 (eBioscience) were used in FACs experiments. Goat anti-mouse IgG-HRP and donkey anti-goat IgG-HRP (Santa Cruz) were used for immunoblotting. NKG2DLs in TRFs were surface stained with NKD2D-Fc

chimera (R&D Systems) followed by anti-human IgG-PE (eBioscience). Human Fc-G1 (BioX-Cell) was used as isotype control. Streptavidin-APC (eBioscience) was used for intracellular secondary staining. For the NK activation assay NKG2A-PE (Z199, Beckman-Coulter) and anti-PE beads (Miltenyi Biotech) were used to magnetically sort $NKG2A^+$ cells from PBMC, and subsequently stained after co-culture with autologous fibroblasts using anti-CD107a-FITC (H4A3), CD8-APC-Cy7 (SK1), IFN-γ-APC (B27) (BD Biosciences), yellow Live/Dead fixable stain (Invitrogen) and NKG2A-PE.

Recombinant Viruses

RhCMV 68-1 (79) was reconstituted by electroporating BAC DNA into TRFs. Recombinant RhCMVΔRh159gag (ΔRh159) was created using *E. Coli* strain EL250, which allows for homologous recombination and has been previously described (495). In brief, Recombination primers containing 50 bp homology to regions flanking the Rh159 ORF (forward mutagenesis primer 5'GGTCGTTTGGTTGTTTCTCACCTATTGCTTGGTACTCTAGCT TCAGTAAG3′ and reverse mutagenesis primer 5'TAGTTTATAAACACACAATCACGTGGTGGT ACTGTGAACCCGCGTCGGTA-3′) were used to amplify SIV mac239 gag followed by a kanamycin (Kan) resistance cassette flanked by FRT sites from plasmid pCP015. The forward primer binding site (5′ACCATGGGCGTGAGAAAC) and reverse primer binding site (5′GTATGTTGTGTGGAATTGTGAG) were added to the 3′ end of mutagenesis primers.

Competent EL250 bacteria containing WT RhCMV BAC were electroporated with the PCR product for recombination. Virus was reconstituted by electroporating purified

recombinant BAC DNA into primary rhesus fibroblasts and characterized by full genome sequencing.

To generate Ad159FL, Rh159 was amplified from purified DNA isolated from TRFs infected with WT RhCMV using AccuPrime Taq DNA polymerase High Fidelity (Life Technologies) and the following primers: 5' CGGGATCCCGCCACCATGGCCTACAACAG 3' (fwd) and 5'GGAATTCCTTACTTATCGTCGTCATCCTTGTAGTCATGAGCTTCACGAC TGCGTT3' (rev). The PCR fragment was cloned into pADTet7, and transfected into 293-CRE cells with psi5 helper virus based on a previously described method (496). RhCMVΔRh186-189gag and RhCMVgag were previously described (21). Adenovirus expressing UL148 and empty vector control were a kind gift from Richard Stanton (Cardiff University) (498).

Immunoblotting and pulse-chase experiments

U373-NKG2DL cells were lysed in PBS 1%NP-40 and HALT protease inhibitor (Thermo Fisher) followed by SDS-PAGE protein separation and transfer to PVDF. Immunoblotted proteins were detected with ECL2 (Thermo Scientific). For Pulse-chase experiments, U373-MICB cells were starved for 1 h in DMEM minus cysteine (cys) and methionine (met) supplemented with 10% FBS (pulse media). After starve period, EXPRE^{35}S^{35}S Protein Labeling Mix (PerkinElmer) was added to pulse media at 150 µCi/10^6 cells for 30 min. Cells were chased for the indicated times then lysed, digested, and immunoprecipitates were separated by SDS-PAGE and detected by autoradiography.

Digestions using EndoH (Roche) or PNGaseF (NEB) were performed according to manufacturer's instructions.

Immunofluoescence assay

Cells were washed 2× with cold PBS and then permeabilized with 1% Triton-X, followed by fixation in 1% formaldehyde (Fisher Scientific) at room temperature (RT) for 10 min. Cells were blocked with 1% bovine serum albumin (Fisher Scientific) and 5 mg/ml glycine (Fisher Scientific) in PBS for 15 min. Cells were then incubated for 1 h with a 1:100 dilution of anti-IE2 antibody. Cells were rinsed 3× with PBS and then incubated for 1 h with a 1:500 dilution Alexa Fluor 594 goat anti-mouse antibody. Cells were rinsed 3× with PBS, stained with DAPI and then mounted onto glass slides (Fisher Scientific) with VECTASHIELD (Vector Laboratories). Slides were visualized on a Zeiss Axioskop 2 Plus fluorescence microscope, and images were produced with AxioVision v4.6 software (Zeiss).

Flow Cytometric Analysis

Cells were harvested, washed in 3% FBS/PBS, incubated with primary antibody followed by fluorophore-conjugated secondary antibody. Cells were then fixed in 1% paraformaldehyde, permeabilized in 0.1% triton-X100/PBS, washed and incubated with biotinylated anti-IE2 antibody (DBX biotin labeling kit, Molecular probes) followed by fluorophore-conjugated streptavidin. Flow cytometry data were acquired on LSR II (BD Biosciences) and analyzed with FlowJo X (v.10.0.7, Tree Star).

NK activation assays

$NKG2A^+$ cells sorted from PBMC were plated overnight in RPMI-1640, 15% FBS, 100 IU/ml of IL-2, and 10 ng/ml of IL-15 (NK media). $NKG2A^+$ cells (2.5×10^6/ml) were incubated with autologous fibroblasts (1.25×10^5 IE-2 expressing cells/ml) for 30 min at 37°C in the presence of anti-CD107/FITC. Brefeldin A (10 µg/ml) and GolgiStop (1 µl/ml; BD Biosciences) were added after 30 min and samples were incubated at 37°C for 8 hrs. Cells were surface stained, fixed with 2% paraformaldehyde, and then permeabilized for intracellular IFN-γ staining with 1x PBS containing 10% FBS and saponin (1 g/L).

Mass Spectrometry

Rh159 was trypsinized and peptides were analyzed by LC/MS-MS using an LTQ Velos Pro linear ion trap (Thermo Scientific) to collect data-dependent MS/MS data (45). Sequest (version 28, revision 12) was used to search MS2 Spectra against a March 2012 version of the Sprot human FASTA protein database, with added sequences from the Uniprot Rhesus Cytomegalovirus and concatenated sequence-reversed entries to estimate error thresholds and 179 common contaminant sequences and their reversed forms. Database processing was performed with python scripts (ProteomicAnalysisWorkbench.com). SEQUEST results were filtered to strict peptide and protein false discovery rates (FDRs), estimated from the number of matches to sequence-reversed peptides, using PAW software (46). Independent FDR control was performed, resulting in a 4.3% FDR for protein discovery (43 identifications to forward sequence proteins and 2 to reverse-sequenced proteins). 9 MS/MS spectra assigned to

forward sequence peptides and none to reverse-sequence peptides from any entry in the database.

Animal Studies

Three male, purpose-bred, Indian-origin RM (*Macaca mulatta*) were used: one animal being RhCMV-positive and two were specific-pathogen free, including RhCMV. ΔRh159 was inoculated subcutaneously at 5×10^6 PFU. For $CD8^+$ cell depletion, RM were treated with 10, 5, 5 and 5 mg/kg of the anti CD8 mab cM-T807 one day prior to inoculation with 5×10^6 PFU ΔRh159 and on days 2, 6, and 9 post inoculation, respectively. SIV-specific $CD4^+$ and $CD8^+$ T cell responses were measured in mononuclear cell preparations from blood by ICCS (19). Briefly, sequential 15-mer peptides (overlapping by 11 amino acids) comprising the SIV_{MAC239}gag were used in the presence of co-stimulatory CD28 and CD49d monoclonal antibodies (BD Biosciences). Cells were incubated with antigen and co-stimulatory molecules for 1h, followed by addition of Brefeldin A (Sigma-Aldrich) for an additional 8h. Co-stimulation without antigen served as a background control. Cells were then stained with fluorochrome-conjugated monoclonal antibodies, flow cytometric data collected on a LSR II (BD Biosciences) and data analyzed using the FlowJo software program (version 8.8.7; Tree Star). Responses frequencies ($CD69^+/TNF^+$ and/or $CD69^+/IFN^{+)}$ were first determined in the overall $CD4^+$ and $CD8^+$ population and then memory corrected (with memory T cell subset populations delineated on the basis of CD28 and CD95 expression).

Ethics Statement

IACUC Project Number 0691, Protocol number IS00002845, and started 05/01/09 The Three purpose-bred male RM (Macaca mulatta) of Indian genetic background were used with consent of the Oregon National Primate Research Center Animal Care and Use Committee, under the standards of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

6. APPENDIX

6.1. MS DATA

on	Link	FiltCoverage	SeqLength	MW	Description	CountsTot	UniqueTot	UniqFrac	Total FRU1188_BAN	Unique FRU1188_BAN	Corrected FRU1188_BANI
HUMAN	IGHA1_HUMAN	11.9	353	37656	Ig alpha-1 chain C region OS=Homo sapiens GN=	5	5	1	5	5	5
UMAN	TRFL_HUMAN	3.2	710	78183	Lactotransferrin OS=Homo sapiens GN=LTF PE=1	2	2	1	2	2	2
HUMAN	S10A8_HUMAN	23.7	93	10836	Protein S100-A8 OS=Homo sapiens GN=S100A8	4	4	1	4	4	4
UMAN	MYL1_HUMAN	10.3	194	21146	Myosin light chain 1/3, skeletal muscle isoform OS	2	2	1	2	2	2
HUMAN	S10A9_HUMAN	14.9	114	13243	Protein S100-A9 OS=Homo sapiens GN=S100A9	3	3	1	3	3	3
UMAN	TPM3_HUMAN	22.2	284	32820	Tropomyosin alpha-3 chain OS=Homo sapiens GN	17	4	0.235	17	4	9.2
UMAN	TPM2_HUMAN	22.2	284	32852	Tropomyosin beta chain OS=Homo sapiens GN=T	16	3	0.188	16	3	6.9
UMAN	TPM1_HUMAN	16.9	284	32710	Tropomyosin alpha-1 chain OS=Homo sapiens GN	16	3	0.188	16	3	6.9
UMAN	GFAP_HUMAN	4.4	432	49881	Glial fibrillary acidic protein OS=Homo sapiens GN	5	1	0.2	5	1	1.022
UMAN	PLAK_HUMAN	7.4	745	81746	Junction plakoglobin OS=Homo sapiens GN=JUP	5	5	1	5	5	5
UMAN	DESP_HUMAN	3.8	2871	331775	Desmoplakin OS=Homo sapiens GN=DSP PE=1 S	11	11	1	11	11	11
UMAN	ITIH1_HUMAN	3.7	911	101390	Inter-alpha-trypsin inhibitor heavy chain H1 OS=Ho	2	2	1	2	2	2
HUMAN	PRDX2_HUMAN	9.6	198	21893	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 P	2	2	1	2	2	2
UMAN	ACTB_HUMAN	13.1	375	41738	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTI	5	5	1	5	5	5
UMAN	DCD_HUMAN	53.6	110	11285	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV	23	23	1	23	23	23
UMAN	DSG1_HUMAN	9.4	1049	113749	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=	7	7	1	7	7	7
UMAN	BPTF_HUMAN	0.7	3046	338263	Nucleosome-remodeling factor subunit BPTF OS=	2	2	1	2	2	2
HUMAN	COBL1_HUMAN	2.6	1204	131788	Cordon-bleu protein-like 1 OS=Homo sapiens GN=	2	2	1	2	2	2
UMAN	FILA2_HUMAN	1.7	2391	248074	Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV	2	2	1	2	2	2
HUMAN	TYW3_HUMAN	13.9	259	29795	tRNA wybutosine-synthesizing protein 3 homolog (	2	2	1	2	2	2
HUMAN	HORN_HUMAN	13.6	2850	282391	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV	16	16	1	16	16	16
HUMAN	AHNK2_HUMAN	0.5	5795	616630	Protein AHNAK2 OS=Homo sapiens GN=AHNAK2	2	2	1	2	2	2
HUMAN	DOCK9_HUMAN	1.2	2069	236447	Dedicator of cytokinesis protein 9 OS=Homo sapie	2	2	1	2	2	2
HUMAN	UBP40_HUMAN	3.2	1235	140131	Ubiquitin carboxyl-terminal hydrolase 40 OS=Hom	2	2	1	2	2	2
HUMAN	CC165_HUMAN	1.3	1896	208750	Coiled-coil domain-containing protein 165 OS=Hor	2	2	1	2	2	2
RHCM6	Q7TFI3_RHCM6	9.8	326	36829	Rh159 OS=Rhesus cytomegalovirus (strain 68-1) I	9	9	1	9	9	9

al Count: 845
minant Count: 280
n factors: 1
rsed

ersed

al Count values performed on Tue Jun 05 09:05:56 2012

.03_Rhesus_Cytomegalovirus_Human_sprot_both.fasta (41316 entries)
iles_list flag
_int flag
_peaks
_counts flag
e_list flag
harge_states_ok flag
ons_ok flag
ntt_per_peptide was
peptide_per_protein
unique_per_protein
le flag
_nterm_acyt flag

6.2. Alignment of RM and HUMAN NKG2DLs

MICA/MIC1

MICA protein [Homo sapiens]

Sequence ID: gb|ABY64781.1| Length: 364 Number of Matches: 1

Range 1: 2 to 322 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
521 bits(1342)	0.0	Compositional matrix adjust.	261/323(81%)	280/323(86%)	2/323(0%)

```
Query  2    HSLRYNVTVLSRDGSVQSGFLAEGHLDGQLFLLYDRQKCRARPQGQWAEDVLGAKTWDTE  61
            HSLRYN+TVLS DGSVQSGFLAE HLDGQ FL YDRQKCRA+PQGQWAEDVLG KTWD E
Sbjct  2    HSLRYNLTVLSWDGSVQSGFLAEVHLDGQPFLRYDRQKCRAKPQGQWAEDVLGNKTWDRE  61

Query  62   TGDLTENGKDLRMTLAHIKGQKGGLHSLQEIKVCEIHEDNSTRGLRHFYYDGELFLSQNL  121
            T DLT NGKDLRMTLAHIK QK GLHSLQEI+VCEIHEDNSTR  +HFYYDGELFLSQN+
Sbjct  62   TRDLTGNGKDLRMTLAHIKDQKEGLHSLQEIRVCEIHEDNSTRSSQHFYYDGELFLSQNV  121

Query  122  ETQEWTELQSSRAQTLALNIRNFWKEDTMKTKTHYRAVQADCLKKLQRYLESGVAVRRTV  181
            ET+EWT  QSSRAQTLA+N+RNF KED MKTKTHY A+ ADCL++L+RYLES V +RRTV
Sbjct  122  ETEEWTVPQSSRAQTLAMNVRNFLKEDAMKTKTHYHAMHADCLQELRRYLESSVVLRRTV  181

Query  182  PPMVNVTHGEASDGNITVTCRASGFYPRNIALTWRQDGVSLNHDAQQWGDVLPDQNGTYQ  241
            PPMVNVT  EAS+GNITVTCRAS FYPRNI LTWRQDGVSL+HD QQWGDVLPD NGTYQ
Sbjct  182  PPMVNVTRSEASEGNITVTCRASSFYPRNITLTWRQDGVSLSHDTQQWGDVLPDGNGTYQ  241

Query  242  TWVATRIRQGEEQRFACYMEHSGNHSTHPVPSGKVLVFQSQWLDIPYVLAVAAAVAAAAA  301
            TWVATRI QGEEQRF CYMEHSGNHSTHPVPSGKVLV QS W    + ++  AA AAAAA
Sbjct  242  TWVATRICQGEEQRFTCYMEHSGNHSTHPVPSGKVLVLQSHWQ--TFHVSAVAAAAAAAA  299

Query  302  AAIFVIILYVLCCKKKTSAAEGP  324
            A   +II YV CCKKKTSAAEGP
Sbjct  300  AIFVIIIFYVCCCKKKTSAAEGP  322
```

MICB/MIC2

MHC class I chain-related protein B [Homo sapiens]

Sequence ID: gb|AAC39850.1| Length: 359 Number of Matches: 1

Range 1: 1 to 355 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
555 bits(1429)	0.0	Compositional matrix adjust.	278/358(78%)	300/358(83%)	10/358(2%)

```
Query  28   PHSLRYNVTVLSRDGSVQSEFLAEGHLDGQLFVRYDRETRRARPQGQWAEAVLG----DQ  83
            PHSLRYN+ VLS+DGSVQS FLAEGHLDGQ F+RYDR+ RRA+ QGQWAE VLG    D
Sbjct  1    PHSLRYNLMVLSQDGSVQSGFLAEGHLDGQPFLRYDRQKRRAKHQGQWAEDVLGAKTWDT  60

Query  84   ETEDLTENAQDLRRTLTHIEGQKGGLHSLQKIKICEIYEDGSTGGFWHFYYDGERFLSLN  143
            ETEDLTEN QDLRRTLTHI+ QKGGLHSLQ+I++CEI+ED ST G  HFYYDGE FLS N
Sbjct  61   ETEDLTENGQDLRRTLTHIKDQKGGLHSLQEIRVCEIHEDSSTRGSRHFYYDGELFLSQN  120

Query  144  LETQKWTVAQSSRAQTLAMN---FWKEDTMKTNTHYRAMRADCLKKLRRYQKSRVAVRRT  200
            LETQ+ TV QSSRAQTLAMN    FWKED MKT THYRAM+ADCL+KL+RY KS VA+RRT
Sbjct  121  LETQESTVPQSSRAQTLAMNVTNFWKEDAMKTKTHYRAMQADCLQKLQRYLKSGVAIRRT  180

Query  201  VPPMVNVTHGEASEGNITVTCRASGFYPGNITLTWRQDGVSLSHDAQQWGDVLPDRNGTY  260
            VPPMVNVT  E SEGNITVTCRAS FYP NITLTWRQDGVSLSH+ QQWGDVLPD NGTY
Sbjct  181  VPPMVNVTCSEVSEGNITVTCRASSFYPRNITLTWRQDGVSLSHNTQQWGDVLPDGNGTY  240

Query  261  YTWVATRIRQGEEQRFACYMEHSGNHSTHPVPSGKPPVLQSERLNLLYVPVAVAVAVVTA  320
             TWVATRIRQGEEQRF CYMEHSGNH THPVPSGK  VLQS+R +  YV  A+   V+
Sbjct  241  QTWVATRIRQGEEQRFTCYMEHSGNHGTHPVPSGKALVLQSQRTDFPYVSAAMPCFVI--  298

Query  321  FIIICVHRCKKKKTSAAEGPELVSLRTLDQHPVGTGDHRDATQLGSQPLMSAPGSTGS  378
             II+CV  C KKKTSAAEGPELVSL+ LDQHPVGTGDHRDA QLG QPLMSA GSTGS
Sbjct  299  IIILCV-PCCKKKTSAAEGPELVSLQVLDQHPVGTGDHRDAAQLGFQPLMSATGSTGS  355
```

ULBP1/RM ULBP1

NKG2D ligand 1 [Homo sapiens]

Sequence ID: gb|AAN40838.1|AF346595_1 Length: 234 Number of Matches: 1

Range 1: 10 to 233 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
421 bits(1083)	6e-149	Compositional matrix adjust.	202/224(90%)	207/224(92%)	0/224(0%)

```
Query  20   SGWSGAGRAVKHCLSYDFIITPKFRPEPRWCEVQGLVDERPFLHYDCVNHKAKAFASLGK  79
            SGWS AG    HCL YDFIITPK RPEP+WCEVQGLVDERPFLHYDCVNHKAKAFASLGK
Sbjct  10   SGWSRAGWVDTHCLCYDFIITPKSRPEPQWCEVQGLVDERPFLHYDCVNHKAKAFASLGK  69

Query  80   KVNVTKTWEEQTETLRDVVDFLKGQLPDIQVENLMPIEPLILQAQMSCEHEAHGHGRGSW  139
            KVNVTKTWEEQTETLRDVVDFLKGQL DIQVENL+PIEPL LQA+MSCEHEAHGHGRGSW
Sbjct  70   KVNVTKTWEEQTETLRDVVDFLKGQLLDIQVENLIPIEPLTLQARMSCEHEAHGHGRGSW  129

Query  140  QFLFNGQTFLLFDSNNRKWTALHPGAKKMKEKWEKNTEVTMFFQKISMGDCKTWLEEFLM  199
            QFLFNGQ FLLFDSNNRKWTALHPGAKKM EKWEKN +VTMFFQKIS+GDCK WLEEFLM
Sbjct  130  QFLFNGQKFLLFDSNNRKWTALHPGAKKMTEKWEKNRDVTMFFQKISLGDCKMWLEEFLM  189

Query  200  YWEQMLDPTKPPSLAQGTTHPKAMATTLIPWSLLIILLCFILAG  243
            YWEQMLDPTKPPSLA GTT PKAMATTL PWSLLII LCFILAG
Sbjct  190  YWEQMLDPTKPPSLAPGTTQPKAMATTLSPWSLLIIFLCFILAG  233
```

ULBP2/ RM ULBP2

NKG2D ligand 2 [Homo sapiens]

Sequence ID: gb|AAN39920.1|AF321606_1 Length: 218 Number of Matches: 1

Range 1: 1 to 218 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
379 bits(974)	1e-132	Compositional matrix adjust.	191/218(88%)	200/218(91%)	0/218(0%)

```
Query  29   DLHSLCYEITIIPKFRPGRRWCAVQGQVDKKTFLHYDCGNKIVTPVSPLGKKLSVTKAWK  88
            D HSLCY+IT+IPKFRPG RWCAVQGQVD+KTFLHYDCGNK VTPVSPLGKKL+VT AWK
Sbjct  1    DPHSLCYDITVIPKFRPGPRWCAVQGQVDEKTFLHYDCGNKTVTPVSPLGKKLNVTTAWK  60

Query  89   AQNPVLREVVDMLTEQLLDIQLENYTPREPLTLQARVSCEQKAEGHRSGSWQFGFDGQVF  148
            AQNPVLREVVD+LTEQL DIQLENYTP+EPLTLQAR+SCEQKAEGH SGSWQF FDGQ+F
Sbjct  61   AQNPVLREVVDILTEQLRDIQLENYTPKEPLTLQARMSCEQKAEGHSSGSWQFSFDGQIF  120

Query  149  LLFDSENRMWTTVHPGARKMKEKWENDKDVTMSFHYISMGDCTRWLKDFLTGTDSTLEPS  208
            LLFDSE RMWTTVHPGARKMKEKWENDK V MSFHY SMGDC  WL+DFL G DSTLEPS
Sbjct  121  LLFDSEKRMWTTVHPGARKMKEKWENDKVVAMSFHYFSMGDCIGWLEDFLMGMDSTLEPS  180

Query  209  AGAPLTMSSGTTQLRATATTLILCCLLIILCCFILAGI  246
            AGAPL MSSGTTQLRATATTLILCCLLIIL CFIL GI
Sbjct  181  AGAPLAMSSGTTQLRATATTLILCCLLIILPCFILPGI  218
```

ULBP3/ RM ULBP3

NKG2D ligand 3 [Homo sapiens]

Sequence ID: ref|NP_078794.1| Length: 244 Number of Matches: 1

▸ See 7 more title(s)

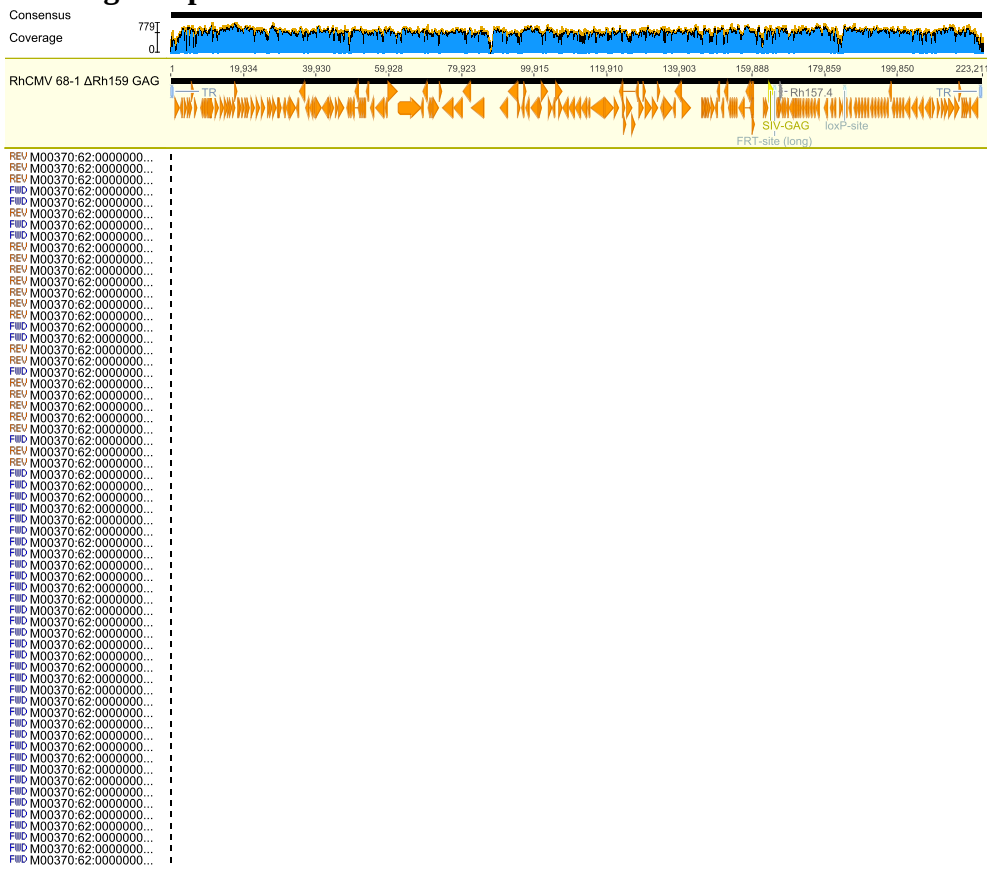
Range 1: 30 to 244 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
380 bits(975)	4e-131	Compositional matrix adjust.	185/215(86%)	190/215(88%)	1/215(0%)

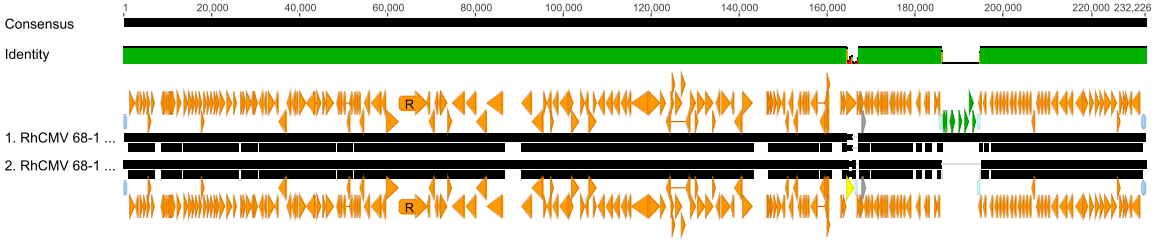
```
Query  57   DTHSFWYNFTIIRLPRHGQQWCEVQVQVDQKNFLSYDCGSDKVLPMGHLEEQLEATDAWG  116
            D HS WYNFTII LPRHGQQWCEVQ QVDQKNFLSYDCGSDKVL MGHLEEQL ATDAWG
Sbjct  30   DAHSLWYNFTIIHLPRHGQQWCEVQSQVDQKNFLSYDCGSDKVLSMGHLEEQLYATDAWG  89

Query  117  KQLEMLREVGQRLRLELADTELEDFTPSGPRTLQARMSCECETDGRIRGSWQFGFDGQKF  176
            KQLEMLREVGQRLRLELADTELEDFTPSGP TLQ RMSCECE DG IRGSWQF FDG+KF
Sbjct  90   KQLEMLREVGQRLRLELADTELEDFTPSGPLTLQVRMSCECEADGYIRGSWQFSFDGRKF  149

Query  177  LLFDSNNRKWTVVQAGARRMKEKWE-DSGLTMFFQVFSMGDCKSWLRDFLMHRKKRLEPT  235
            LLFDSNNRKWTVV AGARRMKEKWE DSGLT FF++ SM DCKSWLRDFLMHRKKRLEPT
Sbjct  150  LLFDSNNRKWTVVHAGARRMKEKWEKDSGLTTFFKMVSMRDCKSWLRDFLMHRKKRLEPT  209

Query  236  EPLTVAPRTAQTKAMATNLSLWSLLIILCFTLPGI  270
             P T+AP  AQ KA+AT LS WS LIILCF LPGI
Sbjct  210  APPTMAPGLAQPKAIATTLSPWSFLIILCFILPGI  244
```

6.3. Next gen sequencing data

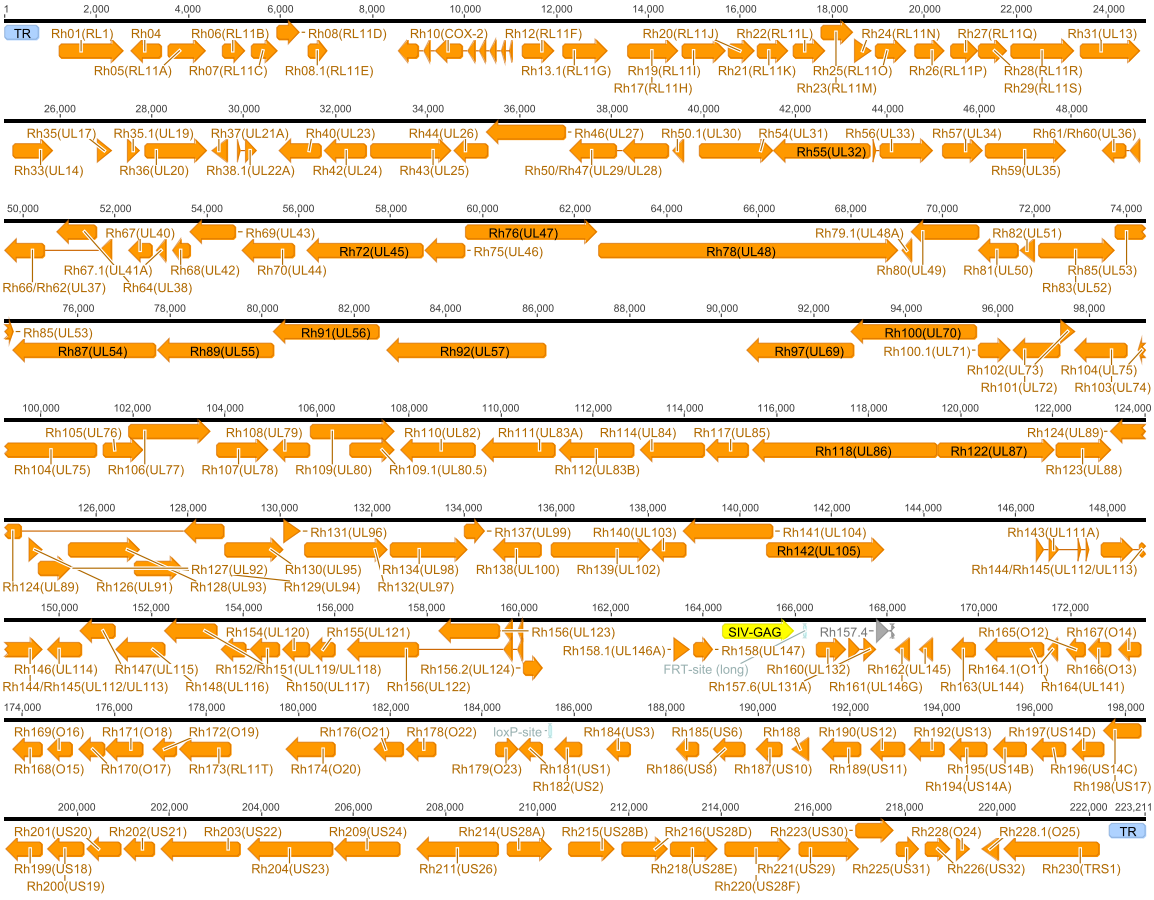
Coverage Map



Genome alignment



Genome Map RhCMV 68-1del159 SIV GAG



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