A RHESUS RHADINOVIRUS VIRAL INTERFERON REGULATORY FACTOR IS VIRION-ASSOCIATED AND INHIBITS THE EARLY INTERFERON ANTIVIRAL RESPONSE

By

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

FIGURES and TABLES

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ABSTRACT

Two closely related gammaherpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV) and rhesus macaque rhadinovirus (RRV), are unique in that they express viral homologues to cellular interferon (IFN) regulatory factors (IRFs), deemed viral IRFs (vIRFs). These vIRF proteins differentially regulate transcription and IFN signaling. The IFN response is an early host immune response dedicated to combating viral infection. Here, we demonstrate a strategy employed by RRV to ensure rapid inhibition of virus-induced type I IFN production. We show that vIRF ORF R6 impedes the IFN response within the first 6 h of poly(IC)-induced stimulation. We also found that RRV vIRF R6 interacts with transcriptional coactivator, CREB-binding protein (CBP), in the nucleus. Consequently, phosphorylated IRF-3, a cellular transcriptional regulator important for the activation of IFNβ transcription, fails to effectively bind to the IFNβ promoter, thus inhibiting the activation of IFNβ genes and causing proteasome-dependent degradation of IRF-3. Additionally, we demonstrate via immunoelectron microscopy that R6 is packaged within RRV virion particles and furthermore that virion-associated R6 is capable of inhibiting the type I IFN response by preventing efficient binding of transcription factors to the IFNβ promoter in the context of infection. The work shown in this thesis is the first example of a virion-associated vIRF in either KSHV or RRV. The presence of this immunomodulatory protein in the RRV virion provides the virus with a rapid immune evasion mechanism, thus perhaps enabling the virus to effectively establish an infection within the host.

CHAPTER 1

INTRODUCTION

I. Human Herpesviruses

A. Classification

Herpesviruses are ubiquitous viruses in nature, highly species specific and very few naturally infect more than one species. To date, there are eight herpesviruses identified that use humans as a primary host: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and *Human herpesviruses 6, 7,* and *8* (HHV-6, HHV-7, HHV-8). Historically, the *Herpesviridae* family was classified based on virion architecture. The classical herpesvirion is composed of a linear double-stranded DNA core, an icosahedral capsid, and an amorphous, extranuclear structure called the tegument, all of which are contained within a glycoprotein-studded membranous envelope. In addition to virion structure, herpesviruses share important biological properties, which include coding for a wide variety of enzymes involved in metabolic processes, nuclear viral DNA replication, ultimate destruction of the infected cell during productive replication and lastly, the ability to remain latent in host cells. As similar as herpesviruses can be they can also differ with respect to their host cell range, duration of their replicative cycle and importantly, in which cell type they choose to remain latent.

Members of the *Herpesviridae* family are further categorized into three subfamilies including the *Alphaherpesvirinae,* the *Betaherpesvirinae*, and the *Gammaherpesvirinae*. This classification was established in the 1970's based on the

biological properties demonstrated by these viruses. Members of the *Alphaherpesvirinae* subfamily have a wide host range as well as a short reproductive cycle. These viruses spread quickly in culture and efficiently destroy infected cells. Additionally, this subfamily establishes latency in sensory ganglia. Members include *Simplexvirus* (HSV-1 and HSV-2) and *Varicellovirus* (VSV), also known as HHV-1, HHV-2, and HHV-3, respectively.

Viruses within the *Betaherpesvirinae* subfamily exhibit strict species specificity and have a relatively long reproductive cycle, resulting in slow spread in culture. A characteristic sign of a cell infected with one of these viruses is cytomegalia, or enlargement of the cell. This family includes *Cytomegalovirus* (HCMV) or HHV-5, as well as HHV-6 and HHV-7. Latent forms of *Betaherpesvirinae* can be found in secretory glands, lymphoreticular cells, T-cells and monocytes (1).

Gammaherpesvirinae have a narrow host range, which is restricted to the taxonomic order of their natural hosts. These viruses are typically specific for T or B-lymphocytes and are able to replicate in lymphoblastoid cells *in vitro*. Latent virus can be found in lymphoid tissue of an infected host. This subfamily contains the genera *γ1-lymphocryptovirus* (HHV-4), also known as Epstein-Barr virus (EBV), and *γ2-rhadinovirus* (HHV-8), also known as Kaposi's Sarcoma Herpesvirus (KSHV).

B. Virus Structure and Genome Organization

Herpesvirus virions can vary in size from 120 nm to 260 nm, depending on the variability in tegument thickness. The core of the mature virion contains viral DNA, which is enclosed in an icosahedral capsid consisting of 4-7 virally encoded proteins (2). Capsids are approximately 100 nm in diameter and are made of 162 capsomers (150 hexons and 12 pentons). Surrounding the capsid is a structure known as the tegument, which is typically asymmetrical and varies in thickness. The size of the tegument is dependent on the location of the accumulating virions; virions found in cytoplasmic vacuoles tend to have more tegument than virions in the perinuclear space. An important purpose of the tegument is to provide the virus with pre-made proteins, so as to help the virus control its environment within the newly infected host cell more rapidly. The final layer of the typical herpesvirus virion is the lipid envelope. The envelope is derived from portions of altered cellular membranes (3) and is studded with viral glycoproteins. The virus encodes 4-17 glycoproteins and a single virus particle can be adorned with over 1,000 individual glycoproteins.

Contained within the herpesvirus virion is a double stranded, linear DNA genome, which upon release into an infected cell, immediately circularizes (4). Genome length between herpesvirus family members varies between 120-250 kbp. However, variation between individual viruses is typically up to 10 kbp. This is due to terminal repeat regions and internal reiterated regions within the genome that may differ in copy number between viruses. Additionally, spontaneous deletions contribute to the variation seen in herpesvirus genome lengths.

The large coding capacity of the herpesvirus genome allows for an estimated 70-200 open reading frames (ORFs). Regardless of size variations among genomes, all herpesvirus encode approximately 40 'core' genes, which are indispensable for viral replication, including virion components such as glycoproteins, capsid and tegument proteins, as well as viral DNA replication proteins and DNA packaging/cleavage proteins (5). The remaining dispensable or "accessory" genes encode proteins involved in processes that allow for the virus to more efficiently replicate in a variety of host cells and in varying conditions. Additionally, these accessory proteins have important roles in immunomodulation and maintenance of latency. The majority of the ORFs are contained within the long unique region of the genome, which is flanked and/or interrupted by terminal or internal repeated regions. These repeat regions contain within them packaging sites and cleavage sites important for the creation of new genomes (6). In the case of KSHV, the terminal repeat regions, which flank the genome, are of particular importance as they are required for the maintenance of episomal DNA in latently infected cells (7). During lytic infection, however, initiation of DNA replication is dependent on sites that serve as lytic origins of replication (*oriLyt)* and these sites have been found in all human herpesviruses (6).

C. Herpesvirus Life Cycle

The first step in infecting a cell is attachment and for most herpesviruses this occurs via binding of viral glycoproteins to heparan sulfate on cell surface proteoglycans (8). Virus entry then occurs by fusion of the virion envelope with the cell membrane; a

process that is mediated also by viral glycoproteins and their interactions with cellular receptors (9). Once the capsid is released into the cytoplasm, it travels along tubulin microtubules, wherein it binds to nuclear pores (10, 11). The linear viral genome is deposited into the nucleus through nuclear pores and immediately undergoes circularization by forming a covalently bonded circular form. Viral gene expression is now ready to proceed. The process of herpesvirus gene expression is a temporally regulated and well-orchestrated event that is initiated and controlled by a transactivator protein delivered into cells as a component of the tegument (6). Viral transcripts can be divided into three sequential groups, immediate-early, early and late transcripts. These groups can be further characterized by their sensitivity to cyclohexamide (CHX) and phosphonacetic acid (PAA). Immediate-early (IE) mRNA expression is insensitive to CHX, an inhibitor of protein translation, as it is not reliant on viral protein. Once expressed, IE genes are important in the regulation of the transcriptional cascade and also play a major role in innate immune evasion (12). Unlike IE genes, early (E) gene expression is sensitive to CHX, as it requires IE protein synthesis to occur. However, E genes, which are expressed independent of viral DNA synthesis, are insensitive to the DNA synthesis inhibitor, PAA (13). The final group of viral transcripts encode for structural proteins and proteins important for viral assembly. These genes are identified as late (L) genes and are dependent on the replication of viral genomes and are therefore sensitive to treatment with PAA.

Shortly after infection and genome circularization, viral genomes are prepared to undergo replication. This replication event initiates at *OriLyt* sites in the genome and

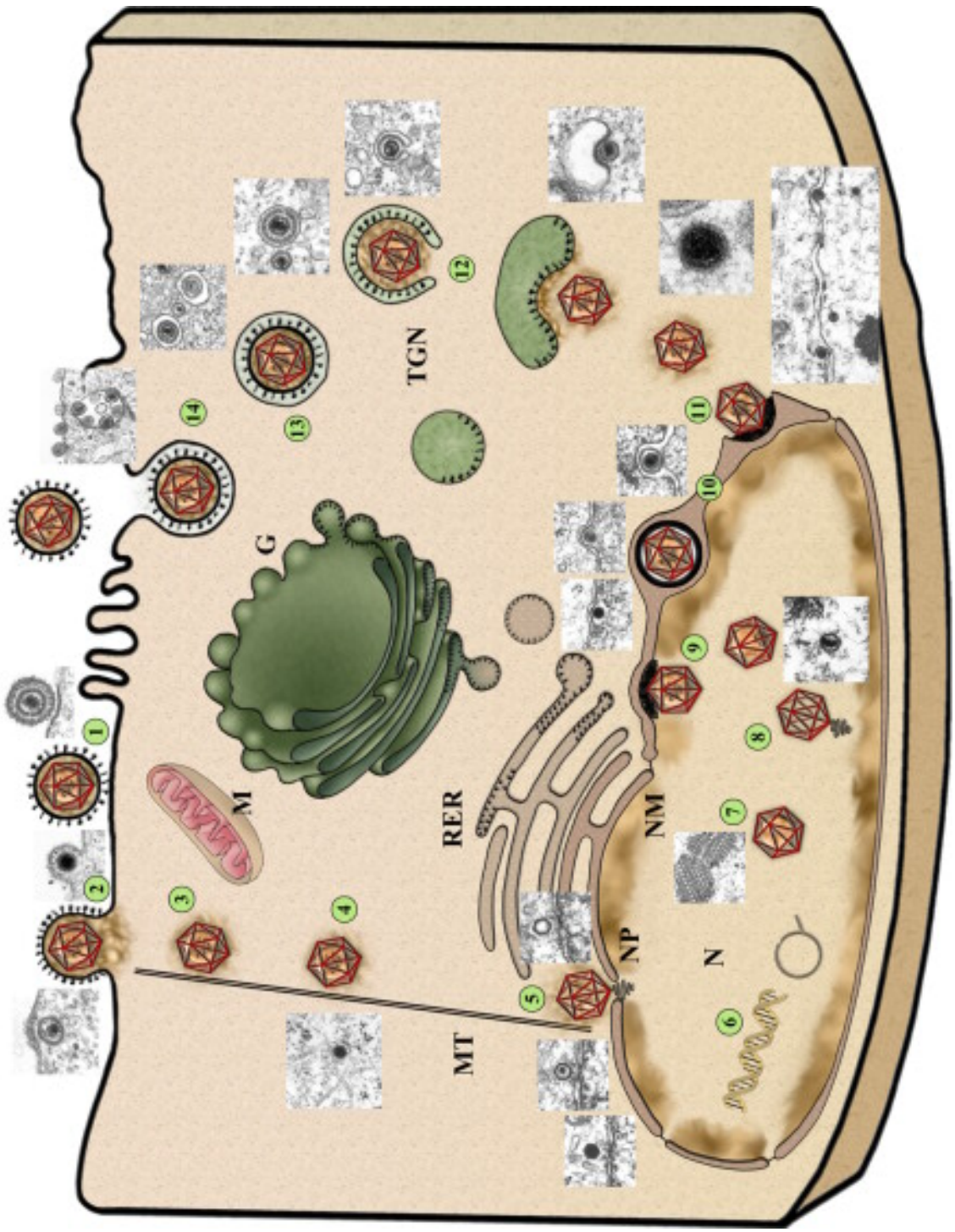
proceeds via theta replication. Subsequently, replication switches to a rolling circle mechanism, yielding long head-to-tail genomes or concatamers. These are then resolved into one unit of full-length genome and packaged into premade capsid structures, all of which occur in the nucleus (14). Capsids also acquire their initial layer of tegument in the nucleus. The matter of nuclear egress has been a topic of controversy for some time, but it is now widely accepted that virions escape the nucleus through a process of envelopment-deenvelopment-reenvelopment. This model suggests that capsids bud through the inner nuclear membrane and gain access into the perinuclear space. They then 'deenvelope' by fusing their primary envelope with the outer nuclear membrane, thus releasing naked capsids into the cytosolic space. Reenvelopment occurs through the trans-golgi network or the ER, wherein a large portion of the tegument is included in the mature, enveloped virion before finally being released from the cell (Figure 1.1) (15, 16).

One of the main distinguishing and highly studied characteristics of herpesviruses is their ability to establish latency in infected cells. Unlike productive infection, latent infection only involves the expression of a small portion of genes that are important for the maintenance of the viral chromosome, evasion of the host antiviral immune response, and to provide a growth advantage to the cells infected with virus. Additionally, cells that are latently infected do not produce viral progeny (2, 17). Cells harboring latent virus, maintain the genome in a multicopy, nonintegrated, circularized form, known as an episome. Like cellular chromosomes, viral episomes are also packaged into chromatin-like structures, which are protected from DNA damage and facilitate tight regulation of gene expression (18). Although the genes important for lytic replication are highly

conserved between herpesviruses, the genes required for maintenance of latency are different even among the herpesvirus subfamilies (17). For example, HSV-1 and HSV-2 only express the LAT (latency-associated transcript) gene during latency, whereas KSHV produces several key latent gene products including LANA-1, LANA-2 (aka. vIRF3), vCyclin, vFlip and Kaposins A and B (2, 17). A key aspect of KSHV latency is its ability to pass on its chromosome to daughter cells after replication. This is dependent on LANA, which tethers the viral episome to the host chromosomal DNA, thus ensuring efficient segregation of the genome to new cells (17). Herpesviruses have developed these important mechanisms for maintaining themselves in a state of latency as a way of establishing a life-long relationship with their host and ultimately securing further propagation.

Figure 1.1. Herpesvirus replication cycle.

A diagram of the replication cycle is shown together with electron micrographs of the respective stages. After attachment (1) and penetration (2), capsids are transported to the nucleus (N) (3) via interaction with microtubules (MT) (4), docking at the nuclear pore (NP) (5) where the viral genome is released into the nucleus. Here, transcription of viral genes and genome replication occur (6). Concatemeric replicated viral genomes are cleaved to unit-length during encapsidation (8) into preformed capsids (7), which then leave the nucleus by budding at the INM (9) followed by fusion of the envelope of these primary virions located in the perinuclear space (10) with the outer nuclear membrane (11). Final maturation then occurs in the cytoplasm by secondary envelopment of intracytosolic capsids via budding into vesicles of the trans-Golgi network TGN (12) containing viral glycoproteins (black spikes), resulting in an enveloped virion within a cellular vesicle. After transport to the cell surface (13), vesicle and plasma membranes fuse, releasing a mature, enveloped virion from the cell (14). RER, rough endoplasmic reticulum; M, mitochondrion; G, Golgi apparatus. *Figure and legend adapted from* (19) *and reprinted with permission.*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D. Human Herpesvirus-associated Diseases

Herpesviruses remain relatively asymptomatic in a host with an intact and healthy immune system. They can, however, become problematic in an immunocompromised host, causing significant clinical manifestations. Primary infection of a seronegative host with HSV-1 or HSV-2, both alphaherpesviruses, occurs through contact with an infected individual who is shedding the virus. In order for primary infection to be initiated, the virus must come in contact with a mucosal surface or broken skin. Both viruses, HSV-1 and HSV-2, target mucosal epithelium for infection and manifest as skin vesicles or mucosal ulcers in the mouth or genitals, respectively (20). Herpes simplex virus is known to be neurovirulent, meaning that it is neuroinvasive and able to replicate in neuronal cells. Not only are HSV-1 and HSV-2 able to replicate in the CNS, but they also establish latency in the dorsal root ganglia (17). Reactivation of the virus, stimulated by stress, physical or emotional, or immune suppression can cause symptoms similar to that of a primary infection. Viral replication can lead to disease, but is rarely life threatening (e.g. encephalitis). The primary outcome of the virus-host interaction is the establishment of latency (20).

VZV, also an alphaherpesvirus, causes varicella (i.e. chickenpox) upon primary infection with the virus and later reactivates, resulting in herpes zoster (i.e. shingles). Infection of a naïve host occurs in mucosal epithelial cells within the upper respiratory tract with infectious virus transmitted by aerosolized respiratory droplets or by contact with a varicella or zoster lesion. Vesicularized skin lesions are caused by infected peripheral blood mononuclear cells (PBMCs), specifically T cells. VZV, like its family

members HSV-1 and HSV-2, is a neurotropic virus and establishes latency in the dorsal root ganglia. Similar to betaherpesviruses HHV-6 and HHV-7, VZV is T-cell tropic, which is a critical step in its life cycle as infected T-cells provide a means of transporting virus from the initial site of infection to the skin for further dissemination. Reactivation of VZV results in herpes zoster, which is characterized by an extremely painful eruption of vesicles on specific areas of the skin supplied by a single nerve ganglion (21). Like varicella, herpes zoster lesions clear after a week, however pain can last several weeks and over 30% of patients develop chronic pain or postherpetic neuralgia (22). In May 2006, a live, attenuated vaccine against VZV was licensed in the United States and has proven to be efficacious and has changed the epidemiology of VZV in the US (23, 24).

CMV is a well-studied betaherpesvirus and while primary infection is largely asymptomatic in immunocompetent individuals, it can cause significant morbidity and mortality in immunocompromised patients as an opportunistic infection. Some important populations susceptible to CMV disease include organ transplant recipients, fetuses and neonates as well as patients with AIDS. Congenital infection occurs upon primary infection or reactivation within the mother and can lead to damage of the CNS, hearing loss, visual impairment and mental retardation. CMV-related diseases and complications in organ transplant recipients and AIDS patients are typically a result of CMV reactivation and can potentially be life-threatening (25). Also included in the betaherpesvirus family are HHV-6 and HHV-7, and collectively known as Roseolovirus. Both viruses are rather ubiquitous and infections typically occur in infants. HHV-6 and HHV-7 are the causative agents of exanthema subitum (ES), a common infection in

infants that causes sudden high fever and a red rash on the trunk, legs and face of the infected individual. The rapid onset of fever can sometimes lead to febrile seizures and in rare cases liver dysfunction can occur (17). HHV-6 has been suggested to be involved in multiple sclerosis (MS), but contradicting data have rendered the contribution of this virus to MS disease unclear (17, 26, 27).

EBV and KSHV, both gammaherpesviruses, have been found to be associated with a number of T-cell and B-cell malignancies. They are lymphotrophic viruses and are capable of establishing latency in B-cells. EBV infection is nearly ubiquitous, with approximately 90% of adults exhibiting seropositivity. Transmission mainly occurs through oral contact, but can also occur through genital transmission, blood transfusions, or organ transplantation. Although primary infection is typically asymptomatic, it is associated with infectious mononucleosis, which is characterized by fever, malaise and fatigue that can last weeks and even months (17). Latency is then established, and can result in B-cell, T-cell, NK-cell and epithelial cell tumors such as Hodgkin's lymphoma, Burkitt's lymphoma, post transplant lymphoproliferative disorders, T cell and NK cell lymphomas, AIDS lymphomas, nasopharyngeal carcinoma and gastric carcinoma (2). It is important to note that the association between EBV and the malignancy is dependent on the degree of immunodeficiency, time between primary infection and tumorigenesis, genetic and geographic factors as well as the malignant tissue (17). KSHV, formally known as HHV-8, was originally discovered in 1994 by Chang et al. (28), making it the most recently identified human herpesvirus (13). The virus was identified as the etiological agent of Kaposi's sarcoma, a highly vascular endothelial tumor that is highly

associated with AIDS. The exact cellular origin of KS remains contensious as spindle cells, the most common cell type in KS lesions, have been shown to express markers of lymphatic endothelium as well as markers of dendritic cells, macrophages, and smooth muscle cells (29). KSHV is also etiologically linked to two B-cell malignancies, primary effusion lymphoma (PEL) (30) and multicentric Castleman's disease (MCD) (31).

II. Kaposi's Sarcoma-associated Herpesvirus

A. Discovery and Classification

Kaposi's sarcoma-associated herpesvirus (KSHV) or HHV-8, was first identified in 1994 by Patrick Moore and Yuan Chang by identifying fragments of the KSHV genome in Kaposi's sarcoma (KS) lesions. This was done by representational difference analysis (RDA), whereby DNA sequences in KS lesions of an AIDS patient were compared to that of normal tissue from the same patient (28). DNA sequencing defined this novel herpesvirus as a member of the gammaherpesvirus family, specifically the γ2 or *rhadinovirus* subdivision, which includes herpesvirus saimiri (HVS) (32). KSHV is the first known member of the *rhadinovirus* genus (33) and is associated with Kaposi's Sarcoma, an endothelial neoplasm, as well as B-cell lymphomas, primary effusion lymphoma (PEL) (34) and multicentric Castleman's disease (MCD) (31).

Seroprevalence of KSHV has been observed all over the world and is greatly varied. The highest seroprevalence, 30-70%, is seen in Africa and parts of the Amazon basin (2, 17, 35). Transmission in these areas is thought to be nonsexual and it occurs largely in childhood. Some infections are spread through vertical transmission (ie. from mother to

child during pregnancy or childbirth) but most are acquired horizontally via contact with saliva from an infected family member (2, 35). Seroconversion slowly increases and plateaus in adolescence/mid-adulthood, likely due to sexual transmission, which is rather inefficient. Western Europe and the United States, on the other hand, both display a different pattern of virus spread. These areas are considered low-prevalence zones, with a seropositivity of 1-7%, and infection is primarily acquired sexually. Prepubescent children are typically uninfected, and the rates of infection increase in adulthood, as does the onset of sexual activity. These non-endemic regions, however, have certain groups within the population that have distinctly high seroprevalences, similar to that of high-prevalence regions. Particularly, male homosexuals display the highest seroprevalence, ranging from 30-60% in HIV-infected homosexual men and 20-30% in HIV-uninfected homosexual men (17, 36). Transmission, in general, occurs in a variety of ways such as sexual contact, blood transfusions, solid organ and bone marrow transplants and vertical transmission, but saliva has been deemed the most important (37).

In infected human, KSHV DNA and transcripts have been found in a multitude of cell types, including epithelial cells, endothelial cells, spindle cells (38), keratinocytes (39), monocytes (40, 41), and B cells (41). Similarly, work done *in vitro* has shown that KSHV can infect human B cells, epithelial cells, endothelial cells, fibroblasts, monocytes and dendritic cell precursors (42). Infection of these cells with KSHV results in expression of latency-associated genes and is now a viable model used to study latency *in vitro* (42-44). Additionally, lytic replication can be chemically induced from latently infected B cells

and endothelial cells, a technique that has been critical in the study of KSHV infection (42, 43, 45).

As previously mentioned, KSHV is capable of infecting a wide variety of cells types *in vitro* and *in vivo*, and as a result the virus has developed different binding and entry mechanisms, using distinct receptors based on the cell type that is the current target of infection. The primary phase of KSHV binding is mediated through interactions between viral glycoproteins, gB, K8.1, ORF4 and gH, with heparin sulfate on human endothelial, epithelial and fibroblast cells (46-48). Several studies also illustrate the importance of host cell surface integrins, α3β1, αVβ3 and αVβ5, in KSHV entry (42). For binding and entry into B cells, dendritic cells and macrophages, however, KSHV makes use of dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which is commonly used by viruses for adherence to target cells (49). It is currently unknown as to the viral glycoprotein that is required for binding to DC-SIGN, but KSHV gB is a potential candidate due to its high levels of mannose. EphA2, a member of the ephrin family of receptor tyrosine kinases, also serves as a KSHV entry receptor and has been shown to directly interact with the gH/gL complex (50). Additionally, another fusion-entry receptor, xCT, renders adherent cells susceptible to KSHV infection. However, the exact role of xCT in KSHV entry has yet to be elucidated (51). KSHV largely gains access into its target cells by clathrin-mediated endocytosis (51). This was demonstrated in human B cells (49), fibroblast (52), epithelial (53) and endothelial cells (54). Additionally, it has been shown that KSHV can utilize the macropinocytosis pathway to enter endothelial cells (55). KSHV's ability to exploit a

variety of cellular receptors is a critical reason for its wide cellular tropism and is evolutionarily advantageous, as it allows the virus to enter an array of cell types thus providing a more extensive platform for virus propagation.

B. KSHV-associated Pathologies

1. Kaposi's Sarcoma

Kaposi's sarcoma (KS), the most prevalent pathology associated with KSHV infection, was first described in Vienna, Austria in 1872 by Mortiz Kaposi as an "idiopathic multiple pigmented sarcoma." These skin cancers were primarily observed in elderly, Jewish men of Ashkenazi origin and considered to be a rare malignancy (56). In 1981, however, physicians in New York and Los Angeles reported an aggressive form of disseminated KS in young, homosexual men (57, 58). This was ultimately an indication of the AIDS epidemic to come. Studies have shown a KS incidence of 1 in 100, 000 in the general population as compared to 1 in 20 in HIV-infected individuals and a whopping 1 in 3 in HIV-infected homosexual men (59). The advent of highly active antiretroviral therapy (HAART) in the 1990's has lead to a precipitous drop in KS incidence in HIV-positive populations where the treatment is available (60). In Sub-Saharan Africa, where 89% of all KS cases occur and HAART availability is limited, KS is still a growing public health problem (61, 62).

KS is characterized as a multicentric angioproliferative cancer of endothelial origin. A typical KS lesion consists of proliferating tumor cells with an elongated shape called spindle cells, which are the driving force behind the histopathology, as well as

inflammatory cells such as B and T cells, plasma cells and monocytes, and significant neovascularity (63). These new vessels are prone to leakage and rupture, which gives KS lesions their characteristic dark color (64). Less than 3% of KSHV-infected cells in KS lesions produce lytic antigens, suggesting that most cells within these lesions are latently infected with the virus (64).

Four clinical forms of KS have been described: 1) classic KS, 2) African (endemic) KS, 3) AIDS-associated (epidemic) KS, and 4) iatrogenic KS. Classic KS is a tumor of elderly men, and most often found in Mediterranean countries. This form of KS is the variant that was originally described by Dr. Kaposi (56). Lesions are found in the lower extremities and disease progression is less aggressive. African or endemic KS, often affecting women and children, was widespread in Central Africa before the AIDS epidemic and it now accounts for approximately 89% of all KS cases. AIDS-associated or epidemic KS is a major AIDS-defining illness and is an aggressive tumor that manifests with disseminated lesions. Disease progression is rapid and the outcome is poor. Iatrogenic KS is associated with immunosuppression in organ transplant patients and lesions may regress after cessation of the immunosuppressive therapy (29, 35, 64).

Because of KS heterogeneity, there is no standard therapeutic protocol for treatment. Instead, therapies differ depending on the disease severity, rate of tumor growth, patient symptoms, the condition of the patients' immune system, and coexisting HIV-related complications. There are currently two types of therapeutic options, local and systemic. Local therapy is reserved for patients with mild disease and includes

excision of lesions, laser therapy, cryotherapy and topical chemotherapeutics. Unfortunately, local therapy is unable to prevent the development of new lesions (35, 65). A newly emerging therapy for cutaneous lesions is electrochemotherapy (ECT). This technique combines the administration of two highly cytotoxic drugs, bleomycin and cisplatin, with electroporation, which facilitates drug delivery into the cell (66-68). HAART is a systemic therapy that uses multiple drugs that act on various viral targets and has been crucial in reducing KS lesions in HIV-infected individuals (65). Patients that do not respond to HAART and/or have widespread and rapid progression of the disease are frequently given systemic chemotherapy. As our current understanding of KS biology increases, investigators are developing therapies that target angiogenesis, oncogenesis, inflammation and cytokine signaling. Some relevant targets include matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF), both of which play a role in angiogenesis. There are currently no therapies in use that target the virus itself.

2. B Cell Malignancies

a. Primary Effusion Lymphoma (PEL)

The identification of KSHV in Kaposi's Sarcoma lesions (28), prompted other groups to search for the virus in a variety of lymphoid malignancies. In 1995, Cesarman et al (30) discovered that KSHV was also present in body-cavity based lymphomas of AIDS patients. These lymphomas, referred to as primary effusion lymphoma (PEL), present as lymphomatous effusion in the pleural, peritoneal or pericardial cavity (69). PEL are extremely rare tumors, only accounting for approximately 3% of AIDS-related

lymphomas. Unfortunately, those afflicted with PEL have a very poor clinical outcome, with the average survival rate being 5 months (69).

PEL cells are derived from post-germinal center B cells (70) and morphologically, these cells display features of immunoblastic plasma cells and the presence of CD45 on their cell surface suggests a state of activation (69). A frequent finding in PEL cells, and in plasma cells, is that these cells express CD138, but exhibit loss of B cell antigen expression (71). Interestingly, transformed PEL cells do not have rearrangements in the c-myc oncogene and also lack Bcl-2, Ras, or p53 gene alterations (69). Typically, it is dysregulation of these oncogenes that plays a significant role in the development of B-cell lymphomas, however since PEL cells do not contain these abberations it is likely that transformation is led by KSHV infection. Virtually all PEL cases harbor KSHV DNA and KSHV infection of PEL cells is predominantly latent. Latently infected cells express five viral proteins, LANA-1 (ORF73), v-cyclin (ORF72), vFLIP (ORF71), vIRF3/LANA-2 (ORF K10.5) and kaposin (K12) (72, 73). Another gene, the viral homolog of IL-6 (vIL-6, ORF K2), although not classically thought of as a latent gene, is found in a small portion (2-5%) of PEL cells (74). Both cellular and viral IL-6 are expressed and secreted by PEL and have been shown to promote the proliferation of B cells (75). This, along with its ability to inhibit tumor-suppressor pathways (76), has made vIL-6 a potentially important cytokine in the development of PEL tumorigenesis.

Unfortunately, there is no clear standard of care established for treatment of PEL patients. Seeing as how PEL develops in patients with advanced AIDS, HAART and

antiretroviral therapies are currently being used and help promote survival of afflicted patients (77). Inhibitors of NF-κB activation and mTOR, Bortezomib and rapamycin, respectively, both lead to apoptosis of PEL cell lines *in vitro* and have thus provided potential treatments for PEL. Additionally, when used in combination with antiviral drugs, valporate, a histone-deacetylase inhibitor, can induce lytic replication of KSHV and subsequently lead to apoptosis of PEL cells (78).

b. Multicentric Castleman's Disease (MCD)

Of the KSHV-associated pathologies, MCD displays the highest number of productively infected cells (79). MCD, occurring in nearly all patients with AIDS, is a particularly aggressive lymphoproliferative disorder that is often found in several lymph nodes and characterized by enlarged germinal centers with B cell and vascular proliferation (74). Tumor cells in MCD are plasmablastic cells that arise from a naïve, pre-plasma B cell. This was determined based on their IgM, IRF4 and Blimp-1 expression, as well as their lack of CD138 expression (80). Additionally, vIL-6 production is a prime feature in the expression program of MCD, as is human IL-6. Many MCD cells are latently infected with KSHV with approximately 10-50% of cells expressing latency antigen, LANA-1. Interestingly, 5-25% of LANA-positive cells have also been found to express lytic antigens, vIRF1 (ORF K9) and vIL-6. This is very suggestive of both lytic and latent transcriptional programs in MCD, something that is not seen in PEL and KS, which are predominantly latent KSHV infections (72).

Seeing as how MCD is associated with the high degree of lytic KSHV replication, antiviral therapies have led to rapid resolution of symptoms and virema (78). Patients that have MCD and HIV simultaneously are given HAART as well as aggressive chemotherapy regimes. A culprit behind MCD pathogenesis is IL-6, making it and the signaling cascade it activates a potential target for MCD therapy. In fact, treatment with monoclonal antibodies against the IL-6 receptor, altizumab, has improved symptoms and abnormalities associated with MCD (81). The most promising treatment of MCD is rituximab, which is a monoclonal antibody against B-cell marker, CD20. Patients undergoing rituximab therapy have observed a decrease in KSHV levels and many have achieved clinical remission (77, 82).

C. Novel KSHV Genes and Proposed Roles in Pathogenesis

The coevolution between KSHV and its host has resulted in KSHV acquisition of genes that causes the selective suppression of host immune responses, thus allowing for a life-long, persistent infection. KSHV has dedicated about a quarter of its genome to immunomodulatory genes, most of which appear to be derived from the host (83). Some of these are viral homologues to cellular genes and include viral interferon regulatory factors (vIRF1, vIRF2, vIRF3, vIRF4 encoded by ORFs K9, K11/11.1, K10.5/10.6, K10/10.1, respectively), CC-chemokine ligands (vCCL1, vCCL2, vCCL3 encoded by ORFs K6, K4, K4.1, respectively), viral interleukin-6 (vIL-6 encoded by ORF K2), a G-protein coupled receptor (GPCR) that is homologous to the IL-8 receptor (vGPCR encoded by ORF74), viral CD200 (vCD200 encoded by ORF K14), a complement regulatory protein (KCP encoded by ORF4), a viral caspase-8 inhibitory protein (vFLIP

encoded by ORF K13), a viral Bcl-2 protein (vBcl-2 encoded by ORF16) and a viral cyclin (vCyc encoded by ORF72) (83, 84). The cellular homologues of these genes have roles in innate immunity including the interferon (IFN) response and the complement system, inflammation, and programmed cell death (PCD) mechanisms, apoptosis and autophagy. Interestingly, *in vitro* studies have shown that many of these proteins function not only within the same pathways as their cellular homologues, but also in distinct pathways, allowing for multifunctional roles of a single protein and increasing breadth of immunomodulation by KSHV. A great example of the multifunctionality of KSHV immunomodulatory proteins are the vIRFs, whose roles include not only inhibition of the host IFN response and IFN-signaling, but also inhibition of apoptosis and downregulation of MHC class I molecules (85), all of which will be further discussed in later sections.

The KSHV genome encodes a number of viral cytokine/chemokine proteins, which are homologous to cellular cytokines/chemokines and also exhibit similar functions. Both cellular IL-6 and vIL-6 bind the gp130 subunit of the IL-6 receptor, and as a consequence, vIL-6 is able to mimic multiple functions of cellular IL-6 including stimulation of IL-6 dependent B cell proliferation, activation of the JAK-STAT pathway as well as stimulation of cells via intracellular signaling (86-88). Being a multifunctional protein, vIL-6 has also been shown to play important roles in the indirect promotion of angiogenesis through induction of vascular endothelial growth factor (VEGF), development of a Th2-polarized response, and inhibition of IFNα-mediated cell cycle arrest (76, 89, 90). Interestingly, vIL-6 is able to inhibit IFN mediated cell cycle arrest, but cellular IL-6 cannot. This is due to the fact that vIL-6 binds solely to the gp130

subunit of the IL-6 receptor, whereas cellular IL-6 requires gp130 as well as gp80, which is downregulated by IFN. Without this need for gp80, vIL-6 can escape the regulatory control of IL-6 signaling by IFN-α. Furthermore, vIL-6 is found to be expressed and secreted from latently infected KSHV-positive B cells and contributes to PEL growth and survival (91-93). KSHV also encodes several chemokine homologues called viral CC-chemokine ligand 1 (vCCL1), vCCL2 and vCCL3, all of which are made during the lytic replication cycle. These vCCL proteins also show homology to macrophage inflammatory proteins (MIPs) and act as chemoattractants primarily for Th2 cells, thus polarizing the adaptive immune response towards a Th2-type response and potentially reducing the efficacy of the host antiviral response towards KSHV (83, 84). Aside from their chemotactic functions, the vCCL proteins have also been found to possess angiogenic and anti-apoptotic functions (94-97), as do vFLIP and vBcl-2 (64), potentially contributing further to KSHV pathogenesis.

Additionally, KSHV encodes several viral homologues that are expressed on the cell surface of infected cells, including vGPCR and vCD200. KSHV vGPCR is a seven-transmembrane, IL-8 receptor homolog that contains a mutation that renders the protein constitutively active (98). Consequently, this protein is capable of constitutively activating specific transcription factors that then turn on the expression of growth factors, inflammatory chemokines and angiogenic factors (99-101). Interestingly, when expressed independent of other viral genes, vGPCR is able to promote an anti-senescence response and transform cells (102). Another immunomodulatory surface protein encoded by KSHV is vCD200, also known as vOX2. This surface glycoprotein exhibits significant

homology to cellular CD200 (OX2), which is broadly distributed on the surface of myeloid cells (103). Both proteins, viral and cellular CD200, are able to bind the CD200 receptor with similar affinity (104). The specific immunomodulatory role of vCD200, however, is controversial. Some have shown that vCD200 promotes the secretion of pro-inflammatory cytokines (103), whereas others have suggested the opposite, showing a reduction in Th1-cell associated cytokine production as well as reduced neutrophil-mediated inflammation (104-106). All in all, it has been concluded that vCD200 can cause immune dysfunction associated with persistent infection by KSHV.

The current KSHV immunomodulatory genes and what is known about how they function are summarized in Table 1.1. KSHV employs a wide variety of mechanisms targeting the immune system, including evasion of both the innate and adaptive branches of immunity as well as inhibition of apoptosis, thus leading to the potentiation of cellular transformation and promoting disease pathogenesis (83, 84). It is important to note, however, that many of these observations have been made *in vitro*, primarily due to the limitations of the current animal models available for the study of KSHV disease progression and pathogenesis (107).

Table 1.1. KSHV Immunomodulatory Gene Products and Their Functions

ORF	ORF/Gene Product	Function
Evasion of Innate Immunity		
ORF45	ORF45	Prevents IRF7 activation
ORF50	RTA	Promotes IRF7 degradation
K9	vIRF1	Inhibits IRF3-mediated transcription
K10.5-K10.6	vIRF3	Inhibits IRF7 DNA binding activity
K11.1-K11	vIRF2	Suppresses IRF1 and IRF3
K8	k -bZIP	Impedes IRF3 binding to IFNβ promoter
ORF4	KCP	Inhibits complement
Evasion of Adaptive Immunity		
K1	K1	Represses BCR signaling
K3	MIR1	Downregulates MHCI as well as CD1d
K5	MIR2	Downregulates MHCI as well as CD1d
K9	vIRF1	Downregulates MHCI
K10.5-K10.6	vIRF3	Downregulates MHCII
K5	MIR2	Downregulates ICAM1 and B7.2
Inhibition of Cytokines/Chemokines		
K2	vIL-6	Homolog to cellular IL-6
K4	vCCL-2	CCR3 and CCR8 agonist; C-, CC-, CXC-, CX_3C-chemokine agonist
K4.1	vCCL-3	CCR4 agonist, Th2 chemoattractant
K6	vCCL-1	CCR8 agonist, Th2 chemoattractant
ORF74	vGPCR	Homolog to cellular IL-8
K14	vCD200	Homolog to cellular CD200
Modulation of Apoptosis		
K13	vFLIP	Inhibits caspase 8 activity
ORF16	vBcl-2	Bcl-2 homolog
K7	vIAP	Links Bcl-2 to effector caspases
K9	vIRF1	Binds p53, ATM kinase and GRIM19
K10.5-K10.6	vIRF3	Binds and inhibits p53
ORF73	LANA	Binds p53

Abbreviations: RTA, replication and transcription activator; bZIP, basic region-leucine zipper protein; KCP, KSHV complement protein; BCR, B-cell receptor; MIR, modulator of immune recognition; MHC, major histocompatibility complex; ICAM, intercellular adhesion molecule; CCL, chemokine ligand; CCR, chemokine receptor; FLIP, FLICE-like inhibitory protein; IAP, inhibitor of apoptosis; LANA, latency-associated antigen. Adapted from (108).

D. Models of KSHV Pathogenesis

A major impediment in moving the field of KSHV biology and pathogenesis forward has been the lack of a suitable animal or cell culture model for *de novo* infection with KSHV. While KSHV is able to infect a variety of primary cells and cell lines, infection typically results in latency and is unable to support the growth of KSHV to high titers. Treatment of latently infected cells with phorbol esters can induce lytic replication in a small percentage (about 20-30%) of cells (45). Phorbol esters such as TPA activate protein kinase C (PKC), which phosphorylates and activates potent transcriptional activators, thus leading to increased expression of genes including lytic genes. Upon treatment with TPA, only approximately 25% of cells complete the lytic cycle and go on to produce virus (109). A complication in studying the lytic gene transcription program in the context of these chemically-induced, latently-infected cells is the background of simultaneous latent gene expression. Conversely, these *in vitro* systems are not ideal for latent studies as approximately 2-5% of cells undergo spontaneous lytic replication in the absence of a chemical stimulus (110, 111). Because of these limitations, much of the research done on lytic genes, which account for the majority of KSHV genes expressed, has been performed outside the context of infection. This alone is limiting to the field of KSHV biology. Another significant constraint on this field has been the development of an animal model, which has proven to be difficult due to the high degree of species specificity of KSHV. Although it has been trying, a significant effort has been made to develop a workable animal model for the study of KSHV pathogenesis.

The first attempt at an animal model for *in vivo* KSHV infection was actually using an immunocompromised, SCID mouse (112-114). One group was able to establish a B cell lymphoma in SCID mice by transferring into the mice KSHV-infected PEL cells, however the tumors were identical to the PEL tumor cells that were initially injected, suggesting that no new B cells were infected with KSHV (114). Another group used SCID-hu Thy/Liv mice, which are SCID mice that have been grafted with human fetal liver and human fetal thymus implants underneath the kidney capsule in order to establish cells of human hematopoetic lineage inside the mouse (112). Dittmer et al. injected purified KSHV into the grafted thymus/liver implants. Both lytic and latent antigens were detected via RT-PCR, but this was restricted to B cells from the transplanted population of cells (112). In addition, Parsons et al. injected NOD/SCID mice with purified KSHV, resulting in lytic and latent infection within a wide array of murine cells and tissue types (113). Not only was LANA protein expression observed, but also KSHV virion production in the murine spleen was visualized 3 months post-infection via electron microscopy. The ability of KSHV to maintain latency in the B cells of these mice is of particular importance, as the B cell compartment is the major site of latency in KSHV-infected humans. Interestingly, in this potential animal model, less that 1% of cells within the mice were actually infected with KSHV and furthermore, none of the mice developed KSHV-associated diseases (113). More recently, a new humanized mouse has led to yet another potential murine model for the study of KSHV infection (115). The hu-BLT (bone marrow, liver and thymus) mouse is generated from NOD/SCID/IL2rγ (NSG) mice. Similar to the SCID-hu Thy/Liv mouse, these mice are transplanted with human thymus and liver cells, in addition to CD34+ hematopoetic stem cells. Hu-BLT mice

harbor high and sustained levels of human immune cells and are also capable of establishing a human mucosal immune system, which is unique to this animal model. These mice are currently being used to study important human pathogens such as HIV and *Plasmodium falciparum* (116). Infection with KSHV via natural routes of infection resulted in the establishment of both lytic and latent infection. Similar to studies conducted in NOD/SCID mice, the hu-BLT mouse model failed to develop KS-like lesions, lymphomas or other pathologies related to infection with KSHV. Although the aforementioned models have provided the field with additional tools for the *in vivo* study of KSHV, they do not allow for the study of virus related pathogenesis as these models often do not develop KSHV-associated diseases, unless engrafted with KSHV-infected human tissue, wherein lesion formation is still only localized to the graft (117). Furthermore, the severely impaired immune systems in these animals provide an added challenge to studying the host immune response to infection and also viral immune evasion mechanisms of KSHV, which are adapted to human immune systems. The current animals model all have their limitations in fully recapitulating KSHV-related pathologies and are thus not ideal for the study of KSHV disease progression or the host immune response to KSHV infection.

The complications in developing a suitable animal model led Renne et al (118) to experimentally inoculate non-human primates with KSHV, in the hopes of establishing infection within an animal more closely related to humans. Simian Immunodeficiency Virus (SIV)-positive and –negative rhesus macaques (RMs) subsequently infected with KSHV did not result in KSHV-specific transcripts or antibodies. Although viral DNA

was detected up to a year after inoculation in expanded peripheral blood mononuclear cells (PBMCs), the animals did not develop any KSHV-associated malignancies. Unfortunately, the low level persistent infection, lack of a humoral response and absence of KSHV-related pathology does not make this animal model suitable for KSHV *in vivo* studies. More recently, the use of common marmosets, a New World primate, as an animal model for KSHV infection has been explored (119). Inoculation of marmosets with KSHV resulted in rapid seroconversion with sustained antibody responses over one and a half years, indicating a robust humoral response. KSHV was able to establish latency in these animals, but detection of lytic transcripts was unsuccessful as was virus recovery from PBMCs (119). Unlike other animal models, however, KS-like lesions were observed, albeit in only one infected marmoset (119). Interestingly, marmosets appear to be more susceptible to infection by various pathogens. Historically, immune protection is conferred by more extensive polymorphisms of major histocompatibility complex class I (MHCI) alleles and marmosets have fairly limited MHCI polymorphism (119). This could be a reason for the increased KSHV susceptibility observed in marmosets. Consequently, the immune components within a prospective animal model are crucial to take into account and it is therefore advantageous to study a pathogen within its natural host for a more accurate representation of disease pathogenesis.

Two other γ-herpesviruses that are closely related to KSHV include murine herpesvirus 68 (MHV-68) and rhesus macaque rhadinovirus (RRV), which are currently being used as models of KSHV infection in their respective natural hosts. Employment of a species-specific model for the study of viral infection and pathogenesis allows for a

more accurate understanding of disease progression, virus infection in the context of an intact immune system as well as specific virus-host interactions. MHV-68 is a natural pathogen of mice, both outbred and inbred strains (120). Sequence analysis and viral genome structure revealed genome colinearity with the KSHV genome (120). Infection of mice with MHV-68 resulted in a persistent infection within the spleen as well as the development of lymphoproliferative diseases in approximately 9% of infected mice (121). Genome-positive cells, indicating the presence of latent virus, were detected in all the analyzed lymphomas, but there was no evidence of lytic antigens, suggesting a lack of viral replication (121). Interestingly, genome analysis demonstrated that MHV-68 lacks important immunomodulators and tumorigenic genes found in KSHV including the vIRFs, vIL-6, vMIP, vFLIP, and vCD200 (120). Furthermore, the pathologies associated with KSHV infection are typically found in immunocompromised patients infected with both KSHV and HIV. A suitable model for the investigation of KSHV pathogenesis should mimic this immune suppression mediated by HIV, as this plays an important part in KSHV disease. Unfortunately, there is no known mouse homologue of HIV that might possibly strengthen the use of MHV-68 as a model for KSHV infection.

The second γ-herpesvirus related to KSHV is RRV. Not only does RRV naturally infect RMs, but its genome is also colinear with the KSHV genome, to a much higher degree than that of MHV-68. Moreover, coinfection of RMs with SIV and RRV provides a better recaputilation of KSHV and HIV coinfection of humans. RRV infection of RMs will be further discussion in the section to follow.

III. Rhesus Macaque Rhadinovirus

A. Identification and General Characteristics

Rhesus rhadinovirus (RRV) was independently isolated from RMs by two separate groups in the early 1990's (122, 123). The first group, located at the New England Primate Research Center (NEPRC), identified an isolate, RRV H26-95, from a colony of healthy monkeys as well as from two other colonies that were tested (122). As opposed to the discovery of RRV from healthy animals at the NEPRC, a homologous RRV isolate, RRV_{17577}, was discovered in SIV-infected macaques at the Oregon Regional Primate Research Center (ORPRC). These animals had developed a lymphoproliferative disorder and other clinical manifestations similar to MCD seen in humans infected with KSHV (124). This group also demonstrated that healthy animals within the same colony were also naturally infected with RRV_{17577} and harbored virus within their B cells (125). Sequence analysis showed considerable homology between the RRV isolates H26-95 and 17577, and with KSHV, and confirmed their classification as gamma-2-herpesviruses (123, 126). It has since been revealed that RRV is endemic in RM breeding populations with approximately 98% of macaques testing positive for RRV (127). Interestingly, infection with RRV_{17577} results in scientifically releveant disease, whereas no disease associations have been reported for strain H26-95 (124, 128, 129), thus making RRV_{17577} a more applicable model to the study of KSHV pathogenesis.

The RRV genome is dsDNA, 131kb in length and its organization is essentially colinear with KSHV (123, 126). Sequence, structural and phylogenetic data has suggested that RRV is the RM equivalent to KSHV (123). RRV encodes 79 ORFs, 67 of

which are found in KSHV, as well as herpesvirus saimiri, another member of the gamma-2-herpesvirus family (123). RRV serves as a unique model for the study of KSHV in both *in vivo* and *in vitro* systems. As described above, infection of RMs with RRV results in clinical manifestations similar to what is seen in humans infected with KSHV, therefore providing a *in vivo* model for RRV biology and pathogenesis. *In vitro* infection of fibroblasts with RRV results in robust lytic replication as well as high titers of virus, which allows for the study of *de novo* infection, in addition to the lytic replication cycle of the virus. Furthermore, the *in vitro* RRV system also permits the quantification of virus via traditional plaque assays, a method that has not been available to the field of KSHV research due to the inefficient lytic replication of KSHV (130). A latent infection system of RRV infection has also been described in B cells (131, 132), however virus production only occurs in B cells immortalized by EBV and yields relatively low titers (132).

B. RRV-associated Pathologies

Even though RRV infection is widespread among the captive macaque population, development of RRV-associated disease is not as common. Immunocompromised RMs coinfected with SIV and RRV display disease manifestation (124, 133), similar to KSHV-infection in HIV-infected individuals. *De novo* infection of RRV-naïve, SIV-infected RMs with RRV_{17577} leads to hyperblastic lymphoproliferative disorder (LPD) resembling MCD, characterized by persistent lymphadenopathy, hepatomegaly, splenomegaly and hypergammaglobulinemia (124, 133). Additionally, coinfected RMs display persistent viremia and a minimal RRV-specific antibody response, in comparison to RMs infected with RRV alone, which results in transient viremia and a strong anti-

RRV antibody response (124) Furthermore, B cells have been determined to be an important site of RRV latency, similar to KSHV (125). Unlike KSHV, however, RRV infection of RMs does not result in KS-like lesions (124, 129). The development of a proliferative mesynchemal lesion referred to as retroperitonal fibromatosis (RF) has been reported in SIV-infected RMs experimentally inoculated with RRV (133). Although RF lesions share morphological and histochemical characteristics with KS lesions, there are notable differences between the two including increased vascularity and the hemorrhagic nature of KS lesions and fibrosis observed in RF lesions.

C. Novel RRV Genes and Their Comparison to KSHV

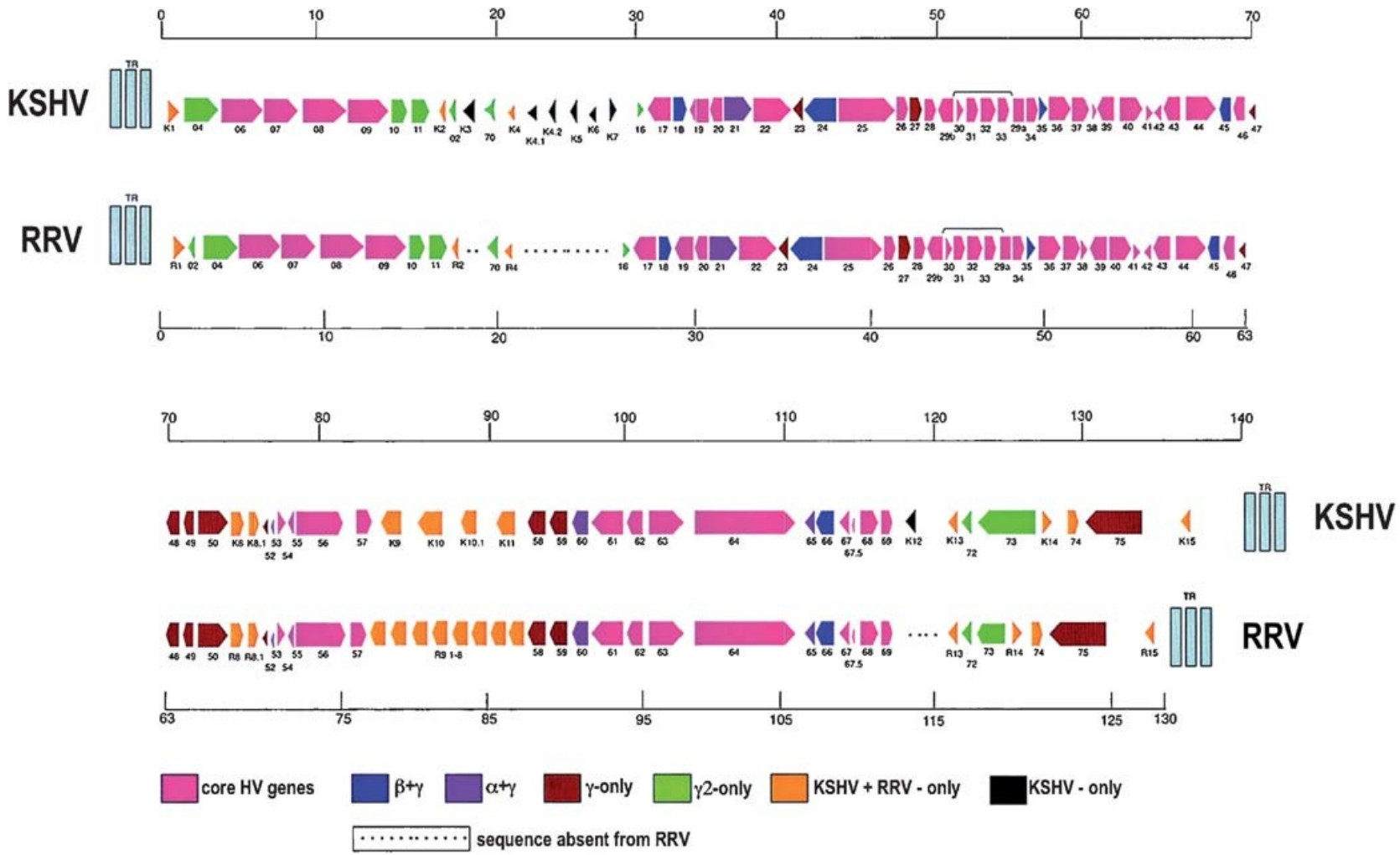
All of the ORFs present in KSHV have at least one corresponding homologue in RRV, expect for four ORFs, which include ORFs K3 and K5 [modulator of immune recognition 1 (MIR1) and MIR2], K7 (viral inhibitor of apoptosis (vIAP)], and K12 (kaposin) as seen in Figure 1.2. RRV encodes one macrophage inflammatory protein (MIP-1) and eight viral interferon regulatory factors (vIRFs) compared to three MIP-1/vCCLs and four vIRF genes in KSHV. Like KSHV, a large portion of the RRV genome is dedicated to immune evasion and pathogenesis, including the novel genes found in both KSHV and RRV (84). The functions of some of the RRV ORFs that are related to KSHV ORFs have been dissected and it has been shown that they are functionally similar as well. For example, both KSHV and RRV encode a homologue to cellular GPCR, the IL-8 receptor. Similar to KSHV GCPR, RRV vGPCR (ORF74) possesses transforming potential both *in vitro* and *in vivo* and induces the secretion of VEGF (134). Additionally, a homologue of cellular CD200 and KSHV vCD200 is encoded by RRV. RRV vCD200

is expressed on the surface of cells, like KSHV vCD200, but RRV vCD200 is also secreted from infected cells, which is unique to this protein. Furthermore, RRV vCD200 downregulates expression of tumor necrosis factor (TNF) in macrophages, similar to both cellular CD200 and KSHV vCD200 (135). Both KSHV and RRV express complement control proteins (CCP), encoded by ORF4, which are both homologous to cellular regulators of complement activation. The KSHV and RRV CCPs are expressed on the surface of virions and infected cells, and they both inhibit the classical (antibody-mediated) complement pathway (136). RRV vIL-6 is also a functional homologue of cellular IL-6, as well as KSHV vIL-6, in that it binds the gp130 subunit of the IL-6 receptor and mediates IL-6 independent B cell proliferation (75, 137). Interestingly, RRV vIL-6 is expressed in lymphomas and retroperitoneal fibromatosis of infected RMs (138), which has also been shown in MCD and PEL tissue samples of KSHV-infected patients (139). Finally, RRV encodes 8 vIRFs, similar to the 4 vIRFs encoded by KSHV, and recent data have shown they are important in disease progression and pathogenesis as well as inhibition in the IFN response in *de novo* infection (140, 141). Further elucidation of these novel RRV proteins *in vitro* and *in vivo* will help define their roles in disease progression and immunomodulation, which will ultimately expand upon our current understanding of their KSHV homologues and how they function *in vivo*.

Previous work on the RRV vIRFs focused primarily on the role of all 8 vIRFs in the context of infection and their effects on the manifestation of disease and the immune response (140, 141). This thesis concentrates specifically on one RRV vIRF, namely R6, and its mechanism of inhibition on the type I IFN response. The overarching advantage

we gain from further dissecting of the mechanisms of these RRV vIRFs is that it will advance our current state of understanding the role of the vIRFs in KSHV infection and pathogenesis.

FIGURE 1.2. Alignment of KSHV and RRV genomes. ORFs are colored according to their presence in specific herpesvirus subfamilies indicated in the code at the bottom. The direction of the arrows signifies the directionality of the genes with the arrow at the 3' end of each ORF. ORF, open reading frame. TR, terminal repeats. *Figure and figure legend are adapted from* (107) *and reprinted with permission.*


KSHV
RRV
TR
core HV genes
β+γ
α+γ
γ-only
γ2-only
KSHV + RRV - only
KSHV - only
sequence absent from RRV

D. RRV Infection of RMs as a Model for the Study of KSHV Pathogenesis

Over the years there has been a vast amount of evidence that supports the use of RRV as a model for studying KSHV pathogenesis. First, RRV shares a high level of both sequence and genome conservation with KSHV (123, 126). Importantly, the genes that are unique to both KSHV and RRV provide a valuable opportunity to study their mechanisms both *in vitro* and *in vivo*. The lytic system of RRV allows for the growth of virus to high titers as well as genetic manipulation of the virus, which is valuable for the analyses of relevant genes of interest. Particularly, the development of a bacterial artificial chromosome (BAC) clone of RRV has provided a more targeted approach to introducing genetic alternations in the RRV genome, allowing for dissection of specific viral genes and their functions both *in vitro* and *in vivo.* In 2012, a new KSHV BAC clone was generated (BAC16), stably propagated and able to robustly produce high titers of infectious virus (142). This provided the field with a tool to better study *de novo* infection of KSHV, which was previously unavailable. Regardless, the RRV BAC system remains relevant, as it is able to span research both *in vivo* and *in vitro*. Aside from the *in vitro* benefits of using RRV to study KSHV, RRV has proven to also be beneficial as an *in vivo* model. RRV and KSHV infection, in their natural hosts, results in comparable clinical manifestations. Moreover, complications that arise in HIV-infected immunocompromised humans also infected with KSHV can be recapitulated by coinfection of RMs with SIV and RRV (124, 133). This, among other genetic components, is an important advantage that the RRV RM model has over the MHV-68 mouse model. RRV also establishes latency in B cells, similar to KSHV, providing a model for the study of latency. Furthermore, both KSHV and RRV are adapted to their

respective natural primate hosts, including their mechanisms of immune evasion, which is an added benefit over the MHV-68 mouse model. Collectively, the reason stated above make RRV infection of RMs an ideal model for the study of viral immune evasion mechanisms of KSHV and ultimately KSHV pathogenesis.

IV. The Immune Response to Primary Herpesvirus Infection

A. Overview of Innate Immunity

The innate immune system is an important defense mechanism in response to infection and is generally distinguished from the adaptive immune response by its ability to respond quickly (ie. 0-4 hours after encountering pathogen), its non-specific pathogen recognition and its lack of immunological memory. An important element to innate immunity often taken for granted, is the epithelia, which defends the surfaces of the body from the external world. Surface epithelia provide mechanical (ie. tight junctions), chemical (ie. enzymes in tears and saliva, fatty acids, pH and defensins), and microbiological (ie. normal microbiota) barriers that must be overcome in order to establish an infection. If this barrier is breached either by a wound or loss of integrity of the epithelia, the pathogen then encounters the complement system, an ancient component of the innate immune system. Pathogens will also come across other cellular components of innate immunity such as Natural Killer (NK) cells, important for killing cells stressed by malignant transformation, or viral or bacterial infection, antigen presenting cells (APCs) and phagocytic cells (143). APCs and phagocytic cells internalize pathogens, leading to the non-specific destruction of the pathogen, wherein these cells mediate an inflammatory response comprised of IFN production, and

proinflammatory cytokine/chemokine secretion. In turn, these components of the inflammatory response stimulate innate effector responses as well as initiate an effective adaptive immune response. The specifics of pathogen recognition, the IFN response and its effects on adaptive immunity will be addressed in later sections.

B. Recognition of Invading Pathogens via PRRs and PAMPs

Pathogen recognition by the innate immune system is mediated by a collection of receptors located within the cytoplasm and on cell membranes of host cells. These germline-encoded receptors, or pattern recognition receptors (PRRs) have evolved to detect cognate pathogen-associated molecular patterns (PAMPs), signatures of "non-self" (144, 145). PAMPs include lipids, lipoproteins, proteins and nucleic acids derived from a variety of sources such as viruses, bacteria, fungi and parasites (144). Importantly, PAMPs are patterns present on or synthesized during replication of microorganisms but are not normally components of host cells. Additionally, the cellular location of the PAMP dictates the type of receptor that initiates recognition. Engagement of PRRs by their cognate PAMPs leads to downstream signaling cascades via different adaptor molecules, leading to the activation of IκB kinases (IKKs) and IKK-related kinase, TBK or Tank-binding kinase. Signals from activated PRRs converge on these kinases, which subsequently activate transcription factors such as nuclear factor-κB (NF-κB) and cellular IRFs. These are then translocated to the nucleus, wherein they mediate transcription of proinflammatory cytokines and type I IFNs (144, 146). NF-κB controls the expression of genes required for inflammation and adaptive immune responses, including IL-1β, IL-6, tumor necrosis factor-α (TNF-α), IL-12 and IL-15 (147). Cellular IRF-3 and NF-κB

cooperatively control the transcription of IFN-β, and both IRF-7 homodimers and IRF-3/IRF-7 heterodimers activate transcription of IFN-α subtypes (148). Induction of type I IFNs further propagates the signal via a positive-feedback loop thus contributing to its effect on the innate immune response and shaping the ensuing adaptive immune response.

Presently, several different classes of PRRs have been identified and include transmembrane and endosomal receptors Toll-like receptors (TLRs) and C-type lectin receptor (CLRs), as well as cytoplasmic receptors Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs) and most recently, cGMP-cAMP synthase (cGAS). The most intensively studied PRRs belong in the TLR family of receptors and are responsible for sensing foreign pathogens outside of the cell and inside the cell in endosomes and lysosomes (144). The extracellular domain of TLRs contains leucine-repeat regions (LRRs) and it is this region that is responsible for the recognition of various pathogens. The cytoplasmic tails of TLRs have a conserved region known as the Toll/IL-1R (TIR) domain, which is crucial for the signaling of TLRs (149). TLRs are expressed on professional APCs such as B cells, monocytes, macrophages and DCs, and also on various nonprofessional APCs like fibroblasts and endothelial cells (150). A total of 10 TLRs have been found in humans and they can be largely divided into two groups depending on their cellular localization and cognate ligands. The group of TLRs that is expressed on the cell surface and recognizes microbial membrane components such as lipids, lipoproteins and proteins is composed of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11. The other group, however, is expressed specifically in intracellular

compartments such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, wherein they detect microbial nucleic acids and this group is comprised of TLR3, TLR7, TLR8, and TLR9. This group of intracellular TLRs is thought to have evolved to combat viral infections (144). During a herpesvirus infection, the most potent immune-stimulating component is viral genomic DNA, which is a ligand for TLR9 and cytoplasmic PRRs. All three subfamilies of herpesviruses, alpha-, beta-, and gamma-herpesviruses, including KSHV, are recognized by TLR9 (151). Specifically, TLR9 recognizes CpG motifs within DNA, which is highly abundant in herpesvirus genomic DNA. Although several studies have shown that viral entry is required for TLR9 ligation and stimulation (152-155), the exact herpesvirus ligand is still unclear. A study performed by Krug et al demonstrated that infection of murine plasmacytoid DCs (pDCs) with UV-inactivated HSV-1 results in TLR9-dependent activation of IFNα, suggesting that DNA replication is not necessary for TLR9 ligation and that viral genomic DNA is the cognate ligand for TLR9 during HSV-1 infection(156). Interestingly, when this same experiment is performed in a human pDC infection with UV-inactivated KSHV, the results are quite opposite and no IFNα is produced (157). These results suggest that the TLR9 ligand may either be dependent on viral replication or that perhaps UV-inactivation renders the TLR9 ligand ineffective.

Other TLRs involved in early detection of herpesviruses are TLR2, TLR3, TLR7 and TLR4. TLR2 recognizes a wide variety of PAMPs on bacteria, viruses, fungi, and parasites, one of which is lipoproteins (147). On the cell surface, TLR2 recognizes virion envelope glycoproteins, gH and gB, which was first demonstrated in HCMV infection

(158). It has been further shown that TLR2 mediates an innate immune response after infection and recognition of HSV-1 (159), HSV-2 (160), MCMV (161), HCMV (162, 163), VZV (164), EBV (165). The ligands for TLR3 and TLR7 are double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), respectively, and are important stimulators of these TLRs during herpesvirus infection as they are commonly byproducts of viral replication (166). It is interesting to note that TLR4, classically known to bind and recognize Gram-negative bacterial component lipopolysaccharide (LPS), is also involved in innate immunity against KSHV (167). The KSHV ligand for TLR4 is unknown, however it is thought that because UV-inactivated KSHV was able to stimulate TLR4-mediated signaling, a virion component such as envelope glycoproteins is likely the TLR4 ligand (167).

In addition to membrane bound TLRs, cytosolic PRRs are also important mediators of an innate immune response following recognition of herpesvirus PAMPs. Particularly, RIG-I and melanoma differentiation-associated gene 5 (MDA-5), both of which contain caspase activation/recruitment domains and RNA helicase domains, thus allowing for the binding of RNA. Although RIG-I and MDA5 share these critical domains, they both recognize distinct RNA species during herpesvirus replication (145). These PRRs have been shown to recognize replication intermediates during HSV-1 and EBV infections (168-170). Given that the herpesvirus genome is DNA, human immune systems have evolved to detect intracellular DNA as well. The first cytosolic DNA sensor to be identified was the DNA-dependent activator of IFN-regulatory factors (DAI also known as ZBP-1) and its was initially found to be involved IFNβ stimulation following HSV-1

detection (171). Thereafter, a variety of cytosolic PRRs have been discovered and their importance, specifically in herpesvirus detection, has been appreciated. These dsDNA-detecting PRRs include DEAH box protein 9 (DHX9) and DHX36, which recognize CpG-containing DNA (172), the absent in melanoma 2 (AIM2) protein (173, 174) and the newly identified, cGAS (175, 176). Detection via cGAS is unique in that cGAS senses dsDNA and catalyzes the synthesis of cyclic GMP-AMP (cGAMP), an endogenous second messenger. This second messenger then binds to an adaptor molecule called stimulator of IFN genes (STING), which promotes downstream transcription of IFN through activation of IFN regulatory factor 3 (IRF3) (177).

Many PRRs trigger signaling cascades that initiate gene transcription by NF-κB, ATF-2, Jun, and IRFs, thus activating transcription of cytokines, proinflammatory proteins and interferons. A subset of NLRs and AIM2-like receptors (ALRs), however, trigger a distinct mechanism to cleave and activate proinflammatory caspases that ultimately leads to the production of proinflammatory cytokines IL-18 and IL-1β, as well pyroptosis, a form of apoptosis that is caspase-1-medited. This occurs via assembly of a protein complex termed the inflammasome (178). Processing of IL-1β by the inflammasome first requires a PRR-triggered priming event that results in NF-κB-mediated expression of pro-IL-1β (179). Recently, KSHV was shown to activate the inflammasome through IFNγ-inducible protein 16 (IFI16), a DNA sensor, within the nucleus of infected cells (180). This is not only the first demonstration of inflammasome activation by KSHV, but it is also the first evidence of inflammasome activation within the nucleus (180).

Coevolution with herpesviruses has caused the human innate immune system to adapt and evolve new mechanisms of pathogen detection, resulting in a wide variety of PRRs that can detect a multitude of pathogen-derived ligands. One might think that cellular PRRs have developed redundancies, however these receptors are differentially expressed in various cell types. TLR3 and TLR9 are prime examples of this as they are primarily expressed in DCs (181) and IFNα-producing cells, pDCs (144), respectively. Therefore, TLR3 and TLR9 stimulation is critical for the induction of an effective immune response again herpesviruses (182, 183).

Table 1.2. PRRs and their cognate PAMPs in human herpesvirus infection

PRR	Herpesvirus	Proposed PAMP
TLR		
TLR2	HSV-1, HSV-2	virion component
	VZV	virion component
	HCMV	gB and/or gH
	EBV	virion component, dUTPase
TLR3	HSV-1	dsRNA
	EBV	EBERs
TLR4	KSHV	envelope glycoproteins
TLR9	HSV-1, HSV-2	genomic DNA
	HCMV	genomic DNA
	EBV	genomic DNA
	KSHV	genomic DNA
RLR		
RIG-I	HSV-1	dsRNA
	EBV	dsRNA
MDA5	HSV-1	replication intermediate
DNA sensors		
DAI	HSV-1	genomic DNA
	HCMV	genomic DNA
IFI16	HSV-1	genomic DNA
	KSHV	genomic DNA
DHX9 & DHX36	HSV	genomic DNA
c-GAS	HSV	genomic DNA

Abbreviations: PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; RLR, RIG-I-like receptors; RIG-I, retinoic acid-inducible gene-I; MDA5, melanoma differentiation-associated gene 5; DAI, DNA-dependent activator of interferon regulatory factors; IFI16, IFNγ-inducible gene 16; DHX, DEAH box protein; HSV, herpes simplex virus; VZV, varicella-zoster virus; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; KSHV, Kaposi's sarcoma-associated herpesvirus. Table data collected from (144, 150, 151).

C. The Antiviral Interferon Response

1. Type I and Type II IFNs

The culmination of downstream signaling through PRRs is an inflammatory antiviral response, largely facilitated by IFNs. These secreted cytokines are vital in mounting a robust innate immune response against infection, as they possess strong antiviral and immunomodulatory properties. The IFN family can be broken down into three main groups, type I, type II and type III IFNs, which are categorized based on sequence homology and receptor complex usage(184). Type I IFNs were described over 50 years ago as the secreted factor responsible for viral replication interference, hence the name interferon (185). The type I IFN family is composed of 14 subtypes of IFNα, IFNβ, IFNε, IFNκ and IFNω (186, 187), IFNα and IFNβ being the most well studied. Almost every cell type produces type I IFNs, including leukocytes, fibroblasts, epithelial cells and endothelial cells. Plasmacytoid DCs, specifically, produce large quantities of IFNα (188). On the other hand, IFNβ is more widely produced by epithelial cells and fibroblasts (187) and this source of IFNβ will be particularly important in later sections. The signaling pathways that result in the induction of type I IFNs vary depending on the stimulus and cell type involved, however the ultimate effect is a potent biphasic IFN response.

The first wave of type I IFN is initiated by engagement of PRRs with their cognate PAMP, leading to activation of common signaling molecules TNF receptor-associated factor 3 (TRAF3), transcription factors IRF3, IRF7, and NF-κB. Dimerized IRF3 and IRF7, along with NF-κB, subsequently translocate to the nucleus, wherein they bind to the promoter regions of IFNα (189, 190) and IFNβ (191-193) genes. The primary

induction of type I IFNs consequently results in a positive feedback loop, thus potentiating further transcription of type I IFNs, primarily IFNα subtypes. This positive feedback loops constitutes the second wave of the type I IFN response. Although both IRF3 and IRF7 are important for a potent IFN response, IRF3 mainly commands the first wave and IRF7 is the driving force behind the second wave. Interestingly, IRF7 is IFN-inducible, thus adding to the positive feedback of IFN production. Both IFNα and IFNβ then bind to the IFNα receptor (IFNAR), which is composed of two subunits, IFNAR1 and IFNAR2. IFNAR engagement leads to the canonical type I IFN signaling pathway, described over 25 years ago, and results in activation of receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (187). Activated JAK1 and TYK2 then phosphorylate cytoplasmic transcription factors signal transducer and activator or transcription 1 (STAT1) and STAT2. Tyrosine-phosphorylated STAT1 and STAT2 heterodimerize, translocate to the nucleus and interact with IRF9 to form the trimeric complex known as the IFN-stimulated gene factor 3 (ISGF3) complex. The ISGF3 complex binds to specific DNA sequences called IFN-stimulated response elements (ISREs), thereby activating the transcription of a host of several hundred antiviral factors deemed IFN-stimulated genes (ISGs). These ISG-encoded proteins establish an antiviral state by inhibiting a multitude of processes employed by pathogens including, but not restricted to viral transcription, translation and replication (194, 195).

The type II family of IFNs includes IFNγ as the sole member, but is absolutely critical in shaping and facilitating a productive adaptive immune response to infection. In contrast to type I IFNs, IFNγ is produced by a more limited subset of cells including T cells in the adaptive phase, and NK cells during the innate phase. IFNγ is not directly induced by pathogen detection; it is instead part of the second wave of the IFN response and is induced in response to receptor activation via APCs or by a number of cytokines such as IL-12, IL-15, IL-18, TNFα and IFNα/β (184). Similar to the type I IFN receptor, the cognate IFNγ receptor (IFNGR) is also a heterodimeric receptor consisting of IFNGR1 and IFNGR2. Binding of the IFNGR with IFNγ leads to downstream signaling via the JAK-STAT pathway. Phosphorylated STAT1 dimerizes, translocates to the nucleus and drives transcription of target genes by binding to specific IFNγ activated sequences (GAS) within the promoter region. Binding to these GAS elements requires the cooperation of a number of transcription factors including NF-κB, nuclear factor activating transcription (NFAT), T-bet and many others (184).

Type III IFNs constitute the third and final member of the IFN family. The cytokines that comprise IFNs are IFNλ1, IFNλ2 and IFNλ3, also known as IL-29, IL-28A, IL-28B, respectively. Similar to type I IFNs, type III IFNs also signal through the same intracellular pathway and play a role in antiviral activity, although the receptor complexes are distinct. Since this family of IFNs was recently discovered in in early 2003, there is still much to be uncovered about the role of type III IFNs in antiviral defense (196).

All three types of IFNs trigger a distinct and partially overlapping pattern of ISGs. Many ISGs encode pattern-recognition receptors to further detect an ensuing viral infection, or transcription factors resulting in an amplified IFN response that could ultimately limit viral replication. Some ISGs, in fact, can have direct antiviral activities that result in the induction of apoptosis, inhibition of viral transcription, degradation of viral RNA and inhibition of protein translation. A few examples of the antiviral ISG-encoded products are protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS) and RNase L, Mx proteins, and viperin. PKR is a protein kinase that is responsible for responding to environmental stresses in order to regulate protein synthesis (197). Upon direct detection of viral RNA, PKR exerts its antiviral function by phosphorylating eukaryotic translation initiation factor 2α (EIF2α) thus halting protein translation, including translation of viral proteins. Foreign RNA is also recognized by OAS and RNase L, which work in combination with one another to mediate degradation of viral RNA. Viral RNA is detected by OAS, leading to the synthesis of 2'5-oligoadenylates, which in turn act as second messengers to activate RNase L (198). Activation of RNaseL results in indiscriminate cleavage of host and viral RNAs. The Mx family of proteins consists of GTPases and so far, of the human Mx proteins, only Mx1 (also known as MxA) has been shown to have antiviral activity. The main target of Mx proteins is viral nucleocapsid-like structures (197). MxA functions by self-oligomerizing and forming ring-like structures to physically surround and trap viral nucleocapsid structures. It is thought that the process of self-oligomerization stimulates the GTPase activity, and potentially redirecting the viral targets for degradation (198). Viperin (virus inhibitory protein, ER-associated, IFN-inducible) is unique in that it has been shown to have various

modes of antiviral action. Viperin can inhibit HIV-1 and influenza A virus by interfering with virus budding. Additionally, viperin is located within lipid droplets, thus allowing it to inhibit RNA replication during HCV infection, which occurs in membranous webs that are also closely associated with lipid droplets (198). These ISGs are just a few among the hundreds of gene products downstream of IFN signaling that contribute to antiviral defense.

2. Interferon Regulatory Factors

The interferon regulator factor (IRF) family of transcription factors was originally identified as inducers of type I IFNs and are now known to mediate the expression of various other genes involved in immunity and oncogenesis (148). The human IRF family is comprised of nine members, IRF-1-9, and each member contains a very well conserved DNA binding domain at the N-terminus of the protein. This region is responsible for binding to ISRE sequences in the promoter regions of genes encoding type I IFNs, IFN-responsive genes, and other genes involved in immunity and oncogenesis (148). The C-terminal end of IRFs, with the exception of IRF-1 and IRF-2, contains protein-binding domains and has been proposed to also contain regulatory properties within this domain. Although all of the IRFs are important in eliciting an immune response, IRF-1, IRF-3, IRF-5 and IRF-7 have been associated with positive regulation of the transcription of type I IFN genes (148).

IRF-1 was the first IRF family member identified to activate the promoter region of type I IFNs (199). IRF-3 and IRF-7 have since been appreciated as the key regulators of

type I IFN induction in response to viral infections (148, 200). IRF-3 is constitutively expressed in most cell types, whereas IRF-7 expression is mediated by type I IFNs in the second wave of the IFN response. Both IRF-3 and IRF-7 reside in the cytoplasm in an inactive state, until they are phosphorylated by IκB kinase (IKK)-related kinases, Tank binding kinase-1 (TBK-1) and IKK-ε. These kinases act in response to PRR ligation and signaling following virus infection. Phosphorylation occurs at specific serine residues at the C-terminal, regulatory region of IRF-3 and IRF-7. Phosphorylated IRF-3 and IRF-7 form homodimers or heterodimers with one another, wherein the dimers are translocated into the nucleus and have differential effects on type I IFN transcription (148). IRF-3 and IRF-7 are not equal in terms of which type I interferons they can induce. IRF-3 is a strong activator of the IFN-β genes, and only the IFN-α4 gene, whereas IRF-7 can activate both IFN-β and IFN-α genes (148).

The initial induction of IFN-β in response to a virus infection is led by IRF-3. IRF-3 is particularly potent due to its constitutive expression in a vast number of cell types. Upon phosphorylation, dimerization and translocation into the nucleus, IRF-3 is then able to bind transcriptional coactivator protein, CBP (cyclin-AMP-responsive-element-binding protein (CREB)-binding protein) or p300, forming a holocomplex within the nucleus. The IFN-β promoters contain 4 regulatory *cis* element called positive regulatory domains (PRDI, PRDII, PRDIII and PRDIV) and IRF-3 binds to two regions that share similar sequences to ISREs, PRDI and PRDIII. The holocomplex then binds to PRDI-III and alters the DNA structure due to the histone acetyltransferase activity of the coactivators. For efficient transcription of target genes including IFN-β, the

enhanceosome, a complex of proteins that assembles at the IFN-β promoter, is formed via the recruitment of transcription factors in addition to IRF-3 such as NF-κB and ATF-2/c-Jun (148), which are also activated downstream of PRR signaling.

Constitutive expression of IRF-7, on the other hand, is limited to lymphoid cells and pDCs, however it is strongly inducible by type I IFN-mediated signaling in many other cell types. Unlike IRF-3, IRF-7 can activate transcription of both IFN-β and IFN-α subtypes and does not require an interaction with CBP, p300 or transcription factor, NF-κB (201-203). Due to it induction after the first wave of type I IFNs, IRF-7 is thought to be important for the propagation of the late phase of the IFN response. Because IRF-7 can be induced by IFN in most cell types, IRF-7 plays a significant role in further amplifying the IFN response to viral infection by activating transcription of important IFN-α subtypes (148, 203, 204).

Other IRF family members that are important for facilitating a robust virus-induced innate immune response are IRF-5 and IRF-9, which act rather distinctly. Although a role for IRF-5 in the induction of type I IFNs has not been found, it has been implicated in the upregulation of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 (148, 205). IRF-5 acts downstream of TLR-4, TLR-5, TLR-7, and TLR-9 stimulation and likely interacts with NF-κB for efficient induction of IL-6 and TNF-α (205). Following TLR-9 ligation, IRF-5 has been shown to bind the ISRE sequence within the promoter of IL-12p40, however the induction of other proinflammatory cytokines via IRF-5 remains to be known (148, 206). IRF-9 is vital in the amplification of the IFN response and acts

downstream of the IFNα/β receptor. Originally discovered as the DNA-binding subunit of ISGF3γ, IRF-9 complexes with STAT1 and STAT2 to drive transcription of ISGs to further potentiate the IFN response (207, 208).

Induction of the type I IFN response is critical for mounting an effective immune response against viral infections. The IRFs as a whole play important and varying roles in regulating the transcription of cytokines/chemokines in response to invading stimuli. The IRFs act distinctly depending on the cell type in which they are expressed and their expression and regulation of the innate immune response is vital for shaping the adaptive immune response.

V. Mechanisms of Immune Evasion

A. General Strategies

In order for herpesviruses to effectively establish a lifelong, persistent infection within a host, they must have mechanisms in place to overcome immune recognition. The coevolution between humans and their herpesviruses has indeed resulted in herpesviruses evolution of successful mechanisms of immune evasion (209). Herpesviruses have a multitude of evasion strategies that target both the innate and adaptive arms of immunity and they include inhibition of the IFN response, inhibition of chemokines and cytokines, inhibition of lymphocyte function via receptor targeting, and finally, inhibition of antigen processing and presentation. The primary immunomodulatory mechanism that will be the focus of this thesis is the inhibition of the IFN response during herpesvirus infection.

B. Evasion of IFN Response during Herpesvirus Infection

1. Inhibition of the IFN response during HSV, VZV, CMV, and EBV infection

Recognition by the innate immune system and the subsequent antiviral response is an important hurdle to overcome as a herpesvirus. Once this is overcome, the virus has a much better chance of establishing a productive infection, as well as a persistent infection for the lifetime of the host. Since the host IFN response is an immediate responder to an infection, herpesviruses must in turn have rapid mechanisms for its evasion or tolerance. In order to respond quickly, herpesviruses encode several immediate early (IE) proteins that act at several points in the IFN signaling pathway and in some cases herpesviruses even prepackage immunomodulatory proteins within the virion particles to ensure fast-acting IFN evasion (209). These viral immunomodulatory proteins impede not only with pathogen detection mechanisms but also with signaling cascades leading to IFN production such as IRF-3 signaling, and also downstream IFN signaling and transcription of ISGs.

HSV-1 encodes several IE proteins that serve to prevent the transcription of IFN-β and do this by targeting multiple cellular proteins involved in this pathway. ICP-0 accelerates the degradation of IRF-3 in the cytoplasm, thereby affecting its nuclear accumulation and ultimately disrupting the induction of type I IFNs (210). Furthermore, ICP-0 can sequester activated IRF-3 and CBP/p300, keeping them from activating IFNβ transcription (211). Similarly, ICP-27 is thought to target IRF-3 and NF-κB in order to downmodulate the IFN response. Although not an IE gene, ICP34.5 forms a complex

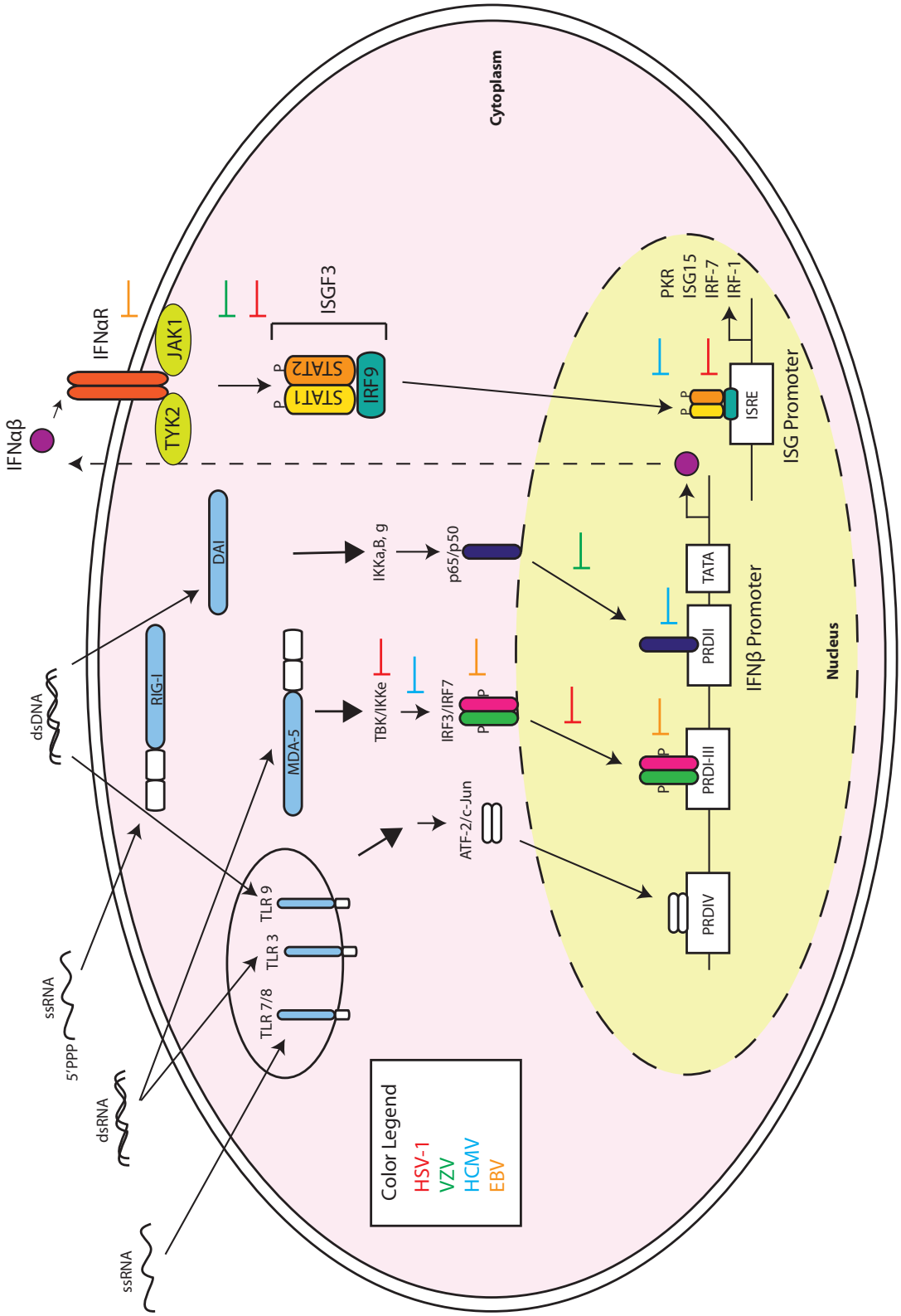
with TBK, and in doing so prevents the interaction with and phosphorylation of IRF-3 (212).

VZV also has mechanisms to avoid immune surveillance. IE-62, an abundant component of the virion tegument inhibits phosphorylation of IRF-3, although the mechanism of inhibition has not been elucidated (213). Additionally, VZV inhibits NF-κB activation by sequestering it in the cytoplasm, therefore preventing its nuclear accumulation and further activation of target genes important for an innate immune response (214). Similarly, CMV, aside from being a master inhibitor of antigen presentation, also targets IFN production by inhibiting NF-κB activity via IE-86. Consequently, expression of chemokines such as RANTES, MIP-1α and IL-8 is blocked as well as IFN-β(215).

EBV targets both IRF-3 and IRF-7 in order to inhibit the type I IFN response. IE protein, BZLF-1, directly interacts with IRF-7 to prevent transcription of IRF-7 targeted genes (216). In addition, LF2, a viral tegument protein, also binds to IRF-7 preventing dimerization and subsequent IFN-α expression (217). Also, viral kinase BGLF4 interacts with IRF-3, but does not interfere with phosphorylation, dimerization, nuclear accumulation or CBP recruitment. Instead, BGLF4 prevents IRF-3 from binding to IRF-3-specific promoter regions (218). Furthermore, EBV protein BRLF1 downregulates transcription of IRF-3 and IRF-7 (219), adding to the various mechanisms to counter the IFN response.

Figure 1.3. Herpesvirus inhibition of PRR-Induced Signaling and IFN-Induced Signaling.

An overview of the inhibition of the IFN signaling pathway by herpesviruses. Herpesviruses encode a multitude of proteins that interfere with both PRR-induced signaling and IFN-induced signaling. Cellular targets are identified with colored bars indicated in the color legend. HSV-1, herpes simplex virus 1; VZV, varicella-zoster virus; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus.


Cytoplasm
IFNαR
JAK1
TYK2
IFNαβ
ISGF3
STAT2
STAT1
IRF9
PKR
ISG15
IRF-7
IRF-1
ISRE
ISG Promoter
DAI
IKKa,B, g
p65/p50
RIG-I
MDA-5
TBK/IKKe
IRF3/IRF7
TATA
PRDII
PRDI-III
PRDIV
IFNβ Promoter
Nucleus
ATF-2/c-Jun
TLR 9
TLR 3
TLR 7/8
dsDNA
ssRNA
5'PPP
dsRNA
ssRNA
Color Legend
HSV-1
VZV
HCMV
EBV

2. Inhibition of the IFN response during KSHV infection

A growing body of evidence shows that KSHV is detected by a number of PRRs, including TLR3 (182), TLR4 (167), TLR9 (157). Because of this, KSHV has the potential to induce a robust innate immune response. As seen with other herpesviruses previously mentioned, KSHV also encodes immune evasion mechanisms in place specifically for the subversion of the IFN response. Of particular interest for this thesis are the vIRFs, which will be discussed further in a later section.

Besides the vIRFs, KSHV encodes other proteins that participate in regulating the IFN-mediated antiviral response. As previously discussed, the ability for a fast-acting evasion mechanism can be beneficial in establishing a productive infection. KSHV makes use of replication and transcription activator (RTA/ORF50) and virion-associated protein, ORF45, for quickly inhibiting parts of IFN induction as they are both present during lytic infection (108). RTA/ORF50 binds IRF7 and inhibits transcription of IFN-α and IFN-β. Inhibition occurs via RTA/ORF50-mediated ubiquitylation of IRF7, thus leading to its degradation (220). Virion-associated ORF45, on the other hand, interacts with IRF7 and prevents its phosphorylation and consequentially, its nuclear accumulation and ability to promote transcription of type I IFNs (12, 221). Another modulator of the IFN response is KSHV basic-region leucine zipper protein (K-bZIP/ORF K8). This protein interferes with IRF3-mediated transcription of type I IFNs by binding to the PRDI/III region of the IFN-β promoter, which is typically the site for IRF-3 binding (222). This, in turn, blocks IRF-3 DNA binding and prevents IFN-β transcription. Interestingly, K-bZIP also interacts with and represses p53, thus blocking apoptosis (223), making K-bZIP a multifunctional

protein, which is a recurring theme with other immunomodulators such as the vIRFs. More recently, an early KSHV protein, regulator of IFN function (RIF/ORF 10) has been shown to form complexes with JAK1, TYK2, STAT and both IFNAR subunits, resulting in an inhibition of kinase activities of JAK1 and TYK2 (224). This ultimately interferes with the downstream signaling otherwise initiated by type I IFNs. Furthermore, another tegument protein, ORF64, suppresses RIG-I-mediated IFN induction by deubiquitinating RIG-I and preventing it from interacting with downstream signaling partners (225).

Although KSHV has been a main focus of research for type I IFN evasion by gammaherpesviruses, another gammaherpesvirus, murine-γ-herpesvirus 68 (MHV68), does have a few mechanisms in place for evasion of the type I IFN response. Most recently, it has been shown that MHV-68 ORF36 interacts with IRF3 within the nucleus, thus preventing effective IFN-β transcription. Furthermore, ORF36 inhibits IRF3-mediated recruitment of the CBP/p300 complex in a dose-dependent manner (226). Interesting, however, MHV68 does not encode viral interferon regulatory factors, making KSHV and RRV unique in their approach to subverting the IFN response.

Subversion of the type I IFN response is critical for the establishment of a primary infection following viral entry. Additionally, later stages in viral assembly and egress benefit from a damped IFN response. Type I IFNs are important not only for shaping the adaptive immune response, but are also vital to potentiate antiviral defense via ISGs. Therefore, these IFN evasion mechanisms employed by gammaherpesviruses are also important for dampening the antiviral defense systems within a cell, which could benefit

the virus both in establishment of infection and in propagation of infection.

Figure 1.4. KSHV inhibition of PRR-Induced Signaling and IFN-Induced Signaling. KSHV stimulates TLR3, TLR4 and TLR9 leading to activation of transcription factors IRF-3 and IRF-7 and downstream induction of type I IFNs. Shown in red are KSHV-encoded viral proteins that inhibit PRR-induced signaling and IFN-induced signaling. vIRF, viral interferon regulatory factor; RTA, replication and transcription activator; K-bZIP, KSHV basic leucine zipper protein; RIF, regulator of IFN function; vIL-6, viral interleukin 6.

KSHV
TLR 4
TLR 9
TLR 3
dsDNA
Cytoplasmic sensor
IFNαβ
IFNαR
JAK1
TYK2
RIF
ISGF3
STAT1
STAT2
IRF9
vIRF2
Cytoplasm
IKKa,B, g
p65/p50
TBK/IKKe
ORF45
RTA
IRF3/IRF7
vIRF3
ATF-2/c-Jun
vIRF2
vIRF1
K-bZIP
PRDIV
PRDI-III
PRDII
TATA
IFNβ Promoter
Nucleus
ISRE
vIRF1
vIL-6
PKR
ISG15
IRF-7
IRF-1
ISG Promoter

VI. Viral Interferon Regulatory Factors

A. KSHV vIRFs

1. vIRF-1

KSHV vIRF-1 was the first vIRF found to repress cellular IFN responses (227, 228). Encoded by ORF K9, vIRF-1 is a 449-amino acid protein with approximately 13% amino acid identity to cellular IRF-3. Although the percent amino acid identity is low, vIRF-1 contains a conserved region that is derived from the IRF family of proteins. This region includes an N-terminal tryptophan-repeat region that confers DNA binding capabilities. The DNA binding region in vIRF-1, however, only includes 2 of the 5 tryptophan residues needed for binding to DNA and driving transcription (229). KSHV vIRF-1 is considered to be an early gene and is detected in latently infected PEL cell lines as well as KS lesions, albeit at low levels (230, 231). Upon induction of KSHV lytic replication in PEL cells, vIRF-1 is detected at much higher levels (229, 232) and is localized to the nucleus (233). Due to the expression patterns of vIRF-1 during lytic and latent KSHV infection, it was thought to have functions critical for KSHV infection and potentially pathogenesis.

Because vIRF-1 exhibits homology to IRF family members, it was thought to regulate transcription in response to IFN and in response to IRF-1. In fact, vIRF-1 inhibits multiple points along the IFN response pathway including inhibition of virus-induced IFN transcription (234, 235) and IFN-induced transcription (227, 228, 236). Specifically, vIRF-1 impairs IRF-1 (228, 235, 236) and IRF-3 (235) mediated transcription. Although inhibition of transcription does occur via vIRF-1, it is not accomplished by direct binding

to ISRE or PRD elements of the IFN-β promoter (228, 234-236). Furthermore, vIRF-1 does not prevent cellular IRFs from binding to DNA, even though several studies have shown vIRF-1 to interact with IRF-1 (235), IRF-2 (235), IRF-3 (234, 235), and IRF-7 (234). Instead, vIRF-1 obstructs interactions between IRF-1 and other cellular proteins such as transcriptional coactivators, CBP and p300. The association with p300 prevents proper formation of IRF-3/CBP/p300 complexes and also interferes with the histone acetyltransferase (HAT) activity of p300, thus inhibiting activation of IFN transcription (234, 237). Interestingly, vIRF-1 binding to p300/CBP to prevent IRF-1 binding is further supported by the lack of inhibition on IRF-7 mediated transcription, which acts independently of p300/CBP in order to activate transcription of IFN genes (234). Since p300 is not limited to interactions with IRF-3, a vIRF-1/p300 interaction may possibly have an effect on the transcription of other cellular genes as well. In fact, vIRF-1, through interactions with p300/CBP has been suggested to prevent basal transcription of MHC-I (85), thereby affecting both NK cells and CD8 T cells. Even though vIRF-1 does not act through DNA binding, one study has demonstrated an exception. It was found that vIRF-1 is able to bind the promoter regions of MIR1 (ORF K3), vIL-6 (ORF K2) and viral dihydrofolate reductase (vDHFR, ORF 2), potentially activating transcription of those viral genes (238). Additionally, vIRF-1 has been found to downregulate TLR-4 transcription as well as surface levels of TLR-4 (167). Not only are cells that lack TLR-4 more susceptible to KSHV infection, but patients that are HIV-infected and contain a mutant TLR-4 allele, are more likely to develop MCD (167), suggesting another important role for vIRF-1 during infection.

vIRF-1 also has oncogenic properties and interferes with apoptosis (239). Expression of vIRF-1 in NIH3T3 cells causes transformation of the cells and causes fibrosarcoma in nude mice (227, 240). KSHV vIRF-1 inhibits apoptosis through multiple mechanisms. First, vIRF-1 interacts with ATM kinase to block its activity and prevent phosphorylation of p53, which in turn increases ubiquitination of p53 and its subsequent degradation (241). Additionally, vIRF-1 directly interacts with p53, preventing acetylation and transcriptional activation of p53 (242, 243). KSHV vIRF-1 also interacts with the protein for retinoid-IFN-induced mortality 19 (GRIM19), both *in vitro* and *in vivo*, inhibiting GRIM19-induced apoptosis (244). Bim, a pro-apoptotic factor induced by cell stress that acts at the mitochondrial membrane, is targeted by vIRF-1 and sequestered in the nucleus as a means of inhibition (245). Furthermore, vIRF-1 inhibits TGF-β-induced apoptosis by specifically targeting tumor suppressor proteins, SMAD3 and SMAD4, which are also transcription factors. These transcription factors normally block transcription of oncogene, c-myc, however vIRF-1 inhibition of SMAD3/4 leads to its production (244). It is evident that vIRF-1 is a multifunctional protein, both in inhibiting the IFN response as well as impeding with cellular apoptosis, likely making it an important viral factor for KSHV infection and pathogenesis.

2. vIRF-2

KSHV vIRF-2 is constitutively expressed and is readily detected in PEL cells and can be induced during lytic replication as well. KSHV vIRF-2 shares 40% amino acid identity to vIRF-1 in the amino-terminal region (246). The KSHV vIRF-2 gene encodes a 2.2 kb spliced transcript representing two exons of ORFs K11.1 and K11 (230). This full-length

vIRF-2 inhibits both IFN-α and IFN-γ driven transcription (247). Specifically, vIRF-2 targets IRF-1, IRF-3, and IFN-stimulated gene factor 3 (ISGF3) mediated transcription for inhibition, as seen in reporter assays transiently expressing vIRF-2 (247). Targeting IRF-3, but not IRF-7, facilitated inhibition of type I IFN transcription, similar to what was observed with vIRF-1. It is thought that the vIRF-2 possesses pleiotropic activity as it can inhibit both early and late type I IFN responses (83).

The underlying mechanism of inhibition employed by vIRF-2 has yet to be defined. However, vIRF-2 can accelerate the degradation of IRF-3 in a caspase-dependent manner (248). This degradation therefore represses IRF-3-mediated transcription of IFN-β. Some of the first studies conducted on vIRF-2, were on the first exon of vIRF-2 (K11.1) or vIRF-2x1 (246, 249, 250). KSHV vIRF-2x1 encodes a 20 kDa protein and primarily localizes to the nucleus (249). It was found to interact with IRF-1, IRF-2, IRF-8, RelA and p300, and any of these interactions could be potentially important for the inhibition of the IFN response. Additionally, the same group demonstrated an interaction between vIRF-2x1 and PKR, an inducible ISG (249). PKR is an important antiviral factor and it acts by blocking protein synthesis during viral infection. Inhibition of PKR by vIRF-2x1 lifts the block, thus allowing for production of viral proteins. Furthermore, vIRF-2x1 was found to inhibit activation-induced cell death (AICD) via downregulation of CD95L, a potent inducer of cell death. Therefore, similar to vIRF-1, vIRF-2 possesses anti-apoptotic functions in addition to inhibiting the IFN response. Interestingly, the expression pattern of vIRF-2 and vIRF-2x1 remains controversial. Several groups were unable to detect the 0.6 kb transcript that corresponds to vIRF-2x1 in PEL cells

stimulated for lytic replication as well as from unstimulated PEL cells (230, 246, 251). However, vIRF-2x1 was detected by western blot analysis in KSHV-positive lymphomas (249). This discrepancy raises the issue of whether vIRF-2x1 is relevant during KSHV infection, if not, then the functions credited to vIRF-2x1 discussed previously may be not be pertinent.

3. vIRF-3

KSHV vIRF-3 produces a 73 kDa protein, which is a 1.7 kb spliced product of ORFs K10.5 and K10.6 (230, 252). The amino terminal region of vIRF-3 has a high degree of amino acid identity to vIRF-2, approximately 25%. KSHV vIRF-3 also shares significant sequence homology to lymphoid-cell specific IRF-4. Additionally, vIRF-3 contains 4 of the 5 tryptophan residues required for DNA binding, however only 2 of these residues are in the same configuration of those in the DNA binding domain of cellular IRFs (252). KSHV vIRF-3 is constitutively expressed in PEL cells as well as in MCD tumors, however it is not present in KS spindle cells (253). Based on its constitutive expression during latency and its nuclear localization, vIRF-3 has also been deemed latency-associated nuclear antigen 2 (LANA2) (253).

Similar to the other KSHV vIRFs described, vIRF-3 is also a multifunctional protein. Unlike vIRF-1 and vIRF-2, however, vIRF-3 has the ability to specifically interact with IRF-7, thus inhibiting the DNA-binding capabilities of IRF-7 and ultimately repressing transcription of IFN-α (254). This function is unique to vIRF-3, and is relevant because of its expression in hematopoetic cells, which also happens to be the primary site of IRF-

7 expression (148, 253). In addition to inhibiting IRF-7 mediated transcription, vIRF-3 also interferes with IRF-3 (252), IRF-5 (255, 256) and NF-κB mediated transcription (257), providing this immunomodulator with a number of targets for repression of the type I IFN response. Contrary to the aforementioned studies, another group demonstrated that vIRF-3 can stimulate the transcriptional activity of IRF-3 and IRF-7 (258). This study was performed in the absence of KSHV infection and was instead carried out following Sendai Virus infection. Furthermore, the cell culture systems of these studies were also different. The disparate infection and cell culture systems could explain the contrasting results of these studies. KSHV vIRF-3 could act differently depending on the cell type in which it is expressed. Also, vIRF-3 could be targeting a KSHV protein for indirect ablation of the IFN response, as an inhibitory phenotype was observed in the context of KSHV infection. Interestingly, vIL-6 contains two ISRE-like elements that can be activated by type I IFNs, thus providing a potential target for vIRF-3, which could result in activation of vIL-6 and B cell proliferation (76). This scenario, however, has yet to be examined.

Aside from its functions as a modulator of type I IFNs, vIRF-3 has also displayed anti-apoptotic characteristics. KSHV vIRF-3 inhibits the pro-apoptotic function of IRF-5 (256), p53 (253) and promotes expression of c-myc (259). Additionally, was shown to be required for the survival of KSHV-infected PEL cells, thus supporting its oncogenic properties (260). Finally, vIRF-3 also downmodulates MHCII in KSHV-infected PEL cells (261, 262). The decreased surface levels of MHCII in vIRF-3-expressing PEL cells

results in a resistance to recognition by KSHV-specific CD4 T cells (262), which could have major implications for vIRF-3 in oncogenesis and KSHV pathogenesis.

4. vIRF-4

The most recently characterized member of the vIRF family is vIRF-4. KSHV vIRF-4 is a 2.9 kb spliced transcript from ORFs K10 and K10.1 (230). Very little is understood about vIRF-4, but it is known to be induced upon lytic replication of KSHV (230). KSHV vIRF-4 may not regulate the IFN antiviral response, but it has been shown to interact with a number of cellular proteins. One such protein poly(A)-binding protein (PABP) (263), however the significance of this interaction has yet to be dissected. KSHV vIRF-4 also interacts with MDM2, a negative regulator of p53, causing enhanced ubiquitination and degradation of p53 and subsequently, decreased levels of apoptosis (264). Furthermore, CSL/CBF1 has been shown to be a binding partner of vIRF-4 (265). CSL/CBF1 is a transcription factor downstream of Notch signaling, a signal transduction pathway that regulates cellular developmental processes and promotes survival of latently infected, KSHV-positive cells (266). The separate interactions between vIRF-4 and MDM2 and CSL/CBF1 imply a role for vIRF-4 in the progression of tumorigenesis. Most recently, vIRF-4 has been shown as a positive coregulator for RTA and potentially a regulator of KSHV reactivation (267).

B. RRV vIRFs

1. Identification and Comparison to KSHV vIRFs

Similar to KSHV, RRV encodes a cluster of genes (ORFs R6-R13) in between ORFs 57 and 58 that correspond to the vIRFs (123, 126). RRV, however, encodes eight vIRFs whereas KSHV encodes four. The vIRF sequences contained in the RRV genome share the highest similarity to that of KSHV vIRF-1, specifically R6, R7, R8, R10 and R11, which share between 26% and 30% similarity. Interestingly, there is no measureable similarity between the RRV vIRFs and other KSHV vIRFs besides vIRF-1 (123). As with the KSHV vIRFs, the eight RRV vIRFs do not contain the conserved tryptophan pentad repeat region found in the DNA binding domain of mammalian IRF proteins, suggesting that most of the RRV vIRFs do not possess DNA binding capabilities (123). Because of this, it is hypothesized that the RRV vIRFs function in a manner that does not involve binding to specific elements within promoter regions of target genes. Further analysis revealed a high degree of similarity between the R6-R9 cluster and the R10-R13 cluster of genes, with similarities falling between 50-62% (123). This suggests a gene duplication event in which an original block of 4 vIRFs was entirely duplicated to result in the final 8 vIRFs encoded by RRV. It is unclear however, which block of 4 vIRFs was duplicated, R6-R9 or R10-R13, and also whether this resulted in functional redundancies between vIRFs. It is important to note that of the 5 vIRFs that share similarity to KSHV vIRF-1, two are the most closely related to vIRF-1, R6 and R10, of which R6 will be the main focus of this thesis.

2. RRV vIRFs in vivo and in vitro

Recent generation of a recombinant RRV devoid of all eight vIRFs has provided a great deal of insight into the contributions of the vIRFs during infection, both *in vivo* and

in vitro (141). Analysis of this mutant virus *in vivo* in infected RMs revealed that the lack of vIRF expression results not only in decreased viral loads and lower levels of both lytic and persistent virus, but also results in earlier and sustained production of proinflammatory cytokines (140). Additionally, the loss of the vIRFs caused an earlier induction of T cell responses, both CD4 and CD8 T cells, and general decrease in B cell hyperplasia, providing strong evidence that the RRV vIRFs contribute to viral pathogenesis and disease progression (140). *In vitro* studies corroborated the data generated *in vivo*. RRV infection in the absence of the vIRFs leads to earlier induction of both type I and type II IFNs (141). Looking more closely at the pathway leading up to the induction of type I IFNs, the lack of vIRFs results in a more rapid nuclear accumulation of IRF-3, as compared to WT RRV infection, indicating that the vIRFs, one or all, are responsible for delaying the accumulation of phosphorylated IRF-3 in the nucleus, which in turn is bound to have a downstream effect on type I IFN production (141). Interestingly, this delay in pIRF-3 nuclear accumulation occurs between 0 and 6 h post-infection, suggesting a very rapid inhibition of IFN during WT RRV infection. Dissection of individual RRV vIRFs exposed vIRF R6 as the most potent inhibitor of IRF-3 mediated transcription of type I IFNs (141).

VII. Thesis Project

A. Summary and Rationale

A vast amount of work has been dedicated to dissecting the role and functions of the KSHV vIRFs. However, the inadequate cell culture systems for lytic replication of KSHV has been an impediment in understanding the role of these immunomodulators

during *de novo* infection (107). The effect of the RRV vIRFs on both the innate and adaptive arms of immunity has provided evidence to their involvement in viral pathogenesis and disease progression (140, 141). To understand the mechanism of inhibition of the type I IFN response by the vIRFs, we chose to further dissect the biochemical mechanism of vIRF R6, which was found to be the most potent inhibitor of IRF-3 mediated transcription of IFNs. With the use of ectopic expression of R6, in the absence of RRV infection, we were able to pinpoint interactions with relevant cellular proteins and specifically determine the function of R6 alone. Recent development of a recombinant RRV expressing R6 with a C-terminal HA tag allowed assessment of the role of R6 during *de novo* infection. Studying the roles of individual RRV vIRFs, in the context of *de novo* infection and exclusively expressed in a transient transfection system, will provide valuable insight into the KSHV vIRFs and their immunomodulatory mechanisms.

B. Author Contributions

All the work presented herein, with the exception of Supplemental Figure 1 (R.D.E), was performed by the author.

CHAPTER 2

A Rhesus Rhadinovirus Viral Interferon Regulatory Factor is Virion-associated and Inhibits the Early Interferon Antiviral Response

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ABSTRACT

The interferon (IFN) response is the earliest host immune response dedicated to combating viral infection. As such, viruses have evolved strategies to subvert this potent antiviral response. Two closely related gammaherpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV) and rhesus macaque rhadinovirus (RRV), are unique in that they express viral homologues to cellular interferon regulatory factors (IRFs), deemed viral IRFs (vIRFs), which are proteins that differentially regulate transcription and IFN signaling. Here, we demonstrate a strategy employed by RRV to ensure rapid inhibition of virus-induced type I IFN induction. We found that RRV vIRF R6, when expressed ectopically, interacts with transcriptional coactivator, CREB-binding protein (CBP), in the nucleus. As a result, phosphorylated IRF-3, an important transcriptional regulator in IFNβ transcription, fails to effectively bind to the IFNβ promoter, thus inhibiting the activation of IFNβ genes. In addition, we found R6 within RRV virion particles via immunoelectron microscopy and furthermore, that, virion-associated R6 is capable of inhibiting the type I IFN response by preventing efficient binding of IRF-3/CBP complexes to the IFNβ promoter in the context of infection. The work shown here is the first example of a vIRF being associated with either the KSHV or RRV virion. The presence of this immunomodulatory protein in the RRV virion provides the virus with an immediate mechanism to evade the host IFN response, thus enabling the virus to effectively establish an infection within the host.

INTRODUCTION

Activation of type I interferons (IFNs) is a fundamental component of a host's antiviral response. Type I IFNs (IFNα and β) are produced by virus-infected cells and are responsible for initiating the primary response again viral infection. IFNα is produced largely by plasmacytoid dendritic cells (pDCs) (188), whereas IFNβ is produced mainly by fibroblasts (268). The induction of IFNs is a tightly regulated process controlled by IFN-regulatory factors (IRFs). Within the broad family of IRFs, IRF-3 and -7 are specifically important for the induction of type I IFNs (204, 208). IRF-3 is constitutively expressed in all cell types, while IRF-7 is induced by type I IFNs. As a consequence of viral infection or treatment with dsRNA, inactive cytoplasmic IRF-3 is phosphorylated, wherein it forms a homodimer and is translocated to the nucleus(204, 268). Phosphorylated IRF-3 (pIRF-3) then interacts with the transcriptional coactivator CBP/p300, and binds the PRDI-III element of the IFNβ promoter thus inducing transcription. To effectively establish infection within a host, viruses have evolved a variety of mechanisms that specifically target cellular IRFs, induction of IFNs and downstream signaling pathways induced by IFNs (84, 146, 269).

Kaposi's Sarcoma (KS)-associated herpesvirus (KSHV) and rhesus rhadinovirus (RRV) are two closely related gamma-2-herpesviruses that are capable of inducing lymphoproliferative disorders in their respective immunocompromised hosts. Both KSHV and RRV encode multiple viral homologues to cellular proteins that play various roles in cellular processes such as apoptosis, cellular growth and differentiation, and immune signaling. These viral proteins have the ability to manipulate the host environment in a manner that maximizes conditions for efficient viral replication.

Particularly, these viral homologues, namely viral CD200 (vCD200), viral IL-6 (vIL-6), viral G protein coupled receptor (vGPCR) and the viral interferon regulatory factors (vIRFs), are critical modulators of the immune response and cell growth and also major players in disease pathogenesis (84). Although it is a common attribute of many viruses to encode genes that subvert the immune system, KSHV and RRV are unique in that they are the only two viruses known to encode vIRFs, which are homologous to cellular IRFs (32, 123, 126, 270). RRV encodes a cluster of 8 vIRFs, localized in the same genomic region as the 4 vIRFs found in KSHV (123, 126). The unique sequence homology shared between the vIRFs and cellular IRFs suggest a possible role for vIRFs in evasion of the IFN response. In fact, it has been shown that 3 of the KSHV vIRFs (vIRF-1, -2 and -3) disrupt functions of cellular IRFs, ultimately altering IFN induction as well as downstream signaling of IFN (227, 228, 234-236, 246, 247, 252, 254, 256, 258, 271, 272). Interestingly, each of these vIRFs has distinct functions in targeting particular elements of the IFN response. For example, KSHV vIRF-1, the most well studied of the IRFs, blocks transcription of type I IFNs in a variety of ways. KSHV vIRF-1 binds transcriptional coactivator proteins, CBP and p300, thus interfering with their binding and function (234, 235, 237, 273). Also, the interaction between vIRF-1 and p300 displaces CBP/p300-asssociated factor (pCAF), a protein that possesses histone acetyltransferase (HAT) activity, effectively inhibiting the HAT activity of p300, which is important for altering chromatin structure and making DNA available for transcription (237). Furthermore, vIRF-1 can displace IRF-3 from CBP/p300, thus inhibiting transcriptional activity of IRF-3 (234). KSHV vIRF-2 blocks both NF-κB and IRF-1-dependent IFNβ transcription (246) and has recently been found to target IFN-stimulated

gene factor 3 (ISGF3) as a means of inhibiting type I IFN signaling (272). Work on KSHV vIRF-3 has been less clear, as some studies have reported an increase in IFNα transcription and expression in the presence of vIRF-3 (258) and others have shown inhibition of IRF-7-mediated signal transduction (254) and decreased IFNα expression (252). Not only do KSHV vIRFs function to limit the IFN response, but they also play a significant role in the modulation of cell cycle and apoptosis. When expressed in NIH3T3 cells, vIRF-1 displayed oncogenic properties and this was further corroborated when vIRF-1-expressing cells formed tumors after injection into nude mice (227). Furthermore, KSHV vIRF-1, -3, and -4 all act as negative modulators of the p53 pathway, a vital pathway involved in the induction of cell cycle arrest or apoptosis and thus important in tumor suppression (241, 242, 253, 264, 273). Additionally, KSHV vIRF-2 downregulates CD95L expression in activated T cells, thus inhibiting activation-induced cell death (250) and providing evidence for yet another immunomodulatory function of the KSHV vIRFs. The aforementioned studies have provided a vast amount of insight into the molecular mechanisms of the KSHV vIRFs, albeit the majority of the work has been done in the absence of *de novo* KSHV infection. This is mainly due to the fact that *in vitro* replication of KSHV is particularly inefficient. RRV, however, displays robust lytic replication *in vitro* and with its 8 vIRFs that share homology to the KSHV vIRFs, RRV provides a suitable model for the study of the function of vIRFs early in *de novo* infection.

Recent studies have tested whether the RRV vIRFs are capable of antagonizing the function of cellular IRFs and IRF-mediated induction of IFN (140, 141). This was done via the construction of an RRV recombinant virus lacking all 8 vIRF open reading

frames (vIRF-ko RRV) (141), the backbone of which is derived from a bacterial artificial chromosome clone of wild type RRV_{17757} ($WT_{BAC}RRV_{17757}$) (128). Infection of rhesus macaques with vIRF-ko RRV resulted in lower viral loads and increased levels of plasma IFNα. Interestingly, although persistence was established in the absence of the RRV vIRFs, decreased levels of persistent virus was detected in B cells, along with decreased development of B cell hyperplasia (140). The decrease in B cell hyperplasia, however, could be due to lower amounts of circulating virus within these animals. *In vitro*, the vIRFs prevented early nuclear accumulation of phosphorylated IRF3 (pIRF3), the activated state of the transcription factor that prompts the induction of type I IFNs (141). No change was observed, however, in total IRF3 levels or in IRF3 dimerization (141), indicating that the mechanism of action of the RRV vIRFs occurs after IRF3 phosphorylation, potentially by interfering with nuclear accumulation of pIRF3 itself. Examination of individual RRV vIRFs in the absence of infection revealed a potential interaction between R6 and IRF3 and also identified R6 as an important inhibitor of IRF3-mediated transcription (141).

To further dissect the potential immunomodulatory role of R6, we examined its effect on the induction of IFN and the signaling cascade leading up to IFNβ transcription. Here we show a delay in IRF3-mediated transcription, in accordance with previous data (141), along with a decrease in the transcription of IFNβ. In terms of mechanism, we found that R6 competes with IRF3 for binding to CBP, resulting in a decrease of IRF3/CBP complexes present on the IFNβ promoter. Remarkably, we detected R6 in purified virus preparations as well as in the context of infection without protein

translation. Our results strongly suggest that R6 is a virion-associated protein that functions to inhibit early type I IFN responses in virus-infected cells.

MATERIALS AND METHODS

Cells, virus, plasmids, and drugs. Primary rhesus fibroblast (RFs) cells, telomerized rhesus fibroblast (tRF) cells, tRF-ISRE cells (274), and HEK 293T/17 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Ogden, UT). All cell culture incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2. Viruses used in these studies include wild-type BAC-derived RRV_{17577} ($WT_{BAC}RRV$) (128), as well as vIRF-ko RRV_{17577} (vIRF-ko RRV) (141) and a wild-type RRV with an HA tag at the C-terminus of the R6 ORF (R6HA RRV). All virus stocks were purified through a 30% sorbitol cushion and resuspended in PBS (unless otherwise stated), and titers in RFs were determined using standard plaque assay. Construction of plasmids, pcDNA3.1-R6HA and pcDNA3.1-R7HA, were described previously (141). Cyclohexamide (CHX; Sigma-Aldrich, St. Louis, MO) stock concentration (in ethanol) was 15 mg/ml, and the working concentration was 75 μg/ml. MG132 (Sigma) stock concentration (in DMSO) was 10 mM, and the working concentration was 5 μM. Cells were pre-treated with the drugs 2 h before infection or transfection, and the drug remained in the medium as the infection or transfection took place, unless otherwise indicated. Poly(IC) (Sigma) was resuspended in PBS and transfected into cells by using TransIT LT1 transfection reagent (Mirus, Madison, WI).

Purification of RRV virions. RFs were infected with the indicated virus at an MOI of 0.01 and allowed to progress to complete CPE. Supernatant and cells were collected and separated by centrifugation at 1,000 x *g* for 15 minutes at 4°C. The supernatant,

containing extracellular virus, and the pellet, containing intracellular and cell-associated virus, were processed separately. The extracellular virus-containing supernatant was further centrifugated at 12,000 x *g* for 1 h at 4°C, wherein samples were resuspended in DMEM and sonicated twice at 30 seconds each. The intracellular virus-containing pellet was freeze/thawed, sonicated 2X at 30 seconds each and centrifuged at 1,000 x *g* for 15 minutes at 4°C and the supernatant was saved for further processing. Supernatants containing either extracellular virus or intracellular virus were then pelleted through a 30% sorbitol cushion in a Beckman SW41 rotor at 18,000 rpm for 1 h at 4°C. Pellets were resuspended in 1 ml 1x PBS and layered on a 20 to 60% sorbitol step gradient and spun in a Beckman SW 41 rotor at 18,000 rpm for 2 h at 4°C. The virus band was collected at the 50-60% interface, which coincides with infectious virus as defined by plaque assay. The gradient purified preparation was diluted in 15 ml cold 1 mM Tris-HCl then pelleted by centrifugation in the SW41 rotor at 18,000 rpm for 50 minutes at 4°C. The virus pellet was then resuspended in Hank's balanced salt solution (HBSS) plus 2% FBS and stored at -80°C.

Plasmid transfections and virus infections of cell cultures. tRFs were transfected with the indicated expression plasmids using the TransIT-LT1 Transfection Reagent (Mirus Bio). Transfections proceeded for 40 h before transfection of poly(IC). For virus infection, a multiplicity of infection (MOI) of 2.5 was used for all virus infections. Infected cells or coverslips were collected at different time points post-infection for further analysis.

Immunoprecipitation (IP), SDS-PAGE analysis, and Western Blot. Cell lysates were immunoprecipitated with rabbit anti-CBP polyclonal antibody (pAb) (A-22; Santa Cruz Biotechnology, Santa Cruz, CA) in native lysis buffer (50 mM Tris-Cl [pH 8.0], 1% NP-40, and 150 mM NaCl supplemented with phosphatase inhibitors [100X cocktail; Sigma] and protease inhibitors [100X cocktail; Sigma]), followed by incubation with protein A/G Plus-agarose (Santa Cruz), and eluted in radioimmunoprecipitation assay (RIPA) buffer (native lysis buffer with 0.1% SDS and 0.5% sodium deoxycholate). Whole cell extracts were collected in RIPA buffer, nuclear and cytoplasmic lysates were collected according to kit protocols (NE-PER; Thermo Scientific). Samples were analyzed by Novex 4%-12% Tris-Bis Mini Gels (Life Technologies Inc, Carlsbad, CA), and proteins were then transferred onto a polyvinylodene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., Hercules, CA) via semidry transfer (30 min at 15 V at RT).

Membranes were blocked for 1 hour in TBS-T (Tris-buffered saline with 0.1% Tween-20) containing 5% Bovine Serum Albumin (BSA), and subsequently probed with the antibodies indicated below. The primary antibodies used in this study are: anti-HA pAb (Y-11) (Santa Cruz), anti-HA mAb (HA-7) (Sigma), anti-CBP pAb (A-22) (Santa Cruz), anti-IRF-3 (SL-12) (Santa Cruz), anti-human phosphor-IRF-3 (Ser396) (4D4G) (Cell Signaling Technology, Beverly, MA), anti-TBK (M-375) (Santa Cruz), anti-human poly(ADP-ribose) polymerase 1/2 (PARP1/2) pAb (H-250) (Santa Cruz), anti-GAPDH mAb (SC-51906) (Santa Cruz), and anti-RRV major capsid protein (MCP) mAb (Monoclonal antibody core, Vaccine & Gene Therapy Institute, Beaverton, OR). Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-

mouse IgG (Santa Cruz) and goat anti-rabbit IgG-HRP (Cell Signaling). Membranes were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence. Cells were grown on glass coverslips in 12-well plates and fixed with 4% paraformaldehyde in PBS (20 min at RT). Cells were then permeabilized and blocked in 5% normal goat serum (NGS)– 0.1% Triton X in PBS (PBST) (1 h at RT) prior to staining, and all subsequent steps were performed with 1% NGS–PBST. Cells on coverslips were stained with anti-human IRF-3 mAb (clone SL012.1) (BD Pharmingen, San Diego, CA) or anti-CBP pAb (A-22) (Santa Cruz) overnight at 4°C and subsequently stained with anti-mouse IgG-Texas Red (Vector Labs, Burlingame, CA) or anti-rabbit IgG-Texas Red (Vector Labs), respectively. Cells were then stained with anti-HA-fluorescein isothiocyanate (FITC) (HA-7) (Sigma) (2 h at RT), and nuclei and/or DNA was detected by using Hoechst 33258 dye. Cells on coverslips were mounted onto slides using Vectashield (Vector Labs) and examined on a Zeiss Axio Imager.M1 microscope (Zeiss Imaging Solutions, Thornwood, NY). Images were acquired by using a Zeiss Axiocam camera (MRm) with Axiovision software (version 4.6) and subsequently processed by using Adobe Photoshop (Adobe Systems, San Jose, CA).

RNA isolation and RT-PCR. RNA was isolated from tRF by using TRI Reagent (Sigma) and DNA endonuclease, RQ1, was used to remove DNA from RNA preparations (Promega, Fitchburg, WI) according to commercial kit protocols. Reverse transcription-PCR (RT-PCR) was performed by using Superscript III one-step RT-PCR with Platinum *Taq* (Life Technologies Inc, Carlsbad, CA). Transcripts were detected with the following

primers: IFNβ 5' primer (5'-GAC GCC GCA TTG ACC ATC TA-3'), IFNβ 3' primer (5'-CCT TAG GAT TTC CAC TCT GAC T-3'), GAPDH 5' primer (5'-GTG GAT ATT GTT GCC ATC AAT-3'), GAPDH 3' primer (5'-ATA CTT CTC ATG GTT CAC ACC-3'). All data was normalized to levels of GAPDH in each sample.

Construction of R6HA RRV. The construction of R6HA RRV was achieved by using the RRV BAC (141) in conjunction with the *galK* recombination system in *E.coli* SW105 cells(275). Initially, a BAC clone lacking R6 was generated by recombination with a *galK* cassette flanked by 50-bp arms homologous to the region outside of the R6 ORF (nucleotides [nt] 76216-80463), and a clone lacking R6 was identified. Next, a DNA cassette possessing C-terminal HA-tagged R6 and 50-bp flanking homology arms was cloned into pcDNA3.1(-) and sequenced. The HA-tagged R6 cassette, along with the homology arms, was excised from the expression vector and used to replace the *galK* cassette. GalK-negative recombinants were selected for resistance to 2-deoxygalactose in minimal media with glycerol as the sole carbon source. Individual R6HA RRV clones were analyzed via restriction digestion in conjunction with Southern blot analysis, and were subsequently analyzed via comparative genome hybridization (CGH) (NimbleGen Systems, Inc. Madison, WI), as described previously (128). Growth curve analysis (MOI = 2.5) revealed no difference between R6HA RRV and WT_{BAC} RRV.

Generation of Doxycycline-inducible stable cell line. The pLVX lentivirus vector system was utilized for constructing a stable doxycycline (Dox)-inducible cell line. The cell lines and vectors used in the construction of this cell line were obtained from Dr.

Victor DeFilippis (Vaccine & Gene Therapy Institute, Beaverton, OR). The pLVX-R6HA plasmid was constructed by subcloning full-length HA-tagged R6 from the pcDNA3.1(-) expression vector, described in reference (141), into the pLVX-Tight-Puro retroviral vector. Replication-defective recombinant retrovirus was produced by transfecting the retroviral vector into HEK 293T/17 cells along with a 2nd generation packaging system (packaging plasmid psPAX2 and envelope plasmid pMD2.g). Supernatant was harvested 48 h later, purified by centrifugation and filtered through a 0.45 μm filter to remove cell debris. Target cells (tRF cells containing a Dox-responsive transactivator [tRF-rtTA cells]) were exposed to the purified retrovirus for 3 h in the presence of Polybrene to facilitate infection and this process was repeated once more 48 h later. Once the cells reached confluence, they were grown in DMEM plus 10% Tet-free FBS containing 1.5 μg/ml puromycin (Sigma) and 150 μg/ml Hygromycin B (Fisher Scientific, Pittsburgh, PA). Cells were continually passaged in the presence of increasing concentrations of puromycin and Hygromycin B (3 μg/ml and 300 μg/ml, respectively) until cells were fully resistant. In order to determine the optimal concentration of Dox and duration of Dox treatment, cells were experimentally treated with Dox at varying concentrations and for various lengths of time. We found that 1 μg/ml of Dox for 24 h yielded the most protein expression.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared after transfection or infection according to kit protocols (NE-PER; Thermo). Equivalent amounts of nuclear extract (20 μg) were assayed for IRF3 and CBP binding in gel shift analysis using a 3' biotin labeled double stranded oligonucleotide corresponding to the

PRDI-PRDIII region of the IFNβ promoter: 5'-GAAAACTGAAAGGAGAACTGAAAGTG-3'. Biotin labeling was performed using a DNA 3' End Biotinylation Kit (Thermo). Complexes were formed by incubating the probe (final concentration of 20 fmol) with 20 μg of nuclear lysate in the presence or absence of the indicated antibodies. The binding reaction (20 μl) contained 2.5% glycerol, 5 mM $MgCl_2$, 0.05% NP-40 and 50 ng/μl poly(dI-dC) to reduce non-specific binding. To demonstrate the specificity of protein-DNA complex formation, 500-fold molar excess of unlabeled wild type oligonucleotide corresponding to the PRDI-PRDIII region of the IFNβ promoter was added before adding labeled probe, or preincubated with anti-CBP (H100) (Santa Cruz) or anti-IRF3 (SL-12) (Santa Cruz). After 20 minute incubation with probe, binding reactions were loaded on a Novex 6% DNA retardation gel (Life Technologies) and run for 1 h at 100 V. Samples were then transferred to a positively charged nylon membrane (Immobilon-Ny+) (Milipore, Billerica, MA) via semidry transfer (30 minutes at 15 V at RT) and detected using streptavidin-HRP chemiluminescence for biotin-labeled probes (LightShift Chemiluminescent EMSA kit, Thermo).

Luciferase Assay. IRF3-mediated transcription was measured by using tRF cells encoding the firefly luciferase gene, driven by the interferon-stimulated response element (ISRE) in the promoter (tRF-ISRE cells) generously provided by Dr. Victor DeFilippis. Firefly luciferase readings were normalized to the constitutive expression of *Renilla* luciferase (pRL-SV40) (Promega). tRF-ISRE cells were transfected for 40 h with 50 ng pcDNA3.1-R6HA or empty pcDNA3.1(-), along with 10 ng pRL-SV40. Cells were then

transfected with 10 μg poly(IC) for 8 h or the indicated time and analyzed with the Dual-Glo luciferase assay according to the manufacturers protocol (Promega).

Immunogold electron microscopy (EM). Gradient-purified virus preparations of extracellular R6HA RRV were fixed in 2.5% gluteraldehyde, pelleted and embedded in resin. Thin sections were made, incubated with anti-HA antibody (Y-11) (Santa Cruz) and subsequently incubated with a 10 nm gold-conjugated secondary goat anti-mouse IgG (Ted Pella Inc., Redding, CA). Percentage of virion-localized gold particles was determined by manually counting gold particles associated with virions as well as total gold particles within three separate fields of view. This was then used to calculate the percentage. Samples were imaged at 120 kV on a FEI Tecnai™ Spirit TEM system. Images were acquired as 2048 × 2048 pixel, 16-bit gray scale files using the FEI's TEM Imaging & Analysis (TIA) interface on an Eagle™ 2K CCD multiscan camera.

Statistical Analysis. Data were analyzed using GraphPad Instat (GraphPad Software, La Jolla, CA), and significant differences were determined using a paired *t-test*, with values of $p \leq 0.05$ considered significant, and p values from 0.05 to 0.1 are considered to be showing a significant trend.

RESULTS

R6 vIRF is sufficient for the inhibition of IFNβ transcription.

Work previously performed on the RRV vIRFs demonstrated a delay in the nuclear accumulation of phosphorylated IRF-3 (pIRF-3) in the presence of the vIRFs (141). This phenomenon was observed at 2, 4, and 6 h pi, with a rebound in nuclear pIRF-3 occurring at 8 h pi. Moreover, it was reported that RRV vIRF R6 can inhibit IRF-3 mediated transcription 8 h after stimulation with poly(IC) (141). Since nuclear accumulation of pIRF-3 was inhibited at time points earlier than 8 h pi, it was important to characterize the early kinetics of R6 on IFN production that was previously observed (141) in order to determine if R6 was indeed responsible for pIRF3 modulation. To achieve this, an IFN-responsive reporter cell line was used in the presence or absence of ectopic expression of R6 and subsequently stimulated with poly(IC) for varying times. Telomerized RFs expressing the firefly luciferase gene under the control of an IFN-stimulated response element (ISRE)-containing promoter (tRF-ISRE) were transfected with an R6 expression clone with a C-terminal HA tag (141) or empty vector as a control, and cells were then stimulated 40 h later with poly(IC) to induce the activation of IRF3. Luciferase levels were analyzed at 2, 4, 6 and 8 h post-poly(IC) stimulation as a means of tracking the early effects on transcription. Relative luciferase units (RLU) were determined by defining 100% RLU as the output of poly(IC)-treated cells transfected with empty vector at 8 hr. A decrease in luciferase in the presence of RRV vIRF R6 was observed as early as 2 h post poly(IC)-stimulation, with a significant reduction occurring 4 h. Similar to previous results, R6 resulted in a 50% reduction in luciferase at all time points post poly(IC)-stimulation when compared to empty vector (Figure 2.1a).

Cellular IRF-3, upon activation and nuclear translocation, induces IFNβ transcription by binding to the IRF element (IRF-E) present in the positive regulatory domain (PRD) of the IFNβ promoter (276). IFNβ then acts in an autocrine and paracrine fashion to stimulate the transcription of IFN-stimulated genes (ISGs). To determine if the effect on IRF-3-mediated transcription by R6 vIRF directly impacts IFN transcription, IFNβ transcripts were analyzed with or without ectopic expression of R6. We found that induction of IFNβ in R6-transfected tRFs was diminished by nearly 50% after 8 h poly(IC)-stimulation, in contrast to cells transfected with empty vector (Figure 2.1b). These data demonstrate that in cells treated with poly(IC) to induce IRF-3 activation, R6 vIRF is sufficient for early inhibition of IFNβ transcription.

R6 affects IRF3 translocation to the nucleus and interacts with CBP.

In order to delineate the mode of action of R6 in the inhibition of IFNβ transcription, the cellular localization of R6 was examined by Western blot analysis as well as immunofluorescence. In transfected cells, R6 vIRF was localized to both the nucleus and the cytoplasm, with a majority of the protein expressed in the nucleus (Figure 2.2a and b). Similar to previous results (141), co-localization was observed between IRF-3 and R6 in poly(IC)-stimulated cells and was not restricted to either the cytoplasm or the nucleus. This was also observed by co-immunoprecipitation of R6 with IRF-3 (data not shown). We also evaluated the intracellular localization of IRF-3 in the presence or absence of R6. Localization was addressed via immunofluorescence over time (Figure 2.2a). Poly(IC)-stimulated cells transfected with a GFP-expressing vector

showed an accumulation of IRF-3 within the nucleus. In the presence of R6 vIRF, however, IRF-3 localization was diffuse throughout the cell and did not accumulate in the nucleus at any time point examined. These data indicate that R6 prevents the accumulation of IRF-3 in the nucleus.

We next wanted to investigate whether R6 interacts with other cellular proteins that associate with pIRF3 in the nucleus. Upon its phosphorylation and dimerization, pIRF-3 translocates to the nucleus, wherein it associates with other components of the enhanceosome (ie. NF-κB and ATF-2/c-Jun) and further recruits RNA polymerase, chromatin-remodeling complexes, and histone-modifying complexes such as CBP/p300 (277). This complex of proteins is referred to as the transcriptional pre-initiation complex and is responsible for the transcription of IFNβ. Interestingly, IRF-3 lacks intrinsic transcriptional activity, therefore the specific interaction between IRF-3 and CBP/p300 is critical for the activation of transcription (193, 278, 279). This complex has been shown to be targeted by both KSHV vIRF-1 (234) and HSV-1 ICP-0 (211) via a direct interaction as a means of inhibiting IFNβ transcription. To examine the possibility of an R6 and CBP interaction, co-immunoprecipitation experiments were performed on whole cell lysates of tRFs transfected with vector expressing HA-tagged R6 vIRF and subsequently stimulated with poly(IC). Lysates were immunoprecipitated with anti-CBP pAb, followed by Western blot analysis using an anti-HA antibody. The results showed that R6 is able to interact with endogenous, cellular CBP at all time points. The specificity of this interaction is supported by the lack of an interaction observed between HA-tagged R7 vIRF and CBP (Figure 2.2c).

R6 prevents formation of a functional IRF3/CBP complex.

Since R6 vIRF was shown to inhibit IRF-3-mediated transcription (141) (Figure 2.1a) and associate with CBP (Figure 2.2c), it was of particular interest to determine if R6 could interact with CBP and impede with CBP/IRF-3 complex formation. To evaluate this, we transfected increasing amounts of HA-tagged R6 into tRF cells and 40 h later, transfected with poly(IC) to stimulate IRF3 translocation. Cells were harvested at 6 h post-poly(IC) and nuclear extracts were then isolated and immunoprecipitated with anti-CBP antibody, followed by Western blot analysis with anti-pIRF-3 and anti-HA antibodies. The co-immunoprecipitation data revealed an interaction between CBP and pIRF-3 as well as between CBP and R6, suggesting two distinct populations of CBP in the cell or a complex that is comprised of CBP/IRF-3/R6. Interestingly, decreased binding of pIRF-3 was observed with increasing amounts of R6, suggesting that, at increased concentrations, R6 can diminish pIRF-3/CBP complex formation (Figure 2.3a).

In order to establish the functionality of IRF-3/CBP complexes in the presence of R6, we examined the DNA binding capability of the complex using the PRDI-III domain of the IFNβ promoter in EMSA experiments (Figure 2.3b). Similar to previous experiments, tRF cells were transfected with HA-tagged R6 or empty vector for 40 h and subsequently stimulated with poly(IC) for 6 h. Nuclear lysates were harvested, then tested in a biotin-labeled PRDI-III oligonucleotide binding assay and subjected to EMSA. In the absence of R6, cells expressing endogenous IRF-3 and CBP displayed a protein-DNA complex indicated by a probe shift (Figure 2.3b, lane 1). To demonstrate the presence of IRF-3 and CBP in the complex, nuclear extracts were pre-incubated with either anti-IRF-

3 or anti-CBP antibodies for 5, 10, or 20 minutes and then allowed to complex to the PRDI-III oligo (Figure 2.3b, lanes 3-8**)**. It is important to note that these antibodies have been previously shown to ablate the ability of IRF-3 and CBP to bind to the PRDI-III domain of the IFNβ promoter (234, 278). A decrease in protein complex binding was observed after a 10 minute pre-incubation with either antibody and even greater decrease after 20 minute pre-incubation, indicating that both IRF-3 and CBP are involved in binding the PRDI-III domain of the IFNβ promoter region. The specificity of the complex binding to the probe was determined by the addition of unlabeled PRDI-III probe, which resulted in loss of binding to the biotin-labeled probe (Figure 2.3b, lane 2). Interestingly, ecoptic expression of R6 resulted in a drastic reduction in probe binding by IRF-3/CBP (Figure 2.3b, lane 9), suggesting that the presence of R6 disrupts the IRF-3/CBP complex from binding the probe. This, along with the coimmunoprecipitation data, shows that in the presence of R6, IRF-3/CBP complexes are less able to effectively bind the PRDI-III oligo. Increasing amounts of R6 did not fully abolish the interaction between IRF-3 and CBP, which could be an effect of less that optimal transfection efficiency, however it did result in decreased IRF-3 bound to CBP. This suggests that R6 displaces IRF-3 from CBP enough to impede its DNA binding activity, thus preventing the transcription of IFNβ.

To determine how R6 mediates a decrease in pIRF-3 and disrupts the IRF-3/CBP complex, we transfected cells with R6-HA that were grown in the presence of proteasome inhibitor, MG132. If R6 facilitates proteasome-mediated degradation, then we would anticipate consistent levels of pIRF-3. Through this analysis, we found that pIRF-3 levels

were unchanged in the presence of MG132, but were decreased in the absence (Figure 2.3c). This effect was specific to pIRF-3 as total levels of IRF-3 remained constant regardless of the addition of MG132.

We also evaluated change in Tank-binding kinase (TBK) levels, as TBK is the main cellular kinase that is responsible for the phosphorylation and subsequent activation of pIRF-3 upon TLR ligation. TBK levels were assessed by Western blot and found to be stable regardless of R6 expression. These experiments demonstrate that R6 is capable of inhibiting IRF-3/CBP complexes from binding to the IFNβ promoter by displacing pIRF-3 from CBP, and that the presence of R6 results in proteasome-mediated degradation of pIRF-3.

R6 is associated with purified RRV virions.

As we observed early inhibition of IFN stimulation, we postulated that R6 must act early to impede IRF-3 activity. Currently, the lack of antibodies specific to the RRV vIRFs makes it particularly difficult to characterize the role of individual vIRFs during infection. To circumvent this shortcoming, we utilized bacterial artificial chromosome (BAC) technology was utilized to generate a recombinant virus in which R6 possesses a C-terminal HA epitope tag, allowing for its detection. Importantly, all 7 other vIRFs are fully intact in this virus and left untagged. Insertion of the HA tag sequence into R6 sequence was accomplished using the RRV BAC coupled with a *galK*-based positive/negative selection system in *E.coli* (275), such that the R6 ORF was initially replaced with a *galK* cassette, then subsequently replaced with a recombinant R6-HA

cassette containing HA sequence at the 3' end of R6. After isolation of an R6-HA containing RRV BAC clone, DNA was isolated and used to produce infectious virus, as previously described (141). The genome of the resultant virus was analyzed via comparative genome hybridization (CGH) in order to compare the genomic sequence of R6HA RRV to WT_{BAC} RRV_{17757} (Supporting Figure 1), confirming the correct insertion of the HA epitope sequence and demonstrating that the remainder of the genomic sequence outside of the modified R6 ORF was identical to the parental WT BAC virus. Finally, the R6 HA virus was analyzed by standard growth curve in RF, and confirmed that modification of the R6 ORF did not have an effect on virus growth kinetics or transcription of neighboring ORFs. (Supporting Figure 1).

To evaluate the role of R6 during early RRV infection, it was first necessary to determine the expression kinetics of R6 in the first 8 h of infection. To that end, a cyclohexamide (CHX) reversal assay was performed (Figure 2.4a). Cells were first treated with CHX, a protein translation inhibitor, and subsequently infected with R6HA RRV at an MOI of 2.5 for 6 h in the presence of CHX. Cyclohexamide was subsequently removed and cells were immediately prepared and analyzed for R6HA expression via immunofluorescence or kept in culture for analysis at later time points for kinetic analysis of expression. It was previously shown that R6 transcripts are detected in RRV infected cells at 6 h post-infection, but not at 3 h pi (141), therefore it was expected that R6 protein would likely only be present at 6 h post infection or later. Remarkably, however, R6 protein was detected in infected cells even in the presence of CHX, suggesting that R6 came into the cells, or the conditions did not prevent *de novo* protein synthesis (Figure

2.4a). After removal of the CHX block we observed R6 at 2 h, and again at 6 and 8 h. R6 signal was noticeably diminished at 4 h after removal of the CHX block, which could suggest that the initial R6 signal was degraded and new R6 expression is due to *de novo* transcription and subsequent translation of the R6 ORF, whereas the first wave could potentially represent R6 protein that enters the cell during infection.

To determine if R6 entered the cell during the infection process, we analyzed equivalent plaque forming units ($1x10^5$ PFU) of gradient-purified virus preparations of extracellular R6 HA RRV, intracellular R6HA RRV, WT_{BAC}RRV and vIRF-ko RRV by SDS-PAGE followed by immunoblot analysis with an anti-HA antibody as well as a control antibody against RRV major capsid protein (MCP) (Figure 2.4b). As expected, MCP was found in all virus preparations. However, R6 was only detected in the extracellular and intracellular virus preparations of R6HA RRV, but not in WT_{BAC}RRV or vIRF-ko RRV preparations. To evaluate this further, we performed immunogold electron microscopy on extracellular R6HA RRV and WT_{BAC}RRV (Figure 2.4c). Gradient purified R6HA RRV preparations were fixed, embedded, sectioned and stained with anti-HA antibody and subsequently with a secondary antibody conjugated with 10-nm-diameter gold particles. Gold particles were seen on and within R6HA RRV particles (Figure 2.4ci), but not in WT_{BAC}RRV (Figure 2.4cii) or in R6HA RRV incubated with secondary gold-conjugated antibody alone (Figure 2.4ciii) to assess the level of background. In three fields of view the number of gold particles that colocalized with virions was manually counted and calculated as a percentage of the total number of gold particles viewed. Approximately, 50% of the gold particles were localized to RRV virion

particles (indicated by white arrows). One gold particle was detected in the WT_{BAC}RRV control (Figure 2.4cii**)**, but was not localized to virus particles. No background was observed in R6HA RRV with secondary antibody alone. Some virions that were detected did not colocalize with gold particles (indicated by black arrow). This could mean that not all virions contain R6 protein, or that the section obtained did not contain R6 even though R6 may have been detected in other sections of the same virion. These data demonstrate that R6 is a virion-associated vIRF.

Virion-associated R6 is functional.

To determine if the virion-associated R6 is functional, we sought to test whether an R6-expressing cell line could complement the lack of R6 in the vIRF-ko virus. To accomplish this, we generated an R6HA-expressing cell line that controls for R6HA expression with a tetracycline-inducible promoter. This doxycycline (Dox)-inducible cell line expressing R6 (tRF-rtTA:R6HA) was constructed to evaluate if R6HA could be packaged in RRV virions to complement a vIRF-ko virus (Figure 2.5a). We choose to treat cells with 1.0 μg/ml Dox for 24 h to allow for the expression of HA-tagged R6 and then infected with vIRF-ko RRV, as we found this concentration of Dox and time post-induction yielded higher R6-HA production (data not shown). A total of 5 μg of gradient purified virus preparations were used to examine the presence of R6-HA by western blot. Analysis of extracellular virus isolated from the Dox-induced tRF-rtTA:R6HA cell line shows that R6 was effectively packaged into vIRF-ko RRV virions, compared to vIRF-ko grown in normal RFs. As a control, we utilized the WT_{BAC}RRV and R6HA RRV grown in normal RFs (Figure 2.5b).

To determine if the R6 protein associated with this complemented virus (vIRF-koR6 RRV) was capable of inhibiting the type I IFN response in infected cells, we infected the tRF-ISRE cells and measured the luciferase units (Figure 2.5c). Infection with either WT_{BAC}RRV or R6HA RRV resulted in a significant decrease in luciferase expression when compared to vIRF-ko RRV. Likewise, vIRF-koR6 RRV inhibited luciferase expression, similar to that seen in WT infection. To determine if this effect was dose dependent, R6 expression was induced in tRF cells with increasing amounts of Dox (0, 0.01, 0.1 and 1.0 μg/ml) and each induced cell sample was subsequently infected with vIRF-ko RRV, thus creating a panel of vIRF-koR6 RRV viruses containing increasing amounts of virion-associated R6. The resultant viruses were then tested in the luciferase assay. The reporter cells were infected with virus at an MOI of 2.5 and as expected, increased Dox-induced R6 within the virion results in more of an inhibitory effect (Figure 2.5d). These data suggest that virion-associated R6 is not only functional, but is also capable of hindering the type I IFN response.

To further support our result, we analyzed the ability of virion-associated R6 to inhibit binding of transcription factors to the IFNβ promoter (Figure 2.5e). Cells were either pretreated with CHX or left untreated prior to and during virus infection. Treatment with CHX ensured that no new proteins would be translated, thus allowing the direct examination of the effects of virion components. Infection with R6HA RRV in the presence of CHX lead to decreased binding to the IFNβ promoter, indicating that intact virus carries within it an inhibitor that can ablate IRF-3/CBP binding to PRDI-III.

Conversely, vIRF-ko RRV infection of cells treated with CHX caused a 3-fold increase in the amount of probe that was shifted. These data, so far, show not only that RRV has a virion-associated protein that interferes with the IFNβ promoter, but also that this virion-associated protein is associated with the region of the vIRF ORFs. Finally, when cells were infected with vIRF-koR6 RRV, the level of protein complex binding to the IFNβ promoter decreased to near levels observed in R6HA RRV infection. Overall, these results indicate R6 is an important virion-associated mediator of the type I IFN response, which at least one function acts to prevent the transcription of IFNβ.

DISCUSSION

Approximately 25% of the KSHV genome is dedicated to immunomodulation (84). Of the 22 immunomodulatory genes encoded by KSHV, the viral interferon regulatory factors are of particular interest as they are unique to both gamma-2-herpesviruses, KSHV and the closely-related RRV (119, 123, 126, 269). The eight vIRFs encoded by RRV display sequence homology to cellular IRFs as well as KSHV vIRF1. In fact, R6, R7, R8, R10 and R11 all have similarities to KSHV vIRF1 ranging from 26-33% (123). These similarities have led to the hypothesis that the RRV vIRFs may be modulators of cellular IRFs, thus interfering with the type I IFN response. This hypothesis was validated when *in vivo* and *in vitro* comparisons of WT_{BAC}RRV and vIRF-ko RRV revealed significant differences on the antiviral state early during infection (140, 141). The RRV vIRFs were found to be responsible for a decrease in gene expression and IFN production, as well as decreased nuclear accumulation of pIRF-3 during *de novo* infection with RRV. The findings on ectopic expression of R6 vIRF and

its significant impact on the inhibition of IRF-3-medited transcription warranted further dissection of its potential immunomodulatory function.

The induction of IFNβ initiated by ligation of TLR3, cytoplasmic sensors such as RIG-I, MDA-5, and c-GAS is largely orchestrated by IRF-3, which is constitutively expressed in most cell types. IRF-3 is rapidly activated after sensing these PAMPs, wherein they accumulate in the nucleus and commence transcription of type I IFNs. A reporter assay expressing firefly luciferase under the control of an ISRE was used to examine the kinetics of IFN induction upon independent expression of R6. These data showed a significant decrease in IFN production by R6 in the soon after poly(IC) stimulation and the decrease was sustained even 8 hr post stimulation. Further analysis of IFNβ transcription revealed a nearly 50% decline in transcripts in the presence of R6, thus supporting the immunomodulatory function of R6 vIRF.

The KSHV vIRFs target a variety of components in the pathways leading up to type I IFN induction. The vIRF-1 protein suppresses transcriptional activity of IRF-3 (234, 235, 237, 273) and IRF-1 (228, 235) by interfering with their binding to transcriptional coactivator p300. vIRF-2 disrupts NF-κB and IRF-1-dependent transcription (246), both of which are required for effective IFNβ transcription. Lastly, the DNA binding function of IRF-7 has been shown to be inhibited by KSHV vIRF-3 (254). Knowing that the KSHV vIRFs are multifunctional and have diverse targets, it was important to first define the cellular localization of R6 vIRF in order to pinpoint its primary mode of action. By Western blot analysis and immunofluorescence, R6 was

found to be concentrated in, but not limited to, the nucleus. R6 was previously shown to coimmunoprecipitate and colocalize with IRF-3 in both the nucleus and cytoplasm. Additionally, the vIRFs in RRV were found to prevent nuclear accumulation of IRF-3 without having an effect on IRF-3 phosphorylation or dimerization (141). Therefore, the effect of R6 on phosphorylated IRF-3 was explored. What was particularly interesting was the lack of pIRF-3 concentration within the nucleus of cells expressing R6, especially at 6 and 8 h post-stimulation. Phosphorylated IRF-3 was likely not being sequestered in the cytoplasm, as there was no observed accumulation of cytoplasmic pIRF-3. Since pIRF-3 was still able to enter the nucleus at early time points, and R6 is primarily localized to the nucleus, it lead to the conclusion that R6 is likely acting on the function of IRF-3 within the nucleus. A vital step in the activation of IFNβ transcription is the binding of pIRF-3 to transcriptional coactivator and histone acetyltransferase, CBP. Similar to KSHV vIRF-1, RRV R6 binds to CBP and IRF-3 and interferes with the DNA binding capacity of these IRF-3/CBP complexes (Figure 2.6). Interestingly, R6 does not inhibit IRF-3 binding to CBP as potently as KSHV vIRF-1 does. However, this may be a result of transfection efficiency.

It is not unprecedented for a virus to prepackage antiviral mediators within the virion in order to quickly dampen the immune response. A prime example is KSHV ORF45, which is not only virion-associated but also inhibits type I IFN induction by specifically blocking the phosphorylation and nuclear accumulation of IRF-7 (12, 221, 280). RRV does in fact encode a homolog of KSHV ORF45 and the RRV and KSHV ORF45 share approximately 24% amino acid identity. RRV ORF45 has also been found

within the tegument of virion particles, but its immunomodulatory functions have yet to be explored (281). Given that ectopic expression of RRV vIRF R6 hampered IFNβ transcription very soon after poly(IC) stimulation, it lead to our investigation that R6 may be a virion-associated protein. In this study, we show that R6 is indeed virion-associated as seen by immunofluorescence and electron microscopy. Furthermore, virion-associated R6 can inhibit IRF3/CBP DNA binding and therefore inhibit IFNβ transcription. The prompt inhibition of the type I IFN response may be especially crucial in the endothelial cells and fibroblasts as these are some of the first cells to be infected and are also key producers of IFNβ. The potent antiviral response mediated by type I IFNs is brought about by further activation of ISGs, whose products inhibit various stages of viral replication. It is, therefore, of utmost importance for RRV to quickly down modulate IFNs. Not only do type I IFNs play a significant role in the innate immune response to infection, but also they are vital in the downstream development of the adaptive immune response to the infection at hand. For example, type I IFNs enhance the expression of major histocompatibility complex I (MHCI), thereby promoting antigen presentation and development of an effective $CD8^+$ T cell response (282). Because epithelial cells and endothelial cells are important sources of IFNβ and are capable of antigen presentation, they are an obvious target for IFNβ inhibition by RRV. In addition, type I IFNs have dramatic effects on natural killer (NK) cells (283, 284), $CD4^+$ and $CD8^+$ T cells (285, 286), as well as dendritic cells (DCs) (287-289). In fact, it was previously shown that complete deletion of all 8 RRV vIRFs initiates an earlier T cell response (140), therefore it could be postulated that the IFNβ inhibition mediated by virion-associated R6 contributes to the delayed T cell response observed in WT RRV infection. It would be

particularly interesting to create an R6-deletion mutant of RRV and determine the phenotype of this virus and the ensuing immune response. These data show that RRV has a prepackaged vIRF for immediate delivery into target cells upon *de novo* infection, thus providing the virus with a direct mechanism to inhibit or slow the innate immune response. We postulate that virion-associated R6 functions to enable RRV to successfully establish infection and further progress with its viral life cycle, including transcription of the remaining vIRFs, without a robust type I IFN response from the host.

Acknowledgements

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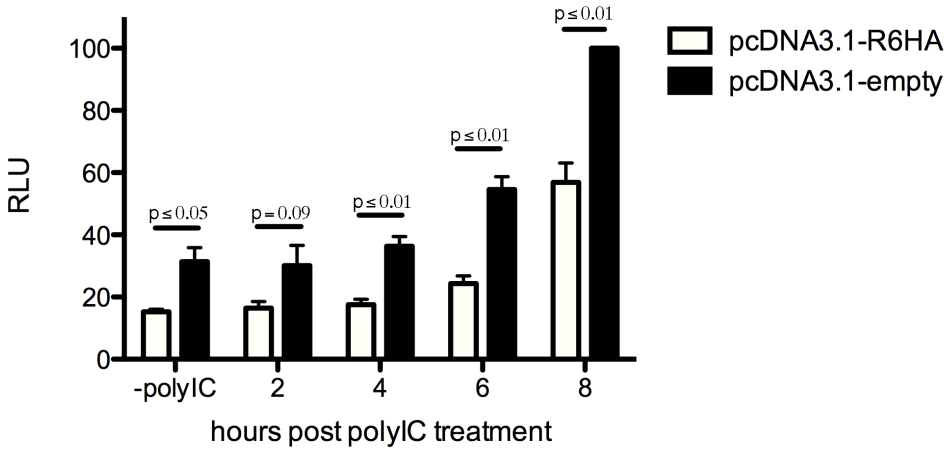
Figure 2.1. R6 inhibits IFN response.

A) tRF-ISRE cells were transfected for 40 h with pRL-SV40 and either pcDNA3.1-R6HA or empty pcDNA3.1. Cells were then transfected with poly(IC) and assayed at the indicated time points. Firefly luciferase levels were normalized to constitutively expressed *Renilla* luciferase levels in each well, and all samples were normalized to the positive control (empty vector plus poly(IC) at 8 h post-poly(IC) treatment). Data are average data (± SEM) from 3 independent experiments. B) Telomerized RFs were transfected with pcDNA3.1-R6HA or mock transfected for 40 h and subsequently treated with poly(IC) for 8 h. RNA was extracted and analyzed by RT-PCR. Values are made relative to GAPDH levels. Data were analyzed using a paired *t* test.

A.


p≤0.01
pcDNA3.1-R6HA
pcDNA3.1-empty
100
80
60
40
20
0
RLU
p≤0.05
p=0.09
p≤0.01
p≤0.01
-polyIC
2
4
6
8
hours post polyIC treatment

B.

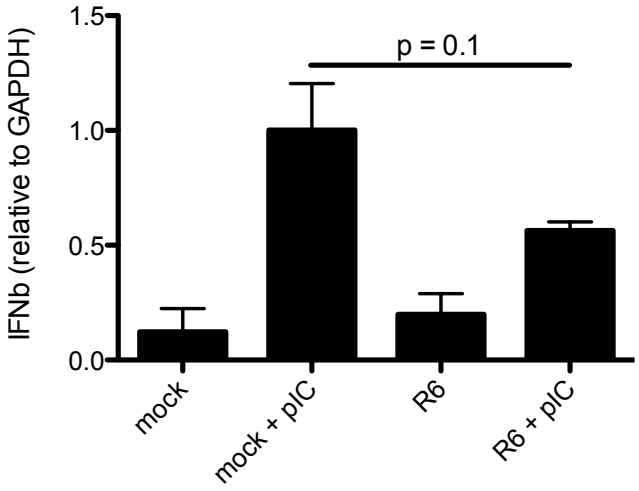
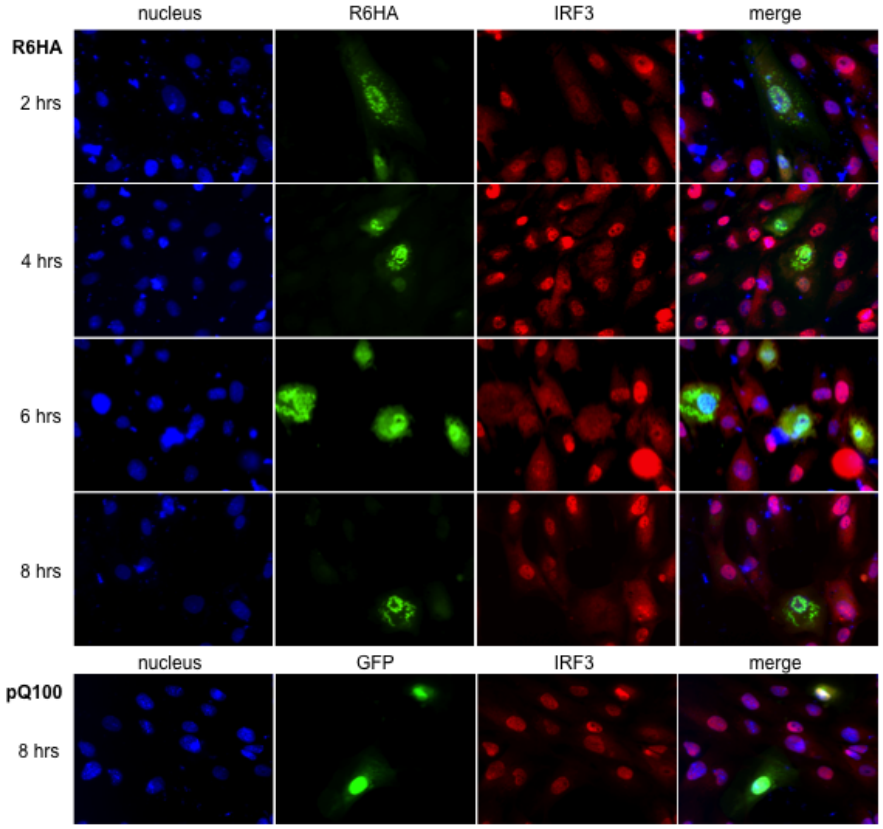

1.5
1.0
0.5
0.0
IFNb (relative to GAPDH)
p = 0.1
mock
mock + pIC
R6
R6 + pIC

Figure 2.2. R6 localizes to the nucleus and prevents nuclear accumulation of pIRF-3.

(A and B) Telomerized RFs were transfected with pcDNA3.1-R6HA or empty pcDNA3.1 for 40 h and subsequently treated with poly(IC) for the indicated times. (A) Transfected cells were fixed and analyzed by immunofluorescence for the detection of R6 (anti-HA) (green) and cellular IRF3 (red) and stained with Hoechst (blue) for the detection of nuclei. (B) Nuclear lysates were immunoprecipitated with anti-CBP antibody, then subjected to SDS-PAGE and probed with anti-HA antibody. Nuclear lysates were probed for HA expression and PARP as a loading control and as a control for purity of nuclear fractionation.

A.


nucleus
R6HA
IRF3
merge
R6HA
2 hrs
4 hrs
6 hrs
8 hrs
nucleus
GFP
IRF3
merge
pQ100
8 hrs

B.

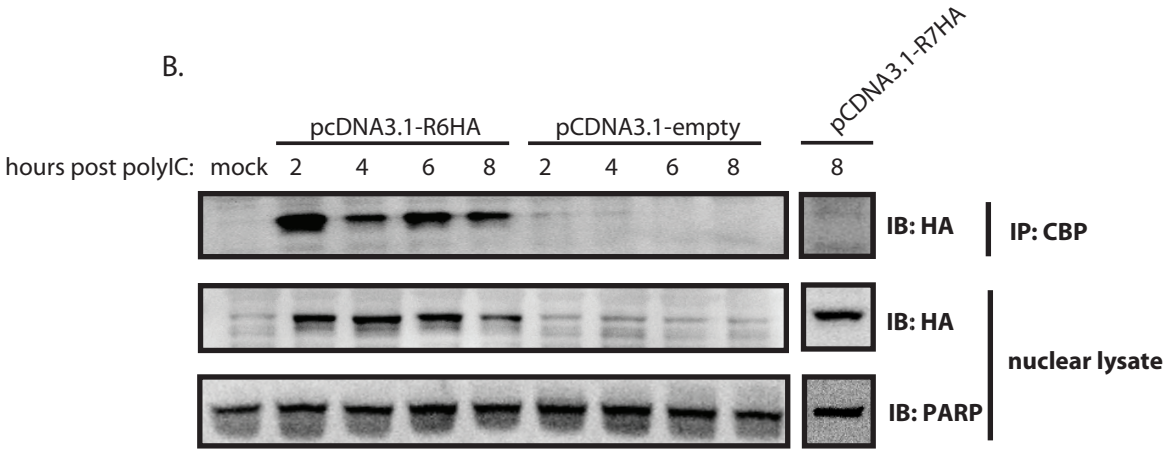
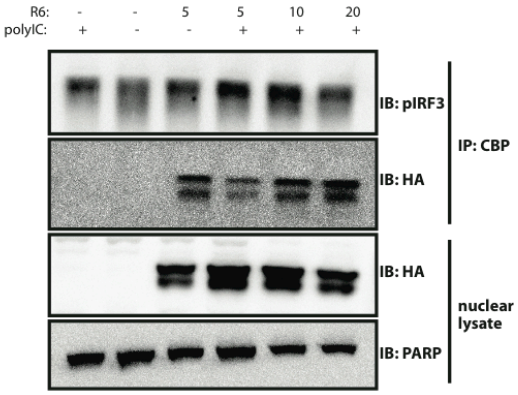

pcDNA3.1-R7/HA
pcDNA3.1-R6HA
pCDNA3.1-empty
hours post polyIC: mock 2 4 6 8 2 4 6 8 8
IB: HA
IP: CBP
IB: HA
nuclear lysate
IB: PARP

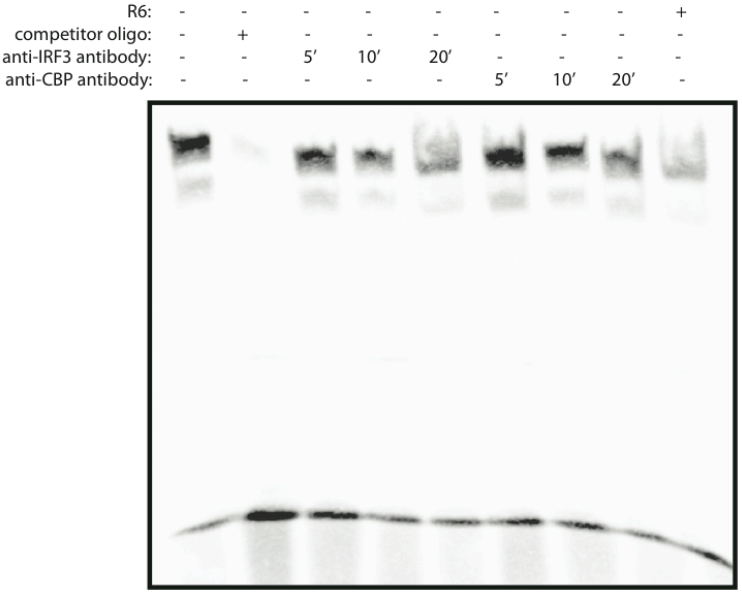
Figure 2.3. R6 competes with IRF3 for binding to CBP.

(A) Telomerized RFs were transfected with pcDNA3.1-R6HA (5, 10 or 20 μg DNA) or empty pcDNA3.1 for 40 h and subsequently treated with poly(IC) for 6 hours. Nuclear lysates were immunoprecipitated with anti-CBP antibody, then subjected to SDS-PAGE and probed with anti-HA antibody or anti-pIRF3 antibody. Nuclear lystates were probed for HA expression and PARP as a loading control and a control for purity of nuclear fractionation. (B) EMSA was performed on whole cell extracts (20 μg) derived from telomerized RFs transfected with pcDNA3.1-R6HA or empty pcDNA3.1. The biotin-labeled probe corresponds to the PRDI-PRDIII motif (5'-GAAAACTGAAAGGAGAACTGAAAGTG-3') of the IFNβ promoter. Anti-CBP antibody and anti-IRF3 antibody were added as indicated to demonstrate the presence of CBP and IRF3 in the DNA-protein complexes. For oligonucleotide competition, a 500-fold molar excess of unlabeled PRDI-PRDIII probe was added as indicated. (C) Telomerized RFs were pretreated with MG132 or left untreated. Cells were then transfected with pcDNA3.1-R6HA or empty pcDNA3.1 for 40 hours and then treated with poly(IC) for 6 hours as indicated. Nuclear lysates were subjected to SDS-PAGE and probed with anti-pIRF3 antibody, anti-TBK or PARP, which served as a loading control and control for purity of nuclear fractionation. Whole cell lysate was probed with anti-IRF3 to gauge total levels of IRF within the cell.

A.


R6: - - 5 5 10 20
polyIC: + - - + + +
IB: pIRF3
IB: HA
IP: CBP
IB: HA
IB: PARP
nuclear lysate

B.


R6: - - - - - - - - +
competitor oligo: - + - - - - - - -
anti-IRF3 antibody: - - 5′ 10′ 20′ - - - -
anti-CBP antibody: - - - - - 5′ 10′ 20′ -

C.

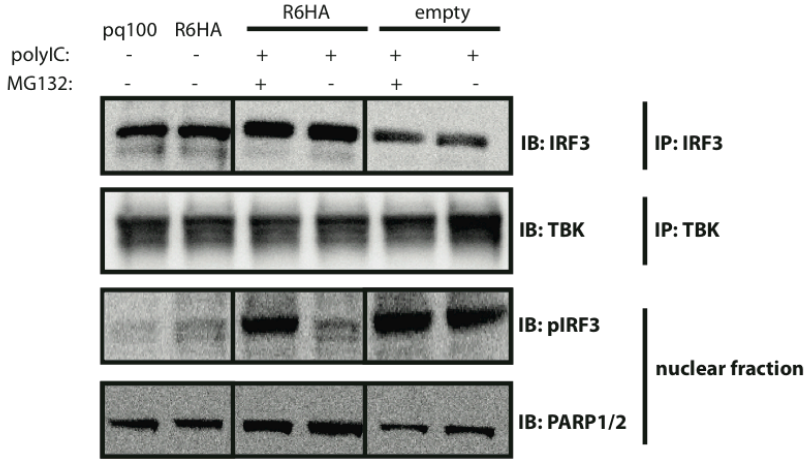
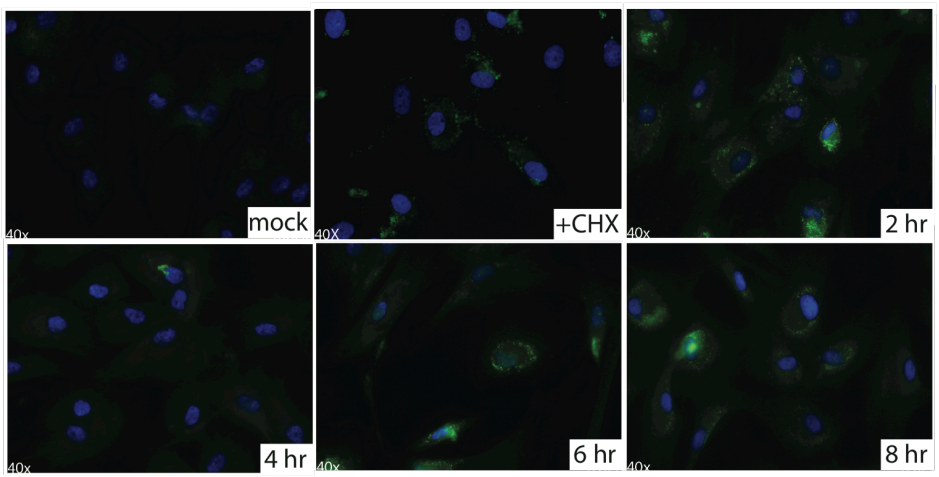

pq100 R6HA
R6HA
empty
polyIC: - - + + + +
MG132: - - + - + -
IB: IRF3
IP: IRF3
IB: TBK
IP: TBK
IB: pIRF3
IB: PARP1/2
nuclear fraction

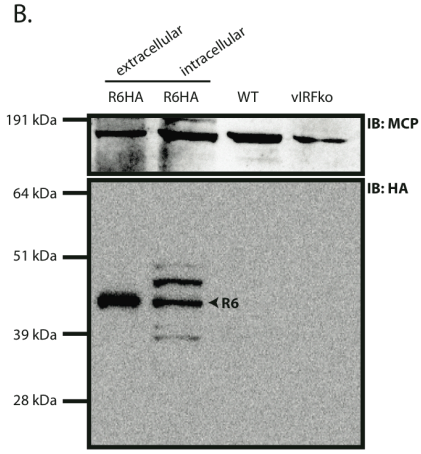
Figure 2.4. R6 is associated with RRV virions.

(A) Primary RFs were pretreated with CHX and subsequently infected with R6HA-RRV at MOI of 2.5. CHX was removed and cells were fixed at the indicated time points and analyzed by immunofluorescence for the detection of R6-HA (anti-HA) (green) and stained with Hoechst (blue) for the detection of nuclei. (B) $1x10^5$ PFU of gradient purified virus samples (extracellular R6HA-RRV, intracellular R6HA-RRV, WT_{BAC}RRV and vIRFko-RRV) were subjected to SDS-PAGE and probed with anti-HA antibody and anti-MCP antibody as a control. (C) (i) Gradient-purified R6HA-RRV was fixed, pelleted and sectioned. Sections were immunogold stained with anti-HA antibody and 10 nm gold-conjugated secondary antibody. (ii) Gradient purified WT_{BAC}RRV was sectioned and immunogold stained with anti-HA antibody and 10-nm gold-conjugated secondary antibody as a control. (iii) R6HA-RRV sections were stained with 10-nm gold-conjugated secondary antibody alone as a control. Virus particles with gold particles are indicated with white arrows and virus particles with no gold particles are indicated with black aarows.

A.


mock
+CHX
2 hr
4 hr
6 hr
8 hr
40x

B.


extracellular
intracellular
R6HA
R6HA
WT
vIRFko
191 kDa
64 kDa
51 kDa
39 kDa
28 kDa
IB: MCP
IB: HA
R6

C.

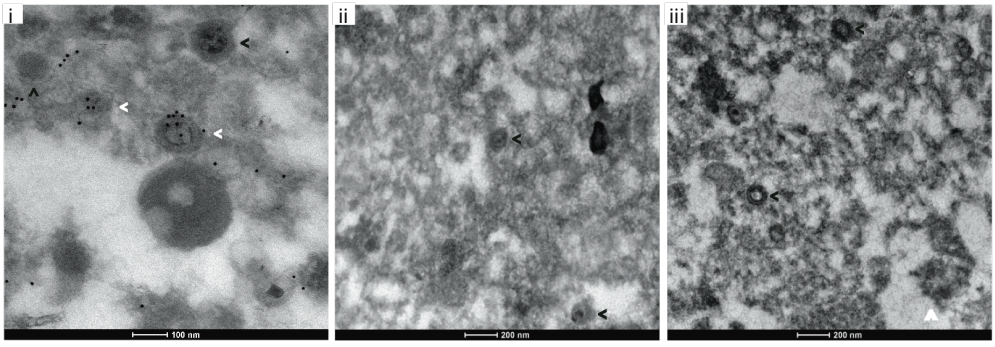
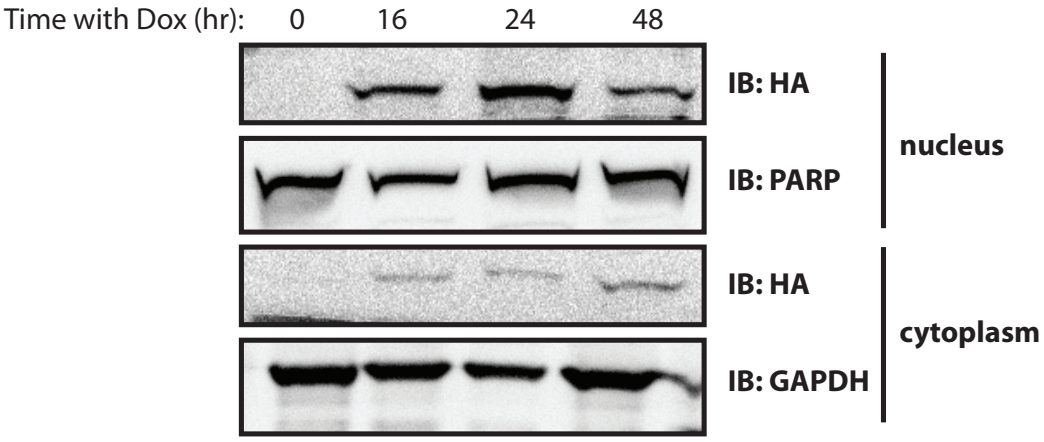

i
ii
iii
100 nm
200 nm
200 nm

Figure 2.5. Virion-associated R6 is functional.

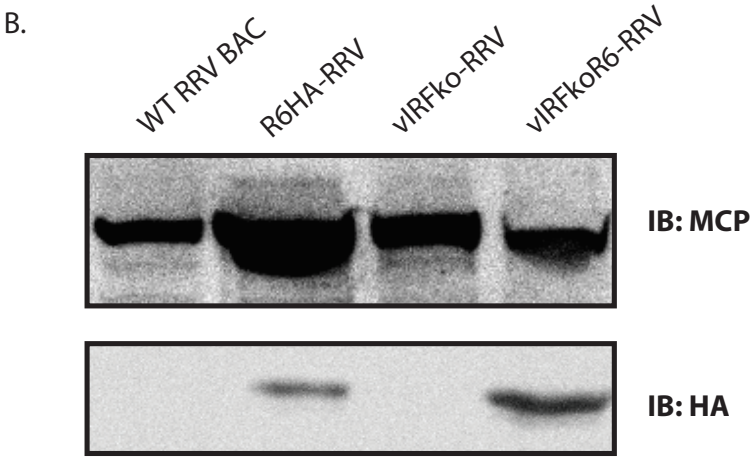
(A) Telomerized RF-rtTA cells stably transduced with R6-HA were treated with Doxycycline for the indicated times. Nuclear and cytoplasmic lysates were subjected to SDS-PAGE and probed with anti-HA antibody. GAPDH and PARP served as loading controls and as controls for purity of fractionation. (B) Telomerized RF-rtTA cells were treated with Doxycycline and infected with vIRFko-RRV at MOI of 0.01. The resultant virus (vIRFkoR6-RRV) was gradient purified from cell supernatants. 5 μg of gradient purified virus (WT_{BAC}RRV, vIRFko-RRV, R6HA-RRV and vIRFkoR6-RRV) was subjected to SDS-PAGE and probed with anti-HA antibody and anti-MCP antibody as a control. (C) tRF-ISRE cells were infected for 4 or 8 h with the indicated virus at an MOI of 2.5. Cells were then assayed for Firefly luciferase activity. Firefly luciferase levels were normalized to constitutively expressed *Renilla* luciferase levels in each well. Data are average data (± SEM) from 3 independent experiments. (D) tRF-rtTA:R6HA cells were infected with vIRFko-RRV at MOI of 0.01 after pretreatment with the indicated amounts of Doxycycline. Virus was then harvested and gradient-purified. The resultant virus was used to infect tRF-ISRE cells at an MOI of 2.5 for 8 h. Cells were assayed for Firefly luciferase activity. Firefly luciferase levels were normalized to constitutively expressed *Renilla* luciferase levels in each well. Data are average data (± SEM) from 3 independent experiments. Total levels of MCP and R6HA in virion preparations were assessed by Western blot analysis with anti-MCP and anti-HA antibodies. (E) Telomerized RFs were infected with the indicated virus at an MOI of 2.5 for 8 h in the presence or absence of CHX. EMSA was

performed on nuclear extracts (20 μg). The biotin-labeled probe corresponds to the PRDI-PRDIII motif (5'-GAAAACTGAAAGGAGAACTGAAAGTG-3') of the IFNβ promoter.

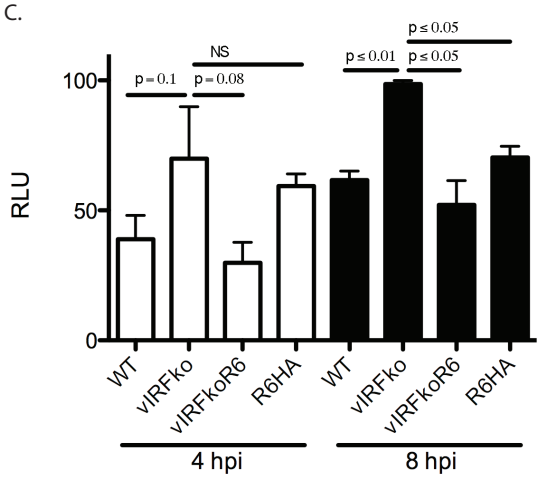
A.


Time with Dox (hr): 0 16 24 48
IB: HA
IB: PARP
nucleus
IB: HA
IB: GAPDH
cytoplasm

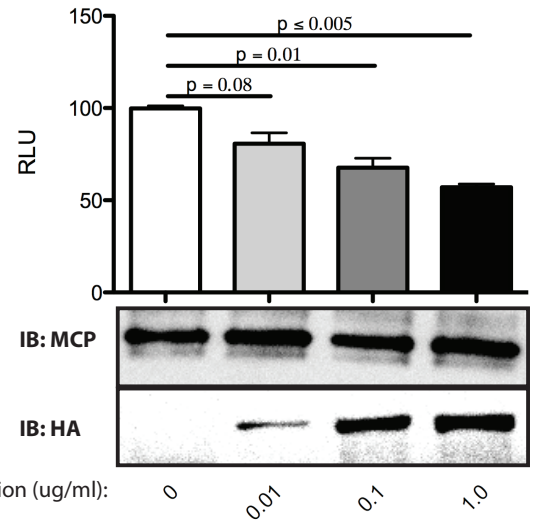
B.


WT RRV BAC
R6HA-RRV
vIRFko-RRV
vIRFkoR6-RRV
IB: MCP
IB: HA

C.


p ≤ 0.05
NS
p ≤ 0.01
p ≤ 0.05
p = 0.1
p = 0.08
100
50
0
RLU
WT
vIRFko
vIRFkoR6
R6HA
WT
vIRFko
vIRFkoR6
R6HA
4 hpi
8 hpi

D.


150
100
50
0
RLU
p ≤ 0.005
p = 0.01
p = 0.08
IB: MCP
IB: HA
Dox concentration (ug/ml):
0
0.01
0.1
1.0

E.

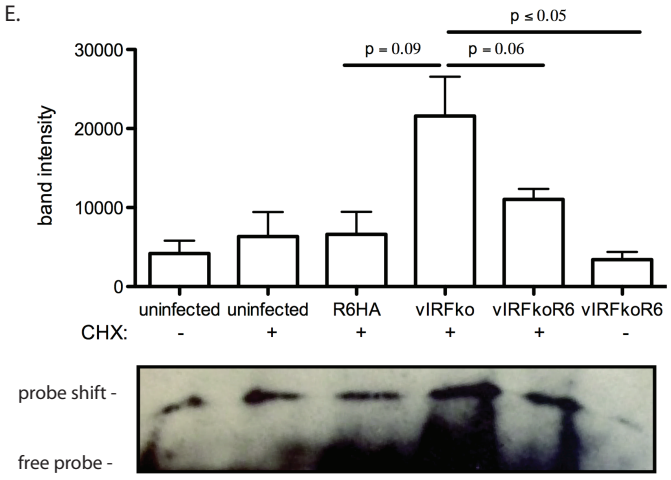
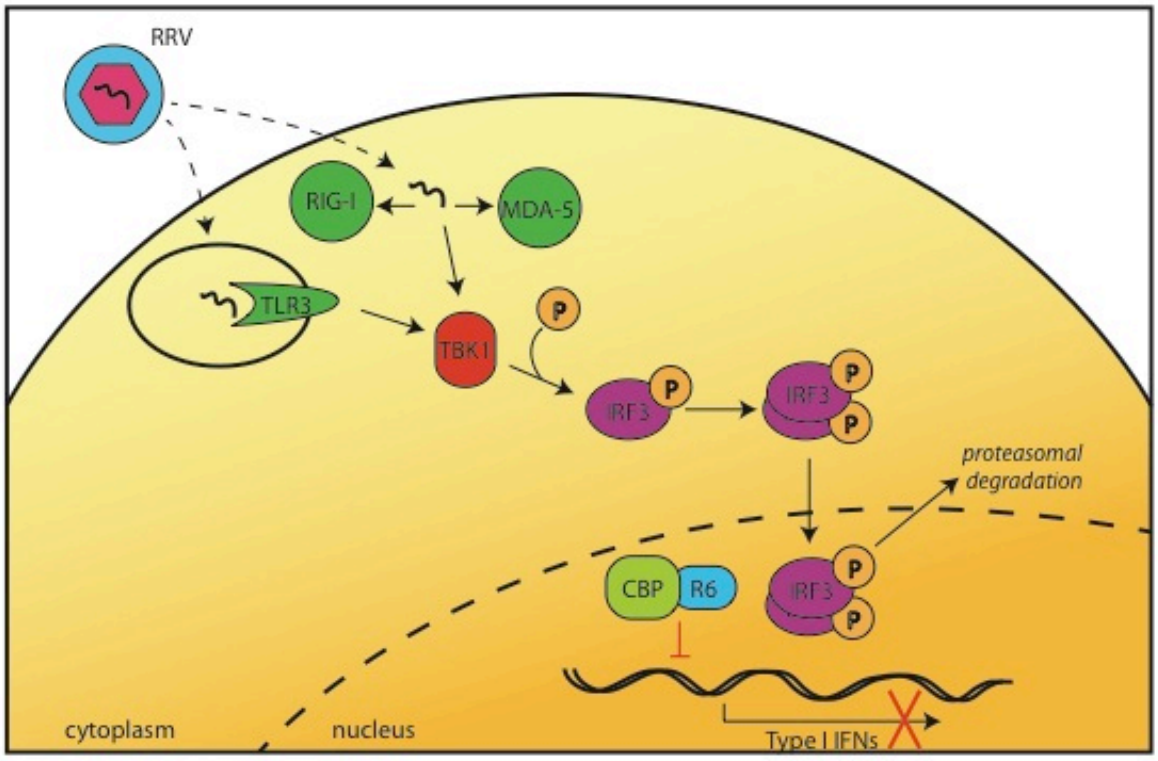

p ≤ 0.05
p = 0.09
p = 0.06
30000
20000
10000
0
band intensity
uninfected
uninfected
R6HA
vIRFko
vIRFkoR6
vIRFkoR6
CHX:
-
+
+
+
+
-
probe shift -
free probe -

Figure 2.6. Potential model of IFN inhibition by R6.

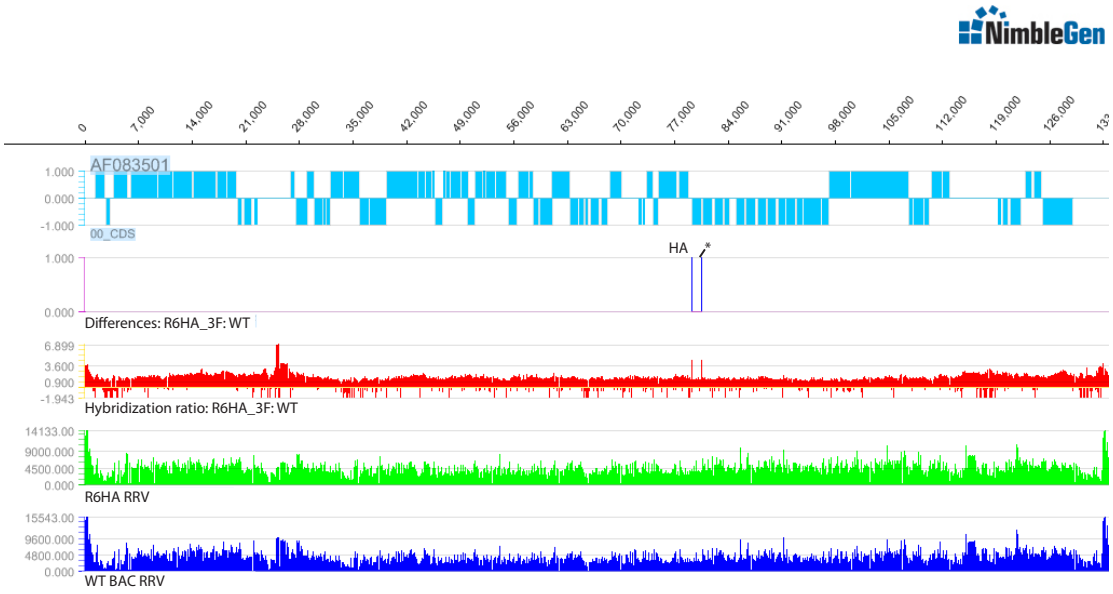
Upon detection of RRV infection by TLR3, RIG-I or MDA-5, TBK1 is activated and subsequently phosphorylates IRF3. Phosphorylated IRF3 (pIRF3) then dimerizes and translocates to the nucleus. Within the nucleus, R6 binds to transcriptional coactivator, CBP. This interaction prevents pIRF3 from binding to CBP, wherein pIRF3 is exported from the nucleus and degraded by the proteasome. Nuclear R6 interferes with complex formation between pIRF3 and CBP and as a result, decreases pIRF3/CBP complex binding to the IFNβ promoter and inhibits IFNβ production.


RRV
RIG-I
MDA-5
TLR3
TBK1
P
IRF3
IRF3
proteasomal
degradation
CBP
R6
IRF3
cytoplasm
nucleus
Type I IFNs

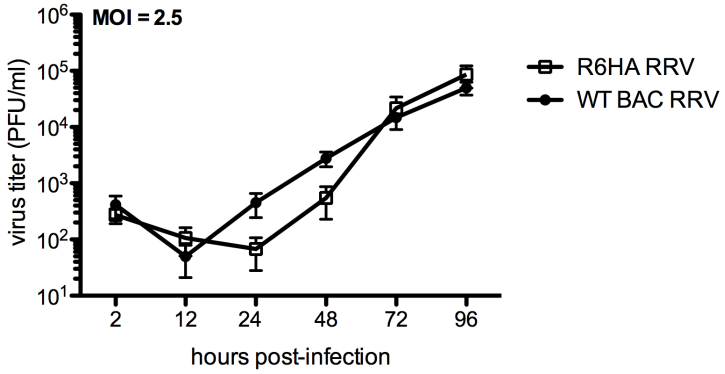
Supporting Figure 1.

(A) Comparative genome hybridization used to directly compare viral DNA from the R6HA RRV recombinant clone to that from WT_{BAC}RRV. Alterations within the R6HA RRV genome resulted in incomplete hybridization to the array, depicted by the ratio of R6HA to WT RRV, and signaled a potential nucleotide mismatch between the two viruses. This comparison identified the HA tag located at the C-terminal end of R6. A second mismatch, indicated with an asterisk (*) was incorrectly identified and the identified sequence was confirmed to be similar to WT via PCR and sequence analysis. (B) RFs were infected with either WT_{BAC}RRV or R6HA RRV at an MOI of 2.5 for growth curve analysis. Infected RFs were harvested at the specified time points and subjected to a serial-dilution plaque assay on RFs to determine viral titers, displayed as PFU/ml. The data from 4 separate experiments were averaged (± standard errors of the means [SEM]).

A.


NimbleGen
0
7,000
14,000
21,000
28,000
35,000
42,000
49,000
56,000
63,000
70,000
77,000
84,000
91,000
98,000
105,000
112,000
119,000
126,000
AF083501
1.000
0.000
-1.000
00_CDS
HA
1.000
0.000
Differences: R6HA_3F: WT
6.899
3.600
0.900
-1.943
Hybridization ratio: R6HA_3F: WT
14133.00
9000.000
4500.000
0.000
R6HA RRV
15543.00
9600.000
4800.000
0.000
WT BAC RRV

B.


MOI = 2.5
virus titer (PFU/ml)
10^6
10^5
10^4
10^3
10^2
10^1
2
12
24
48
72
96
hours post-infection
R6HA RRV
WT BAC RRV

CHAPTER 3

SUMMARY & CONCLUSIONS

I. Mechanism behind modulation of the IFN response by vIRF R6

A. Biochemical mechanism of R6 in the absence of RRV infection

1. vIRF R6 inhibits the early IFN response

Previous findings suggest R6 as an important viral mediator of IFN inhibition (141). These experiments were performed using transient R6 expression and the effect of R6 on the IFN response was determined at 8 h post-stimulation with poly(IC). During WT RRV infection, however, inhibition of pIRF-3 nuclear accumulation occurs between 0 and 6 h post-infection, indicating that this effect is an early attack against the host antiviral response (141). Because of this, we chose to observe the inhibitory effects of R6 at time points spanning 0 and 8 h post-stimulation. Additionally, so as to dissect the role of R6 without confounding results due to effects of other vIRFs, we analyzed R6 in the absence of viral infection. This allowed us to look specifically at the inhibitory potential of R6 and to examine its interactions with cellular proteins. In doing so, we found that R6 significantly inhibits the IFN response, not only at 8 h post-stimulation, but as early as 4 h post-stimulation. The luciferase assay used is an indirect measure of IRF-3-mediated transcription. The IFNβ that is produced in these reporter cells upon poly(IC)-stimulation is a transcription product downstream of the IRF-3 transcription factor. IFNβ then stimulates transcription of luciferase, which is controlled by an ISRE sequence (141). Therefore, a decrease in luciferase could mean that IFNβ is decreased via inhibition of

IRF-3-mediated transcription, or that inhibition of the signaling pathway downstream of IFNβ is the cause of decreased luciferase transcription. Since we observed a measurable decrease in IFNβ transcription in the presence of R6, we deduced that IRF-3-mediated transcription is the target of inhibition by R6.

2. Mechanism of IFNβ inhibition mediated by R6

R6 and R10 are the RRV vIRFs most closely related to KSHV vIRF-1 (123), and similar to vIRF-1, we found that R6 is also found in the nucleus of transfected cells in large quantities. Functionally, R6 and vIRF-1 both inhibit type I IFN production by way of impeding with IRF-3 (141, 227, 234, 236). Therefore, we wanted to determine if the mechanisms were also the same. Expression of R6 results in reduced levels of nuclear pIRF-3 over time, indicating that the turnover of nuclear pIRF-3 is much more rapid in the presence of R6. It is known that virus infection triggers phosphorylation of IRF-3. IRF-3, in this phosphorylated state, then enters the nucleus wherein it interacts with transcriptional coactivator, CBP and induces transcription of type I IFNs (147, 204). IRF-3 contains a nuclear export signal (NES), which is masked by the interaction with CBP, thus causing nuclear retention of pIRF-3 (290). If pIRF-3 is not bound by CBP, however, pIRF-3 is then shuttled out of the nucleus and is promptly degraded in a proteasome-dependent manner (192). Because of this and because of the fact that vIRFs are known to bind many cellular proteins, we wanted to determine if R6 interacts with CBP in the nucleus and found that it indeed does bind to CBP. Similar to vIRF-1, R6 prevents the formation of pIRF-3/CBP complexes, but does so in a different way. KSHV vIRF-1 directly competes with IRF-3 for binding to CBP and is a powerful competitor, leading to

almost complete abrogation of pIRF-3/CBP complexes. R6, on the other hand, prevents the formation of functional pIRF-3/CBP complexes. R6 only slightly displaces pIRF-3 from these complexes, but pIRF-3/CBP DNA binding capabilities are severely impaired by R6, indicating that although complexes form, R6 still prevents complex binding to PRDI-III elements of the IFNβ promoter. The idea that R6-R9 and R10-R13 are the result of a duplication event lends itself to the possibility that the RRV vIRFs may have redundant functions, but they may complement one another as well. It would be interesting to determine the function of R10 as well and compare its functions to that of R6. Also, coexpressing R6 and R10, the two most potent inhibitors of IFN, could help determine if the two proteins together recapitulate the phenotype observed with KSHV vIRF-1. Perhaps R6 would be a more effective competitor of IRF-3 binding to CBP with the help of R10.

Another interesting finding was that the decrease in pIRF-3 was due to proteasome-mediated degradation of pIRF-3. A decrease in pIRF-3 could also be attributed to a decrease in phosphorylation of IRF-3 by an upstream kinase, such as TBK. Nevertheless, TBK levels are stable in the presence of R6, indicating that the change in pIRF-3 levels is not a factor of TBK degradation. Whether IKKε is affected by R6 is unknown, however it is unlikely because IKKε is activated downstream of intracellular DNA and RNA sensors, such as DAI and RIG-I, respectively (291), and these receptors are not thought to be major players in poly(IC)-induced activation of IRF-3. As with KSHV vIRF-1, R6 has proven to be a multifunctional protein in that it can bind to a number of cellular proteins

including IRF-3 (141), CBP (Figure 2.3a), and TBK (Figure A.1). The significance of the R6/TBK interaction is not yet known and requires further evaluation.

B. Characterization of R6 in the context of RRV infection

The recent generation of a recombinant RRV encoding R6 with a C-terminal HA tag (RRV R6HA) has provided us with a tool for visualization of R6 in the context of infection. The lack of available antibodies against RRV vIRFs has been a severe limitation on dissecting the role of individual vIRFs during infection. Analysis of R6 expression in *de novo* infection revealed early detection of R6, prior to the detection of R6 transcripts, suggesting R6 protein is deposited into infected cells from RRV virions. In fact, we demonstrated that R6 is detected in gradient purified RRV R6HA virus preparations and appears to be localized to the virion tegument. This finding is not trivial; it is the first demonstration of a virion-associated vIRF in either RRV or KSHV. The presence of an immunomodulator within a virus particle is not unprecedented, however, as KSHV carries ORF45 within the virion, which acts to inhibit type I IFNs by blocking IRF-7 phosphorylation and nuclear translocation (12, 221, 280). In fact, RRV does encode an ORF45 (123), however, it appears to be distinct from the KSHV ORF45. Therefore, virion-associated R6 could provide the virus with an immediate inhibitor of the rapid IFN response.

Using a virus construct lacking all eight vIRFs and grown in cells expressing HA-tagged R6, we were able to determine if virion-associated R6 was sufficient for the modulation of the IFN response. Indeed, R6 prepackaged within virus particles inhibits

the type I IFN response at the level of transcription of IFNβ. Our vIRF-koR6 virus, containing virus-associated R6, displayed more of an inhibitory effect on IFN than R6HA RRV. This could be due to an abundance of R6 in vIRF-koR6 virions derived from the R6-expressing cell line, whereas R6HA RRV may contain less R6, but still enough to modulate the IFN response. Furthermore, when determining if virion-associated R6 could prevent protein complex formation on the IFNβ promoter, we noticed a more profound decrease in binding in the absence of CHX. Similarly, uninfected cells without CHX treatment displayed less binding to the IFNβ promoter as compared to uninfected cells with CHX treatment. CHX and other inhibitors of protein synthesis have, in fact, been previously shown to induce some IFN (292), which would explain this discrepancy. These results suggest that R6 appears upon *de novo* infection, before the expression of viral transcripts, and again after viral transcripts are produced. Since R6 interacts with several cellular proteins, the different forms of R6 (ie. virion-associated R6 and R6 produced *de novo*) could provide the virus with different functions at different times during infection.

The early IRF-3-mediated IFN response is an important pathway to target in order to allow RRV to establish a productive infection. Therefore, carrying along an inhibitor of this pathway in the virion would be a powerful tool for the virus. The immediate inhibition of type I IFNs in cells such as fibroblasts, epithelial cells and endothelial cells would not only downregulate IFNβ, but would also decrease antigen presentation, thus allowing for the evasion of immune detection by NK cells and CD8 T cells. Furthermore, suppression of the type I IFN response would have an important downstream effect on

the production of ISG proteins. A decrease in ISGs would result in less transcription of PRRs and therefore, less efficient detection of PAMPs. Additionally, transcription of important antiviral proteins such as viperin, Mx1, OAS, RNase L and ISG15 would decrease as well, thus allowing for better propagation of the infection to occur with fewer cellular defense mechanisms in place. Interestingly, *de novo* synthesized R6 appears around 6 h post-infection, consistent with the timing of R6 transcription (141), and is unlikely to be involved in the inhibition of pIRF-3 nuclear translocation, as this phenotype is observed between 0 and 6 h post-infection. IRF-3 may still be a target of R6 at later time points, as we observed a biphasic interaction with IRF-3 at 4 h and then again at 8 h post-stimulation with poly(IC) (Figure A.2). Because the IRF-3 binding elements in the IFNβ promoter are similar to those found in ISRE sequences of ISG promoters, IRF-3 is known to directly regulate a subset of ISGs, namely ISG54 and ISG56 (293, 294). This function of IRF-3 could potentially serve as a target for R6 as well and would contribute to the overall inhibition of the IFN response. It would, therefore, be of interest to determine the function of this newly synthesized R6.

II. Future Directions

The discoveries presented in this thesis demonstrate the mechanism by which one of the RRV vIRFs functions to effectively inhibit the early IFN response. These data contribute to our ultimate goal of understanding how the vIRFs function both *in vitro* and *in vivo* and how they participate in disease progression. This thesis provides the first description of how a single RRV vIRF functions biochemically and we are the first to demonstrate that a vIRF is a virion-associated immunomodulatory protein. Aside from these important contributions, we continue to have more questions that are worth investigating.

An important tool that needs to be generated in order to better evaluate the role of R6 during RRV infection is an R6-specific antibody. This would allow us to examine R6 during WT RRV infection, instead of using a recombinant virus. Furthermore, any confounding effects of an HA tag on protein-protein interactions would be eliminated and would provide a more realistic view of how R6 functions on a biochemical level.

In regards to looking deeper into how R6 functions, it will be important to elucidate other potential binding partners of R6 like TBK, for example, which will provide insight into additional functions of R6. We've shown that R6 appears first in a virion-associated form upon infection and again at 6 h post-infection following *de novo* production of R6. Since IRF-3 functions not only as an activator of IFN, but also as an inducer of ISGs, downstream of IFN signaling, it would be interesting to determine if R6 is capable of blocking both functions of IRF-3 over the course of infection. Therefore, the properties of

R6 during the course of infection should be further examined as well as its potentially differential effects on the IFN response. Additionally, dissecting the functions of the other seven RRV vIRFs *in vitro* will also be an important task to undertake.

Understanding the biochemical mechanism of R6 is valuable, but even more valuable is how R6 functions *in vivo*. Previous data on the vIRFs in the context of RRV infection of RMs has shown that the vIRFs, as a whole, have an immediate impact on the antiviral immune response, both innate and adaptive (140). What we do not know, however, is if the vIRFs work in a coordinated fashion *in vivo*, or if they can function individually to make a significant impact on the host immune response. It is most likely that the vIRFs have a more dramatic effect on the immune response as a whole, as opposed to individually. However, understanding the individual functions of the vIRFs *in vivo* would be invaluable information, as KSHV does not currently have a suitable animal model to study the vIRFs. Utilizing BAC technology, a panel of individual RRV vIRF deletion mutants will need to be created in order to fully characterize the individual functions of the vIRFs and their influence on the immune response and their roles in the development of disease during RRV infection.

Another important facet of the vIRFs that has yet to be explored is whether the RRV vIRFs have redundant functions *in vivo*. It has been hypothesized that R6-R9 and R10-R13 arose due to a duplication event (123, 126) and because of this they may display redundant functions. We currently have an RRV deletion mutant that lacks R6-R9 and only expresses vIRFs R10-R13. Also, construction of an R10-R13 deletion mutant is

currently underway. These tools provide us with a way to further investigate the vIRFs in the context of RRV infection of RMs. We will not only be able to compare the effects on the IFN response, but we can also compare potentially differential effects on the ensuing adaptive immune response and development of B cell malignancies. Due to the varying effects of the individual vIRFs on inhibiting the IFN response *in vitro*, it is unlikely that the vIRFs are redundant, thus understanding the individual functions of all eight vIRFs will be pivotal in determining their contribution to immune evasion of RRV.

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APPENDIX

SUPPORTING FIGURES

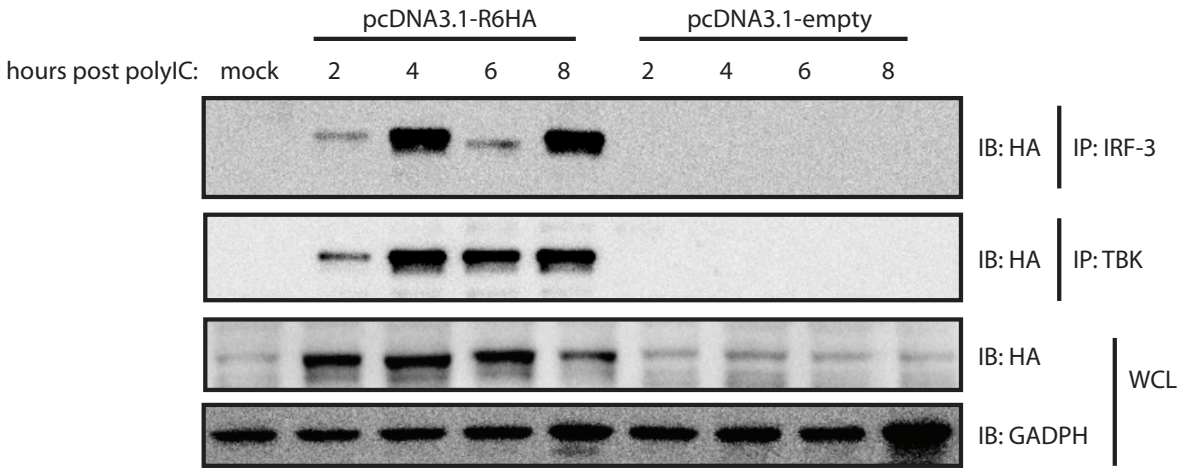

Figure A.1. R6 interacts with IRF-3 and TBK.

Telomerized RFs were transfected with pcDNA3.1-R6HA or empty pcDNA3.1 for 40 h and subsequently treated with poly(IC) for the indicated times. Whole cell lysates (WCL) were immunoprecipitated with either anti-IRF-3 antibody (FL425) or anti-TBK antibody. Samples were then subjected to SDS-PAGE and probed for HA. GAPDH was used as a loading control.

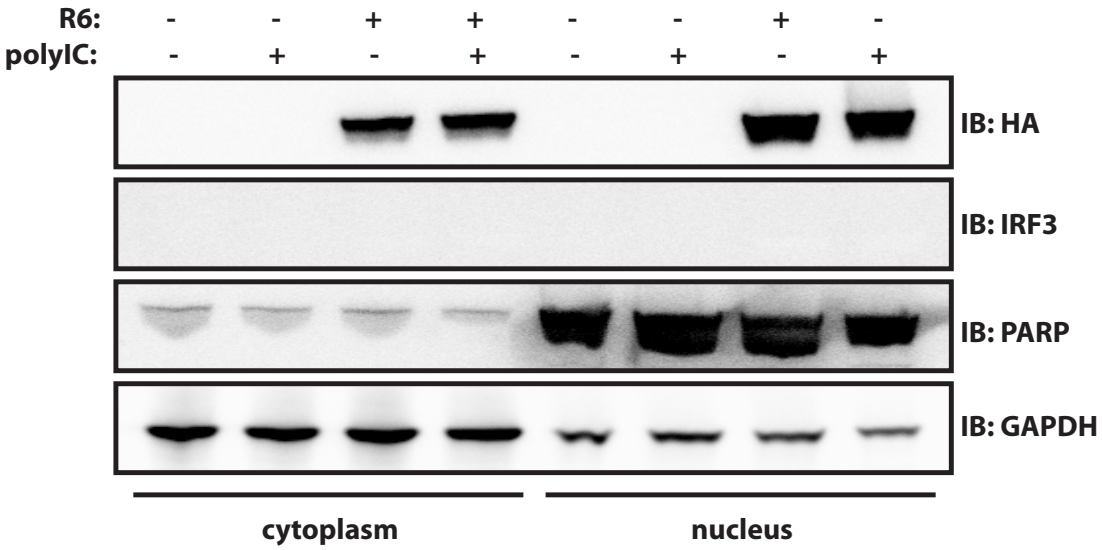

Figure A.2. R6 enters the nucleus independent of IRF-3.

IRF3-/- RFs were transfected with pcDNA3.1-R6HA or empty pcDNA3.1 for 40 h and were either treated with poly(IC) for 8 h or left unstimulated. Cytoplasmic and nuclear fractions then subjected to SDS-PAGE and probed for HA and IRF-3 (FL425). GAPDH and PARP were used as loading controls and to verify purity of fractionation.