RHESUS MACAQUE RHADINOVIRUS R12 IS REQUIRED FOR PROMYELOCYTIC LEUKEMIA NUCLEAR BODY DISRUPTION AND INHIBITS INNATE IMMUNE RESPONSES

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CERTIFICATE OF APPROVAL

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SELECTED ABREVIATIONS

α: Alpha

β: Beta

γ: Gamma

κ: Kappa

λ: Lambda

ART: Anti-Retroviral Therapy

BAC: Bacterial Artificial Chromosome

EBV: Epstein Barr Virus

GAS: Gamma-Activated Sequence

HCMV: Human Cytomegalovirus

HHV: Human Herpesvirus

HSV: Herpes Simplex Virus

IE: Immediate early

IFN: Interferon

IFNAR: Interferon Alpha Receptor

IRF: Interferon Regulatory Factor

ISG: Interferon Stimulated Gene

JAK: Janus Kinase

KS: Kaposi Sarcoma

KSHV: Kaposi Sarcoma Associated Herpesvirus

MCD: Multicentric Castleman's Disease

MHV68: Murine Herpesvirus 68

ORF: Open Reading Frame

PAMP: Pathogen-Associated Molecular Pattern

PML: Promyelocytic Leukemia

PML-NB: Promyelocytic Leukemia Nuclear Body

PRR: Pattern Recognition Receptor

RM: Rhesus Macaque

RhIFNα2: Rhesus Macaque Interferon Alpha 2

RRV: Rhesus Macaque Rhadinovirus

SIM: SUMO-Interacting Motif

SP100: Speckled 100 kDa

STAT: Signal Transducer and Activator of Transcription

SUMO: Small Ubiquitin-like Modifier

vIRF: Viral Interferon Regulatory Factor

VZV: Varicella Zoster Virus

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ABSTRACT

Kaposi sarcoma herpesvirus (KSHV) is the infectious etiological agent of several malignancies, lymphoproliferative disorders, and immune-dysfunction syndromes with significant morbidity and mortality in HIV-infected and immune-compromised individuals. While incidences of AIDS-associated malignancies caused by KSHV have declined considerably since the implementation of highly active antiretroviral therapies (ART), those individuals who develop KSHV-associated diseases have few, and often ineffective, treatment options. This underscores the continued need for research to understand KSHV pathology and development of new therapies.

Intrinsic and innate immunity are the first lines of cellular defense that viruses must overcome in order to successfully establish infection. Herpesviruses are a very successful group of viruses, as they not only infect a wide variety of species; they also produce lifelong infections that are never cleared from the infected hosts. A large and growing number of studies have helped to define the virally-encoded strategies utilized by herpesviruses to overcome the intrinsic, innate, and adaptive immune responses and explain their great success at persisting in their respective hosts. Unique among the herpesviruses, the gamma-2 herpesviruses such as KSHV and the closely related rhesus macaque rhadinovirus (RRV), encode open reading frames (ORFs) with homology to cellular interferon regulatory factors (IRFs). These viral IRFs (vIRFs) have been shown to play roles in innate and adaptive immune evasion, as well as oncogenesis and apoptosis. Several studies have now described how

promyelocytic leukemia nuclear bodies (PML-NBs) function as intrinsic immune barriers that can potently inhibit viral replication. Much has been learned about how the alpha- and beta- herpesviruses are able to circumvent PML-NBs. However, only a few studies have begun to describe the interactions between gamma-2 herpesviruses and PML-NBs. Understanding how gamma-2 herpesviruses overcome the immediate restriction imparted by PML-NBs will provide valuable insight into the establishment of productive infections and could identify possible therapeutic targets to prevent infections.

This dissertation will present the current research findings in the fields of herpesvirus activation of and evasion of intrinsic and innate immunity, with a particular focus on PML-NBs and the gamma-2 herpesviruses, KSHV and RRV. I report for the first time that an RRV vIRF (R12) is involved in the disruption of PML-NBs upon *de novo* lytic infection. R12 not only localizes to PML-NBs but also complexes with PML protein. Additionally, R12 expression inhibits the interferon (IFN)-signaling pathway downstream of type I IFN engagement of the type I IFN receptor. These functions of R12 inhibit the induction of IFN stimulated genes (ISGs) during *de novo* lytic infection and ultimately aid RRV replication at early times post infection. R12 increases RRV infection efficiency in the presence of type I IFN signaling, such as would be encountered during natural *in vivo* infection, and provides evidence that R12 could help RRV to efficiently infect rhesus macaques.

CHAPTER 1

Introduction

1.1 Human Herpesviruses

Human herpesviruses are highly successful viruses that have co-evolved with humans and establish persistent lifelong infections. Diseases associated with the various human herpesviruses range in severity from mild rashes to severe birth defects and cancer development. Thus, the human herpesviruses pose a significant threat to human health.

1.1.1 Herpesvirus Classification

The *Herpesvirales* order contains three families, *Malacoherpesviridae*, *Alloherpesviridae*, and *Herpesviridae* (1). The *Malacoherpesviridae* family infects molluscs. *Alloherpesviridae* members infect fish and amphibians and the *Herpesviridae* family infects mammals, birds, and reptiles. The *Herpesviridae* family is further divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Eight herpesviruses are known to infect humans and can be found in each subfamily of *Herpesviridae*. Herpes simplex virus 1 [HSV-1, also known as human herpesvirus 1(HHV-1)], herpes simplex virus 2 (HSV-2, HHV-2), and varicella-zoster virus (VZV, HHV-3) are members of the *Alphaherpesvirinae*. Human cytomegalovirus (HCMV, HHV-5), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7) are classified within the *Betaherpesvirinae* subfamily. Lastly, the *Gammaherpesvirinae* subfamily is divided into *gamma-1 lymphocryptoviruses* and *gamma-2*

rhadinoviruses, of which Epstein-Barr virus (EBV, HHV-4) and KSHV (HHV-8) are members (respectively).

Herpesviruses, including the human herpesviruses, infect a narrow host range and have adapted to their respective hosts so that infections are never cleared, but display two distinct phases; a lytic phase marked by active gene transcription and viral replication, and a latent phase with a very limited gene transcription program and no viral replication (2). The subfamilies of *Herpesviridae* are separated based on cellular tropism, host range, replication kinetics, and phylogenetic relatedness based on genetic sequence. Alpha-herpesviruses show rapid kinetics of replication and during latency they display tropism for sensory ganglia. Beta-herpesviruses have slow replication kinetics and establish latency in myeloid-lineage cells. Lastly, the gamma-herpesviruses are lymphotropic viruses with replication kinetics similar to beta-herpesviruses (3). The same factors that help delineate the different subfamilies of human herpesviruses also influence the research models used to study each virus, as discussed below.

1.1.2 Herpesvirus Structure

One of the main criteria for inclusion into the *Herpesvirales* order is virion morphology (Figure 1.1) (4). All herpesviruses have linear double-stranded (ds) DNA genomes packaged within an icosahedral capsid. The size of the genomes varies across the different herpesviruses, ranging from 125kbp to 240kbp and encoding around 40 functionally conserved genes and many more non-conserved genes (5). The capsid is composed of 12 pentons and 150 hexons

which themselves consist of 5 or 6 major capsid proteins, respectively. Pentons and hexons are interconnected by triplexes that consist of two proteins, a monomer and a dimer (6). Surrounding the capsid is an unstructured area called the tegument, which contains functional proteins that are important for initial virus infection (7). The outer-most layer of the mature virion is the envelope, which contains lipid membrane derived from the Golgi and/or endosomes of the infected cell. Along with the cellular proteins and lipids, the envelope also contains viral glycoproteins necessary for attachment, fusion, and entry into the next target cell (7).

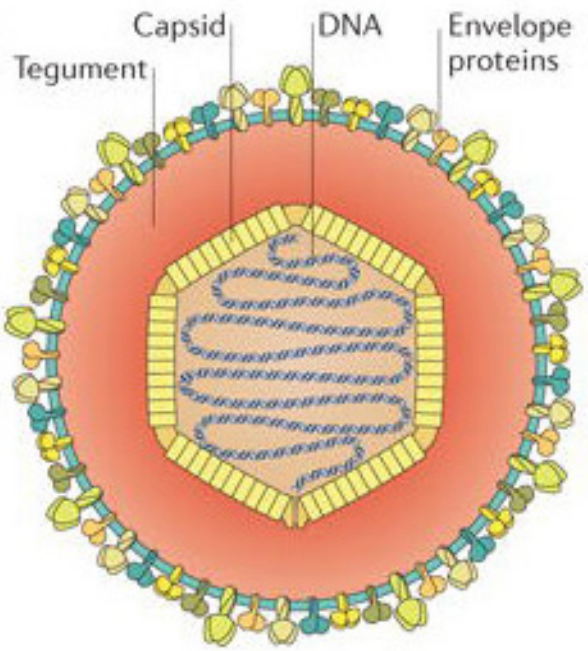

Figure 1.1 Herpesvirus Virion
With permission from Zerboni et al. 2014 Nature Reviews Microbiology

1.1.3 Herpesvirus lifecycle

Entry into cells by the human herpesviruses can occur by fusion at the plasma membrane or fusion with endocytic vesicles to deliver capsid and tegument proteins into the cytoplasm (8). Once in the cytoplasm, the capsid is transported to the nucleus along microtubules. At the nucleus the viral DNA is ejected from the capsid through the capsid portal and into the nucleus through nuclear pores (9). Once inside the nucleus, the viral DNA circularizes and, during lytic replication, begins a highly ordered gene-expression program (9). A set of genes termed immediate early (IE) are transcribed first and do not require previous viral protein synthesis before their activation. Some of the IE genes function as transactivators of subsequent viral genes. The early (E) genes are transcribed next and require IE gene transcription to have occurred prior, but do not require viral DNA synthesis. Several of the E gene products are involved in genome synthesis and, therefore, viral genome replication can begin at this point. The late (L) genes are the last set of genes to be transcribed during the acute phase of viral replication. The transcription of L genes requires that viral DNA synthesis has occurred. These gene products include the structural proteins necessary for assembly of the new progeny virions (10). After the capsid proteins are synthesized in the cytoplasm they move back into the nucleus to assemble, the newly synthesized genomes are packaged into the capsids and must next exit the nucleus. This is thought to be achieved by budding through the inner nuclear membrane into the space between the two nuclear membranes. The virus particle now has a lipid membrane envelope surrounding the capsid, which

will fuse with the outer nuclear membrane so that the capsid loses the envelope as it enters into the cytoplasm. The viral capsid acquires viral and cellular proteins that will make up the tegument as it travels through the cytoplasm and gains the final envelope from the modified membranes of the Golgi or endosomes during the exit from the cell (Figure 1.2) (8, 11).

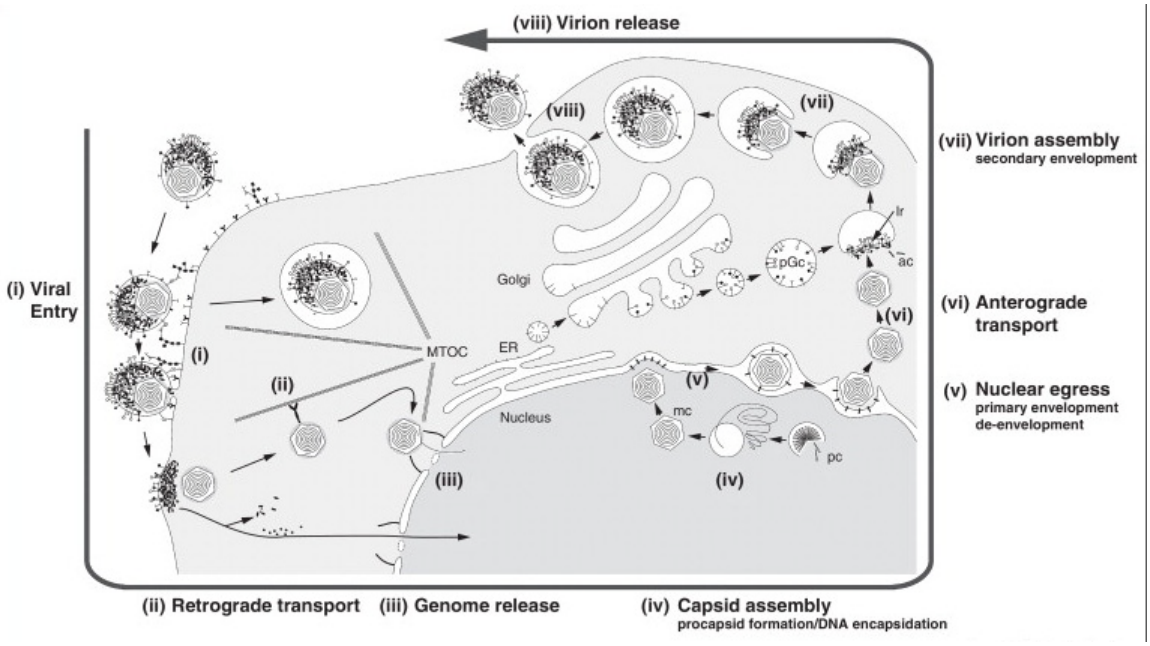

Figure 1.2 Herpesvirus Lifecycle
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1.1.4 Herpesvirus Pathogenesis and Animal Models

1.1.4.1 Human alpha-herpesviruses

HSV-1, HSV-2, and VZV cause ulcerative sores and vesicles during active replication. They initially infect mucosal epithelium before establishing latency in the dorsal root ganglia that innervate sites of active replication (3). However, there are several important distinctions to be made in the pathogenesis of these viruses.

HSV-1 and HSV-2 are transmitted person to person from physical contact of infected mucosal sites where viral shedding is occurring (3). These viruses will infect the mucosal epithelium and produce pustules and ulcers at the site of infection (oral for HSV-1 and genital for HSV-2). Eventually the viruses will infect the innervating ganglia and become latent. Reactivation of the virus occurs following stress (physical or emotional) or immune suppression. Active virus will travel down the nerve (called anterograde transport), back to the mucosal sites of initial infection and cause blistering and sores, viral replication, and virus shedding (3). Currently, no vaccines are in use for preventing or treating HSV-1 or -2 infections. Three antiviral therapies are approved by the Food and Drug Administration (FDA) for the treatment of herpes simplex outbreaks; Acyclovir, Valacyclovir, and Famciclovir, which are all nucleoside analogues that inhibit the viral DNA polymerase.

HSV-1 and HSV-2 are more promiscuous viruses in that they are able to infect several species such as mouse, rabbit, guinea pig, rat, owl monkey, marmoset, and rhesus macaque (RM) (12). Infection of mice with HSV-1/2 quickly establishes latency in ganglia, and provides a model to study acute infection, latency, and neuroinvasion (12, 13). Guinea pigs and rabbits provide a useful model for studying reactivation, as this does not occur as reliably in the mouse model (12).

Unlike HSV-1/2, VZV infects individuals through the upper respiratory tract via aerosolized virus particles (14). After the mucosal epithelium of the upper respiratory tract becomes infected, a 10-21 day incubation period can be

followed by varicella disease (chickenpox) (14). The rash first appears on the trunk and soon spreads to the face and extremities and can be accompanied by fever. Following primary infection, the innervating ganglia can become infected and VZV will become latent within these cells (15). Reactivation can occur and results in herpes zoster (shingles) disease. Very painful lesions on isolated areas of the skin that coincide with the innervating ganglia experiencing viral reactivation mark this disease (15). Similar to primary infection, lesions will usually clear in about a week. However, nerve pain can last for several more weeks and around 30% of infected individuals will develop chronic pain or post-herpetic neuralgia (16). Three vaccines are in use within the U.S. for prevention of VZV-related diseases. Varivax is approved for children as young as 12 months to prevent varicella and Zostavax is approved for adults aged 50 or older to prevent herpes zoster reactivation (17). Both of these vaccines are live attenuated strains of VZV. Recently, in October of 2017, a new recombinant herpes zoster subunit vaccine (Shingrix) was approved by the FDA in the U.S. (17).

Animal models for VZV include the use of guinea pigs, mice, and rats for *in vivo* studies, although these models have limitations. Guinea pigs can only become infected with a guinea pig adapted strain of VZV, obtained through serial passaging within fetal guinea pig cells (18). Infection of guinea pigs with the adapted strain results in seroconversion but pathogenesis is limited and reactivation does not occur (19). Experimental VZV infection of mice and rats will similarly result in seroconversion without any clinical disease manifestations (19).

Severe-combined immunodeficient (SCID)-humanized (SCID-hu) mice provide a better mouse model for VZV pathogenesis. Transplantation of fetal human thymus and liver tissue, human skin grafts, or human fetal dorsal root ganglia into SCID mice provides a model for VZV infection and pathogenesis (14, 20). Simian varicella virus (SVV) is a non-human primate (NHP) alpha-herpesvirus with 70-75% DNA similarity to VZV (21). SVV infection of NHPs such as cynomolgus macaques and patas monkeys results in more severe disease manifestations including death, providing a model for severe VZV disease complications (22-24). RMs infected with SVV do not die and present a similar pathogenesis to that seen in most human infections with VZV including rash, viremia, and T and B cell involvement, followed by latency in ganglia (25). Therefore, while limited availability and expensive costs can make RM models more difficult to work with, the SVV model is very important for studying VZV-like disease and pathogenesis *in vivo*.

1.1.4.2 Human beta-herpesviruses

Pathogenic human beta-herpesviruses can be grouped into two classes, HCMV and Roseolaviruses (HHV-6 and HHV-7). HCMV infection occurs through contact with infected bodily fluids (such as saliva, urine, and breast milk), organ transplantation with a HCMV positive donor, or by vertical transmission from mother to fetus (3). HCMV can infect a wide range of cellular targets however it is now believed that hematopoietic cell lineages, especially monocytes, provide the latency reservoir for HCMV (26, 27). When latently infected monocytes traffic to

tissues and differentiate into macrophages or monocyte-derived dendritic cells, the virus can reactivate (26).

Infection of immune-competent individuals is usually asymptomatic but fever, sore throat and swollen glands may also occur. In rare cases, some healthy individuals may develop a HCMV mononucleosis or hepatitis (28).

HCMV infection of a fetus or immune-compromised individual can result in significant morbidity and mortality. In immune-compromised individuals, such as AIDS patients, primary HCMV infection or reactivation can lead to retinitis, gastrointestinal disease, pneumonia, and central nervous system disease (including peripheral neuropathy and myelitis) (26, 29, 30). Another population at risk for severe HCMV infection outcomes is transplant patients undergoing immunosuppression. The highest risk population is HCMV seronegative patients receiving a transplant from a HCMV positive donor (31). CMV syndrome, characterized by fever, low white blood cell and blood platelet counts, and elevated liver enzyme levels, can develop 3 to 4 weeks post transplant (31). Other diseases like hepatitis, pneumonia, gastrointestinal disease, and retinitis can occur in the HCMV-transplant setting (31). Finally, HCMV disease is associated with allograft rejection (31, 32). Another immune-compromised population at risk of severe outcomes following HCMV infection is the unborn fetus. If a pregnant woman experiences a primary infection (or more rarely, a reactivation) with HCMV, the virus can infect the fetus (33, 34). *In utero* infection affects multiple organs and can cause a multitude of birth defects including growth and mental retardation, sensorineural hearing loss, hepatosplenomegaly,

and microcephaly (26). Presentation of severe pathology in the context of multiple types of immune-deficiency underscores the balance between immune protection and persistent HCMV infection.

Characteristic of beta-herpesviruses, HCMV displays restricted species tropism, only infecting humans. Humanized mouse models provide a means to study HCMV *in vivo*, but the data that can be obtained from such animal models depends on how the mice are humanized. SCID mice lacking B and T cells can be engrafted with human fetal tissues (liver, thymus, lung, colon, skin, retina) to study HCMV replication in the transplanted tissues (35). More recently, knockout of interleukin-2 receptor γ-chain locus in SCID mice (NSG mice) provides mice deficient in B, T, and NK cells (36). When human fetal bone marrow, liver, and thymus (BLT) are transplanted into these mice there is reconstitution of human myeloid lineage, NK cells, B cells, and T cells. Human $CD34^+$ hematopoietic progenitor cells have also been transplanted into NSG mice. These newer models of humanized mice provide the ability to examine HCMV latency, reactivation, and immune responses (26). Other mammals harbor their own versions of CMV; murine CMV (MCMV), rat CMV (RCMV), guinea pig CMV (GPCMV), and RM CMV (RhCMV) provide valuable models to study HCMV-like infections, pathogenesis, and vaccine development (37-39). However, the study of vertical transmission and subsequent birth defects can only be studied using the guinea pig and RM models, as MCMV and RCMV do not cross the placental barrier in their respective hosts (38, 40, 41).

HHV-6 and HHV-7 are also known as roseoloviruses due to a characteristic rash that can present after infection with either virus. HHV-6 is further divided into two species, HHV-6A and HHV-6B. HHV-6 and HHV-7 infection routes are thought to be primarily through saliva (42). *In vivo*, HHV-6 can infect several cell types and organs (central nervous system tissue, salivary glands, liver, endothelial cells and monocyte/macrophages). HHV-7 displays a more restricted cell tropism *in vitro* than HHV-6, which may suggest different *in vivo* cell tropism. In fact, latency appears to be established in monocyte/macrophage cells by HHV-6 and in $CD4^+$ T cells by HHV-7 (43, 44). One unique feature of HHV-6 (both A and B) that is not shared by the other human herpesviruses is the ability of HHV-6 to integrate its genome into human chromosomes. Up to 1% of the human population harbors HHV-6 in their genome and this can be passed to offspring through germ-line cells (45). Additionally, the integrated HHV-6 genome can reactivate (46). Primary infection with HHV-6 and HHV-7 typically occurs in early childhood and can be asymptomatic or result in a sudden high fever followed by a rash (exanthema subitum) (42). More severe disease outcomes rarely occur and may be due to underlying factors such as genetic abnormalities or immune dysregulation (43).

Similar to HCMV, HHV-6 and HHV-7 display a restricted species tropism. A marmoset model for HHV-6A and HHV-6B infection has been developed with limited clinical manifestations (47). Humanized mouse models have also been utilized for HHV-6 and HHV-7 *in vivo* studies (47). Pig-tailed macaques have been shown to be susceptible to HHV-6A infection with evidence of viral

replication and similar disease manifestations observed in humans (48). Additionally, viral homologs to HHV-6 and HHV-7 have very recently been described for pig-tailed macaques (MneHV-6 and MnHV-7) and mice (murine roseolovirus, MRV) and provide the opportunity to investigate these beta-herpesviruses *in vivo* (49, 50).

1.1.4.3 Human gamma-herpesviruses

The human gamma-herpesviruses have the distinction of being oncogenic viruses and include EBV and KSHV. KSHV will be discussed in detail in the following section 1.2. EBV is transmitted through infected saliva and first infects mucosal epithelial cells where active replication generally takes place. Virus is then transmitted to B cells and becomes latent in memory B cells (51). There are three latency programs for EBV. Latency III helps drive B cell proliferation and expresses the full repertoire of EBV latency genes and non-coding RNAs. Latency II only expresses Epstein-Barr nuclear antigen 1 (EBNA1) and the latent membrane protein (LMP) transcripts to help the virus evade the immune system. Finally, latency I expresses only EBNA1 (52). Infection of young children with EBV is usually asymptomatic while infection of adolescents can result in infectious mononucleosis (fever, sore throat, swollen lymph nodes, and fatigue) (52). A minority of (often immunocompromised) individuals infected with EBV will go on to develop EBV-associated malignancies. These malignancies include B-cell lymphoproliferative disease, Burkitt's lymphoma, Hodgkin lymphoma, primary effusion lymphoma, diffuse large B-cell lymphoma, nasopharyngeal carcinoma, and gastric adenocarcinoma (53, 54).

Just like the beta-herpesviruses, human gamma-herpesviruses do not readily infect other species. Humanized mouse models provide one method for studying EBV infections *in vivo* (55). Closely related viral homologs afford another means to study EBV-like infections. Murine gamma-herpesvirus 68 (MHV68) is closely related to EBV and is also utilized as a mouse model for EBV-like infections (56). Many NHPs are also infected with host-specific viruses that are closely related to EBV. RMs (Rhesus lymphocryptovirus, RhLCV), marmosets (maLCV), and cynomolgus macaques (cyLCV), provide additional animal models for EBV (57).

As described in this section, human herpesviruses are pernicious viral threats to human health. Their ability to establish life-long persistent infections presents a distinct obstacle that must be overcome in order to reach a cure. The various animal models and related animal viruses described above are indispensable for the development of therapeutics and for research into potential cure strategies.

1.2 KSHV

KSHV (HHV8) was the most recently identified human herpesvirus. With the onset of the AIDS epidemic in the United States, a focus on research to discover the cause of KS in AIDS patients began. Roughly 10 years after the AIDS epidemic in the U.S. began, KSHV was isolated from KS lesions and identified as the etiologic agent of KS (58). The prevalence of KSHV infection varies from region to region across the world with high-level endemic areas (sub-Saharan Africa and some regions of the Middle East) reporting 30%-70% of

adults infected, intermediate-level endemic regions (Mediterranean area) with 10%-25%, and non-endemic areas (the Americas, northern Europe, and Asia) observing less than 10% of the general population infected (59, 60). Immunocompetent individuals infected with KSHV generally remain latent and asymptomatic. In the event of immune dysregulation or immune suppression, KSHV can reactivate and lead to several malignancies and lymphoproliferative disorders.

1.2.1 KSHV Genome

The genome of KSHV is packaged into virions as linear dsDNA, 165-170 kbp in length, which remains linear during lytic replication but forms circular episomes during latency (61). A ~137 kbp long unique region (LUR) is flanked on either side by terminal repeat sequences of 30 kbp in size (62, 63). The LUR contains the over 90 ORFs encoded by KSHV and 12 pre-micro RNAs (pre-miRNAs) that give rise to 25 mature miRNAs (64, 65). Genes that were initially identified as unique to KSHV were designated K1 through K15. KSHV encodes at least 14 viral genes with homology to cellular genes including a viral Interleukin-6 (vIL-6, K2), viral B cell lymphoma-2 (vbcl-2, ORF16), viral Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein (vFLIP, ORF71), and vIRF-1 though vIRF-4 (K9-K11.1, see section 1.6 below) (66). The terminal repeats on either end of the KSHV genome contain ~800 bp repeat units with a G+C content of 84.5% (compared to the 53.5% G+C content of the LUR). These terminal repeats contain potential packaging and cleavage sites and are required for episomal DNA maintenance during latency (61, 66).

1.2.2 KSHV Tropism and Lifecycle

1.2.2.1 Tropism

KSHV is like several other human herpesviruses in that it possesses a strict species-specificity for humans. KSHV can infect many different human cell types including fibroblasts, endothelial cells, B cells, $CD34^+$ hematopoietic progenitor cells, monocytes, epithelial cells, and DCs (61, 67). It is widely believed that the main latency reservoir for KSHV *in vivo* is memory B cells, although latency may establish in other cell types including monocytes (67). In addition to human cells, KSHV can infect some non-human cells *in vitro* like hamster BHK-21 and CHO cells, mouse fibroblasts, and owl monkey kidney cells (67).

1.2.2.2 Infection and Active Replication

The primary infection route for KSHV is not fully elucidated as of yet, but is believed to occur mainly through saliva and to a lesser extent through sexual contact, or blood (68). Binding receptors and entry receptors on target cells allow KSHV to attach and then enter the cell. Heparin sulfate on the cell surface serves as a binding receptor for KSHV to first attach to the target cell (69). KSHV glycoproteins gB, gpK8.1A, ORF4, and gH enable the initial binding of KSHV to heparin sulfate (70). Several entry receptors utilized by KSHV have been identified and the receptor used depends on the cell type. KSHV gB contains an integrin binding motif and the $\alpha 3\beta 1$, $\alpha V\beta 3$, $\alpha V\beta 5$, and $\alpha 9\beta 1$ integrins have been

shown to serve as entry receptors on endothelial cells, fibroblasts, and monocytes (70-72). A 12-transmembrane glutamate/cysteine exchange transporter (xCT) has also been identified as a KSHV fusion-entry receptor that may work alongside integrins to facilitate viral fusion (73, 74). To date, the KSHV glycoprotein that interacts with xCT has not been identified. DC-SIGN on myeloid dendritic cells (DCs), macrophages, THP-1 monocytes, and activated B cells is another entry receptor for KSHV, through binding with KSHV gB (70, 72) (75). Lastly, ephrin type-A receptor 2 (EphA2R) on endothelial and fibroblast cells can be bound by gH/gL of KSHV, leading to phosphorylation of EphA2R and internalization of the KSHV virion (76). Once bound to the target cell, KSHV is internalized through macropinocytosis or clathrin-mediated endocytosis (70, 72). Following internalization, the KSHV replication cycle is typical for herpesviruses with temporal expression of viral genes, replication in the nucleus, mature capsids budding from the nucleus, obtaining tegument proteins in the cytoplasm, and acquiring an envelope from endosomes or golgi, before exiting the cell (70, 77, 78).

1.2.2.3 Latency

When KSHV becomes latent, it must maintain its genome throughout cell division. This is achieved by tethering the episome (packaged into chromosome-like structure) to cellular chromosomes using the viral protein latency-associated nuclear antigen 1 (LANA-1, ORF73) (79). The terminal repeat sequence is the origin of latent replication (*Ori-P*) and is necessary for genome persistence (78). 50 to 100 copies of KSHV genomes are maintained in a latently infected cell, and

replication of the viral genomes occurs alongside cellular genome replication (80, 81). During latency only a small subset of viral genes and miRNAs are expressed (62).

1.2.2.3.1 Latency Associated Genes

KSHV genes expressed during latency include LANA-1, vCyclin, vFLIP, K12, and vIRF-3 (82). As described above, LANA-1 helps to maintain the viral episome in dividing cells. LANA-1 also possesses anti-apoptotic, cellular proliferation, and maintenance of latency functions through its interactions with other cellular proteins, viral proteins, and viral promoters (82). ORF72/vCyclin promotes cell cycle progression and proliferation by activating cyclin dependent kinase 6 (83). ORF71/vFLIP activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and promotes cell survival and viral latency (84, 85). The K12 locus produces three proteins, Kaposin A, B, and C with functions that contribute to tumorigenesis, angiogenesis, and pro-inflammatory responses (82, 86, 87). vIRF-3 has immune-modulatory, oncogenic, and anti-apoptotic functions and is discussed in detail in section 1.6.

1.2.2.3.2 KSHV miRNAs

Micro RNAs (miRNAs) are noncoding single-stranded RNAs 19-22 nucleotides in length that can post-transcriptionally repress target genes and prevent protein synthesis. Biogenesis of miRNAs begins in the nucleus where RNA polymerase II transcribes the pri-miRNAs (up to kilobasepairs long). Drosha and DiGeore syndrome critical region 8 (DGCR8) proteins process the pri-

miRNAs into pre-miRNA that are subsequently exported from the nucleus. Once in the cytoplasm, Dicer further cleaves pre-miRNAs into 19-22 nucleotide duplexed miRNAs. One strand of the mature miRNA is loaded onto the RNA-induced silencing complex (RISC) by binding to the Argonaute 2 protein. The sequence of the miRNA seed region (8bp long) directs the RISC complex to target mRNAs and results in translation inhibition or mRNA degradation.

KSHV encodes 12 pre-miRNAs that are processed into 25 mature miRNAs (88). Ten of the KSHV pre-miRNAs are located in the genomic region between K12 and ORF71 while two are located within K12 itself (pre-miR-K10 and pre-miR-K12) (89). As stated above, all of the KSHV miRNAs are expressed during latency. However, miR-K10a-5p, miR-K10a-3p, miR-K10b-3p, miR-K12-5p, and miR-K12-3p are also expressed during lytic infection (90, 91). Single miRNAs are able to target multiple genes and the KSHV miRNAs are no exception. KSHV miRNA target identification and validation has revealed maintenance of latency, anti-apoptotic, angiogenic, cell cycle regulation, and immune evasion roles for these miRNAs (88). For example, miR-K9-5p and miR-K7 target the KSHV lytic switch, replication and transcription activator (RTA), to help maintain latency (92-94). miR-K7 also targets major histocompatibility complex (MHC) class I polypeptide-related sequence B (MICB), which reduces attack of the infected cell by NK cells (95). miR-K11 targets I-kappa-B kinase epsilon [IKKε, a kinase that can phosphorylate/activate interferon regulatory factor 3/7 (IRF3/IRF7) transcription factors to induce IFN transcription] to inhibit type I IFN signaling (96). miR-K1, miR-K3-3p, miR-K6-3p, and miR-K11 target

thrombospondin 1, an anti-angiogenic and anti-proliferative protein (97). Caspase 3-induced apoptosis is suppressed by miR-K1, miR-K3, and miR-K4-3p (98). Thus, the KSHV miRNAs are a tool to modulate many cellular and viral targets in order to maintain latent viral infection within a suitable cellular environment.

1.2.3 KSHV-Associated Diseases

KSHV is associated with three malignancies and two acute inflammatory diseases. The first association of KSHV and a malignancy was the discovery that KSHV was the causative agent of Kaposi's sarcoma (KS). KS was first described by Dr. Moritz Kaposi in 1872 as benign lesion of the extremities found in elderly Mediterranean and eastern European men (99). There are now four recognized epidemiological forms of KS, and the one described by Dr. Kaposi is termed classic KS. Classic KS rarely develops into an aggressive malignancy (59, 99). The other three forms of KS are African endemic KS, iatrogenic KS, and AIDS-associated KS (59, 99). African endemic KS is found in sub-Saharan Africa where severe disease mainly afflicts children. Furthermore, African endemic KS is more aggressive than classic KS with significant mortality rates. Iatrogenic KS, or post-transplant KS is associated with the immunosuppressive therapies that transplant patients receive. Lastly, AIDS-associated KS is the most aggressive form of KS and is considered an AIDS defining illness. AIDS-associated KS is also the most common cancer associated with human immunodeficiency virus (HIV) infection. KS lesions are usually cutaneous or mucosal in location but have also been found on lymph nodes and visceral organs (100). The lesions are often polyclonal or oligoclonal (arising independently rather than through disseminated

metastasis) but instances of monoclonality have also been observed. These lesions are purple, brown, or red in color due to their high angiogenic nature. Vascular leakage is characteristic of KS lesions and cellular infiltration as well as extravasation readily occurs. Strikingly, over 95% of KS lesions contain KSHV DNA and the virus is often found to be latently infecting the cells. KS tumors contain a spindle-shaped cell that is believed to be of endothelial origin. Both blood endothelial and lymphatic endothelial cell markers are found on KS spindle cells and transcriptional reprogramming may be the cause (99, 100).

Primary Effusion Lymphoma (PEL) is another malignancy associated with KSHV. PEL is a non-Hodgkin lymphoma of B cell origin. Malignant B cells of PEL are monoclonal in nature and found in the pleural, pericardial, and peritoneal cavities of the body (101). PEL cells contain 50-100 copies of latent KSHV genomes and are often co-infected with EBV (101). PEL is an aggressive lymphoma with poor prognosis having an average of 5 to 6 months survival post diagnosis (99, 102).

Multicentric Castleman disease (MCD) is a lymphoproliferative disorder where proliferative tumors are found in lymph nodes and other lymphatic organs (99). While not all types of MCD are caused by KSHV infection, the plasma cell version of MCD is KSHV positive (99, 103). MCD entails an abnormal proliferation of (often) polyclonal immunoglobulin M (IgM) λ-plasmablasts in the mantel zone of B cell follicles (104). In contrast to KS and PEL, KSHV-positive MCD cells are often lytically infected (105). Fever, hypergammaglobulinemia, high levels of IL-6 and vIL-6, and autoimmune phenomenon are symptoms of

MCD (99, 105). MCD can cause autoimmune hemolytic anemia and sometimes leads to non-Hodgkin lymphoma, both of which can be fatal (99, 106).

Acute inflammatory diseases associated with KSHV include KSHV-associated inflammatory cytokine syndrome (KICS) and immune reconstitution inflammatory syndrome (IRIS). KICS is an inflammatory disease that lacks lymphadenopathy (as observed in MCD). KICS is characterized by high KSHV viral loads and increased levels of cellular and viral cytokines including human IL-6, IL-10, and vIL-6 (107). IRIS occurs in a subset of patients with advanced HIV infection, following the onset of ART treatment, and manifests as either a worsening of an opportunistic infection or the unmasking of a previously unknown infection. IRIS is believed to occur due to an increase in $CD4^+$ T cells (following ART treatment) that recognize pathogens or autoantigens which had been previously unrecognized and dysregulation of the reconstituted immune system. Worsening of KS lesions following the start of ART as well as unmasking of KS lesions and MCD following ART have been described (108-111).

KSHV diseases often, although not always, occur in a context of immune dysfunction. Understanding how KSHV contributes to this immune dysfunction and pathological outcomes is crucial for the development of effective antiviral therapeutics.

1.2.4 Therapies for KSHV-Associated Diseases

Following administration of ART, the incidence of KS, PEL, and MCD has declined in HIV positive individuals. However, AIDS-associated and non-AIDS-associated KSHV malignancies are still a real problem in parts of Africa and for

transplant patients. Treatments for established KS involve excision of cutaneous lesions if possible as well as administration of radiation, chemotherapeutic agents, cytokines, and antivirals. For visceral or disseminated KS, chemotherapy (doxorubicin, etoposide), IFNα, and IL-12 are options for treatment (99, 112-114). Very limited efficacy is achieved with the use of viral DNA polymerase inhibitors (ganciclovir, cidofovir, foscarnet), however prophylactic use may prevent the development of KS in immune-compromised individuals (99). Drugs to treat KS by targeting non-viral proteins have recently been developed. Imatinib inhibits the tyrosine kinase c-Kit, which is expressed in KS tumor cells and showed promising results in a phase II clinical trial (115). (115). The KSHV encoded G protein-coupled receptor (vGPCR) is a viral oncogene that helps to transform cells by activating the phosphoinositide 3-kinase and mammalian target of rapamycin (PI3K-mTOR) pathway, which positively regulates cellular proliferation and protein production (116). Rapamycin and analogs of rapamycin inhibit the PI3K-mTOR pathway and have been used both orally and topically to treat KS (116, 117).

MCD treatment also involves chemotherapy and use of viral DNA polymerase inhibitors have increased efficacy in MCD patients (compared to KS patients) due to the lytically replicating virus (99, 118). Rituximab (anti-CD20 monoclonal antibody) used alone or in conjunction with chemotherapy is another therapeutic option (118). However, rituximab treatment for MCD may worsen a concurrent case of KS and in at least five instances, HIV negative/KSHV positive individuals receiving rituximab for autoimmune or transplant reasons developed

KSHV induced tumors (4 cases of KS and 1 case of solid PEL) (119). IL-6 or IL-6 receptor monoclonal antibody therapy has also been used to alleviate symptoms of MCD (99).

PEL treatments mainly include chemotherapy and ART as well as radiation in limited cases (120). No standard of care is established for PEL and prognosis remains poor.

Therapies for KICS, KSHV-IRIS, and unmasking-IRIS are also not well defined but antivirals (cidofovir), chemotherapies, and other supportive care appear to show some efficacy. In some cases, KS-IRIS is self limiting and resolves without therapeutic intervention (110).

Limited treatment options and high costs illustrate the need for continued KSHV research and development of better therapeutics. Further characterization of how KSHV manipulates infected cells can help identify both viral and cellular targets for antiviral therapies.

1.2.5 Animal Models for KSHV

Animal models for KSHV infection and pathogenesis studies have been difficult to develop due to the high species specificity exhibited by the virus. Attempts to infect immune-compromised mice, specifically the SCID mouse, by transplanting KSHV infected human B cell lymphomas along with human peripheral blood mononuclear cells (PBMCs) resulted in lymphomas derived from the transplanted B lymphoma cells but infection of the co-transplanted human PBMCs was not observed (121). KSHV infected $CD34^+$ hematopoietic progenitor cells implanted into the NOD/SCID mouse model had detectable KSHV infected

B cells in the bone marrow and spleen. Additionally, 3 of the 10 mice developed a pleural effusion with the presence murine B cells (displaying *in vitro* immortalization) and KSHV infected human monocytes (122). Another group showed that injection of KSHV into NOD/SCID mice has also been found to result in the establishment of productive infection and long-term persistence of the virus; however, no KSHV-associated diseases developed within these mice (123). Humanized BLT mice infected with KSHV displayed a similar response as the NOD/SCID mice where both latent and lytic KSHV gene expression could be detected up to 3 months post infection but no KSHV-associated diseases developed (124). However, infection routes believed to be important for human transmission, such as through the oral mucosa and intravaginally, established successful infections in the humanized BLT mice. NGS mice (NOD/SCID/IL-2R γ-chain knockout) provide a human tumor xenograft model utilized to study possible anti-tumor therapeutics. Successful engraftment of KSHV infected PEL and KS tumors into NGS mice has been reported. However, no infectious virions are generated in the mice following engraftment due to latent infection predominating in the tumors (125). Thus, humanized mouse models can recapitulate only some aspects of KSHV infection and very limited pathogenesis.

As described in section 1.1.4, MHV68 is a murine gamma-herpesvirus that is phylogenetically related to both EBV and KSHV. MHV68 genome is essentially collinear with both EBV and KSHV genomes and provides a model to study tropism, latency, and immune responses to gamma-herpesviruses (125). However, there are some important differences between KSHV and MHV68.

MHV68 does not encode several immune-modulating and tumor-promoting genes encoded by KSHV including vCD200, viral macrophage inflammatory protein (vMIP), vFLIP, vIL-6, and the vIRFs (126). While lymphoproliferation has been observed in mice infected with MHV68, skin lesions and lymphomas do not develop in wildtype mice infected with MHV68 (125). Conversely, immune deficient mice infected with MHV68 do develop B cell tumors and inflammatory disorders (127). Whether MHV68 is a better model for EBV or KSHV is a matter or debate, however, the lack of important viral homologs to cellular genes that are present in KSHV is a drawback of this model. Additionally, KSHV-associated malignancies are often associated with HIV co-infection and no HIV homolog has been described for mice, precluding the development of a co-infection model in mice.

NHPs provide useful animal models for human diseases due to the close evolutionary relationship between NHPs and humans. Experimental infection of RMs with KSHV [both simian immunodeficiency virus (SIV) positive and SIV negative] does not result in robust KSHV infection (128). While KSHV viral DNA was detected in PBMCs a year after infection, no KSHV transcripts or KSHV-specific antibodies were detected in the animals and no KSHV-associated diseases developed (128). KSHV infection of the common marmoset produced a sustained antibody response, establishment of latency, and one of two orally-infected animals developed lesions similar to KS (129). These results could be due to the marmoset's limited MHC-I polymorphisms, which may restrict the breadth of the marmoset's adaptive immune response, and should be taken into

account when using this animal model. A gamma-2 herpesvirus was identified, by DNA sequencing, in retroperitoneal fibromatosis lesions that spontaneously arose in pig-tailed macaques and RMs at the Washington Regional Primate Research Center and was subsequently named retroperitoneal fibromatosis-associated herpesvirus (RFHV) (130). Retroperitoneal fibromatosis in macaques is a lesion that is similar to KS, containing spindle-shaped cells, high vascularization, and strong co-incidence with simian retrovirus 2 infection (and simian acquired immunodeficiency syndrome) (130). However, the origin of the spindle-shaped cells of retroperitoneal fibromatosis are believed to be fibroblasts, while an endothelial cell origin for the KS spindle-shaped cells has been characterized (131). Genomic comparisons between RFHV, KSHV, and RRV (discussed below) revealed that RFHV is more closely related to KSHV than RRV is to KSHV (132). Unfortunately, the inability to culture RFHV has limited its use as a model for KSHV-like infections and pathogenesis (133). The discovery of RRV, another gamma-2 herpesvirus closely related to KSHV that naturally infects RMs and is able to be propagated in tissue culture, has provided a very useful animal model and will be discussed in the next section.

1.3 RRV

RRV was isolated from two separate RM colonies by Dr. Scott W. Wong at the Oregon Regional Primate Research Center and by Dr. Ronald C. Desrosiers at the New England Regional Primate Research Center (134, 135). Sequence analysis revealed that this newly identified virus is a gamma-2 herpesvirus closely related to KSHV (Figure 1.3) (136). The two viral isolates, H26-95 (from

New England) and 17577 (from Oregon), were very similar genetically but only 17577 displayed pathogenesis in RMs co-infected with SIV, perhaps owing to the genetic divergence of the glycoproteins (in particular gB). Today, both isolates are studied and provide a means to investigate *in vivo* gamma-2 herpesvirus infections.

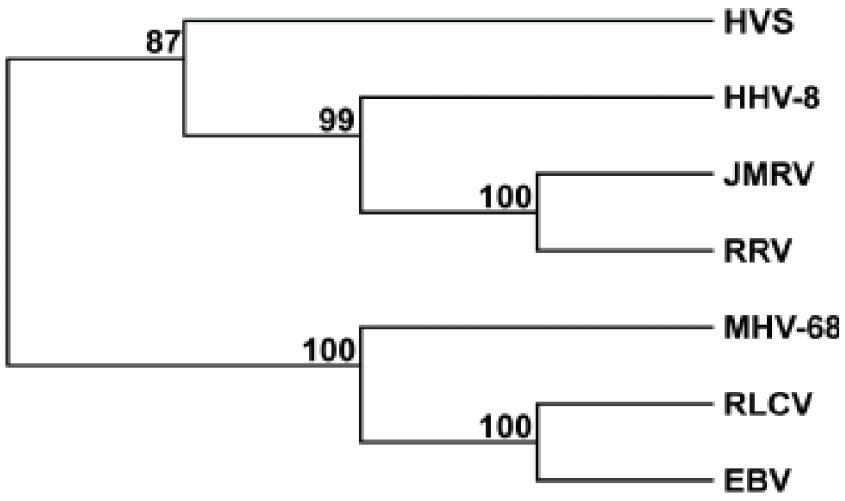

Figure 1.3 Phylogenetic Tree Displaying KSHV (HHV8) and RRV relatedness
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1.3.1 RRV Genome

The genome of RRV is essentially collinear to that of KSHV. As is the case for KSHV, RRV genome structure consists of terminal repeats on either ends of a LUR, and the 52.2% G+C content of the RRV LUR is comparable to KSHV LUR (53.5%) (137). Notable differences between KSHV and RRV include the genomic location of ORF2 (DHFR), a lack of K3, K4.2, K5, K7, and K12 homologs in RRV, and the number of vMIP (3 for KSHV and 1 for RRV) and vIRF (4 for KSHV and 8 for RRV) genes (137, 138). Sequence alignments between RRV isolates H26-95 and 17577 found mostly silent mutations and amino acid sequence identities of less than 95% were only found for 4 of the 84 ORFs (137,

139). One noteworthy difference was in the gB sequence, where 19 amino acid differences were identified, 7 of which changed the charge of side chains. The difference in gB sequence between H26-95 and 17577 may indicate that they are separate strains of RRV (137). The divergent gB sequences could contribute to the differential pathogenicity observed for H26-95 compared to 17577, as herpesvirus gB proteins are often immunogenic and help determine cellular tropism. However, it is clear that both H26-95 and 17577 provide a model to study KSHV-like infections.

1.3.2 RRV Tropism and Lifecycle

Multiple cell types can be infected with RRV *in vitro* including primary and telomerized rhesus fibroblasts, primary and immortalized rhesus B cells, rhesus T cells, rhesus monocytes, rhesus DCs, human BJAB cells, human lymphatic endothelial cells, and human vascular endothelial cells (140-143). *In vivo*, the major latency reservoir for RRV is B cells (144). RRV can use Ephrin receptor tyrosine kinases (Ephs) for entry into B cells and endothelial cells, by engagement of RRV gH/gL with Ephs followed by receptor mediated endocytosis (141, 142). Other entry receptors may exist, as blocking Ephs mediated entry did not completely inhibit RRV entry and infection of cells (142). However, integrins are not believed to be used as RRV gB does not contain the integrin binding motif (Arg-Gly-Asp) found in KSHV gB (145). Another study revealed that RRV entry into fibroblasts occurs through clathrin-mediated endocytosis, but no cellular receptor was identified (146). Following entry, viral capsids are trafficked to the nucleus on microtubules with the help of dynein motor proteins (147). RRV

follows the same general infection cycle as other herpesviruses with replication and capsid assembly occurring in the nucleus and then acquisition of the tegument and final envelope membrane within the cytoplasm as the virus traffics out of the cell (148-150).

1.3.3 RRV-associated Diseases

RRV infection of immunocompetent RMs does not usually cause disease. However, when RMs are co-infected with RRV and SIV or simian retrovirus 2, incidences of pathological outcomes increases. RRV 17577 was first isolated from an SIV infected RM that displayed a multicentric lymphoproliferative disorder (134). Experimental infection of RMs with RRV 17577 and SIV resulted in the development of a plasma cell variant MCD-like hyperplastic B cell lymphoproliferative disorder (LPD) (134). Hyperplastic B cell LPD is characterized by B cell hyperplasia, elevated IgG levels, lymphadenopathy, enlarged spleen, presence of vIL-6, and viremia (151). RMs infected with RRV alone displayed a transient B cell hyperplasia around 2 weeks post infection (134). However, if the RMs were co-infected with SIV and RRV, the B cell hyperplasia was larger in magnitude and was sustained over several more weeks. Lymphomas and retroperitoneal fibromatosis lesions (positive for RRV and vIL-6, negative for RFHV) have also been found in RRV/SIV co-infected RMs, and at least one case of lymphoma in an RRV-infected (but SIV negative) RM has been described (152).

The similarity in observed pathologies as well as the fact that disease incidence is predominantly in the context of dysfunctional immune systems further demonstrate the close relatedness between RRV and KSHV.

1.3.4 RRV as a model for KSHV

RRV and KSHV display some differences during *in vitro* infections, which provide valuable insights into different aspects of gamma-2 herpesvirus lifecycles. *In vitro* infection with KSHV will produce abortive replication and quickly establish latency in most cell lines. Use of chemical stimuli (phorbol esters and sodium butyrate) will induce reactivation of the virus in a subset of cells (153, 154). Exogenous expression of KSHV ORF50/RTA in cells infected with KSHV can also induce reactivation (155, 156). The development of an immortalized dermal microvascular endothelial cell line (DMVEC), immortalized with human papilloma virus 16 (HPV16) E6 and E7 genes, that is able to be infected with KSHV and sustains latent KSHV infection has been indispensible in the study of KSHV biology (157). In addition, telomerase-immortalized microvascular endothelial (TIME) cells, immortalized with the telomerase reverse transcriptase subunit (hTERT), provide long-lived endothelial cells for study of long term KSHV infections (158). Primary dermal endothelial cells as well as lymphatic endothelial cells can be infected with KSHV *in vitro*, however their tissue culture life-span is much shorter than the immortalized endothelial cells and can affect the study of latency and reactivation (159). B cells have been more refractory to *in vitro* infection with KSHV, however some recent advances

have been made. Infection of B cells has been achieved by treating B cells with CD40L and IL-4 (to up-regulate DC-SIGN and render B cells permissive to infection) prior to infection (160). Another method to infect B cells involves co-culturing B cells with KSHV lytically infected Vero or iSLK cells (lytic infection achieved by ectopic expression of ORF50 and sodium butyrate) (161, 162). The current *in vitro* systems for KSHV investigation provide models for latency and reactivation, but lack the ability to adequately study *de novo* lytic infection. In contrast to KSHV, *in vitro* RRV infection of a wide variety of both rhesus and human cell lines results in a predominantly lytic infection (163). Additionally, a RRV bacterial artificial chromosome (RRV-BAC) technology has also been developed and provides the means to mutate or remove specific RRV ORFs in order to study their function in the context of viral infection (164). Therefore, *in vitro* infection with KSHV provides a model for latent or persistent infections while RRV provides a model for lytic/productive replication.

As described above in section 1.5.3, in vivo RRV infection of SIV-infected RMs can result in pathologies that are very similar to those seen in KSHV/HIV co-infected humans. This animal model, along with the RRV-BAC system, provides a valuable model to study the involvement of specific viral- and host-factor contributions to disease development and viral persistence. Furthermore, RMs and RRV afford the ability to develop and test antiviral therapeutics and vaccines *in vitro* and *in vivo*.

1.4 Immune System

In order to maintain homeostasis and defend against harmful pathogens, including herpesviruses, vertebrates possess a multi-layered immune system. From ever-present restriction factors to the delayed but highly potent cytotoxic T cells, invading pathogens must overcome many obstacles to establish infections and replicate. The three layers of the immune system are described in this section, with emphasis on intrinsic and innate immunity as well as how type I interferon impacts the adaptive immune response.

1.4.1 Innate and intrinsic immunity

The first layer is called the intrinsic immune response. Intrinsic immunity is often categorized within innate immunity, but is characterized by a readiness to restrict infection by pathogens at all times. Components of the intrinsic immune response are constitutively expressed (although they may be further induced upon stimulation) and are termed restriction factors because they can limit replication of the pathogen directly without the need for signaling cascades (165, 166). With respect to viral infections, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G; restricts HIV-1 replication), bone marrow stromal antigen 2 (BST-2; inhibits viral egress of several enveloped viruses), and PML-NBs (restrict viral replication of a variety of RNA and DNA viruses) are examples of the mediators of intrinsic immunity (165, 166).

Innate immunity on the other hand involves the sensing of pathogens followed by the rapid induction of signaling cascades to orchestrate a broadly antimicrobial state mediated by innate immune cells, the complement system, cytokines, and chemokines (165). Innate immune responses do not rely on

specificity for the pathogen (as is the case with adaptive immunity, described below), instead reacting to anything non-self or danger-associated that is found within the body. Innate immunity is triggered through pattern recognition receptors (PRRs) expressed by the host cell that can detect pathogen associated molecular patterns (PAMPs) present on bacteria, fungi, parasites, and viruses. The innate immune response can also be triggered by danger associated molecular patterns (DAMPs), which are intracellular molecules such as heat shock proteins, adenosine triphosphate (ATP), or mRNA, that are released by a host cell in response to cellular damage (167). Once in the extracellular spaces, neighboring cells will recognize the DAMPs and initiate an innate immune response. Many cell types are involved in innate immunity include monocytes/macrophages, mast cells, neutrophils, eosinophils, basophils, fibroblasts, DCs, natural killer (NK) cells, innate lymphoid cells, and more (168). PRRs include the toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and Nod-like receptors (NLRs) (169, 170). There are other PRRs that recognize bacterial and viral nucleic acids, including but not limited to DNA-dependent activator of IRFs (DAI), cyclic GMP-AMP synthase (cGAS), interferon-γ-inducible protein 16 (IFI16), DEAD-box protein 41 (DDX41), DEAH-box protein 36 (DHX36), DNA-dependent protein kinase (DNA-PK), absent in melanoma 2 (AIM2), 2’5’-oligoadenylate synthetase (OAS), DHX9, and protein kinase-R (PKR) (171, 172).

Important for viral recognition and induction of the innate immune response are the trans-membrane TLRs and cytoplasmic RLRs. TLRs 2, 3, 4, 7,

8, and 9 recognize different viral infections, with TLR2 and TLR4 detecting viruses at the cell surface and the rest detecting viral components within endosomes (173). TLR2 and TLR4 can detect viral envelope proteins or viral proteins released from cells, TLR3 can detect viral dsRNA, TLR7 and TLR8 can detect viral single-stranded (ss) RNA, and TLR9 can detect viral dsDNA (174-179). The RLRs include RIG-I, MDA5, and LGP2. These PRRs can recognize cytoplasmic viral dsRNA (produced during RNA and some DNA virus infections) that contain certain moieties such as 5'-triphosphate, 5'-diphosphate, poly(U/UC) rich, and 2'-O-methylation (172, 180). In addition, MDA5 is thought to recognize longer dsRNA while RIG-I detects shorter dsRNA (181-183). Signaling by TLRs and RLRs results in the production of IFNs, pro-inflammatory cytokines and chemokines.

PKR can also detect cytosolic RNA produced during viral infection and will phosphorylate eukaryotic translation initiation factor 2A (eIF2a) to inhibit both viral and cellular protein synthesis (184, 185).

NLRs are known to sense bacterial infections directly, but no direct viral sensing by NLRs has been demonstrated to date (186). However, some NLRs are part of the inflammasome, which can be activated following viral infections (187, 188). Inflammasomes are multiprotein complexes that activate caspase-1, which will go on to cleave and activate pro-interleukin-1β (pro-IL-1β) and pro-IL-18 cytokines, as well as activating pyroptosis (186). IL-1β and IL-18 are important cytokines that induce many pro-inflammatory genes, enhance IFNγ production by and proliferation of T helper 1 (T_H1) cells, and cause fever (187).

The DNA sensing PRRs are important for the antiviral responses to infection with DNA viruses. For example, sensing of viral DNA by DAI will signal through the transcription factors NFκB and IRF3 to induce the production of type I IFNs, cytokines, and chemokines (189). IFI16 can function through multiple mechanisms, including inhibition of viral replication, induction of apoptosis, synergistically cooperates with cGAS to activate the stimulator of interferon genes (STING) pathway, or activation of the ASC-dependent inflammasome (190-193). When cGAS binds to viral DNA in the cytosol, cGAS produces the second messenger cyclic GMP-AMP (cGAMP). STING will detect cGAMP and signal through TANK-binding kinase 1 (TBK-1) to activate IRF3 and induce type I IFN production (194, 195).

In the event that intrinsic immune defenses are unable to stop infection or replication of invading pathogens, innate immune responses can come into play. Many of the innate immune pathways induced by PRRs result in the production of type I IFNs which go on to induce an IFN signaling pathway that activates the production of antiviral effectors. While intrinsic and innate immunity are defined as distinct branches of the immune system, they do influence each other and can establish positive feed-back loops.

1.4.1.1 Promyelocytic Leukemia Nuclear Bodies (PML-NBs)

Promyelocytic Leukmia Nuclear bodies [PML-NBs; also known as nuclear domain 10 (ND10) and PML oncogenic domains (PODs)] are dynamic subnuclear structures. PML-NBs were first discovered by electron microscopy as dense spheres within the nucleus of cells and by immunofluorescence

microscopy using autoantibodies from primary biliary cirrhosis patients which identified a 100 kDa nuclear protein (SP100) that formed a speckled or punctate dot pattern in the nucleus (196, 197). The promyelocytic leukemia (PML) gene was identified in acute promyelocytic leukemia (APL) cells where a translocation event between chromosomes 15 and 17 fused the PML gene to the retinoic acid receptor alpha (RARα) gene (198). Subsequent studies showed that the localization of PML protein was in punctate structures in the nucleus, and this structure was disrupted in APL cell lines (198, 199). Additionally, the clinical treatment for APL (all-trans retinoic acid) results in remission of the leukemia and reformation of PML-NB structures (200). Thus, the discovery of PML-NBs also provided the first insights into the significant role of these important subnuclear structures in human disease.

PML-NBs are now known to be constitutively expressed structures, expressed in most cell types, ranging in number from 1 to 30 PML-NBs per nucleus (201-204). PML protein is a member of the tripartite motif (TRIM) protein family and is also designated TRIM19. As a member of the TRIM family, PML protein contains a RING finger, two B boxes, and a coiled-coil (RBCC) domain (196, 205-207). The PML gene consists of 9 exons and through alternative splicing, produces at least 7 PML protein isoforms with differing carboxy-termini (Figure 1.4) (208, 209). All isoforms of PML protein contain the RBCC domain and hetero- and homo-dimerization between PML isoforms can occur through the coiled-coil domain (205, 208). PML isoform I contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) and is found in both the

cytoplasm and the nucleus. PML isoforms II-VI contain only the NLS and mainly localize to the nucleus while PML isoform VII lacks the NLS and is cytoplasmic. However, due to the ability of PML isoforms to heterodimerize, it is believed that all isoforms could shuttle between the nucleus and cytoplasm by binding to PML isoform I.

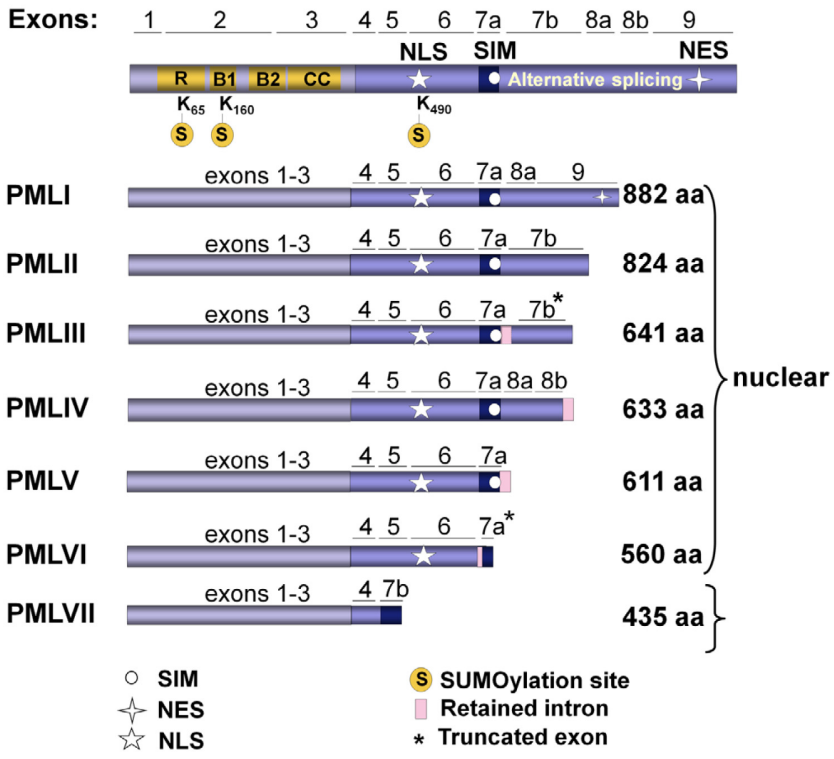

Figure 1.4 PML Protein Isoforms
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Formation of PML-NBs requires PML protein, but many other proteins are found to localize to PML-NBs either permanently or transiently, depending on the phase of the cell cycle, if the cell is damaged or under stress, or if the cell is infected with a pathogen (196). SP100, death domain-associated protein (Daxx), and alpha thalassemia/mental retardation syndrome X-linked (ATRX) are

considered more permanent residents at PML-NBs while p53, CREB-binding protein (CBP), checkpoint kinase 2, and many other proteins will transiently localize to PML-NBs (208). Once localized to PML-NBs, PML-NB resident proteins are often post-translationally modified [modified by small ubiquitin-like modifier (SUMOylated), phosphorylated, acetylated, ubiquitinated] which affects their functions and stability (210). Investigations into the mechanism of PML-NB formation have produced multiple conflicting results. PML protein, itself, can be post-translationally modified through phosphorylation, acetylation, and SUMOylation (211-213). SUMOylation of PML occurs on three lysine residues (K65, K160, K490) and there is also a SUMO interacting motif (SIM: ^{508}VVVI^{511}) therefore it was hypothesized that PML-NBs formed through SUMO-SIM interactions between PML proteins (211, 214). Some groups reported that overexpression of mutant PML proteins lacking either the SUMOylation lysines or the SIM failed to form PML-NBs and concluded that SUMO-SIM interactions were responsible for PML-NB formation (211, 214, 215). However, others have reported that SUMO and SIM deficient PML proteins can still form PML-NBs and instead the RBCC domain is responsible for the formation of PML-NBs (Figure 1.5) (196, 216-219). While the role of SUMO/SIM interactions in the multimerization of PML protein is contested, there has been consensus that SUMO/SIM interactions are necessary for other protein partner (such as SP100 and Daxx) recruitment to PML-NBs (217). Studies of PML-NB structure have revealed that these are not static or homogenous structures but are highly dynamic and diverse in composition and protein-protein interactions.

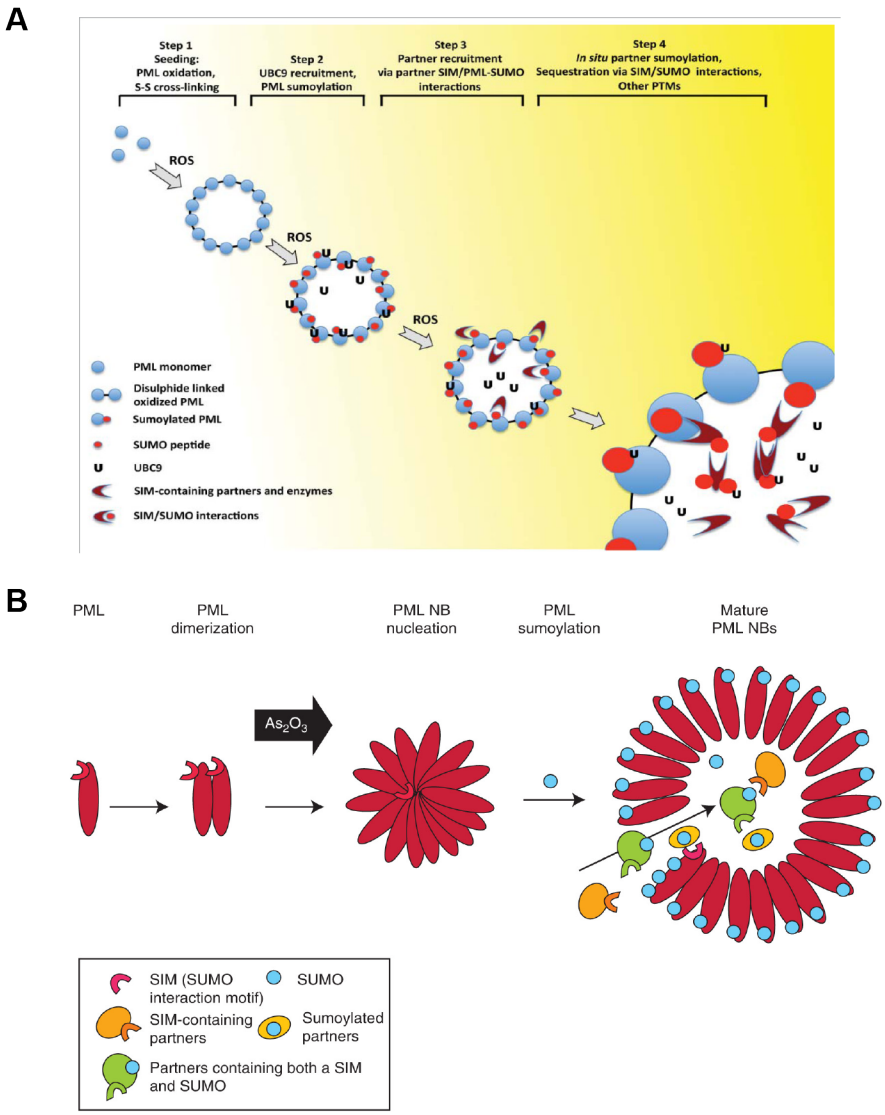

Figure 1.5 Proposed PML-NB Structures

A) Diagram of PML oligomerization occurring through disulfide bonds.
B) Diagran of recruitment of proteins to PML-NBs through SUMO/SIM interactions.
With permission from (A) Sahin et al. 2014. Nucleus and (B) Lallemand-Breitenbach et al. 2010. Cold Spring Harbor Perspectives in Biology

Because of the wide range of proteins that can localize to PML-NBs, and wide range of functions attributed to PML-NBs, it has been suggested that there is more than one kind of PML-NB structure (210, 220, 221). PML-NBs are believed to be involved in transcriptional regulation of cellular genes as multiple transcription factors and transcription regulators localize to PML-NBs [SP100,

activating protein 1 (AP-1), CBP, TRIM24] and nascent RNA can be found adjacent to PML-NBs (221, 222). Additionally, PML-NBs may play a role in chromatin structure and therefore have an indirect effect on transcription. PML-NBs can be found near highly acetylated chromatin (such as at actively transcribed MHC class I gene cluster) and many chromatin remodeling proteins can localize to PML-NBs including heterochromatin protein 1 (HP1), ATRX, Daxx, histone regulator A (HIRA), and Bloom's syndrome protein (BLM) (208, 221, 223). Transcriptional activation and repression have both been attributed to PML-NBs, which may be due to the stage of the cell cycle, cell type, or due to the fact that PML-NBs are heterogeneous structures.

PML-NBs also play a role in apoptosis and tumor suppression. Loss of PML-NBs results in cells that are unable to initiate apoptosis in response to several stimuli (224, 225). P53 and several p53 regulatory enzymes can localize to PML-NBs where p53 is post-translationally modified and activated (224-226). Retinoblastoma (Rb) protein and E2F transcription factors colocalize with PML-NBs and result in repression of E2F target gene transcription, many of which are involved in cellular proliferation (227).

Activation of the AKT- mTOR pathway within the hypoxic environment of a tumor can lead to tumor neoangiogenesis through upregulation of hypoxia-inducible factor 1-alpha (HIF1α) and subsequently vascular endothelial growth factor (VEGF). VEGF expression can result in increased vascularization of the tumor and is associated with tumor progression and a poor prognosis. PML-NBs have been shown to inhibit the AKT-mTOR pathway, under conditions of

hypoxia, by sequestering mTOR within the nucleus so that mTOR cannot be activated in the cytoplasm by the small GTPase Rheb (228). Loss of PML-NBs relieves this inhibition of the AKT-mTOR-HIF-1α-VEGF pathway within the hypoxic microenvironment of tumor (229). This results in increased tumor neoangiogenesis as evidenced by (1) increased tumor growth and microvessel density when $PML^{-/-}$ murine embryonic fibroblasts (MEFs) were injected into nude mice compared to wildtype MEFs, and (2) the correlation between PML loss and increased microvessel density that is observed in human prostate cancer samples (229). Finally, several cancers exhibit loss or very low levels of PML protein expression consistent with a tumor-suppressor role for PML-NBs (230). However, it has also been reported that certain cancers show an overexpression of PML that leads to aberrant, pro-survival, functions of PML (231). Thus, further investigations are needed to determine the exact role of PML-NBs in oncogenesis.

Importantly, PML-NBs have been extensively studied for their role in the intrinsic/innate immune response. Through the study of PML-NBs during viral and, to a much lesser extent, bacterial infection, the innate immune functions of PML-NBs have begun to be deciphered. PML-NBs and their role in the intrinsic immune response has been supported by the fact that PML-NBs can, in the absence of viral PML-NB antagonistic genes, epigenetically silence incoming viral genomes (HSV-1 and HCMV). Additionally, knockdown of many PML-NB resident proteins results in the increased replication of several DNA (HCMV, adenovirus, HSV-1) and some RNA (avian sarcoma virus, lymphocytic

choriomeningitis virus, influenza A) viruses (232-238). PML-NBs have also been shown to physically restrict the virus life cycle. For example, PML isoform IV interacts with the capsid protein ORF23 of VZV to encapsulate newly assembled VZV nucleocapsids into PML-NBs and prevent egress (239). The role of PML-NBs in the innate immune defense against bacteria has not been as extensively studied as PML-NB defense against viruses. However, several recent studies have shown that PML-NBs are important for protection against bacteria. Using $PML^{-/-}$ mice, researchers found that these mice were no longer susceptible to the lipopolysaccharide (LPS) lethality observed in wildtype mice. Additionally, macrophages from these $PML^{-/-}$ mice did not produce high levels of IL-6 following LPS treatment, providing the first evidence that PML-NBs were involved in the NFκB pathway (240). PML-NB defense against invading bacteria was demonstrated both *in vitro* and *in vivo* as PML-$NB^{-/-}$ cells and mice displayed increased *Listeria monocytogenes* replication, while *Shigella* infection has been shown to increase the number of PML-NBs (241, 242).

The involvement of PML-NBs in innate immunity has been extensively studied in regards to the IFN pathway. While PML-NBs are constitutively expressed, their number and size increases following type I or type II IFN treatments (243). This is a result of the induction of PML and SP100, as both of these genes contain IFN-stimulated response element (ISRE) and gamma-activated sequence (GAS) elements (244, 245). More direct evidence for the involvement of PML-NBs in the IFN pathway was discovered when specific isoforms of PML were studied. Following recognition of viral components or

PAMPs, PRRs initiate signaling cascades that activate transcription factors in the cytosol, such as IRF3 and NFκB (among others), which can then translocate to the nucleus to bind to target gene promotor regions and initiate the transcription of IFNβ and other antiviral genes. Following IFNβ production, IFNβ can signal through the type I IFN receptor to induce a second signaling cascade to activate the interferon stimulated gene factor 3 (ISGF3) transcription complex comprised of Signal Transducer and Activator of Transcription 1 (STAT1), STAT2, and IRF9. Once assembled, ISGF3 will translocate to the nucleus and induce transcription of additional innate immunity and antiviral genes. PML isoform II is necessary for the efficient and robust induction of IFNβ and certain ISGs, as it stabilizes and prolongs the occupancy of IRF3, NFκB, and ISGF3 transcription complexes at their target gene promoters (246). PML isoform IV enhances the activity of IRF3 by recruiting the IRF3 antagonist Pin1 into PML-NBs (247). Increased transcription of PML-NB components by type I IFN, and the enhanced induction of IFNβ by PML-NBs can thus produce a positive feedback loop and connect the intrinsic and innate immune responses.

While the importance of PML-NBs is still being elucidated, it is important to note that knockout of the PML gene in cells or mice is not a lethal mutation, suggesting that PML is not required for development. However,as discussed above, $PML^{-/-}$ mice have an increased susceptibility to infections and the loss of PML contributes to tumor development under certain circumstances (248, 249).

1.4.1.2 IFN Signaling

IFNs are important molecules in the innate immunity arsenal. The human IFN proteins range in size from 165 amino acids to 208 amino acids (250). There are 22 human IFN genes categorized into three types based on amino acid sequence and receptor usage: type I, type II, and type III. Type I IFNs consist of 14 IFNα subtypes, and a single subtype each of IFNβ, IFNε, IFNκ, and IFNω. IFNγ is the only IFN within the type II IFN group while type III IFNs contain IFNλ1, IFNλ2, IFNλ3, and IFNλ4 (250, 251). IFNs can signal in autocrine and paracrine manners to exert wide-ranging effects.

Type I IFNs are produced by and can act on most cell types, and are vital to antiviral defenses. The type I IFNs are involved in establishing a cellular environment that is refractory to pathogen growth, promoting antigen presentation and NK cell functions, and modulating cell cycle progression, apoptosis, and autophagy (252). Type I IFNs also activate the adaptive immune response, thus linking the innate and adaptive arms of the immune system (252). Induction of type I IFN transcription is regulated by NFκB and several IRF transcription factors (253). The receptor that recognizes type I IFNs is a heterodimer that consists of the IFNα receptor 1 (IFNAR1) and IFNα receptor 2 (IFNAR2) chains. Once IFNα or IFNβ engage the IFNAR1/2 receptor, oligomerization of IFNAR receptors occurs to allow two tyrosine kinases [Janus kinase 1(JAK1) and tyrosine kinase 2 (TYK2)] that are associated with the cytoplasmic tail of the receptor to trans-phosphorylate each other (252). Once activated, JAK1 and TYK2 can phosphorylate STAT1 and STAT2, which will then form a trimeric complex with IRF9, termed ISGF3, and translocate to the nucleus.

This transcription complex will bind to ISREs on the promoters of target genes and induce the transcription of ISGs (Figure 1.6) (254). Because the transcriptional program induced by type I IFNs can harm the host if allowed to progress unchecked (due to the proinflammatory functions of IFNs which can result in toxicity, tissue damage, autoimmune disorders, etc.), negative feedback loops to turn off this pathway are present (255). Type I IFN signaling induces the transcription of suppressor of cytokine signaling (SOCS) proteins and ubiquitin carboxy-terminal hydrolase 18 (USP18) which will either compete with STATs for binding to IFNAR or displace JAK1 from IFNAR2, respectively (256). Additionally, IFNAR receptors can be downregulated on cell surfaces and host miRNAs can target components of the type I IFN pathway (miR-302d targets IRF9, miR-29a targets IFNAR1) (257-260).

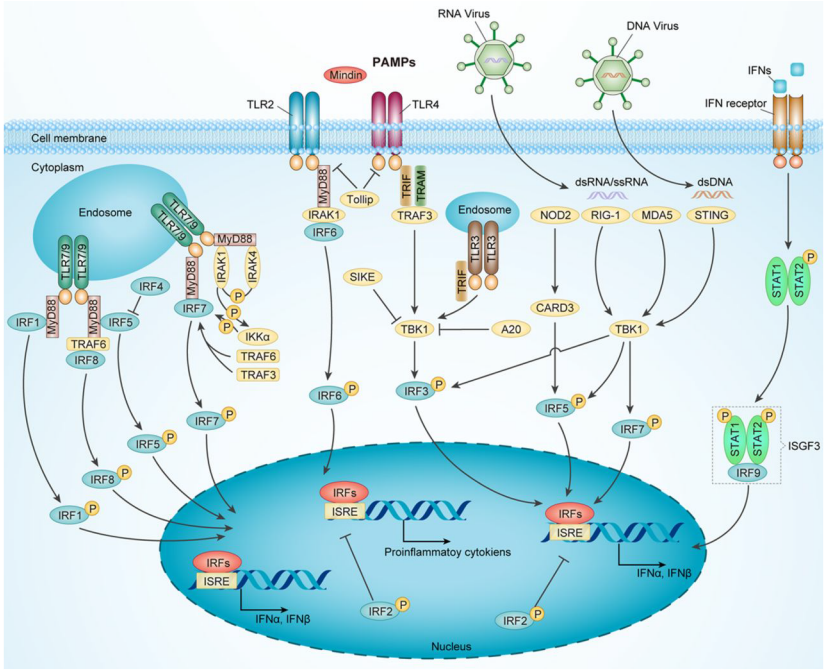

Figure 1.6 Induction of Type I IFN and Type I IFN Signaling
With permission from Zhang et al. 2015. Hypertension

Type III IFNs (IFNλ1, IFNλ2, IFNλ3, and IFNλ4) signal through a distinct receptor, IFNλR, comprised of a heterodimer of IL28R/IL-10R chains (261). IFNλ receptor expression was initially thought to be limited to epithelial cells and hepatocytes in humans, but new studies are discovering that this receptor may be expressed on a wider repertoire of cells (neutrophils, DCs, and macrophages) (262). Once the IFNλ receptor is engaged it appears that the JAK/STAT pathway, utilized by type I IFNs, is also triggered by type III IFNs (262).

The single type II IFN (IFNγ) is mainly produced by activated T and NK cells, but is also produced by B cells, NKT cells, and professional antigen presenting cells (APCs) (263). Similar to IFNAR and IFNλR, the IFNγ receptor (IFNGR) also consists of two chains, IFNGR1 and IFNGR2, and oligomerizes upon engagement with IFNγ (263). In contrast to the type I and type III IFN receptors, IFNGR signals through JAK1 and JAK2 to phosphorylate STAT1 and results in STAT1 homodimer formation (264). STAT1 homodimers then move into the nucleus and bind to a GAS encoded in the promoters of target genes. Although it appears that the main signaling cascade induced by IFNγ involves STAT1, STATs 2, 3, and 5 can also be activated following IFNγ stimulation (265). Additionally, other non-canonical IFNγ signaling can occur through mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) (264). IFNγ signaling results in the transcription induction of IFNγ-inducible genes. The effects of IFNγ include immunoglobulin class switching in B cells, activation of macrophages, and increased expression of MHC class II on immune and non-immune cells (266).

The IFN family of cytokines exert pleiotropic effects that help produce an anti-microbial state in both the innate and adaptive immune responses. Proper regulation of these cytokines is important to protect the host against pathogens and to prevent aberrant IFN signaling that could also harm the host. Hence, a family of transcription factors help to regulate IFN and ISGs. These transcription factors are described in the next section.

1.4.1.3 IFN Regulatory Factors

IFN regulatory factors (IRFs) are a family of transcription factors involved in innate immune signaling, hematopoietic cell differentiation, and cell growth and apoptosis (169, 267). There are nine members of the IRF family designated IRF1 through IRF9. Common to all IRF proteins is the amino (N)-terminal DNA binding domain, which includes five conserved tryptophan-rich repeats. The carboxy (C)-terminal region of the IRF proteins is more diverse and confers the specificity of each IRF for their interacting partners (other IRFs, transcription factors, and cofactors). The C-terminus of IRF3-IRF9 contains the IRF-associated domain 1 (IAD1) while IRF1 and IRF2 contain IAD2 in their C-terminus (169, 267). Because the IRFs regulate the production of IFNs and ISGs, they play a central role in the innate immune response to viral infections (Figure 1.6) (254).

IRF1 expression is variable depending on the cell type and although basal levels are constitutively present, it can be further induced upon stimulation by IFNγ, IFNβ, DNA damage, and viral infections (267-269). Transcription of IRF1 is induced by IFNγ, but is only fully activated with the help of IRF9 signaling (presumably through phosphorylation), at which point IRF1 translocates to the

nucleus to activate target genes such as IFNβ and IL-12p35 (270). IRF1 can translocate to the nucleus without MyD88 involvement, but with slower kinetics (270). More recently, it has been shown that IRF1 acts as a transcription factor for IFNλ1 in airway epithelial cells in response to several viruses (271, 272). IRF1 is also involved in DNA damage-induced apoptosis, suppression of oncogenesis, NK cell development, and differentiation of T_H1, $CD8^+$ T cell, and regulatory T cell (T_{reg}) differentiation (267, 273). A recent study on gamma-herpesvirus driven lymphoproliferative disorders implicated a role for IRF1 in B cell differentiation at germinal centers (274). Thus IRF1 can regulate type I, II, and III IFNs as well as help regulate cellular development and differentiation.

IRF2 competes for the same DNA binding sites as IRF1, but does not activate transcription of IFN or ISGs and thereby negatively regulates type I IFN production and signaling (275). However, there have also been reports of IRF2 cooperating with IRF1 to induce the transcription of certain genes including IL-12p40 and Cox-2, as well as a role for IRF2 in the defense against some viral infections (276-279). IRF2 is also involved in the normal development of several types of immune cells including NK cells, DCs, B cells, T cells, and basophils (267, 280). In addition, IRF2 expression along with IRF1 promotes T_H1 and suppresses T_H2 polarization (281). Oncogenic functions of IRF2 may be due in part to its antagonistic activity towards IRF1, however IRF2 also activates the transcription of histone H4 (involved in cell-cycle progression) (282-284). While IRF2 was first characterized as antagonizing IRF1, it is now clear that IRF2 has

many other functions including antiviral defense and the regulation of immune cell development.

IRF3 has been extensively studied for its role in the defense against pathogens. IRF3 is constitutively expressed but resides in the cytoplasm until it is activated by phosphorylation (285). Both TLR3 and TLR4 can signal through the TRIF adaptor protein to activate TBK-1, which will phosphorylate IRF3. The cytoplasmic PRRs, RIG-I, MDA5, and cGAS/STING can also signal through IRF3 (169). When cytoplasmic RNA is detected by RIG-I, or MDA5, they signal through the adaptor molecule mitochondrial antiviral-signaling protein (MAVS) which can activate TBK-1 to phosphorylate IRF3. cGAS can detect cytoplasmic DNA and produces cyclic di-nucleotides which are then sensed by STING, and STING brings TBK-1 and IRF3 into close proximity of each other so that TBK-1 can phosphorylate IRF3 (169). IKKε can also phosphorylate IRF3 (286). Once IRF3 is phosphorylated it can translocate to the nucleus and interact with CBP and P300. IRF3 will then dimerize with another IRF3 molecule or with IRF7 and this complex (CBP/P300/IRF3 dimer or IRF3/IRF7) will bind to the promoters of type I IFN genes and other target genes. IRF3 induces a subset of ISGs both distinct and similar to those induced by other IRFs, for example IRF3 can induce IFNβ, RANTES, and IFNα1 and IFNα2 transcription (287). However IRF3 does not induce transcription of several other IFNα subtypes (which are instead activated by IRF7) (287). IRF3 is also involved in apoptosis as it transcriptionally induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which can trigger cell death by engaging death receptor (DR) 4 and DR5 (288, 289).

Following RLR activation and signaling through TNF receptor associated factor (TRAF) 2 and TRAF6, linear ubiquitin chains are added to IRF3, which switches the role of IRF3 from transcription factor to apoptosis mediator. Ubiquitinated IRF3 binds to the pro-apoptotic protein Bax and together they translocate to the mitochondria and trigger cytochrome C release and intrinsic apoptosis follows (290). Thus, IRF3 plays a central role in the defense against viruses and can work cooperatively with IRF7.

IRF4 binds to the same region of myeloid differentiation primary response 88 adaptor protein (MyD88) that IRF5 binds, and as such, IRF4 competitively inhibits IRF5 signaling (291). In this manner IRF4 is able to negatively regulate the production of pro-inflammatory cytokines induced by IRF5. Similar to IRF3, IRF4 is constitutively expressed but only in a subset of cells including DCs, macrophages, and B cells. Additionally, transcription of IRF4 can be induced upon antigen stimulation in T cells. Aside from the IRF5 antagonistic role, IRF4 is involved in cellular development. Differentiation of the $CD4^+$ DC subset and T_H2 cells requires IRF4 expression (292, 293). IRF4 is also involved in several aspects of B cell regulation including at the pre-B stage to develop immunoglobulin light chains, plasma cell differentiation, and formation of germinal centers (294, 295). Similar to IRF2, IRF4 has been described to perform antagonistic functions against another IRF while also possessing cellular development and differentiation functions.

IRF5 is another key transcription factor activated following viral infections. Several cell types, including B cells, conventional and plasmacytoid DCs, and

macrophages, have been shown to express IRF5 (296). Following engagement of TLR9 and activation of MyD88, IRF5 binds to the central region of MyD88 resulting in activating phosphorylation and translocation to the nucleus. Phosphorylated IRF5 will form a dimer and interact with CBP/P300, similar to IRF3 and IRF7 (267, 280, 297). The transcriptional program induced by IRF5 includes proinflammatory cytokines, such as IL-6, IL-12, and TNFα (169, 298). In myeloid DCs, it has been shown that IRF5 (as well as IRF3 and IRF7) can be activated through the adaptor protein MAVS and then induce transcription of type I IFNs (299). IRF5 can induce cell cycle arrest and transcription of several pro-apoptotic genes such as Bak, Bax, caspase 8, and p21 (300). The stability of IRF5 protein in uninfected or otherwise quiescent cells has recently been discovered to be involved with the constitutive photomorphogenesis 9 (COP9) signalosome (297). The COP9 signalosome is a multi-protein complex that is homologous to the regulatory subunit of the 26S proteasome and controls proteasome-mediated protein degradation of several immune signaling components (231). A component of the COP9 signalosome, CSN3, has been identified as a binding partner for IRF5 and when the COP9 signalosome is knocked down, IRF5 protein levels rapidly drop in a proteasome-dependent manner (297, 301). Ultimately, IRF5 is involved in the induction of a robust antiviral state along with IRF3 and IRF7.

Less is known about **IRF6** compared to the other IRFs. Similar to other IRFs, in response to poly (rI:rC) treatment, IRF6 translocates from the cytoplasm into the nucleus (302). Expression of IRF6 has recently been demonstrated in

macrophages, neutrophils, and DCs, with an anti-inflammatory role observed in macrophages. It was discovered that IRF6 is a negative regulator of TLR4 signaling and inhibits the activation of NFκB, thereby protecting against LPS-induced endotoxic shock (303). The majority of work on IRF6 has revealed the important function of IRF6 in keratinocyte development and differentiation. Mutation of IRF6 in humans has been linked to two cleft lip/palate syndromes, Van der Woude syndrome and Popliteal Pterygium syndrome, and this is linked to the role of IRF6 in keratinocyte development. IRF6 is also expressed in mammary epithelial cells and oral epithelial cells, and is involved in cell cycle arrest of mammary epithelial cells. Mice displaying a deficiency in IRF6 also show abnormal skin, limb, and craniofacial development. Therefore, it appears that IRF6 has important functions in normal development.

IRF7 is only constitutively expressed in B cells and DCs, but is induced in other cell types upon IFN signaling or viral infection (304). Activating phosphorylation of IRF7 can occur following RLR/MAVS/TBK-1/IKKε, TLR3/TRIF/TBK-1/IKKε, and cGAS-dependent and independent signaling cascades through STING and TBK-1 (267). Such as is observed with IRF3 and IRF5, IRF7 will translocate to the nucleus, interact with CBP/P300, and homo-dimerize or form heterdimers with IRF3 before inducing transcription of target genes. While IRF7 can often induce the same set of ISGs as IRF3, it can induce a larger set of unique ISGs. For example, IRF7 can induce several IFNα subtypes that IRF3 cannot induce, such as IFNα4, IFNα7, and IFNα14 (287). In addition to the above-mentioned signaling cascades involved in IRF7 activation,

a pathway known to activate NFκB also targets IRF7. The IL-1 receptor-associated kinase 4 (IRAK4), IRAK1, and IKKα kinase-signaling pathway can phosphorylate and activate IRF7 (305). Thus, IRF7 plays an indispensable role in the host defense against pathogens.

IRF8 is expressed in hematopoietic cells and is either constitutively expressed or IFNγ inducible depending on the cell type. In DCs, unmethylated CpG DNA triggers the TLR9 signaling pathway and this activates NFκB, which is dependent on IRF8 expression (306). IRF8 has also been shown to enhance type I IFN induction by stabilizing the recruitment of transcription complexes onto IFN promoters (307). Additionally, IRF8 transcriptionally regulates PML gene expression and is required for the formation of PML-NBs in myeloid cells (260). Many roles for IRF8 in cell development and differentiation have been discovered. IRF8 is required for the proper differentiation of certain DC subsets, macrophages and T_H1 cells (308). Additionally, IRF8 is involved in B cell and germinal center development. Hence, IRF8 possess a wide variety of roles in both defense against pathogens and the proper development and differentiation of several cell types.

IRF9 functions downstream of receptor binding by type I and type III IFNs, to activate different subsets of ISGs. Following type I or III IFNs binding to their respective receptors, the JAK/STAT pathway is activated. STAT1 and STAT2 are phosphorylated as a result and can complex with IRF9 to form the ISGF3 transcription complex (261, 309, 310). However, IRF9 can also bind to a STAT2 homodimer or complex with STAT2 and STAT6. These different complexes may

induce different ISG programs in response to different stimulation (311). IRF9 can also play an indirect role in apoptosis as type I IFNs signal through ISGF3 to activate p53 (312). Thus, IRF9 is necessary for the expression of antiviral genes that will ultimately exert protective effects for the host cell.

In conclusion, the IRF family of transcription factors has a central role in the IFN response and defense against pathogens including viruses. However, the continued research on this family of transcription factors has revealed a much larger and still-expanding role for the IRFs.

1.4.2 Adaptive immunity

If intrinsic and innate immunity fail to control a pathogen, then the adaptive immune response is triggered. Adaptive immunity, in contrast to intrinsic and innate immunity, is a specific response to a particular antigen and results in immunological memory, ensuring that a more rapid and robust immune response can occur in the event that the host encounters the same antigen again (313). Innate immunity helps to activate the adaptive immune response by driving differentiation of adaptive immune cells, as well as the maturation and migration of APCs to lymph nodes. In addition to their important role in innate immune responses, type I IFNs also influence the adaptive immune response.

DCs are professional APCs that will present antigens on MHC class I or class II cell surface molecules to $CD8^+$ or $CD4^+$ T cells, respectively. When T cells bind to antigen-presenting MHC molecules and costimulatory molecules such as CD40, CD80, or CD86, they become fully activated. Exposure of DCs to type I IFN results in an increased expression of MHC class I and class II as well

as the costimulatory molecules CD40, CD80, and CD86 (314-317). DCs will also release CXC motif chemokine receptor 3 (CXCR3) ligands [CXC motif chemokine ligand 9 (CXCL9), CXCL10, and CXCL11] in response to type I IFN (317). Activated T and B cells express the CXCR3 receptor and thus T and B cells can be recruited to sites of infection by DCs that are exposed to type I IFN (318). Therefore, type I IFN matures DCs into professional APCs that are capable of recruiting and activating adaptive immune cells.

When $CD8^+$ T cells become activated following recognition of antigen on MHC class I, they secrete cytokines such as TNFα and IFNγ, as well as perforin, and granzymes, which collectively have antiviral and pro-apoptotic effects on the infected cell (313). $CD8^+$ T cells can also induce apoptosis of infected cells through Fas/FasL signaling. The presence of type I IFN during primary $CD8^+$ T cell responses to viral infections enhances the proliferation, clonal expansion, and generation of memory $CD8^+$ T cells (319-321). For example, it was shown that during lymphocyte choriomeningitis virus (LCMV) infection, $CD8^+$ T cells that lacked the type I IFN receptor had significant impairment in their capacity to expand and differentiate into effector cells (320). In this manner, a strong $CD8^+$ T cell response relies on the type I IFN response.

$CD4^+$ T cells (also known as helper T cells) aid in the clearance of microbes through their abilities to help B cells produce antibodies, enhance macrophage activation, produce cytokines and chemokines, and recruit other cells to sites of infection (neutrophils, basophils, eosinophils) (313). Naïve $CD4^+$ T cells will further differentiate upon antigen stimulation. Depending on which

cytokines are present at the time of differentiation, the type of APC activating the T cell, the amount of antigen, and the costimulatory molecules that are activated, naïve $CD4^+$ T cells can differentiate into one of at least seven different lineages (322). Lineages that naïve $CD4^+$ T cells can differentiate into include T helper 1 (T_H1), T_H2, T_H17, T_H22, T_H9, follicular helper T cells (Tfh), and regulatory T cells (Tregs) (323). Type I IFNs have been shown to help drive a T_H1 $CD4^+$ T cell differentiation program, both indirectly by enhancing DC secretion of IL-12 and directly by upregulating transcription factors in $CD4^+$ T cells that drive T_H1 differentiation (324-326). It has also been demonstrated that type I IFN can suppress T_H2 and T_H17 differentiation of $CD4^+$ T cells (325, 327, 328). T_H1 cells secrete cytokines that activate macrophages and $CD8^+$ T cells (322). T_H2 cells promote IgG1 and IgE secretion by B cells, and T_H17 cells activate several cell types (endothelial cells and stromal cells for example) to produce proinflammatory molecules (322). Hence, type I IFN can skew $CD4^+$ T cell differentiation in a way that would benefit the clearance of intracellular pathogens, such as viruses, by macrophages and $CD8^+$ T cells.

B cells provide additional defense in the adaptive immune response. When mature follicular B cells recognize an antigen through their B cell receptor, and then receive help from $CD4^+$ T cells they can become plasma cells producing IgM. B cells can also go through proliferation, affinity maturation, and class switching in germinal centers to become memory B cells and plasma cells with high affinity B cell receptors and can produce other classes of immunoglobulins (IgG, IgA, IgE, IgD) (313). These antibodies can lead to multiple

outcomes including prevention of pathogen entry into cells (by interfering with virus binding to cellular receptors, preventing the uncoating of genomes, or aggregation of viral particles), activation of the complement system (a cascade that will produce inflammation, enhance phagocytosis, and attack bacterial membranes), enhanced phagocytosis of antibody coated pathogens (opsonization), and antibody-dependent cell-mediated cytotoxicity (increased destruction of infected cells by macrophages, eosinophils, and NK cells). Type I IFN effects on B cells are complex and appear to be context dependent. Differentiation of B cells into antibody-secreting B cells can be regulated indirectly by the type I IFN activation of DCs to secrete IL-6 (329, 330). Additionally, there is evidence that type I IFN directly affects B cells resulting in enhanced B cell receptor signaling (331). Using type I IFN receptor knockout mice, and chimeric mice lacking type I IFN receptors on B cells only, it has also been shown that type I IFN signaling is required for (1) an early antibody response during primary influenza virus infection and (2) plasma cell development and IgM production following vesicular stomatitis virus infection (332, 333). However, in the context of certain chronic viral infections, type I IFN can result in the loss of virus-specific B cells and broadly neutralizing antibodies. During persistent LCMV infection there is a loss of LCMV-specific B cells that is dependent on type I IFN and $CD8^+$ T cells (334). As mentioned above, type I IFN suppresses the T_H2 response, which in turn inhibits specific antibody production by B cells. In the case of T_H2 driven allergic IgE production, type I IFN treatment reduced this allergic response and showed therapeutic benefits for asthma

patients treated with IFNα (335, 336). While several factors (duration of IFN signaling, viral infections, stage of B cell development, effects on other immune cells) influence the final effect on B cells, it is evident that type I IFNs play a pivotal role in B cell responses.

Innate and adaptive immune responses are distinct arms of the immune system, however they are not isolated from each other. The pleiotropic effects that type I IFN can exert on the adaptive immune system, as described above, evidence this fact. Following clearance of a pathogen, long-lived B and T cells will continue to provide surveillance and protection from that specific pathogen (called immunological memory) (313).

1.5 Herpesvirus Innate Immune Evasion

While many species have developed an intricate immune system to guard against microbial infection, herpesviruses have co-evolved the ability to circumvent or antagonize many of these immune responses and establish life-long infections within their host species. This section will focus on what is known about the induction and evasion of the IFN response and evasion of PML-NBs following infection with human herpesviruses and their closely related primate-model herpesviruses.

1.5.1 IFN induction by herpesvirus infection

Herpesviruses can induce IFN upon cellular recognition of viral envelope glycoproteins, DNA, and RNA. Viral binding and entry into a host cell triggers the first signaling cascades that lead to IFN production. HCMV envelope glycoprotein B (gB) can trigger IRF3 activation and IRF3-dependent induction of IFN and

ISGs. While soluble HCMV gB can elicit this response, whole HCMV virions induce a much greater response (337). Similarly, purified soluble KSHV envelope glycoprotein K8.1 could elicit the transcription of IFNβ and ISGs in fibroblasts, and IFNα in HEK 293T cells (338, 339). HSV-1 virion binding and entry induce IRF3 activation and ISG induction, however soluble HSV-1 gB is not sufficient for this induction (340-342). IFNβ activation can also occur through the activation of NFκB and HSV-1, HCMV, and KSHV are capable of activating this pathway upon infection (343-345). Additionally, HSV-1 envelope protein gD can induce IFNα production in lymphocytes and myeloid dendritic cells (346). Interestingly, KSHV infection induces TLR4 signaling (usually associated with recognition of bacterial LPS) and type I IFN production (347). UV treated KSHV virions could also activate TLR4 implying that the PAMP recognized by TLR4 may be a viral envelope glycoprotein. Research to date has thus revealed that all three subfamilies of human herpesviruses can elicit an IFN response with several viral glycoproteins.

Viral DNA sensing by cellular PRRs (such as IFI16, cGAS, ZBP1, DDX41, and TLR9) can also trigger the IFN response. Most human herpesviruses have now been shown to activate TLR9 and induce type I IFNs following infection (348). VZV infection of plasmacytoid DCs induces the production of IFNα by TLR9-dependent and –independent mechanisms (349). A recent study on VZV infection of dermal cells revealed a role for STING in the induction of IFNβ and IFNλ following infection, although the exact mechanism of viral recognition is unknown (350). KSHV and HSV-1 *de novo* infections, as well as KSHV and EBV

latent infections, are sensed in the nucleus by IFI16 complexed with breast cancer type 1 susceptibility protein (BRCA1) and histone H2B. This viral DNA recognition results in the activation of the IFI16-inflammasome complex and is dependent on IFI16 and BRCA1 but not H2B (351). Meanwhile, following viral DNA sensing in human vascular endothelial cells, the IFI16-BRCA1-H2B complex translocates to the cytoplasm where it interacts with both cGAS and STING resulting in the phosphorylation of TBK-1 and IRF3, and induction of IFNβ transcription (351, 352). As a reminder, cGAS is a cytoplasmic DNA sensor that produces 2'3'-cGAMP following detection of DNA in the cytoplasm. 2'3'-cGAMP is a strong activating ligand for STING, which will then recruit and activate TBK-1 and IRF3, followed by IRF3-regulated induction of IFNβ. Interestingly, a recent study revealed that several human B cell lines (primary and EBV-negative) lack STING protein and do not produce type I IFN upon cytoplasmic DNA exposure (353). This has implications for the ability of B cell tropic viruses such as EBV and KSHV to infect B cells. This study also found that reconstitution of STING protein within B cells did not rescue IFN production in response to cytoplasmic DNA exposure suggesting further dysfunctions in cytoplasmic DNA sensing pathways (353). Of course there are other DNA sensors such as TLR9 and IFI16 (described above) that sense EBV and KSHV genomes. However, this study revealed that the cGAS-STING pathway is defective in human B cells. HCMV infection of monocyte-derived DCs and macrophages induces type I IFNs through cGAS while HCMV infection of plasmacytoid DCs induces type I IFNs through TLR9 (354). HCMV is also detected by the DNA sensor, Z-binding

protein 1 (ZBP1, also known as DAI), and induces IFNβ expression through IRF3 (355). It follows that the human herpesviruses, with their DNA genomes, will trigger several DNA-sensing PRRs. This has now been elucidated and revealed that IFN production results from this viral DNA sensing by the cell.

IFNs can also be induced following the sensing of viral RNA, even in the context of DNA virus infections. HSV-1 and KSHV infection can induce type I IFN through TLR3 activation, perhaps due to the production of viral dsRNA during the virus lifecycle (356-358). EBV infection can also activate TLR3 and induce type I IFN production, however this is due to virally encoded small RNAs (EBERs) (359). A role for RIG-I and MAVS has been demonstrated for HSV-1 induction of type I IFNs (360, 361). Cellular RNA polymerase III is able to transcribe viral DNA into RNA possessing a 5'-triphosphate, which is then recognized RIG-I (362). EBERs RNAs can also activate RIG-I in addition to TLR3, resulting in type I IFN induction (363). KSHV is also able to activate RIG-I signaling and IFNβ production following infection or reactivation, however the viral PAMP responsible is currently unknown (364). While less is known about the mechanisms of herpesvirus-RNA sensing it is clear that this pathway is triggered during infections with several herpesviruses, and results in the production of IFN.

1.5.2 IFN evasion

While the herpesviruses clearly induce the IFN pathway, they also encode many strategies to evade this innate immune response. Numerous studies have revealed that these large DNA viruses encode diverse mechanisms that act at multiple locations along the IFN pathway. Herpesvirus-encoded mechanisms to

inhibit the production of IFN as well as IFN signaling have been characterized. Additionally, to evade the initial signaling cascades that induce type I IFN upon virus binding and entry, herpesviruses often incorporate immune evasion proteins in their teguments so that they can act immediately upon infection.

Inhibition of IFN Production by Alpha-herpesviruses

Virally encoded mechanisms to inhibit the induction of IFNs are critical for the establishment of a robust infection. HSV-1 tegument protein US3 (a viral protein kinase) is able to reduce the expression of TLR3, inhibit TLR2 signaling through its interactions with TRAF6, and hyperphosphorylate IRF3 and the p65 subunit of NFκB to inhibit IRF3 and NFκB activation (365-368). HSV-1 infected cell protein 0 (ICP0) is another tegument protein with multiple immune modulatory effects. As an E3 ligase, ICP0 is able to reduce levels of several cellular proteins including MyD88, toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP), the p50 subunit of NFκB, and IFI16 (369-372). Other HSV-1 proteins inhibiting IFN induction include US11 (inhibits RIG-I and MDA5 signaling), UL36 (deubiquitinates TRAF3 preventing TBK1 recruitment), ICP34.5 (sequesters TBK1), UL41 (degrades cGAS mRNA), VP24 (inhibits TBK1 and IRF3 interaction), ICP27 (inhibits IRF3 activation), and VP16 (inhibits IRF3 and CBP interactions) (373-380).

At least three VZV proteins target IRF3; ORF61 can subvert the type I IFN response by inducing degradation of phosphorylated IRF3, IE62 (ORF62) blocks activating phosphorylation of IRF3, while ORF47 aberrantly phosphorylates IRF3 to prevent homodimerization (381, 382). ORF61 of VZV and the related primate

virus, SVV, has also been shown to block the activation of NFκB by preventing the ubiquitination of IκBα (383).

Inhibition of IFN Production by Beta-herpesviruses

Although HCMV infection results in IFN induction, the virus is able to dampen the IFN response as evidenced by comparative studies between wildtype HCMV and loss of function or gene knockout mutants. HCMV encodes multiple genes to inhibit IFN induction such as IE2, which can block NFκB transcription of IFNβ (384). Another mechanism to inhibit IFNβ transcription, at late times post infection, is through the HCMV glycoprotein US9. US9 is expressed at its highest levels 48 hours post infection and is able to reduce mitochondrial MAVS levels and disrupt STING/TBK-1 complex formation, both of which result in reduced IRF3 activation and IFNβ transcription (385). Two viral homologs of human IL-10 (hIL-10) are encoded by HCMV, cmvIL-10 and latency associated (LA) cmvIL-10 (386). While cmvIL-10 has been shown to bind the hIL-10 receptor and function in the same anti-inflammatory capacity as hIL-10 (including inhibition of type I and II IFN production), LAcmvIL-10 does not exert the full repertoire of hIL-10 functions (387-389). LAcmvIL-10 is a truncated version of cmvIL-10 and does not possess the ability to suppress pro-inflammatory cytokines (390-392). However, LAcmvIL-10 can upregulate protein levels of hIL-10 and may exert anti-inflammatory functions indirectly through hIL-10 during latency (393). Although the role of pp65 in HCMV innate immune evasion has been controversial in the past, a recent publication demonstrated that pp65 binds to cGAS and inhibits the release of cGAMP, preventing

downstream activation of STING and IFNβ (394). Another HCMV gene product, pp71, can inhibit the STING/IFNβ pathway by inhibiting the trafficking of STING and preventing STING/TBK-1/IRF3 complex formation (395). HCMV miRNAs can also modulate the IFN response. The HCMV miRNA Hcmv-miR-UL112 has been shown to down-regulate type I IFN secretion, perhaps by targeting IRF1 (396, 397). While HCMV infection results in ISG induction, RhCMV infection does not. Contrary to what occurs during HCMV infection, RhCMV infection does not result in IRF3 dimerization or translocation to the nucleus (398). This result highlights that while many similarities may exist between human and nonhuman primate herpesviruses, key differences also exist.

Significantly less is known about the IFN evasion mechanisms of HHV6 and HHV-7. It has been shown that IE1 of HHV 6A and 6B inhibits IRF3 translocation to the nucleus and subsequent induction of IFNβ (399).

Inhibition of IFN Production by Gamma-herpesviruses

Several mechanisms for the inhibition of IFN production have been discovered for EBV. EBV BZLF1 can transcriptionally repress IFNγ, BPLF1 inhibits TLR signaling, LF2 binds to IRF7 preventing IRF7 dimerization and IFNα induction, BGLF4 inhibits IRF3 from binding to DNA, and BRLF1 down-regulates IRF3 and IRF7 transcription inhibiting type I IFN induction (400-404). EBV encodes miRNAs, some of which have been implicated in modulating the IFN response. EBV miR-BART6-3p inhibits IFNβ induction by targeting RIG-I mRNA and suppressing the RIG-I pathway (405).

Several genes encoded by KSHV help suppress IFN induction. ORF45 competitively inhibits IRF7 phosphorylation by IKKε and TBK-1 (406). ORF50/RTA encodes a ubiquitin E3 ligase activity that polyubiquitinates and targets IRF7 for proteasome-dependent degradation (406, 407). ORF50 can also recruit a cellular ubiquitin E3 ligase, RTA-associated ubiquitin ligase (RAUL), to ubiquitinate and target both IRF3 and IRF7 for proteasome-dependent degradation (408). Lastly, ORF50's E3 ubiquitin ligase activity has been shown to promote the ubiquitination and proteasome-dependent degradation of MyD88 and TRIF (409, 410). KSHV LANA competitively inhibits IRF3 DNA binding to the IFNβ promoter to inhibit IFNβ transcription (411). K-bZIP/ORF8 displaces IRF3 from target-gene promoters, inhibiting IRF3-dependent gene transcription including IFNβ (412). Three PRRs are also inhibited by KSHV. TLR4 transcription is down-regulated by vGPCR and vIRF1, RIG-I signaling and IFNβ induction are inhibited by ORF64 (possibly through the deubiquitinase activity of ORF64), and cGAS enzymatic activity (ability of cGAS to produce cGAMP) is inhibited by ORF52 (347, 364, 413). ORF36 of KSHV can inhibit TBK-1-dependent IFNβ production, which may occur through the disruption of IRF3 activity (414). Similar to EBV, KSHV encodes miRNAs with diverse targets and functions (see section 1.2.2.3.2 above). KSHV miRNAs miR-K9, miR-K5, and miR-K11 have some effect on reducing type I IFN production. miR-K9 targets IRAK1 while miR-K5 targets MyD88, two components of type I IFN-induction cascades (415). miR-K11 targets IKKε, a kinase responsible for activating IRF3 and IRF7, down-regulating the transcription of type I IFNs (96). Finally, all four KSHV vIRFs can inhibit the

IFN pathway by multiple mechanisms including antagonizing cellular IRFs and PRRs. The vIRFs are discussed in detail in section 1.6.

Due to the diverse and potent effects of IFNs, it is not surprising the herpesviruses have evolved several strategies to inhibit IFN synthesis. Not only do the herpesviruses encode multiple effectors to inhibit the production of IFN, some herpesviruses even utilize multiple viral effectors to target the same cellular protein (i.e. miR-K5 and ORF50 targeting of MyD88). However, in the event that IFN is produced, herpesviruses have also developed mechanisms to inhibit IFN-induced signaling pathways.

Evasion of IFN Signaling by Alpha-herpesviruses

The ability to inhibit signaling events downstream of IFN production will also aid viral infection and spread. As a reminder, the canonical type I IFN signaling pathway involves engagement of IFNAR1/2 with type I IFN which will trigger the phosphorylation of JAK1 and TYK2 and the recruitment and phosphorylation of STAT1 and STAT2, followed by phospho-STAT1 and phospho-STAT2 complexing with IRF9 to form the ISGF3 transcription complex that translocates to the nucleus and activates transcription of ISGs.

HSV-1 encodes ICP27 which can inhibit STAT1 phosphorylation and translocation to the nucleus following IFN signaling (416). Infection with HSV-1 induces an increased expression of SOCS1 and SOCS3, negative regulators of the JAK/STAT pathway (417). This up-regulation of SOCS expression following infection was dependent on UL13 (418).

As for VZV, the growth of a VZV mutant virus lacking ORF63 was inhibited in the presence of IFNα but not IFNγ, suggesting a role for ORF63 in the inhibition of type I IFN signaling (419). It was later shown that infection with VZV and the nonhuman primate homologous virus, SVV, results in reduced levels of IRF9 and inhibition of STAT2 phosphorylation (420). ORF63 of both VZV and SVV was able to down-regulate levels of IRF9 but had no effect on STAT2 phosphorylation, therefore another viral gene product may be involved (420). SVV is able to inhibit the signaling of IFNγ by reducing protein levels of STAT1, JAK1, and JAK2 (421). Similar to HSV-1, VZV infection also up-regulates SOCS3 to inhibit type I IFN signaling (422).

Thus, alpha-herpesviruses share common strategies for inhibiting the IFN signaling pathway by (1) antagonizing components of the JAK/STAT pathway and (2) upregulating the SOCS proteins involved in negative feedback of the IFN signaling pathway.

Evasion of IFN Signaling by Beta-herpesviruses

Infection with HCMV results in the inhibition of type I and type II IFN signaling. HCMV infection can block IFNα induced phosphorylation of IFNAR1, JAK1, TYK2, STAT1, and STAT2, and also reduce the levels of JAK1 and IRF9 (423). Unfortunately, the mechanisms and viral factors involved in these phenotypes have yet to be identified. One HCMV gene product known to inhibit type I IFN signaling is IE1. IE1 expression inhibits ISGF3 from binding to target genes and IE1 binds to STAT1 and STAT2 to relocalize these two components of ISGF3 to PML-NBs (424-426). The STAT binding (and type I IFN signaling)

function of IE1 is presumed to be separate from the PML-NB disruption function as a mutated IE1 that could no longer bind to STATs was still able to disrupt PML-NBs (425). HCMV UL23 inhibits STAT mediated transcription of IFNγ stimulated genes by inhibiting Nmi and STAT interactions (Nmi interactions with STATs enhance the recruitment of CBP/P300 to STAT transcriptional complexes) (427). IE1 of HCMV can also inhibit IFNγ stimulated gene transcription (428). Although the mechanism is not fully elucidated, IE1 expression reduces STAT1 homodimer binding to target genes without directly binding to STAT1.

It is clear that HCMV is able to inhibit IFN signaling at multiple steps along the pathway. However, much remains to be elucidated about the mechanistic details of this process and identification of the viral effectors involved.

Evasion of IFN Signaling by Gamma-herpesviruses

EBV can inhibit IFN signaling by inducing the degradation of IFN receptors, an effect that is dependent on LMP2A and LMP2B (429). LMP-1 of EBV can inhibit the Tyk2 phosphorylation event following the binding of IFNα to its receptor, abrogating IFN signaling (430). EBV miR-BART16 targets CBP, an important transcriptional coactivator in the IFN pathway, and inhibits ISG transcription following IFNα treatment (431).

Impairment of IFN signaling has been described for four KSHV gene products. ORF10 interacts with IFNAR and the receptor associated kinases TYK2 and JAK1, inhibiting their phosphorylation and the phosphorylation of STAT2, and thus inhibiting ISGF3 formation (432). KSHV vIL-6 also prevents

TYK2 phosphorylation and ISGF3 formation (433). vIRF-2 can inhibit ISGF3-mediated transcription and will be discussed further in section 1.6 (434). Lastly, KSHV ORF54 inhibits the IFN signaling pathway and while the mechanism is currently unknown, the MHV68 homolog of KSHV ORF54 induces the degradation of IFNAR1 (435).

A growing body of evidence is revealing the strategies used by gamma-herpesviruses to inhibit the IFN signaling pathway. These strategies involve multiple viral proteins as well as noncoding RNAs to antagonize every step of the IFN signaling cascade. This multi-pronged attack (by members of every subfamily of human herpesviruses) to stop the induction of IFN and ISG transcription indicates how detrimental the ISGs are for viral infections. Consequently, these viruses expend a large amount of their genomic coding capacity towards evading this one pathway.

1.5.3 PML-NB Evasion

PML-NBs provide a strong and immediate defense against herpesvirus infections. Because herpesviruses are so successful at establishing infection and persisting in the host, it follows that these viruses encode a means to modulate or disrupt PML-NBs. Examples of herpesviruses in each subfamily of the *Herpesvirales* family have been found to modify or disrupt PML-NBs.

PML-NB Evasion by Alpha-herpesviruses

The alpha-herpesvirus HSV-1 encodes the tegument protein ICP0 to disrupt PML-NBs. ICP0 is a SUMO-targeted ubiquitin ligase (STUbL) and is able to induce the proteasomal degradation of SUMO-conjugated PML and SP100

proteins, thereby disrupting PML-NB structures (436, 437). ICP0 homologues in other alpha-herpesviruses (bovine herpesvirus 1, equine herpesvirus 1) had a similar effect on PML-NBs (438). While the group that investigated ICP0 homologs in other alpha-herpesviruses did not find the ICP0 homolog of VZV (ORF61) to be able to disrupt PML-NBs, they admitted that expression levels of their ORF61 construct were low and may explain their results. Another group was later able to show that ORF61 does disrupt PML-NBs following infection with VZV using an ORF61 mutant VZV (439).

The alpha-herpesviruses use homologous tegument-associated proteins to antagonize PML-NBs, a theme that has also been described for gamma-herpesviruses. In this manner, viral protein expression does not have to occur before the virus can disrupt these restrictive sub-nuclear structures.

PML-NB Evasion by Beta-herpesviruses

HCMV is the most studied beta-herpesvirus in regards to PML-NBs. HCMV tegument protein pp71 binds to Daxx and localizes to PML-NBs where it is able to displace (but not degrade) ATRX from PML-NBs and also induces the proteasome-dependent degradation of Daxx (440). Daxx has the ability to silence the major immediate early promoter (MIEP) of HCMV, thus the targeting of Daxx allows for activation of MIEP and the viral immediate early protein 1 (IE1) is transcribed and translated (440-442). IE1 is then able to bind to PML and induces the removal of SUMOs from PML protein resulting in the dispersal of PML-NBs but not the degradation of PML protein (443, 444).

Another beta-herpesvirus, HHV-6, appears to modify PML-NBs but does not completely abolish the PML-NB structures from infected cells. Upon infection, PML-NB structures become fewer in number but larger in size, while PML protein levels increase (445). The HHV-6 IE1 protein can localize to PML-NBs but does not disperse them. Additionally, SUMOylation of IE1 may stabilize this viral protein (445, 446). Furthermore, the HHV-6 ORF U19 was shown to colocalize with PML-NBs and to have transcriptional transactivating functions on the RANTES promoter, which was suppressed by PML expression (447). The authors theorize that U19 transactivates transcription broadly of both viral and cellular genes and PML protein can suppress this activity to inhibit viral transcription. They postulate that this may contribute to the slow viral replication observed during HHV-6 infection compared to HSV-1 and HCMV, which are able to disrupt PML-NBs. Direct evidence for this claim has not been provided. Furthermore, investigation of other PML-NB resident proteins important for inhibiting viral replication (hDaxx, SP100) has not been done and may explain how HHV-6 replicates without abolishing PML-NB structures.

Why HCMV infection does not result in the degradation of PML protein (as occurs with HSV-1 infection), or how HHV-6 replicates without apparent degradation of PML-NB structures, are unresolved questions. However, these viruses show that manipulation of PML-NB structures is sufficient to evade the restrictive functions of these sub-nuclear structures, and that PML protein degradation is not required.

PML-NB Evasion by Gamma-herpesviruses

All gamma-herpesviruses studied to date encode a viral homolog of the cellular enzyme phosphoribosylformylglycineamide amidotransferase (FGARAT), an enzyme involved in *de novo* purine biosynthesis. While the viral FGARATs (vFGARATs) have not been shown to have purine biosynthesis capabilities, they all display functions in the disruption of PML-NBs. The EBV vFGARAT, BNRF1, binds to Daxx at PML-NBs and inhibits Daxx from binding to ATRX (448). Thus BNRF1 is able to alleviate the transcriptional repression of EBV by PML-NBs. Intriguingly, knocking down Daxx and ATRX in latently EBV-infected lymphoblastoid cells resulted in the reactivation of the virus, suggesting that the virus may utilize PML-NB components to regulate the lytic-to-latent switch (448). A subsequent analysis of the interaction domains between BNRF1 and Daxx revealed that mutating the residues necessary for this interaction also inhibited the expression of viral latent genes and B cell proliferation (449). The KSHV vFGARAT, ORF75, also interferes with the Daxx-ATRX complex. ORF75 expression results in the loss of ATRX protein and dispersal of Daxx from PML-NBs (450). Attempts to make a KSHV mutant virus with stop codons in ORF75 failed to produce infectious virus and lytic gene expression could not be detected (450). In addition to ORF75, KSHV also encodes 4 ORFs with homology to cellular IRFs, termed vIRFs. One of these vIRFs, vIRF-3 (also known as LANA2), is able to antagonize PML-NBs by increasing the SUMO 2/3 modification on PML proteins which leads to ubiquitination and subsequent proteasomal degradation (451, 452). Animal gamma-herpesviruses also antagonize PML-NBs through vFGARATs. MHV68 encodes ORF75c (a ubiquitin E3 ligase) that induces the

proteasomal degradation of PML protein, thereby disrupting PML-NBs (453, 454). MHV68 mutants with non-functional ORF75c are still able to replicate and it was found that a second protein, ORF61, has PML-NB modifying functions. ORF61 expression resulted in a change to PML-NB morphology whereby the PML-NB dots became elongated tracks, however the mechanism and consequence to PML function have yet to be elucidated (455). Herpesvirus saimiri encodes ORF3 to degrade SP100 protein, however PML protein and PML-NB structures remain (albeit lacking SP100) (456). Lastly, RRV infection results in the dispersal of PML-NBs by 24 hours post infection with a loss of SP100 protein levels by 8 hours post infection and loss of PML protein levels by 24 hours post infection (457). ORF75 of RRV was shown to be responsible for the loss of SP100 proteins in human iSLK cells and rhesus fibroblasts, while conflicting data showed ORF75 was responsible for PML protein loss in iSLK cells but not rhesus fibroblast cells (457).

While all gamma-herpesviruses use a vFGARAT to antagonize PML-NBs, the mechanisms have diverged across the viruses within this subfamily. Similar to the alpha-herpesviruses and HCMV, gamma-herpesviruses utilize a tegument-associated protein to quickly subvert PML-NBs. Furthermore, gamma-2 herpesviruses may also share another common strategy by encoding vIRFs to help disrupt PML-NBs. KSHV vIRF-3 is also required for PML-NB loss and the research presented in chapter 2 of this thesis provides evidence of a role for the RRV vIRF R12 in PML-NB subversion. Another gamma-2 herpesvirus, Japanese macaque rhadinovirus (JMRV), also encodes vIRF proteins and it would be

interesting to investigate whether similar PML-NB related functions exist for the JMRV vIRFs. However, there have not been any reports on the function of these proteins or the ability of JMRV to disrupt PML-NBs to date.

1.6 Viral interferon regulatory factors

Both KSHV and RRV encode vIRF ORFs with homology to cellular IRFs (Table 1). KSHV encodes four vIRFs (vIRF-1 through vIRF-4) from four ORFs, while RRV encodes eight vIRFs (R6 through R13) from eight ORFs (63, 137, 458). The vIRFs of KSHV have been studied and shown to possess transcriptional regulatory, oncogenic, cell survival, and immune evasion functions (451, 459-462). The RRV vIRFs have thus far been studied mainly for their role in immune evasion (140, 463, 464). Because of their wide-ranging effects, further investigation into the functions of vIRFs is warranted and may reveal important information for combating gamma-2 herpesviruses.

Table 1. Comparisons of vIRFs and cellular IRFs

		Cellular IRF	KSHV vIRF	RRV vIRF
KSHV	vIRF-1	IRF8 [a] 15% / 23%	---	R10 15% / 28%
	vIRF-2	IRF4 [a] 21% / 31%	---	R11 11% / 27%
	vIRF-3	IRF4 [a]* 16% / 36%	---	R9 19% / 25%
	vIRF-4	None	---	R12 16% / 26%
RRV	R6	IRF8 [b] 16% / 21%	vIRF-1 15% / 25%	R10 12% / 45%
	R7	IRF8 [b] 16% / 22%	vIRF-1 16% / 23%	R11 18% / 45%
	R8	IRF8 [b] 14% / 23%	vIRF-1 15% / 21%	R12 17% / 52%
	R9	IRF8 [b] 19% / 25%	vIRF-3 * 16% / 25%	R13 11% / 53%
	R10	IRF8 [b] 14% / 23%	vIRF-1 13% / 28%	---
	R11	IRF8 [b] 16% / 24%	vIRF-1 15% / 24%	---
	R12	IRF8 [b] 16% / 23%	vIRF-2 20% / 23%	---
	R13	IRF8 [b] 16% / 21%	vIRF-1 * 14% / 23%	---

Protein-protein BLAST comparison using NCBI database. Results presented as %Identity / %Similarity. [a] denotes comparison with human genome, [b] denotes comparison with macaca mulatta genome, * denotes those comparisons that required a position-specific iterated BLAST (PSI-BLAST) algorithm to produce result.

1.6.1 KSHV vIRFs

1.6.1.1 vIRF-1

vIRF-1 is a lytically expressed gene with early kinetics (transcripts detected 8-12 hours post induction in PEL cells) and there is some evidence for low level expression during latency (458, 465). A conserved DNA binding domain found in cellular IRFs was identified in vIRF-1 and the capacity to bind DNA was confirmed (462). Additionally, vIRF-1 was shown to bind to the KSHV viral promoter region of K3, viral dihydrofolate reductase (vDHFR), and vIL-6, and can function as a viral transcription factor activating this promoter (462). It is perhaps not surprising that a viral gene displaying homology to a cellular transcription

factor can function as such. However, several more diverse functions have now been described for vIRF-1 and are described below.

Anti-apoptotic functions of vIRF-1

Several diverse functions have been described for KSHV vIRF-1. vIRF-1 can inhibit apoptosis by binding to and sequestering Bim (a BH3-only pro-apoptotic Bcl-2 family member involved in the initiation of apoptosis) in the nucleus, binding to other BH3-only proteins, and by binding to gene associated with retinoid-IFN-induced mortality 19 (GRIM19) to inhibit IFN/retinoic acid-induced cell death (466-468). KSHV can also inhibit p53 mediated cell cycle arrest and apoptosis through vIRF-1. P53 is a tumor suppressor and transcription factor for many genes involved in cell cycle arrest and apoptosis, and is normally maintained at very low levels in a cell. Another cellular protein, ubiquitin-specific protease 7 (USP7), can increase stability of p53 by deubiquitinating p53 to avoid proteasome-mediated degradation. Two separate studies have revealed that vIRF-1 binds to both p53 and USP7, inhibits USP7 deubiquitination functions, inhibits p53 acetylation as well as p53 transcriptional transactivation, and results in reduced protein levels of p53 (469, 470). The study that showed decreased p53 protein levels utilized overexpression of vIRF-1 or USP7-refractory vIRF-1 constructs transfected into U2OS osteosarcoma cells (469). A recent publication analyzing vIRF-1 and USP7 interactions did not find altered levels of p53 protein when vIRF-1 was knocked down using short hairpin (sh) RNA in KSHV infected BCBL-1 cells or using vIRF-1-null or USP7-refractory vIRF-1 mutant viruses (471). This recent study did, however, find that latently infected PEL and BCBL-1

cells displayed increased proliferation and reduced apoptosis dependent on the vIRF-1 interaction with USP7. They also found increased viral replication in lytically reactivated cells when vIRF-1 was present and able to interact with USP7. Therefore, the functions of vIRF-1 may be cell type specific with regard to effects on p53, or the endogenous levels of vIRF-1 do not result in biologically significant regulation of p53. In line with the hypothesis that endogenous levels of vIRF-1 do not result in regulation of p53, a report by Pozharskaya *et al.* characterized the expression of vIRF-1 during infection of BCBL-1 cells. They found only transient high levels of vIRF-1 early after reactivation, a protein half-life of 3 hours for vIRF-1, and low levels of vIRF-1 during latency and at later times during lytic replication (472). Lastly, vIRF-1 was shown to inhibit transforming growth factor-β (TGF-β) signaling and Smad mediated transcription (such as inhibiting p21 transcription) as well as promoting cell growth (473). While vIRF-1 can inhibit apoptosis, the exact contribution of vIRF-1 *in vivo* remains to be fully defined.

Innate immune modulation by vIRF-1

Several innate immune evasion activities have been described for vIRF-1. vIRF-1 interacts with two adaptor molecules of the innate immune system, STING and IPS-1 (also known as MAVS). The interaction of vIRF-1 and STING (demonstrated by transient expression of vIRF-1 *in vitro*) inhibits the cGAS-STING pathway by preventing the phosphorylation of STING and the ability of TBK1 to bind to STING (474).

RIG-I and MDA5 are cytosolic proteins capable of binding and detecting viral dsRNA in the cytosol (475). The inhibition of KSHV infection and reactivation by the RIG-I and IPS-1 signaling pathway has been reported, however, it is currently unknown how KSHV is able to activate this pathway (476). Regardless, vIRF-1 was recently reported to inhibit IPS-1 signaling during reactivation of KSHV (477). IPS-1 is membrane bound on the surface of mitochondria and vIRF-1 was shown to localize to mitochondria in an IPS-1-dependent manner, in reactivated KSHV-infected PEL and BCBL-1 cells. Additionally, vIRF-1 was found to co-immunoprecipitate with IPS-1. vIRF-1 expression during KSHV reactivation was able to inhibit the aggregation (and activation) of IPS-1 and disrupt the antiviral activity of IPS-1 (e.g., IFNβ production and induction of apoptosis) (477).

Several studies have also confirmed that vIRF-1 interacts with or otherwise inhibits cellular IRFs, as was hypothesized due to their homology to cellular IRFs. While two reports presented conflicting data as to whether *in vitro*-translated vIRF-1 was able to bind to *in vitro*-translated IRF1, both reports demonstrated that exogenous expression of vIRF-1 was able to inhibit IRF1 transactivating and antiviral functions (478, 479). In addition to IRF1, vIRF-1 also inhibits the functions of IRF3. When protein levels of vIRF-1 were reduced (using peptide-conjugate phosphorodiamidate morpholino oligomers which block translation of target RNA sequences) in KSHV-infected and TPA-induced BCBL-1 cells, protein levels of both IRF3 and STAT1 increased (480).

vIRF-1 has also been shown to interfere with the CBP/p300 transcriptional coactivator complex (481, 482). Specifically, when vIRF-1 was over-expressed in

Sendai virus-infected 293 cells and immunoprecipitated, vIRF-1 was found to bind to CBP and p300 and reduce the ability of IRF3 to bind the CBP/p300 complex. Decreased levels of IRF3, in conjunction with impaired binding to transcriptional coactivators, results in the specific block of IRF3 signaling by vIRF-1 following viral infection (482).

The overall effect of vIRF-1 modulation of cellular IRFs and transcriptional co-activators is the inhibition of the transcription of type I IFNs, as well as IFN-stimulated genes. Activation of both the IFNα and IFNβ gene promoters was shown to be greatly diminished by transient transfection of vIRF-1 (477, 478). Additionally, in response to Sendai virus infection, exogenous vIRF-1 inhibited the transcription of IFNα, IFNβ, and the ISGs ISG15, RANTES and IP-10 (77, 483, 484).

Aside from the inhibition of transcriptional activation, ectopically expressed vIRF-1 has also been shown to co-immunoprecipitate with HERC5, an E3 ligase responsible for conjugating the ubiquitin-like ISG15 protein onto target proteins (485). Overexpression of vIRF-1 reduced the levels of ISG15 conjugation to cellular proteins, perhaps through its interaction with HERC5. Because knockdown of ISG15 by shRNA resulted in increased KSHV reactivation, it has been suggested that vIRF-1 may play a role in the reactivation of latent KSHV infections (485).

vIRF-1 has thus evolved to antagonize multiple innate immune pathways to help the virus evade innate antiviral responses.

Adaptive immune modulation by vIRF-1

vIRF-1 expression can inhibit the adaptive as well as the innate immune response. For example, Lagos *et al*. demonstrated that overexpression of vIRF-1 results in reduced transcription and cell surface expression of MHC I, thereby preventing recognition of KSHV-infected cells by $CD8^+$ T cells *in vitro*. The authors went on to show that inhibition of MHC class I expression was mediated through the interaction of vIRF-1 with the transcriptional coactivator p300, as removal of the p300 binding sequence from vIRF-1 prevented vIRF-1-mediated downregulation of MHC class I in transfected cells (486). Thus, vIRF-1 is capable of regulating the adaptive immune response, in addition to its roles in modulating the innate immune response.

1.6.1.2 vIRF-2

KSHV vIRF-2 is encoded by K11.1 and K11 and evidence for both latent and lytic expression of vIRF-2 exists (458, 487). Splicing results in a two exon, full length vIRF-2 protein while a shorter vIRF-2 protein translated from just the first exon (K11.1) is also produced (488). Functions for both forms of vIRF-2 have been demonstrated. Short form vIRF-2 (but not full length vIRF-2) can interact with the NFκB consensus binding site while full-length vIRF-2 is able to bind DNA and function as a transcription factor for certain target genes (487, 489). One gene target of full length vIRF-2 is *PIK3C3*, the catalytic subunit of phosphatidylinositol 3-kinase (PI(3)K), and vIRF-2 was capable of up-regulating a promoter-reporter for *PIK3C3 (489)*. Another group identified that vIRF-2 activated the PI(3)K/Akt pathway resulting in the inhibition of transcription factor FOXO3A (490). FOXO3A regulates the expression of Fas ligand, which is

involved in apoptosis. Therefore, vIRF-2 transcriptionally up-regulates the catalytic subunit of PI(3)K, and activates the PI(3)K/Akt pathway to inhibit FOXO3A transcriptional activities, which results in diminished apoptosis of KSHV infected cells. Additional innate immune evasion functions for vIRF-2 have been described and are reported below.

Innate immune modulation by vIRF-2

Like vIRF-1, vIRF-2 is able to interact with cellular IRFs. One report demonstrated that vIRF-2 interacts with IRF1, IRF2, and IRF8 in *in vitro* pull-down assays, while a second report discovered an interaction between vIRF-2 and IRF3 as well (487, 491). However, only a down regulation of the IRF1- and IRF3-induced transactivation of IFNα4 and IFNβ gene promoters has been demonstrated (434, 487, 491). The vIRF-2 inhibition of IRF3 was shown to occur through the simultaneous binding of vIRF-2 to both activated IRF3 and procaspase-3 (491). Exogenous expression of vIRF-2 was able to induce the loss of IRF3 even in the presence of the proteasome inhibitor MG132, while treatment with a general caspase inhibitor was able to inhibit the vIRF-2-induced loss of IRF3 protein. This implicates caspase 3 (and perhaps other caspases) in the vIRF-2-induced degradation of IRF3. Interestingly, this same study found a caspase 3-independent mechanism for vIRF-2 inhibition of IRF3 function; however, the mechanism has not been fully elucidated (491).

Additionally, a GST-vIRF-2 fusion protein was found to bind to the transcriptional coactivator p300 as well as to the p65 component of NFκB. In fact, His_6-vIRF-2 homodimers were found to bind to the NFκB consensus-binding site

and exogenous expression of vIRF-2 was able to inhibit NFκB transactivation of IFNβ (487).

vIRF-2 is also able to inhibit signaling through the ISRE promoters as induced by IFNα or the IFNλ family members IFNλ1 and IFNλ2. vIRF-2 is able to block ISRE signaling and ISG induction by reducing protein levels of IRF9 and phosphorylated STAT1, which prevents the formation of ISGF3 (492).

Lastly, it has been demonstrated that ectopically expressed vIRF-2 can bind to and inhibit the kinase activity of PKR, resulting in the inhibition of the IFNα-induced translational block (493). PKR expression is induced upon IFN stimulation and when PKR binds to dsRNA it is activated and can phosphorylate eIF2α leading to a block in cellular translation. This block in translation can lead to programmed cell death as a means to clear viral infection (494). The binding of vIRF-2 to PKR inhibited the phosphorylation of eIF2α by PKR following dsRNA treatment (493). However, eIF2α is not the only target of PKR. Under certain circumstances PKR can also phosphorylate IκBα, the negative regulator of NFκB resulting in degradation of IκBα and activation of NFκB (495). However, there is no direct evidence that vIRF-2 can inhibit the PKR-mediated activation of NFκB.

vIRF-2 is thus able to inhibit the production of IFN by multiple mechanisms as well as inhibit type I and III IFN signaling and PKR induced translation inhibition.

1.6.1.3 vIRF-3

KSHV vIRF3 is also known as LANA2 and is expressed during latency and with late kinetics during lytic reactivation (465, 496). Several reports

determined that vIRF-3 is exclusively expressed in B cells, however vIRF-3 has now been found in PEL and MCD B cells as well as in KS spindle cells of lymphatic endothelial cell origin (497-501).

Anti-apoptotic functions of vIRF-3

Multiple mechanisms have now been described for vIRF-3 mediated inhibition of apoptosis. Similar to vIRF-2, vIRF-3 can inhibit PKR-dependent phosphorylation of eIF2α (502). This study also demonstrated that vIRF-3 could inhibit PKR-induced activation of caspase-3, thus inhibiting the FADD/caspase 8 apoptotic pathway. Interestingly, vIRF-3 does not inhibit NFκB or caspase 9 activation by PKR. Therefore, vIRF-3 does not completely inhibit PKR activation or activities, but instead selectively inhibits the PKR/eIF2α pathway and the extrinsic apoptotic pathway (502). Several groups have now demonstrated that vIRF-3 can also inhibit p53-induced transcription and p53 stability, resulting in inhibition of apoptosis (497, 503). Post-translational modifications can also affect the function of p53; indeed, vIRF-3 was found to inhibit the SUMOylation of p53 and this correlated with reduced cellular senescence in response to SUMO2 overexpression or IFN treatment (504). A recent publication calls into question whether endogenous vIRF-3 (or vIRF-1, as described above in section 1.6.1.1) alters p53 protein levels (471). Using knockdown of endogenous vIRF-3 in KSHV infected cells, or vIRF-3-null mutant viruses, these authors found that vIRF-3 had no effect on p53 stability. Instead, the authors showed that vIRF-3 interacts with USP7, resulting in enhanced cell viability of latently infected PEL cells and reduced virus production during reactivation (471). Thus vIRF-3 may help

maintain latency. As stated before, the authors of this study suggested that their different p53 results may have to do with the use of over-expression systems rather than endogenous levels of vIRF-3, or may be due to different cell lines used in each study. Lastly, vIRF-3 has been shown to inhibit the FOXO3A transcription factor, blocking G_2/M cell cycle arrest (which can lead to apoptosis) (505).

Role of vIRF-3 in oncogenesis

Several studies have focused on elucidating a role for vIRF-3 in the development of KSHV driven malignancies. PEL cell survival has been shown to require vIRF-3 expression, as knockdown of vIRF-3 resulted in the activation of caspase-3 and -7, as well as reduced proliferation of the PEL cells (506). Additionally, vIRF-3 disrupts PML-NBs in PEL cells resulting in the up-regulation of the survivin gene, as PML protein is a negative transcriptional-regulator of survivin (451). Survivin is a member of the inhibitor of apoptosis (IAP) protein family and is highly expressed in many cancers (507). Another prototypical oncogene is c-Myc, which has roles in regulating cell growth and survival. Transcription mediated by c-Myc is repressed by myc modulator-1 (MM-1) (508). vIRF-3 can bind to MM-1α (the most abundant and most repressive isoform of MM-1) and inhibit its interactions with c-Myc (509, 510). Additionally, vIRF-3 interacts with S-phase kinase-associated protein 2 (Skp2) to enhance c-Myc mediated transcription and increase the half-life of c-Myc (511). Other possible connections between vIRF-3 and malignant progression, which have not been fully elucidated, include: 1) vIRF-3 interacts with and inhibits the SUMOylation of

the pocket proteins, Rb, p107, and p130 (involved in tumor suppression), 2) vIRF-3 inhibits T-cell factor (TCF)-dependent transcriptional activities (deregulation may contribute to oncogenesis), and 3) interaction of vIRF-3 with histone deacetylase 5 (HDAC5) in lymphatic endothelial cells results in spindle cell morphology and hyper-sprouting formation (501, 512, 513).

Innate immune modulation by vIRF-3

Similar to vIRF-1 and vIRF-2, vIRF-3 is also able to interact with cellular IRFs. The outcome of these interactions, however, is not as clear as with vIRF-1 or -2. Both exogenously and endogenously expressed IRF5 protein co-immunoprecipitates with endogenous vIRF-3 protein in multiple KSHV-infected cell lines (514, 515). In reporter cell lines, exogenous vIRF-3 expression inhibits IRF5 mediated activation of IFNα and IFNβ promoters. In this same system, it was found that overexpression of vIRF-3 also results in reduced type I IFN production following Newcastle disease virus infection, indicating that vIRF-3 can prevent virally-induced production of IFN (514). vIRF-3 was found to inhibit the transactivating function of IRF5 by preventing it from binding to DNA promoters (514, 515).

Additionally, vIRF-3 has been shown to modulate the activities of IRF3 and IRF7. One report demonstrated a repressive function for vIRF-3, in which co-transfection of vIRF-3 with either IRF3 or IRF7 (in mouse NIH3T3 cells) resulted in reduced activation of the IFNα4 promoter following virus-stimulation, when compared to transfection of either IRF3 or IRF7 alone (496). However, a second report from the same group presented data that vIRF-3 enhances activation of

IFNα and IFNβ promoters in human cell lines (516). The authors of this study showed that exogenous vIRF-3 complexes with endogenous IRF3 and IRF7; this interaction does not inhibit these cellular IRFs from binding to CBP/p300, and the presence of vIRF-3 increases the DNA binding affinity of this complex. When vIRF-3 was over-expressed, the transactivation of IFNα and IFNβ promoters by IRF3 and IRF7 following Sendai virus infection was increased compared to IRF3 and IRF7 alone (516).

Like vIRF-2, vIRF-3 has also been found to be capable of modulating NFκB signaling. As stated above, NFκB must first dissociate from IκBα before it can translocate and act in the nucleus. This dissociation is partly regulated by the IκB Kinase (IKK) complex, of which IKKβ is a member (517). Transiently expressed vIRF-3 selectively binds to and inhibits IKKβ kinase activity leading to repression of the NFκB transactivating functions (518).

Additionally, vIRF-3 is able to inhibit PKR signaling. Ectopic expression of vIRF-3 inhibited the PKR-mediated block in translation as well as apoptosis; however, no direct interaction between vIRF-3 and PKR was found (502). Therefore, vIRF-3 likely utilizes a different mechanism than vIRF-2 to inhibit PKR signaling. Interestingly, exogenous expression of vIRF-3 was not able to inhibit PKR activation of NFκB (502).

Adaptive immune modulation by vIRF-3

In addition to its innate immune evasion functions, vIRF-3 also plays a role in the evasion of the adaptive immune response. While vIRF-1 functions to down modulate MHC class I molecules, vIRF-3 has been found to play a role in the

inhibition of MHC class II expression (519, 520). MHC class II transcription is induced by the MHC class II transactivator protein, CIITA, and CIITA transcription is induced by IFNγ (521). Overexpression of vIRF-3 inhibits the transcription of CIITA by inhibiting the production of IFNγ, which results in the reduced levels of MHC class II transcripts and protein (519, 520). Additional data also suggests a CIITA-independent mechanism for the downregulation of MHC class II transcription. While overexpression of vIRF-3 in a KSHV-negative B cell line resulted in the reduction of both CIITA and MHC class II transcripts, the knock down of vIRF-3 in a KSHV-positive B cell line resulted in a reduction in MHC class II transcripts without a change in CIITA transcript levels (520). The CIITA-independent mechanism of vIRF-3 regulation of MHC class II expression has not yet been elucidated.

To date, vIRF-3 has been demonstrated to have the most functions of all the vIRFs. vIRF-3 is an attractive therapeutic target because it is expressed during both latent and lytic infection as well as having pleitropic effects.

1.6.1.4 vIRF-4

Work on KSHV vIRF-4 has revealed an important role in reactivation and lytic replication. vIRF-4 is expressed during lytic replication and, until very recently, had no immune modulatory functions attributed to it. RTA /ORF50 is the regulator of the switch to lytic replication in KSHV infection, and vIRF-4 works with RTA to induce efficient reactivation, at least with regards to a subset of lytic viral genes (522). KSHV lytic gene expression is further enhanced by vIRF-4 mediated suppression of cellular IRF4 expression (523). Cellular IRF4 negatively

regulates B-Cell lymphoma 6 (BCL6), a gene shown to enhance the expression of lytic KSHV genes. Thus, when vIRF-4 suppresses cellular IRF4, there is an up-regulation of BCL6 and enhanced KSHV lytic gene expression. The suppression of IRF4 also results in the downregulation of c-Myc. While c-Myc is important for malignant progression and maintaining latency, efficient reactivation and lytic replication requires the loss of c-Myc expression. Therefore, multiple genes regulated by the vIRF-4 repression of cellular IRF4 may change the cellular environment to favor lytic replication. vIRF-4 can further modulate the cellular environment to favor KSHV lytic replication. First, vIRF-4 can inhibit the G1-S transition in the cell cycle, and this enhances virus replication. It is hypothesized that viruses promote inhibition of the G1-S transition so that they do not have to compete with cellular genome replication for free nucleotides during viral replication. Second, vIRF-4 can interact with murine double minute 2 (MDM2) to inhibit the degradation of MDM2 and enhance p53 degradation. An interaction with USP7 (also known as HAUSP) was also found in this study, however the vIRF-4/MDM2 interaction resulted in greater p53 degradation than the vIRF-4/USP7 interaction. The enhancement of p53 degradation inhibits apoptosis so that lytic viral replication can continue. Lastly, an interaction between vIRF-4 and IRF7 was discovered, which inhibits IRF7 dimerization and subsequent induction of type I IFN transcription (524). Innate immune evasion is critical in the early stages of lytic infection, so that robust replication can occur and additional immune-modulatory viral proteins can be synthesized. Overall, it

appears that vIRF-4 is critical for the latent to lytic switch and establishing a robust lytic reactivation.

KSHV vIRF research to date has revealed many important functions that contribute to many aspects of the viral lifecycle as well as disease progression. The ability to test the vIRF functions during *in vivo* infection would further elucidate their importance for the virus as well as provide a means to test the therapeutic targeting of these ORFs for the treatment of KSHV infection. One way to achieve this is with the use of the RRV/RM model.

1.6.2 RRV vIRFs

The eight RRV vIRFs (encoded by ORFs R6 through R13) are located in the same region of the genome as the KSHV vIRFs (137, 139). Due to the sequence identities between the first 4 RRV vIRFs (R6-R9) and the last 4 (R10-R13), it has been hypothesized that a genetic duplication event may have given rise to the 8 vIRFs encoded by RRV (137). Regardless of how the RRV vIRFs arose, mounting evidence suggests that the vIRFs have functionally diverged from each other (140, 463). Six of the RRV vIRF protein sequences share some level of identity with KSHV vIRF-1 (21%-28%) while R9 and R12 share some level of identity with KSHV vIRF-3 (25%) and vIRF-2 (23%), respectively (Table 1). Unlike KSHV, RRV displays robust lytic replication *in vitro*, allowing for the unique opportunity to study the function of vIRFs during *de novo* infection (101, 525-527). A complete analysis of the function of all 8 RRV vIRFs is still forthcoming; however, much has already been learned about the function of several of the vIRFs both *in vitro* and *in vivo*.

1.6.2.1 RRV vIRF immune modulation *in vitro*

Analysis of individual vIRFs *in vitro*

Analysis of the RRV vIRF R6 has been performed using both *in vitro* overexpression systems and virological approaches (463). Using a luciferase reporter rhesus fibroblast cell line and transient transfection of RRV R6, it was shown that R6 could inhibit the activation of the ISRE promoter by poly(I:C). This inhibition correlated with the reverse transcription (RT)-PCR results showing that transient transfection of RRV R6 could reduce IFNβ transcripts by 50% in poly(I:C)-stimulated cells compared to empty vector. Further, it was found that R6 achieves this inhibition of the IFNβ response by binding to CBP and phosphorylated IRF3. Due to this interaction, R6 prevents the CBP/p300/IRF3 complex from binding to DNA, resulting in the shuttling of phosphorylated IRF3 out of the nucleus, followed by proteasomal degradation. Finally, using both exogenously expressed R6 and an infectious bacterial artificial chromosome (BAC)-derived form of RRV encoding a form of R6 tagged with an HA epitope tag, R6 was shown to act early during infection, and was also demonstrated to be packaged within the virion. As a result, this virion-associated form of R6 can function immediately upon infection to inhibit IFNβ transcription (463).

Transient transfection of either R10 or R11 inhibited the poly(I:C)-stimulated secretion of type I IFN, although to a lesser extent than R6 (140). Interestingly, transient transfection of R7 into a rhesus fibroblast luciferase reporter cell line, followed by transfection of poly(I:C) stimulation, revealed that

R7 does not inhibit type I IFN transcription or secretion. In fact R7 appeared to enhance the stimulation by poly(I:C) compared to empty vector (140).

Thus initial investigations into the RRV vIRFs *in vitro* have found some overlapping functions but also some distinctions that necessitate further investigation.

Analysis of the complete set of RRV vIRFs *in vitro*

Using an infectious BAC of RRV strain 17577, a recombinant virus was generated in which all eight vIRF-encoding ORFs were deleted from the viral genome, resulting in the production of a vIRF knockout virus ($RRV_{vIRF\text{-}KO}$) (140). Analysis of this recombinant $RRV_{vIRF\text{-}KO}$ virus indicated that it displayed similar growth kinetics in rhesus fibroblasts *in vitro* when compared to wild-type BAC-derived RRV (WT-BAC RRV). However, *in vitro* infection of RM PBMCs or splenocytes with $RRV_{vIRF\text{-}KO}$ indicated that this virus was less efficient at infecting these cells than WT-BAC RRV, implying that vIRFs help RRV establish infection.

It was suggested that this differential infection efficiency between the two viruses was due to differential inhibition of the IFN response. This was confirmed by the analysis of transcript levels in infected telomerized rhesus fibroblasts and rhesus PBMCs, where it was found that $RRV_{vIRF\text{-}KO}$ virus induced higher levels of both type I and type II IFNs compared to WT-BAC RRV between 6 and 72 hours post-infection. One cell type that was found to display similar infection efficiencies for both $RRV_{vIRF\text{-}KO}$ and WT-BAC RRV was plasmacytoid DCs. In rhesus plasmacytoid DCs, intracellular cytokine staining showed that $RRV_{vIRF\text{-}KO}$ virus infection resulted in longer and sustained IFNα production compared to

WT-BAC RRV. These results suggest a role for the RRV vIRFs in the suppression of both the type I and type II IFN response, early during RRV infection. In addition, Western blot analysis and immunofluorescence microscopy showed that $RRV_{vIRF\text{-}KO}$ infection of rhesus fibroblasts resulted in increased levels of phosphorylated IRF3 within the nucleus compared to WT-BAC RRV infection (140). This implies that vIRF modulation of IRF3 may be one mechanism by which RRV suppresses the type I IFN response.

Taken together, these findings all clearly demonstrate that the vIRFs function in inhibiting IFN production during RRV infection.

1.6.2.2 RRV vIRF immune modulation *in vivo*

Analysis of the effects of vIRF deletion on *in vivo* infection and immune regulation was accomplished by infecting RMs with $RRV_{vIRF\text{-}KO}$ virus and comparing this to RMs infected with WT-BAC RRV. In these studies, expanded specific pathogen-free RMs seronegative for RRV were infected intravenously (*iv*) with $5x10^6$ PFU of either WT-BAC RRV or $RRV_{vIRF\text{-}KO}$ (464). These studies were the first to analyze the function of any viral vIRFs during *de novo* infection *in vivo*. The results of this work indicated that the RRV vIRFs aid in the initial infection, replication, and persistence of the virus. Specifically, infection of RMs with $RRV_{vIRF\text{-}KO}$ virus resulted in lower viral DNA loads (measured in whole blood) and less viremia compared to WT-BAC RRV infection. Additionally, levels of RRV genomes were below the limit of detection by qPCR in $CD20^+$ B cells isolated from $RRV_{vIRF\text{-}KO}$ -infected RMs at 3, 6, and 9 months post infection (and could

only be detected by the more sensitive nested PCR) compared to WT-BAC RRV-infected RMs where RRV genomes were detectable by qPCR (464).

Innate immune modulation *in vivo*

Following experimental IV infection of RMs with either WT-BAC RRV or $RRV_{vIRF\text{-}KO}$, the plasma from each RM was incubated with type I IFN reporter cells to quantify the amount of type I IFN in the plasma. This sensitive assay revealed that the vIRFs are important for inhibiting the type I IFN response early during infection (within the first two weeks). Specifically, 75% of $RRV_{vIRF\text{-}KO}$ -infected RMs had measurable levels of type I IFN in their plasma at one day post-infection, and all animals displayed sustained type I IFN levels in their plasma within the first two weeks of infection (464). In comparison, only 50% of the WT-BAC RRV-infected RMs had any measurable type I IFN in their plasma during the first two weeks, and only 33% of RMs had sustained type I IFN levels in their plasma during the first two weeks of infection (464). These results suggest the RRV vIRFs play a role in the suppression of the type I IFN response at early time-points during *in vivo* infection.

Adaptive immune modulation *in vivo*

In addition to their effects on innate immune responses *in vivo*, the RRV vIRFs were also found to inhibit the development of RRV-specific T cell responses, especially within the first two weeks of infection. $RRV_{vIRF\text{-}KO}$ infection of RMs resulted in the detection of RRV-specific $CD4^+$ and $CD8^+$ T cells 7 to 14 days earlier than with WT-BAC RRV-infected RMs, perhaps due to the increased type I IFN response within the $RRV_{vIRF\text{-}KO}$ infected RMs (464). In line with these

differences in T cell responses, IFNγ and IL-12p40 (two cytokines important for development of a T_{H1} response) were detected in plasma of $RRV_{vIRF\text{-}KO}$ -infected RMs at elevated levels throughout the first week of infection (IFNγ), or became detectable two weeks earlier (IL-12p40) than in WT-BAC RRV-infected RMs (464). IFNγ was detected in the plasma of $RRV_{vIRF\text{-}KO}$ -infected RMs during the first 7 days post-infection, while WT-BAC RRV-infected RMs only displayed detectable levels of IFNγ in plasma at day 1 post-infection. IL-12p40 was detected in plasma of $RRV_{vIRF\text{-}KO}$ -infected RMs one day after infection, but was not detected at similar levels in the plasma of WT-BAC RRV-infected RMs until day 14 post-infection (464). Thus, the RRV vIRFs also affect the development of adaptive immune responses. This could be the result of the vIRF suppression of type I IFN responses, or direct effects on adaptive immune pathways. Detailed *in vitro* characterization of each RRV vIRF would no doubt elucidate the mechanism of adaptive immune suppression. A summary of the KSHV and RRV vIRF roles in immune evasion are depicted in Figure 1.7 and Table 2 below.

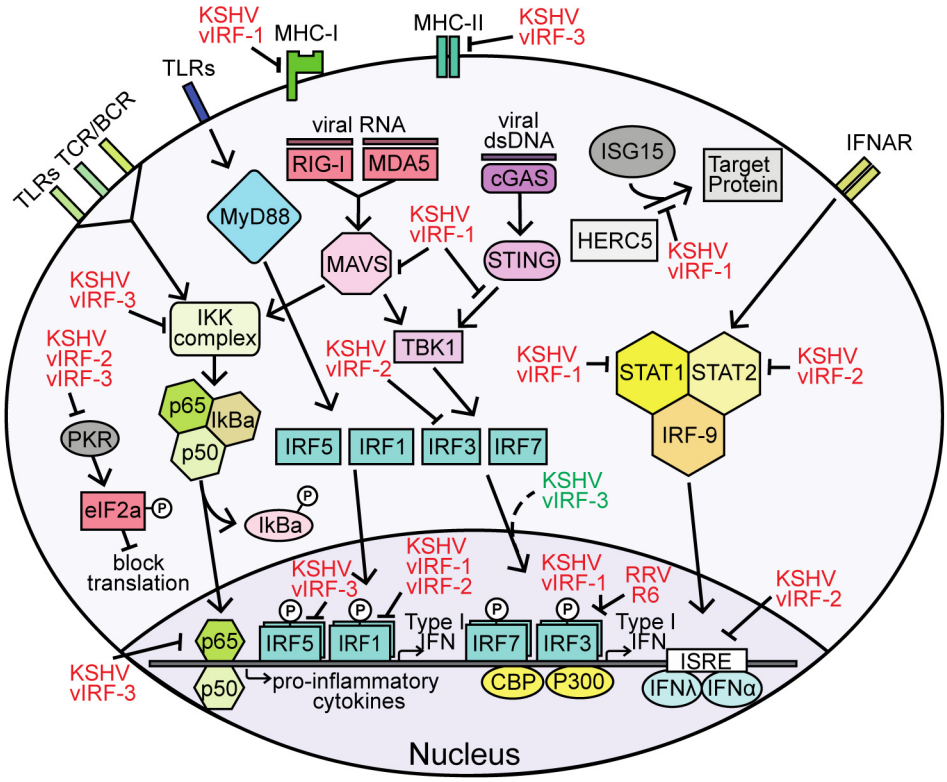

Figure 1.7 Immune Evasion Functions of KSHV and RRV vIRFs

Table 2. Effects of RRV vIRFs *in vitro* and *in vivo*

	In Vitro	*In Vivo*	
Innate Immune Evasion	vIRFs reduce transcript levels of type I and type II IFNs between 6-72 hpi in telo-RhFs and PBMCs	vIRFs reduce IFNa levels in plasma during the first two weeks of infection	Innate Immune Evasion
	vIRFs inhibit the production of IFNa by plasmacytoid DCs	vIRFs inhibit sustained production of IFN gamma in plasma during the first two weeks of infection	Adaptive Immune Evasion
	R6 binds CBP and phosphorylated IRF3, prevents complex binding and inhibits IFN beta production	vIRFs inhibit the appearance of IL-12p40 in plasma during the first two weeks of infection	
	R10 and R11 both inhibit poly(I:C) stimulated secretion of type I IFN in HEK293 cells	vIRFs delay the RRV-specific $CD4^+$ and $CD8^+$ T cell response	

1.7 Concluding remarks

Analysis of the vIRFs encoded by KSHV and RRV has shown that these viral proteins act in multiple ways to affect the immune response to viral infection (Figure 1.7). Data obtained thus far on the RRV vIRFs suggests similar roles, and perhaps mechanisms, for immune evasion as compared with the proposed functions of the KSHV vIRFs (Table 2). RRV *in vivo* studies have also demonstrated that vIRFs have a functional consequence on *de novo* viral infection, aiding in the establishment of infection in the host by suppressing both the innate and adaptive immune responses. This thesis addresses the role of RRV vIRFs and one in particular, R12, in the evasion of type I IFN signaling. *In vivo*, RRV will undoubtedly encounter type I IFNs produced by infected cells or neighboring uninfected cells. The ability of the virus to dampen or turn off type I IFN signaling to prevent ISG induction would subvert the expression of antiviral effectors and enable replicating virus to more efficiently spread throughout the host even in the presence of type I IFN.

In chapter 2, I demonstrate that the RRV vIRFs aid the disruption of PML-NBs during infection, reduce ISG transcription, and enhance viral replication in the presence of type I IFN signaling. I further characterize the specific vIRF R12, as having a crucial role in this evasion of type I IFN signaling through its interaction with PML-NBs. This work demonstrates for the first time that a RRV vIRF plays a role in the disruption of PML-NBs and can modulate the signaling cascade downstream of type I IFN binding to receptors.

CHAPTER 2

Rhesus Macaque Rhadinovirus Encodes a Viral Interferon Regulatory Factor to Disrupt Promyelocytic Leukemia Nuclear Bodies and Antagonize Type I Interferon Signaling.

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Running Title: Viral IRF of RRV disrupts PML nuclear bodies

- Manuscript was written by Laura Springgay.
- All experiments were performed by Laura Springgay except:
  - Cell sorting by flow cytometry was performed by Eric McDonald with the help of Kristin Fitzpatrick.
  - The WT RRV_{17577} BAC used in the construction of the RRV R12 mutants was originally constructed by Ryan Estep.
  - The $RRV_{vIRF\text{-}KO}$ virus was constructed by Bridget Robinson.

2.1 Abstract

Interferon (IFN) production and the subsequent induction of IFN stimulated genes (ISGs) are highly effective strategies utilized by cells to protect against invading pathogens, including viruses. Promyelocytic Leukemia Nuclear Bodies (PML-NBs) are sub-nuclear structures that are critical for the development of a robust IFN response. As such, PML-NBs serve as an important hurdle for viruses to overcome to successfully establish an infection. Both Kaposi sarcoma Herpesvirus (KSHV) and the closely related rhesus macaque rhadinovirus (RRV) are unique for encoding viral homologues to IFN regulatory factors (termed vIRFs) that can manipulate the host immune response by multiple mechanisms. In our current study we demonstrate that the RRV vIRF R12 aids viral replication in the presence of the type I IFN response. This is achieved in part through the disruption of PML-NBs and the inhibition of robust ISG transcription.

2.2 Importance

KSHV and RRV encode a unique set of homologs to cellular IFN regulatory factors, termed vIRFs, which are hypothesized to help these viruses evade the innate immune response and establish infections in their respective hosts. Our work elucidates the role of one RRV vIRF, R12, and demonstrates that RRV can dampen the type I IFN response downstream of IFN signaling, which would be important for establishing a successful infection *in vivo*.

2.3 Introduction

All viruses must employ tactics to evade or counteract the host immune response, if they are to successfully establish an infection. The cell's first line of

defense against infection is the innate immune system. Recent studies have found that viruses can antagonize the innate immune system by disrupting Promyelocytic Leukemia Nuclear Bodies (PML-NBs) (208, 232, 528). PML-NBs are constitutively expressed, multi-protein punctate structure located within the nucleus of most cell types (196). Proteins found in PML-NB are either permanent residents (PML, SP100, Daxx) or transiently present (CBP, P300, p53) (196, 214, 232, 528-531). The PML proteins are absolutely required to form PML-NBs and evidence suggests that SUMOylation of PML and some of the other PML-resident proteins is important to form these structures (211, 221). Multiple functions have been attributed to PML-NBs including cell-cycle regulation, antiviral defense, as sites where proteins are SUMO modified, and transcriptional regulation (208, 232, 528). The anti-viral functions of PML-NBs have been shown to restrict herpesvirus infections. PML-NBs can epigenetically silence herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) viral genomes once they enter the nucleus and PML-NBs composed of PML isoform IV can trap varicella zoster virus (VZV) nucleocapsids to prevent nuclear egress (166, 204, 239, 532). More generally, PML-NBs enhance the induction of interferon (IFN)-stimulated genes (ISGs) aiding the establishment of an anti-viral state within the cell (246). While PML and SP100 are constitutively expressed, type I IFN does upregulate their protein expression (243, 244). Several viruses, including human herpesviruses, have been shown to modulate or disperse PML-NBs in order to evade antiviral defenses, regulate viral gene transcription, and replicate (232, 440, 441, 450, 451, 457, 533, 534). For example, HSV-1 encodes ICP0 which

disrupts PML-NBs by degrading SUMO-1 modified forms of PML and SP100 proteins (437). HCMV encodes pp71 and IE1 which both aid in the dispersal of PML-NBs (166, 440). Gamma-herpesviruses have also been reported to antagonize PML-NBs. Kaposi sarcoma herpesvirus (KSHV), a human gamma-2 herpesvirus (γ2HV) encodes ORF75 and viral IFN regulatory factor 3 (vIRF3) to mediate displacement of hDaxx from PML-NBs and induce degradation of PML protein respectively (450, 451). ORF75 homologs of other gamma-herpesviruses [murine gamma-herpesvirus 68 (MHV-68), Epstein-Barr virus (EBV), Herpesvirus Saimiri (HVS), and RRV] have also been implicated in the disruption of PML-NBs (448, 453, 456, 457, 535).

KSHV infection is generally asymptomatic in healthy individuals; however, the virus can promote the development of Kaposi sarcoma (KS), primary effusion lymphoma (PEL), multi-centric Castleman's disease (MCD), and some non-Hodgkin lymphomas (NHL) in immune-compromised individuals, including AIDS patients (103, 536-538). Unfortunately, establishing animal models for KSHV has proven difficult due to species-specific tropism displayed by KSHV. Infection of rhesus macaques (RMs) with KSHV proved ineffective, with no KSHV transcripts or pathologies being detected in infected animals (128). Several mouse models have also been explored with limited success (124, 539, 540). *In vitro*, KSHV produces a predominantly latent infection in cell culture, making the study of virus replication and expression of viral genes difficult (526, 527). A viable alternative is to study an animal virus that can induce similar disease manifestations in its natural host, such as the γ2HV rhesus macaque rhadinovirus (RRV), which

naturally infects RMs (135, 137). RRV provides a powerful animal model with which to study infection and KSHV-like disease development, as experimental RRV infection of SIV-infected RMs can lead to the development of MCD, NHL and retroperitoneal fibromatosis (a mesenchymal proliferative lesion that possesses cellular features that resembles KS) (134, 152). *In vitro*, RRV establishes a robust lytic infection in primary RM fibroblast cells, facilitating the growth and propagation of the virus as well as the study of the lytic infection cycle (135, 525, 541, 542). Importantly, the genomes of KSHV and RRV are essentially collinear with both viruses encoding vIRFs. KSHV encodes four vIRF ORFs (vIRF-1 through vIRF-4) and RRV encodes eight (R6 through R13). The vIRFs display as much as 21% identity to cellular IRFs, which are transcription factors involved in regulating IFN and ISG expression (137, 140, 459, 461, 463, 464, 543). Research on the KSHV vIRFs, which has typically involved over-expression systems or chemically induced reactivation of KSHV from latently-infected cells *in vitro*, has revealed multiple functions of these molecules, including an ability to antagonize both the innate and adaptive immune responses, and the inhibition of apoptosis (459, 544). RRV provides the opportunity to investigate the function of vIRFs not only during *in vitro* infection, but also during infection *in vivo* in an established non-human primate (NHP) model system. Previously, an RRV bacterial artificial chromosome (BAC) system was used to investigate the vIRF ORFs in the context of *de novo* lytic infection both *in vitro* and *in vivo* (140, 464). It was shown that while WT RRV and an RRV BAC-derived mutant lacking all 8 vIRF ORFs ($RRV_{vIRF\text{-}KO}$) grew similarly *in vitro*,

there was a significant growth defect observed *in vivo* with $RRV_{vIRF\text{-}KO}$ early during infection, indicating that the type I IFN response is overcome more efficiently when the vIRF ORFs are expressed during viral infection *in vivo* (140, 464). In this current study we show that the RRV vIRFs help to establish a more efficient infection in the presence of type I IFN through the disruption of PML-NBs. Further, we show that the vIRF R12 is necessary but not sufficient for RRV disruption of PML-NBs.

2.4 Results

2.4.1 RRV vIRFs enhance infection in the presence of IFN and are required for the dispersal of PML-NBs.

To determine whether the difference in viral growth between WT RRV and $RRV_{vIRF\text{-}KO}$ observed *in vivo* is due to the type I IFN response, a single-step growth curve was performed using primary rhesus fibroblast (RhF) cells that were treated with RhIFNα2 for 18 hours prior to infection (Fig. 2.1A). In the presence of RhIFNα2, an almost one-log growth reduction was measured during $RRV_{vIRF\text{-}KO}$ infection as compared to WT RRV infections at 12, 24, and 48 hours post infection (hpi). This indicated that vIRFs are capable of inhibiting the negative effects of IFN on viral lytic replication *in vitro*, but did not restore viral growth to levels attained in the absence of IFN. Concentrations of IFN that carried over to the plaque assay plates were confirmed to be insufficient to affect the viral growth assays.

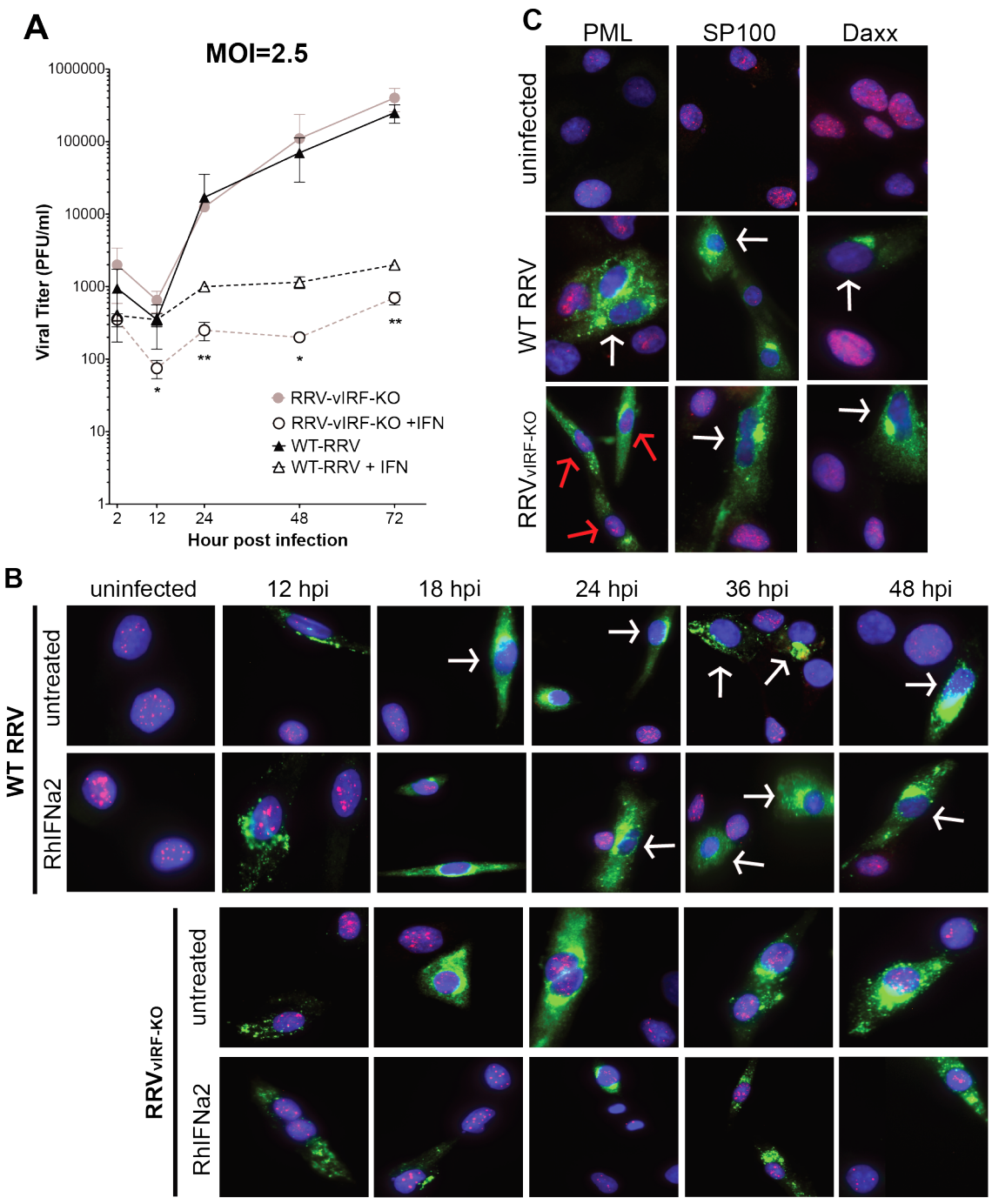
Since PML-NBs enhance the type I IFN response, we investigated whether RRV infection affected the functions of PML-NBs in order to evade the

type I IFN response. Telomerized rhesus fibroblast (Telo-RhFs) cells were infected at an MOI of 2 and infected cells were analyzed over a time-course from 6 to 48 hpi by immunofluorescence analysis (IFA) for PML and RRV-gB protein (Fig. 2.1B). We observed that by 18 hpi the punctate PML-NB staining pattern present in uninfected cells was no longer detectable in WT RRV-infected cells while cells infected with $RRV_{vIRF\text{-}KO}$ retained PML-NB staining even by 48 hpi. These results demonstrate that WT RRV can disrupt PML-NBs and suggest that one or more of the RRV vIRFs are necessary for this disruption. We next investigated whether RhIFNα2 treatment affected the kinetics of the loss of PML-NB and found that RhIFNα2 treatment delayed the loss of PML-NBs during WT RRV infection by 6 hours (PML-NB loss observed at 24 hpi), while PML-NBs remained intact throughout the $RRV_{vIRF\text{-}KO}$ infection. Additionally, we investigated whether SP100 and Daxx, two PML-NB resident proteins, remain associated with PML-NBs. Following 24 hours of WT RRV or $RRV_{vIRF\text{-}KO}$ infection at an MOI of 2, infected cells were analyzed for SP100 and Daxx by IFA. As expected, the punctate PML staining was absent within the WT RRV infected cells, but was still present in the $RRV_{vIRF\text{-}KO}$ infected cells. However, both SP100 and Daxx were absent in the WT RRV and $RRV_{vIRF\text{-}KO}$ infected cells, indicating an RRV protein other than the vIRFs is responsible for the loss of SP100 and Daxx (Fig. 2.1C). Interestingly, the staining pattern for Daxx changed in the bystander (RRV-gB negative) cells as well. In the uninfected samples, Daxx displayed punctate structures in the nucleus along with some diffuse nuclear staining. However, in WT RRV and $RRV_{vIRF\text{-}KO}$ infected samples, the bystander cells only displayed a

diffuse nuclear staining pattern (right panel, Fig. 2.1C). This could be due to abortive replication occurring within the “bystander” cells, cells that are infected with RRV but the gB protein is not expressed, or a factor secreted by the infected cells may affect the localization of Daxx within the bystander cells. To quantify the loss of PML-NBs observed by IFA, the number of gB positive cells with and without PML-NBs was counted at 36 hpi (several hours after the PML-NB disruption by WT RRV had begun but before cytopathic effects of viral infection could obscure results) with either WT RRV or $RRV_{vIRF\text{-}KO}$ (Fig. 2.1D). Following WT RRV infection 11% of the gB positive cells still contained PML-NBs. However, following $RRV_{vIRF\text{-}KO}$ infection 90% of gB positive cells retained PML-NBs. The difference in gB positive cells containing PML-NBs between WT RRV and $RRV_{vIRF\text{-}KO}$ infections was statistically significant ($p<0.0001$). RhIFNα2 treatment did not alter this phenotype as 21% of WT RRV infected cells had PML-NBs while 96% of $RRV_{vIRF\text{-}KO}$ infected cells contained PML-NBs. In addition, the number of PML-NBs within nuclei of gB-positive cells in the same 36 hpi samples was also counted and revealed a statistically significant ($p < 0.01$) difference between the two virus infected cultures in the absence or presence of RhIFNα2. Specifically, there were less PML-NB punctate structures per nucleus in WT RRV-infected cells (average of 2 PML-NBs per nuclei of RhIFNα2 treated cells and an average of 7 PML-NBs per nucleus in the absence of RhIFNα2) as compared to samples infected with $RRV_{vIRF\text{-}KO}$ (averages of 16 or 21, with or without RhIFNα2, respectively) (Fig. 2.1E).

To determine whether the loss of PML-NBs observed by IFA was due to a

loss of PML proteins or to dispersal of PML-NBs, telo-RhFs were infected with a GFP expressing WT RRV (WT RRV-GFP) or $RRV_{vIRF\text{-}KO}$ ($RRV_{vIRF\text{-}KO}$-GFP) at an MOI of 5 for 24 hours, followed by sorting the infected cells based on GFP expression. The MOI was increased to 5 in order to attain a greater percentage of RRV infected cells as the infection of Telo-RhF cells by RRV is not efficient. Nuclear and cytoplasmic protein extracts were prepared from the GFP+ cells and resolved by SDS-PAGE for Western blot analysis (Fig. 2.1F). This revealed that 24 hpi with WT RRV-GFP, there was a reduction in PML isoforms I and II protein level by approximately 75% compared to mock infected cells. This differed from $RRV_{vIRF\text{-}KO}$-GFP infection where an increase in the levels of PML protein isoforms I and II was detected compared to mock infected cells. This suggests that expression of one or more of the vIRFs induces the degradation of PML proteins.


A
MOI=2.5
Viral Titer (PFU/ml)
1000000
100000
10000
1000
100
10
1
2
12
24
48
72
Hour post infection
RRV-vIRF-KO
RRV-vIRF-KO +IFN
WT-RRV
WT-RRV + IFN
C
PML
SP100
Daxx
uninfected
WT RRV
RRVvIRF-KO
B
uninfected
12 hpi
18 hpi
24 hpi
36 hpi
48 hpi
WT RRV
untreated
RhIFNa2
RRVvIRF-KO
untreated
RhIFNa2

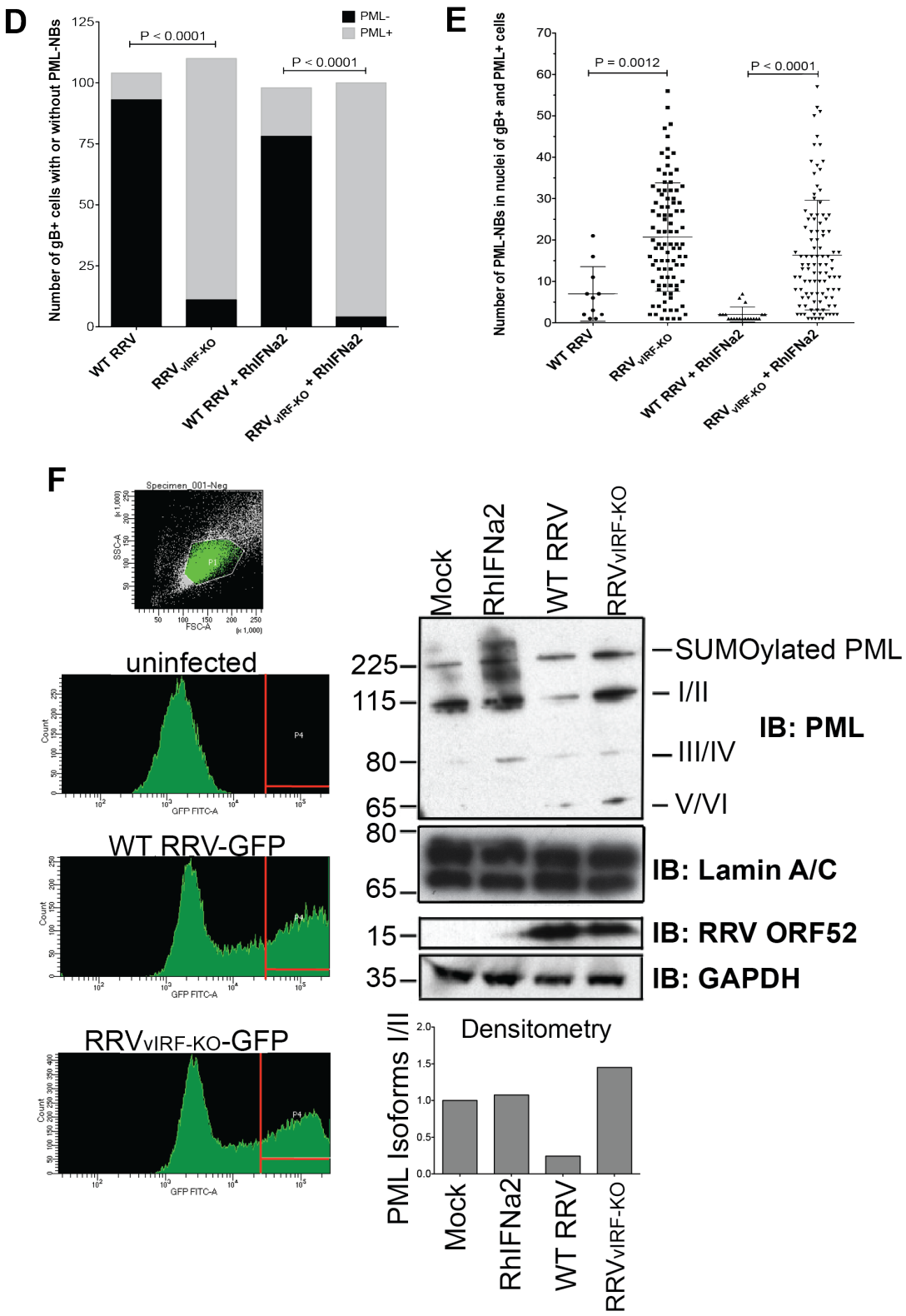

D
Number of gB+ cells with or without PML-NBs
PML-
PML+
P < 0.0001
P < 0.0001
WT RRV
RRV vIRF-KO
WT RRV + RhIFNa2
RRV vIRF-KO + RhIFNa2
E
Number of PML-NBs in nuclei of gB+ and PML+ cells
P = 0.0012
P < 0.0001
F
uninfected
WT RRV-GFP
RRVvIRF-KO-GFP
Mock
RhIFNa2
WT RRV
RRVvIRF-KO
SUMOylated PML
I/II
III/IV
V/VI
IB: PML
IB: Lamin A/C
IB: RRV ORF52
IB: GAPDH
Densitometry
PML Isoforms I/II

Figure 2.1. RRV vIRFs increase viral titers in the presence of IFN and are necessary for PML-NB disruption. A) Primary RhFs were infected with WT RRV or $RRV_{vIRF\text{-}KO}$ at an MOI of 2.5 in the presence or absence of 100U/ml RhIFNα2. Viral titers were measured at the indicated times post infection (pi) by plaque assay and presented as plaque forming units (PFU) per milliliter (mL). Assays were performed in duplicate, replicates averaged, and data were analyzed by unpaired student t test. P values of less than 0.05 were considered significant and asterisks denote the following significant p values: * ($p \leq 0.05$) and ** ($p \leq 0.01$). B) Telo-RhFs were infected with WT RRV or $RRV_{vIRF\text{-}KO}$ at an MOI of 2 in the presence or absence of 100U/ml RhIFNα2. At the indicated times pi, cells were fixed with methanol and stained with PML (red) and RRV-gB (green) specific antibodies while nuclei were stained with DAPI (blue). White arrows indicate infected cells that have lost PML-NB structures. C) Telo-RhFs were mock infected or infected with WT RRV or $RRV_{vIRF\text{-}KO}$ at an MOI of 2 for 24 hours. Cells were fixed with methanol and stained with PML (red), SP100 (red), Daxx (red), and RRV-gB (green) specific antibodies. Nuclei were stained with DAPI (blue). White arrows indicate infected cells that have lost PML, SP100, or Daxx punctate structures. Red arrows indicate infected cells that still contain PML-NB structures. (B-C) Experiments were performed twice in duplicate and representative images are shown. D) PML/RRV-gB double positive cells (grey) as well as RRV-gB positive/PML negative cells (black) were counted at 36hpi from cells treated as in part (B). Data were analyzed by two-tailed Fisher's exact test and p values of less than 0.05 were considered significant. E) Of the RRV-gB positive cells that were positive for PML-NBs in part (D), the number of PML-NBs was counted within the nuclei of each cell. Data were analyzed by unpaired student t test with p values less than 0.05 considered significant. (D-E) Experiments were performed twice

and one representative experiment is shown. F) Telo-RhFs were infected with WT RRV-GFP or $RRV_{vIRF\text{-}KO}$-GFP at an MOI of 5 for 24 hours, before sorting the GFP positive cells by flow cytometry. Proteins from sorted infected cells, untreated cells, and cells treated with 100U/ml RhIFNα2 for 18 hours, were harvested. Cytoplasmic and nuclear proteins were separated before analysis by SDS-PAGE and probed with PML, lamin A/C, RRV-ORF52, and GAPDH specific antibodies. Densitometry on the PML isoform I/II band was preformed and normalized to the lamin A/C loading control. Experiment was performed three times and one representative experiment is shown.

2.4.2 The RRV vIRF R12 co-localizes with PML-NBs.

Previous unpublished work from our laboratory showed that during transient transfection assays, R12 localized to the nucleus in punctate structures (data not shown) while the other seven vIRFs were localized diffusely throughout the nucleus and/or cytoplasm. Thus, we first investigated R12 as a potential vIRF involved in the disruption of PML-NBs. A doxycycline (dox)-inducible telo-RhF cell line that expressed R12 tagged with a C-terminal FLAG-epitope was constructed to assess R12 involvement in PML-NB disruption. After 18 hours of dox treatment, IFA and z-stack analysis revealed that R12-FLAG protein is expressed, co-localizes with PML-NBs, and appears to be encased in PML protein (Fig. 2.2A). The optimal dox concentration was determined to be 2ug/ml and was used for all experiments with the R12-inducible cell line, and R12 protein was expressed as early as 4 hours post treatment (Fig. 2.2 B and C). While R12 localizes to PML-NBs, the expression of R12 alone (in the presence or absence of RhIFNα2) does not lead to the disappearance of PML

immunoreactivity, even up to 72 hours post dox treatment (Fig. 2.2D). However, there was a statistically significant change in the number of PML-NBs, as untreated cells had an average of 16 PML-NBs per nuclei while the cells expressing R12-FLAG averaged 7.5 PML-NBs (Fig. 2.2E). A reduction in the number of PML-NBs following R12 expression was also observed in the presence of RhIFNα2 treatment with an average of 19.5 PML-NBs in RhIFNα2 treated cells and 13 PML-NBs in R12-FLAG and RhIFNα2 treated cells ($p<0.0001$). While the number of PML-NBs per nucleus declined in the presence of R12 protein expression, the size of the PML-NBs appeared to increase, suggesting that R12 may promote the re-organization of these sub-nuclear structures (Fig. 2.2D). Western blot analysis of R12-FLAG-inducible cells showed that un-SUMOylated PML isoforms I and II decreased when R12 was expressed (Fig. 2.2F, first 4 lanes from the left). RhIFNα2 treatment further revealed that while R12 expression decreased the un-SUMOylatd PML I/II isoforms, there was also an increase in SUMOylated versions of PML (Fig. 2.2F, last 6 lanes). The anti-FLAG western blot revealed that in addition to the presence of a protein of the predicted size of R12 (37kDa), additional larger protein species were also observed at around 45kDa and 75kDa (Fig. 2.2F). While this may be a non-specific cross-reaction with the FLAG antibody, it is also conceivable that R12 protein is post-translationally modified. Analysis of the R12 protein sequence reveals possible SUMOylation, farnesylation, and palmitoylation sites.

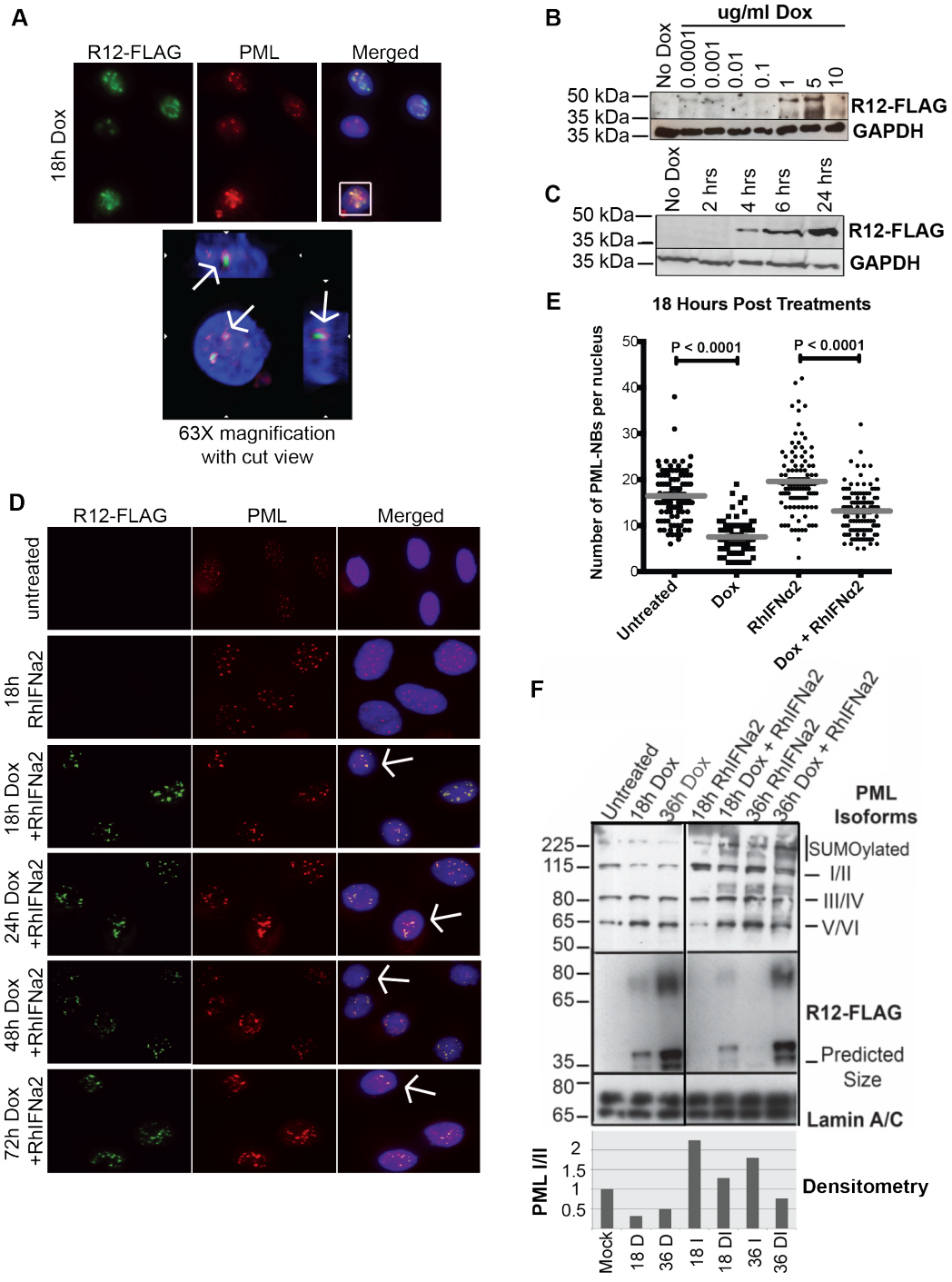


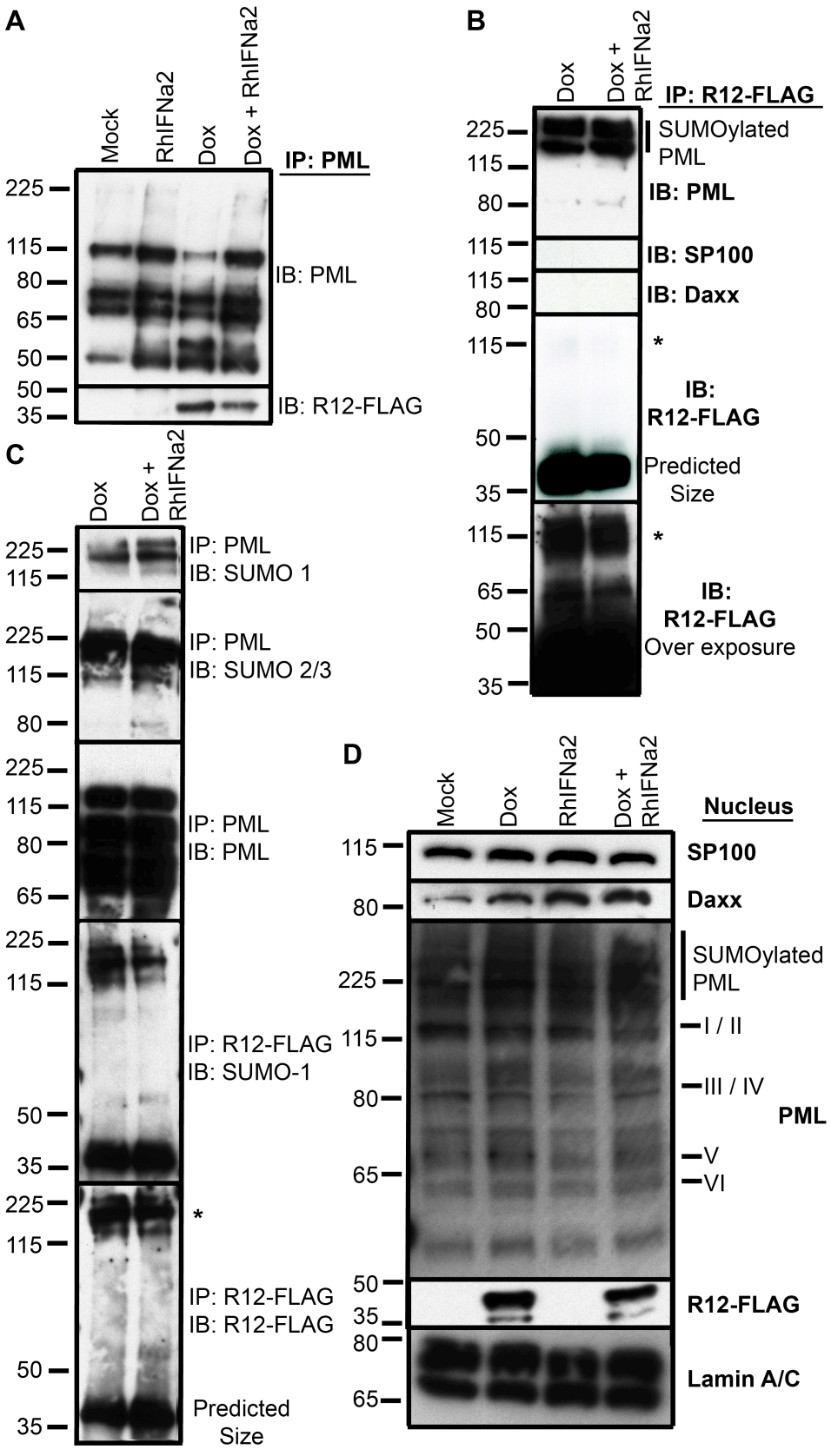
Figure 2.2 Exogenously expressed vIRF R12 co-localizes with PML-NBs. A) R12-FLAG inducible telo-RhF cells were treated with 2ug/ml dox for 18 hours and then fixed with methanol and stained with FLAG (green) and PML (red) specific antibodies. Images

at 40X magnification are presented along with a 63X magnification and cut view of the cell highlighted by the white box. White arrows indicate R12 protein surrounded by PML protein. B) Indicated concentrations of dox were added to telo-RhF cells for 18 hours before protein lysates were harvested and analyzed by SDS-PAGE and probed with anti-FLAG and anti-GAPDH specific antibodies. C) Telo-RhF cells were treated with 2ug/ml dox for the indicated hours before protein lysates were analyzed by SDS-PAGE and probed with anti-FLAG and anti-GAPDH specific antibodies. D) Telo-RhFs that were dox inducible for R12-FLAG expression were left untreated, treated with 100U/ml RhIFNα2 for 18 hours, or treated with 2ug/ml dox and 100U/ml RhIFNα2 for the indicated hours before fixation with methanol. Fixed cells were stained with FLAG (green) and PML (red) specific antibodies while nuclei were stained with DAPI (blue). White arrows indicate examples of cells with large PML-NB structures. Images at 40X magnification are presented. (A and D) Experiments performed three times and representative images are shown. E) Number of PML-NBs within nuclei of untreated cells or cells treated with 2ug/ml dox, 100U/ml RhIFNα2, or both dox and RhIFNα2 for 18 hours is presented in the graph. Data were analyzed by unpaired student t test with p values less than 0.05 considered significant. Experiments were performed twice and representative experiment is shown. F) R12-FLAG inducible telo-RhF cells was left untreated, treated with 2ug/ml dox, 100U/ml RhIFNα2, or dox and RhIFNα2 for the indicated times, or pretreated with dox for 18 hours before 10 hours of RhIFNα2 treatment. Nuclear protein lysates were analyzed by SDS-PAGE and probed with PML, FLAG, and lamin A/C specific antibodies. Densitometry on the PML isoform I/II band was performed and normalized to the lamin A/C loading control (D=dox and I=RhIFNα2). Experiments were performed three times and representative experiments are presented.

2.4.3 R12 co-immunoprecipitates with PML protein.

To determine whether the localization of R12 with PML-NBs was due to an interaction between PML protein and R12 protein, we performed co-immunoprecipitation (co-IP) assays. Using the dox-inducible R12 cell line, cells were treated with or without dox to induce R12 expression and with or without RhIFNα2 to up-regulate PML protein expression. Following separation of cytoplasmic and nuclear lysates, PML protein was immunoprecipitated from the nuclear lysates, resolved by SDS-PAGE, and then analyzed by Western blot analysis for R12 protein. By this analysis, we observed FLAG-tagged R12 protein co-immunoprecipitated with PML protein only after dox induction, plus or minus RhIFNα2 (Fig. 2.3A). The immunoprecipitation of FLAG-tagged R12 also resulted in the detection of high molecular weight PML protein (possibly SUMOylated PML) by western blot analysis (Fig. 2.3B). Importantly, the interaction of R12 with PML protein appears to be specific, as R12-FLAG does not co-purify with SP100 or Daxx (Fig. 2.3B). To further investigate the high molecular weight PML bands that co-purify with R12-FLAG protein, we harvested R12-FLAG cells treated with dox and immediately boiled the cell lysates. We immunoprecipitated PML proteins and performed Western blot analysis with SUMO-1 and SUMO-2/3 specific antibodies. High molecular weight protein bands (the same size as was observed in figure 2.3B) were detectable by anti-SUMO-1 and anti-SUMO-2/3 antibodies (Fig. 2.3C). This is consistent with previous publications that have shown that SUMOylation of PML is necessary for PML to interact with other proteins and to form PML-NBs (219). Additional studies have also found that a

SUMO-interacting motif (SIM) contained within the PML protein may be necessary for the interaction of PML with other SUMOylated proteins (545). While prediction software identified four possible SIMs in the R12 protein sequence, our analysis of immunoprecipitated R12-FLAG protein found that R12 protein was SUMO-1 modified (data not shown and Fig. 2.3C). Western blot analysis of total nuclear lysates, to control for total protein input levels, revealed that SP100 protein levels were not altered by the expression of R12-FLAG while Daxx protein levels may be slightly increased following R12-FLAG expression and RhIFNα2 treatment (Fig. 2.3D).


A
Mock
RhIFNa2
Dox
Dox + RhIFNa2
IP: PML
225
115
80
65
50
50
35
IB: PML
IB: R12-FLAG
B
Dox
Dox + RhIFNa2
IP: R12-FLAG
225
115
80
115
115
80
115
50
35
SUMOylated PML
IB: PML
IB: SP100
IB: Daxx
*
IB: R12-FLAG
Predicted Size
115
65
50
35
*
IB: R12-FLAG
Over exposure
C
Dox
Dox + RhIFNa2
225
115
IP: PML
IB: SUMO 1
225
115
80
IP: PML
IB: SUMO 2/3
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115
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IB: PML
225
115
50
35
IP: R12-FLAG
IB: SUMO-1
225
115
50
35
*
IP: R12-FLAG
IB: R12-FLAG
Predicted Size
D
Mock
Dox
RhIFNa2
Dox + RhIFNa2
Nucleus
115
80
225
115
80
65
50
35
80
65
SP100
Daxx
SUMOylated PML
I / II
III / IV
PML
V
VI
R12-FLAG
Lamin A/C

Figure 2.3. Ectopically expressed R12 protein co-immunoprecipitates with PML protein.

A) R12-FLAG inducible telo-RhF cells was untreated or treated for 18 hours with 100U/ml RhIFNα2, 2ug/ml dox, or both RhIFNα2 and dox. Nuclear protein lysates were immunoprecipitated (IP) with a PML specific antibody before resolving proteins on SDS-PAGE. Western blots were probed with PML and FLAG specific antibodies. B) R12-FLAG inducible cell line was treated for 18 hours with 2ug/ml dox or for 18 hours with 100U/ml RhIFNα2 and 2ug/ml dox. Nuclear protein lysates were IP with a FLAG specific antibody before resolving the proteins on SDS-PAGE. Western blots were probed with PML, SP100, Daxx, and FLAG specific antibodies. (*) denotes high molecular weight R12-FLAG protein. C) R12-FLAG inducible cell line was treated for 18 hours with 2ug/ml dox or for 18 hours with 100U/ml RhIFNα2 and 2ug/ml dox. Cells were lysed in RIPA buffer with 1% SDS and boiled for 5 minutes before IP with PML or FLAG specific antibodies. IP lysates were resolved on SDS-PAGE and Western blots were probed with PML, SUMO-1, SUMO-2/3, and FLAG specific antibodies. D) R12-FLAG inducible cell line was treated for 18 hours with 100U/ml RhIFNα2, 2ug/ml dox, RhIFNα2 and dox, or left untreated. Protein input control from total nuclear lysates were also subjected to SDS-PAGE and Western blots were probed with SP100, Daxx, PML, FLAG, and Lamin A/C specific antibodies. Experiments were performed at least twice and representative Western blots are shown.

2.4.4 Ectopic R12 protein expression during $RRV_{vIRF\text{-}KO}$ infection results in the loss of PML-NBs.

Because $RRV_{vIRF\text{-}KO}$ infection did not result in the loss of PML-NBs and R12 appears to interact with PML-NBs, we next asked whether exogenous expression

of R12 could rescue the WT RRV phenotype during infection with $RRV_{vIRF\text{-}KO}$. To test this, the dox-inducible R12 cell line was infected with $RRV_{vIRF\text{-}KO}$ in the presence or absence of dox (Fig. 2.4). We found that only when R12-FLAG expression was induced by dox treatment did the $RRV_{vIRF\text{-}KO}$ infection result in a loss of PML-NBs, similar to what is observed during WT RRV infection, demonstrating R12 is sufficient for this phenotype.

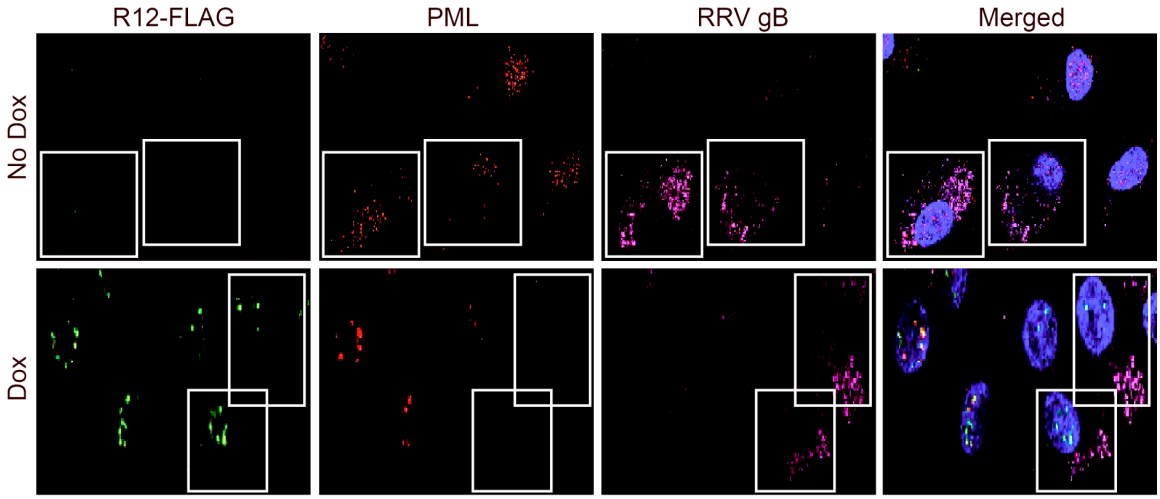


Figure 2.4. Exogenous expression of R12 protein during $RRV_{vIRF\text{-}KO}$ infection results in a loss of PML-NB structures. R12-FLAG inducible cell line was treated with dox for 18 hours, or left untreated, before infection with $RRV_{vIRF\text{-}KO}$ at an MOI of 2 for 24 hours (dox was kept on through out the infection). Cells were fixed with methanol and stained with antibodies specific for PML (red), FLAG (green), RRV-gB (purple) and nuclei stained with DAPI (blue). Boxes indicate the infected cells. 63x magnification images were obtained by confocal microscopy. The experiment was performed four times and representative images are shown.

2.4.5 Construction of recombinant R12 mutant RRV.

In order to examine the contribution of R12 towards the disruption of PML-

NBs within the context of RRV infection, we constructed two recombinant viruses using the RRV_{17577} BAC (RRV BAC) system. An R12 mutant RRV (RRV_{R12ns}) converting the start codon into a stop codon in the R12 ORF and encoding a FLAG epitope tag at the C-terminus of the R12 ORF was constructed. Additionally, an R12 rescue mutant RRV ($RRV_{R12FLAG}$) encoding FLAG tagged R12 but lacking any other mutations was constructed so that endogenous R12 could be detected following infection (Fig. 2.5A). Following the construction of the mutant RRV-BAC DNA clones using recombination to insert the mutated R12 sequences, the clones were screened by restriction digestion and Southern blot analysis (Fig. 2.5B-C). Clones were also screened by PCR and sequencing to confirm the correct insertion into the RRV BAC, and the resulting clones were used to generate infectious recombinant virus by transfection into RhF cells. The BAC cassette was removed from the recombinant viruses using CRE-recombinase and the loxP sites flanking the BAC cassette. After growth and purification of recombinant viruses, Western blot analysis was performed on nuclear lysates from telo-RhFs infected with both recombinant viruses, which indicated that $RRV_{R12FLAG}$ expresses an R12 FLAG-tagged protein, and that the RRV_{R12ns} virus lacks this R12 protein expression (Fig. 2.5E). Furthermore, RT-PCR analysis of RNA from cells infected with $RRV_{R12FLAG}$ or RRV_{R12ns} indicates that the ORFs immediately upstream and downstream of R12 (R11 and R13) are expressed, and that the altered R12 sequence in both viruses does not affect transcription of neighboring genes (Fig. 2.5D). Lastly, one-step and multi-step growth curve analysis of WT RRV, $RRV_{vIRF\text{-}KO}$, RRV_{R12ns}, and $RRV_{R12FLAG}$

revealed that all four viruses display the same growth kinetics *in vitro*, indicating the mutations do not affect growth of virus *in vitro* (Fig. 2.5F).

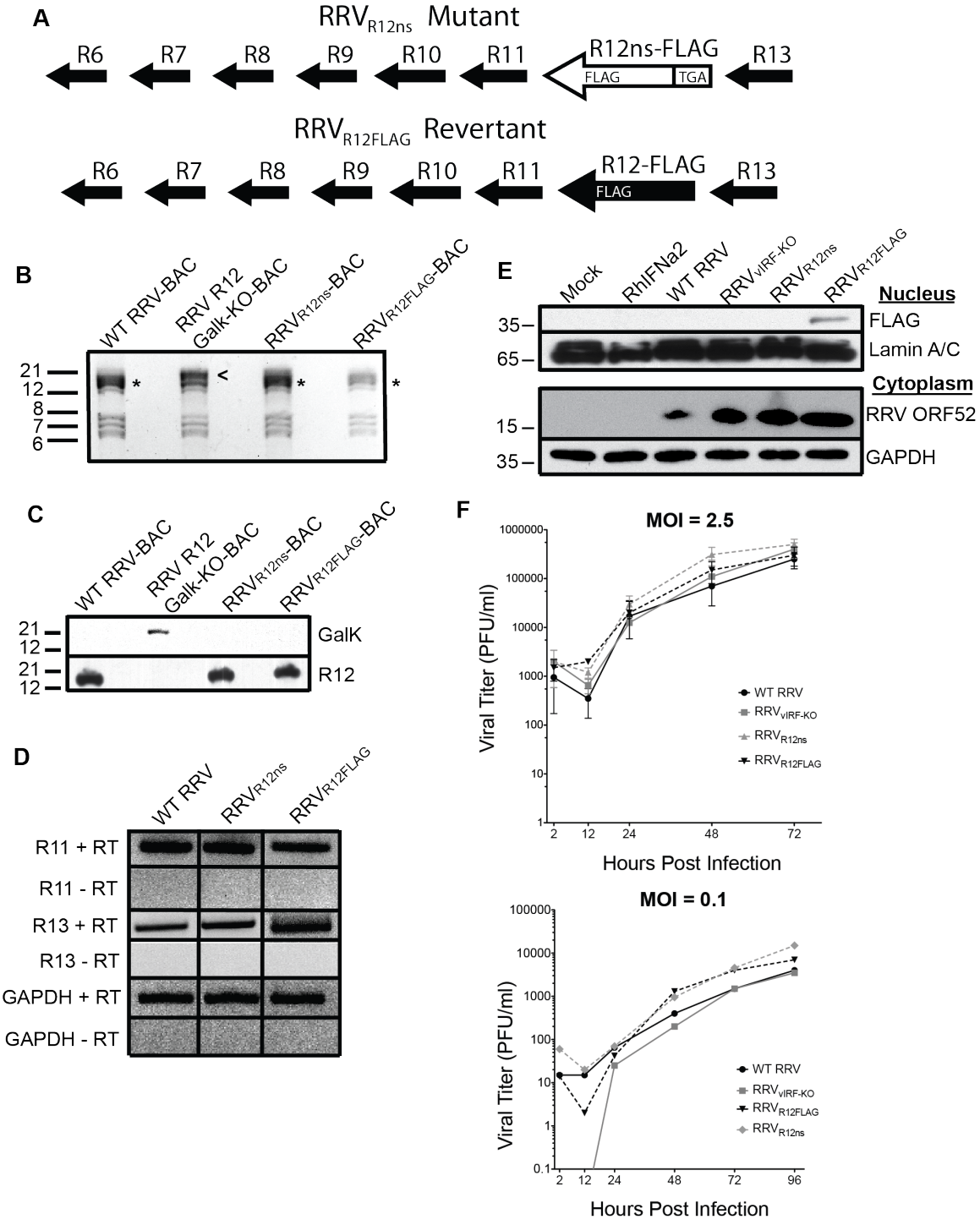


Figure 2.5. $RRV_{R12FLAG}$ and RRV_{R12ns} virus construction and characterization. A)

Schematic of the mutations introduced into the RRV genome to generate RRV_{R12ns} and $RRV_{R12FLAG}$ viruses. B) BamHI restriction digests of WT RRV-BAC, RRV-R12-GalK-KO BAC, RRV_{R12ns}-BAC, and $RRV_{R12FLAG}$-BAC DNA clones. (*) denotes location of the digest band containing R12 sequence and (<) denotes the increased digest band size that contains the GalK cassette. C) The BamHI digested BAC DNA clones from part (A) were subjected to Southern blot analysis and probed with GalK and R12 specific probes. D) RNA from telo-RhF cells infected with WT RRV, RRV_{R12ns}, or $RRV_{R12FLAG}$ at an MOI of 2.5 for 24 hours was purified. RT-PCR was performed with or without reverse transcriptase enzyme using R11, R13, and GAPDH specific primers and PCR products were subjected to agarose gel electrophoresis. E) Telo-RhFs were left untreated, treated with 100U/ml RhIFNα2 for 18 hours, or infected with WT RRV, $RRV_{vIRF\text{-}KO}$, RRV_{R12ns}, $RRV_{R12FLAG}$ at an MOI of 2.5 for 24 hours. Nuclear and cytoplasmic protein lysates were separated, analyzed by SDS-PAGE, and probed with FLAG, lamin A/C, RRV-ORF52, and GAPDH specific antibodies. F) Primary RhF cells were infected with WT RRV, $RRV_{vIRF\text{-}KO}$, RRV_{R12ns}, or $RRV_{R12FLAG}$ at an MOI of 2.5 (one-step growth curve) or 0.1 (multi-step growth curve) for the indicated times. Viral titers at each time point were determined by plaque assay and presented as plaque forming unit (PFU) per milliliter. Experiments were performed twice and representative experiments are shown except for panel (F) where the average titer from both experiments is graphed.

2.4.6 Endogenous R12 localizes to PML-NBs and is necessary for PML-NB disruption during viral infection.

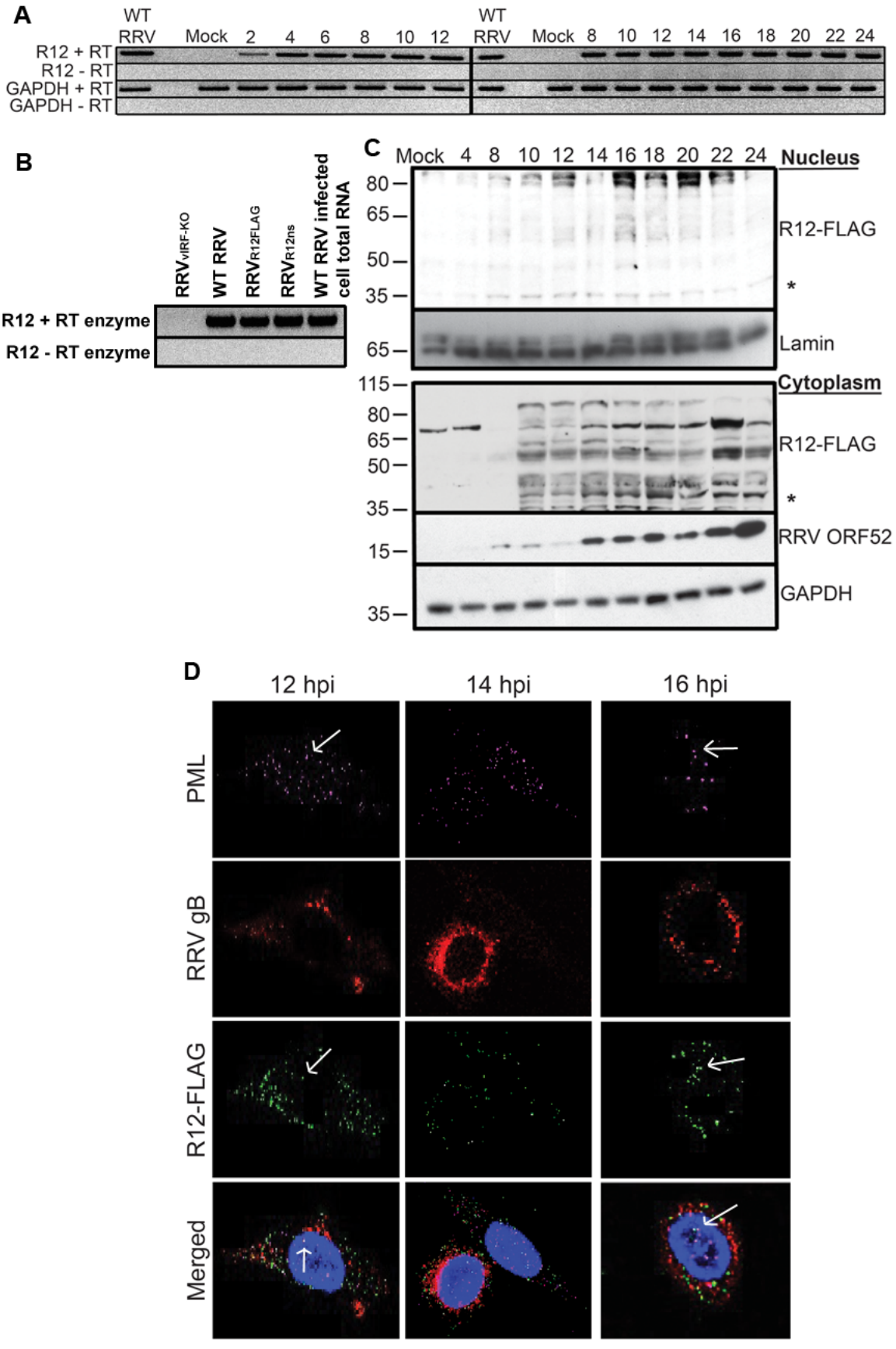
To characterize the kinetics of endogenous R12 expression during RRV infection we infected telo-RhFs with $RRV_{R12FLAG}$ virus at an MOI of 2.5 and harvested RNA and protein from infected cells every two hours during the first 24

hours of infection. RT-PCR revealed R12 transcripts at every time point tested, even as early as 2 hpi (Fig. 2.6A). To determine if R12 transcripts could be packaged within RRV virions, RNA was purified from WT RRV, $RRV_{R12FLAG}$, RRV_{R12ns}, or $RRV_{vIRF\text{-}KO}$ virions, that were initially treated with micrococcal nuclease to eliminate contaminating infected cell RNA that co-purifies with virus. Purified virion-associated RNA was isolated and subjected to RT-PCR for R12 transcripts. This analysis revealed that RRV RNA specific for R12 is packaged within R12-encoding RRV virions, and that the $RRV_{vIRF\text{-}KO}$ negative control did not contain R12 transcripts (Fig. 2.6B). Analysis of R12 protein expression by Western blot revealed that endogenous R12 protein ranges in size from the predicted 37 kDa to above 80 kDa, and displays a laddering effect indicative of post-translational modifications (such as SUMOylation). R12 protein expression within the nucleus was first detected at 8hpi and peaked 16-20 hpi, while R12 protein expression within the cytoplasm was first detected 10 hpi (Fig. 2.6C). It should be noted that ectopic R12 expression within the dox-inducible R12 cell line was strictly nuclear. Interestingly, endogenous R12 protein (produced during RRV infection) levels within the nucleus appeared to oscillate while R12 protein levels within the cytoplasm remained constant (Fig. 2.6C). Using IFA we found endogenous R12 located in both the cytoplasm and the nucleus, and observed that R12 could co-localize with PML-NBs (Fig. 2.6D).

To determine whether endogenous R12 protein produced during RRV infection interacts with PML protein, co-IP assays were performed in $RRV_{R12FLAG}$ infected cells. We found that when PML was immunoprecipitated using a PML-

specific antibody, R12 protein could be detected in the immunoprecipitation lysates (Fig. 2.6E). The R12 protein that co-purifies with PML displays a laddering pattern of different molecular weights. Again, a high molecular weight R12 protein (150-200kDa) was undetectable at 14 hpi in the total nuclear lysates (input controls), but was observed again at 16 hpi (Fig. 2.6E). This may suggest that certain forms of R12 protein can shuttle between the nucleus and cytoplasm.

To investigate whether endogenous R12 is necessary for PML-NB disruption by RRV, we infected telo-RhFs with the RRV_{R12ns} virus for 24 hours followed by IFA. We observed that the RRV_{R12ns} virus was unable to disrupt the PML-NB protein punctate structures similar to the $RRV_{vIRF\text{-}KO}$ virus (Fig. 2.6F). RRV_{R12ns} infection did result in a loss of SP100 and Daxx punctate structures with in the nucleus of infected cells (Fig. 2.6F). This result supports our observations in figure 2.1 and suggests that a viral gene product other than a vIRF is responsible for the loss of SP100 and Daxx localization to PML-NB structures.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WT RRV
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2
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6
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WT RRV
Mock
8
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16
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22
24
R12 + RT
R12 - RT
GAPDH + RT
GAPDH - RT
B
RRVvIRF-KO
WT RRV
RRVR12FLAG
RRVR12ns
WT RRV infected cell total RNA
R12 + RT enzyme
R12 - RT enzyme
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Cytoplasm
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GAPDH
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12 hpi
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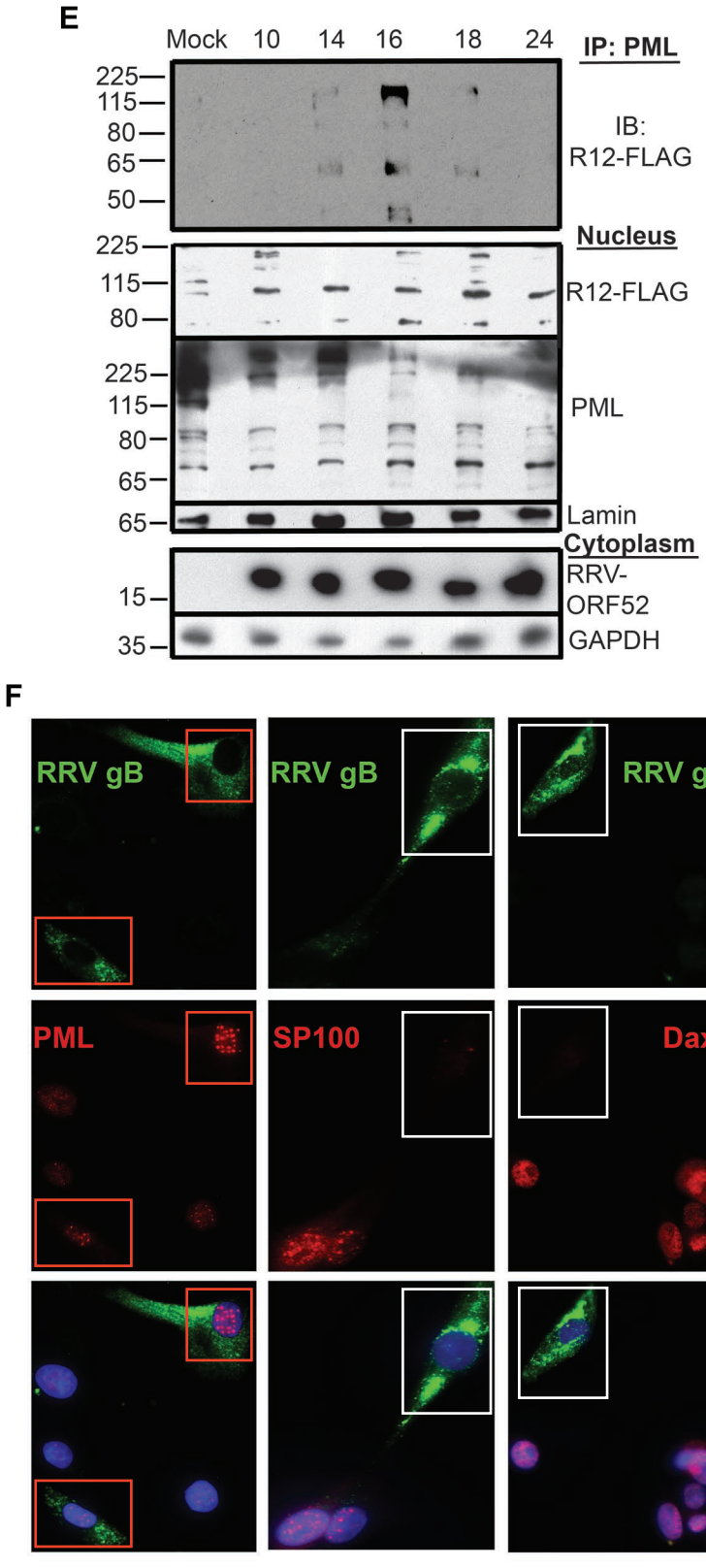
16 hpi
PML
RRV gB
R12-FLAG
Merged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RRV-
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GAPDH
F
RRV gB
RRV gB
RRV gB
PML
SP100
Daxx

Figure 2.6. Endogenous R12 can colocalize with PML-NBs, co-immunoprecipitates with PML protein, and is required for RRV disruption of PML-NBs. A) RNA was purified from telo-RhFs mock infected, infected with WT RRV for 24 hours, or $RRV_{R12FLAG}$ for the indicated hours at an MOI of 2.5. RT-PCR was performed with or without reverse transcriptase enzyme using R12 and GAPDH specific primers and PCR products were subjected to agarose gel electrophoresis. B) RNA was purified from viral stocks of $RRV_{vIRF\text{-}KO}$, WT RRV, $RRV_{R12FLAG}$, and RRV_{R12ns}. As a positive control, a sample of $RRV_{vIRF\text{-}KO}$ virions treated with micrococcal nuclease had purified total cellular RNA from a WT RRV infected cell culture spiked into the sample after inactivation of the nuclease (by EGTA) but before virion lysis and RNA purification of virion packaged RNA. 40ng of RNA from each sample was reverse transcribed and PCR amplified using R12 specific primers in the presence or absence of reverse transcriptase. Resulting amplified DNA was resolved on a 1% agarose gel. C) Telo-RhF cells were mock infected or infected with $RRV_{R12FLAG}$ at an MOI of 2.5 for the indicated hours. Nuclear and cytoplasmic protein lysates were separated, resolved by SDS-PAGE, and probed with FLAG, lamin A/C, RRV-ORF52, and GAPDH specific antibodies. Asterisk (*) indicates the predicted size of R12 protein. D) Telo-RhF cells were infected with $RRV_{R12FLAG}$ at an MOI of 2.5 for 12, 14, or 16 hours. Cells fixed with methanol and stained with FLAG (green), RRV-gB (red), and PML (purple) specific antibodies while nuclei were stained with DAPI (blue). White arrows indicate R12 co-localization with PML-NBs. 63x magnification images were obtained by confocal microscopy E) Telo-RhF cells were mock infected or infected with $RRV_{R12FLAG}$ at an MOI of 2.5 for 10, 14, 16, 18, and 24 hours. Protein lysates were immunoprecipitated with a PML specific antibody, resolved by SDS-PAGE and analyzed by Western blot using FLAG specific antibodies. Input controls were analyzed by SDS-PAGE and Western blot using total nuclear and total cytoplasmic lysates and staining with FLAG, PML, lamin A/C, RRV-ORF52, and GAPDH specific antibodies. F) Telo-RhF

cells were infected with RRV_{R12ns} at an MOI of 2.5 for 24 hours before fixation with methanol. Fixed cells were stained with RRV-gB (green) and either PML (red), SP100 (red), or Daxx (red) specific antibodies while nuclei were stained with DAPI (blue). Red boxes indicate infected cells with PML-NB staining pattern in nuclei, white boxes indicate infected cells that lack SP100 or Daxx staining. Experiments in part (A) were performed twice, and experiments in parts (B-E) were performed at least three times. Representative experiments and images are shown.

2.4.7 Disruption of PML-NBs during RRV infection inhibits ISG induction and aids viral infection.

As our data suggests that PML isoform II is decreased following WT RRV infection, we wanted to define the downstream effects on the cell. Previous reports state that PML isoform II is necessary for efficient ISG transcription, in particular targets of the ISGF3 transcription complex (246). As such, we analyzed ISG induction following infection with WT RRV, $RRV_{vIRF\text{-}KO}$, $RRV_{R12FLAG}$, and RRV_{R12ns}. First we infected cells with WT RRV-GFP or $RRV_{vIRF\text{-}KO}$-GFP at an MOI of 5 for 24 hours and then sorted for the GFP positive-infected cells. Total RNA was purified from the sorted cells and transcripts of four ISGs known to be targeted by ISGF3 and reliant on PML isoform II (IRF-7, Mx1, IP-10, and ISG54) were measured by qPCR (Fig. 2.7A). We found that $RRV_{vIRF\text{-}KO}$ infection failed to suppress ISG transcription for all four compared to WT RRV infection. Induction of Mx1 transcripts was two-fold while IP-10 was over 100 times greater in $RRV_{vIRF\text{-}KO}$ infected cells than in WT RRV infected cells (Fig. 2.7A).

Inhibition of ISG transcription can occur by blocking IFN production and/or

blocking the signaling cascade induced when IFN binds the IFN receptor. To test if the block in ISG induction was downstream of IFN binding to the IFN receptor, we infected telo-RhFs with WT RRV, $RRV_{R12FLAG}$, $RRV_{vIRF\text{-}KO}$, or RRV_{R12ns} for 18 hours at an MOI of 5. At 18 hpi, 100U/ml of RhIFNα2 was added to the culture media and the infection was continued for an additional 8 hours before harvesting total RNA to determine the induction of a prototypical ISG, IP-10 (Fig. 2.7B). WT RRV and $RRV_{R12FLAG}$ viruses were able to suppress the induction of IP-10 following the addition of type I IFN to the infected cells with only 5- and 6-fold induction (respectively) over mock-infected cells (Fig. 2.7B light grey and dark grey bars). However, both $RRV_{vIRF\text{-}KO}$ and RRV_{R12ns} infections were unable to suppress the induction of IP-10 following IFN treatment with 41- and 39-fold induction (respectively) over mock-infected cells (Fig. 2.7B vertical and horizontal striped bars). This revealed that the block in ISG induction that we observed for WT RRV but not for $RRV_{vIRF\text{-}KO}$ infection can occur after IFNα signals through the IFNα/β receptor. Moreover, this block is downstream of IFN signaling and was dependent on R12 expression as the RRV_{R12ns} infection displayed similar results to the $RRV_{vIRF\text{-}KO}$ infection (Fig. 2.7B).

Because R12 was able to inhibit the IFN signaling cascade to result in reduced ISG transcription in the context of RRV infection, we next wanted to determine if R12 could function similarly in the absence of viral infection. To test this, we utilized our dox-inducible R12 cell line. Cells were treated with dox for 12 hours, RhIFNα2 for 6 hours, or treated with dox for 6 hours followed by 6 hours of dox and RhIFNα2, before purifying total cellular RNA. Transcript levels of IP-

10 were again analyzed, using quantitative RT-PCR. Following RhIFNα2 treatment, R12 expression alone was able to inhibit ISG induction as compared to the positive control (RhIFNα2 treatment without dox treatment) (Fig. 2.7C). This experiment was repeated in the telo-RhF parental cell line and confirmed that dox treatment (without R12 expression) does not affect ISG transcript levels (Fig. 2.7D). Thus, our results from the R12 cell line were due to R12 expression. These results suggest that R12 protein is able to antagonize the type I IFN signaling pathway (downstream of IFN receptor binding) without the aid of any other RRV viral factors to inhibit ISG transcription.

Lastly, we wanted to know whether the expression of R12 would aid viral replication in the presence of IFN. Thus, we performed one-step growth curve analysis in the presence RhIFNα2 with WT RRV, $RRV_{vIRF\text{-}KO}$, RRV_{R12ns}, and $RRV_{R12FLAG}$ (Fig. 2.7E). Both $RRV_{vIRF\text{-}KO}$ and RRV_{R12ns} growth curves had significantly lower viral titers (1/2 to 1 log less) at 12, 24, and 48 hpi compared to the WT RRV and $RRV_{R12FLAG}$ growth curves.

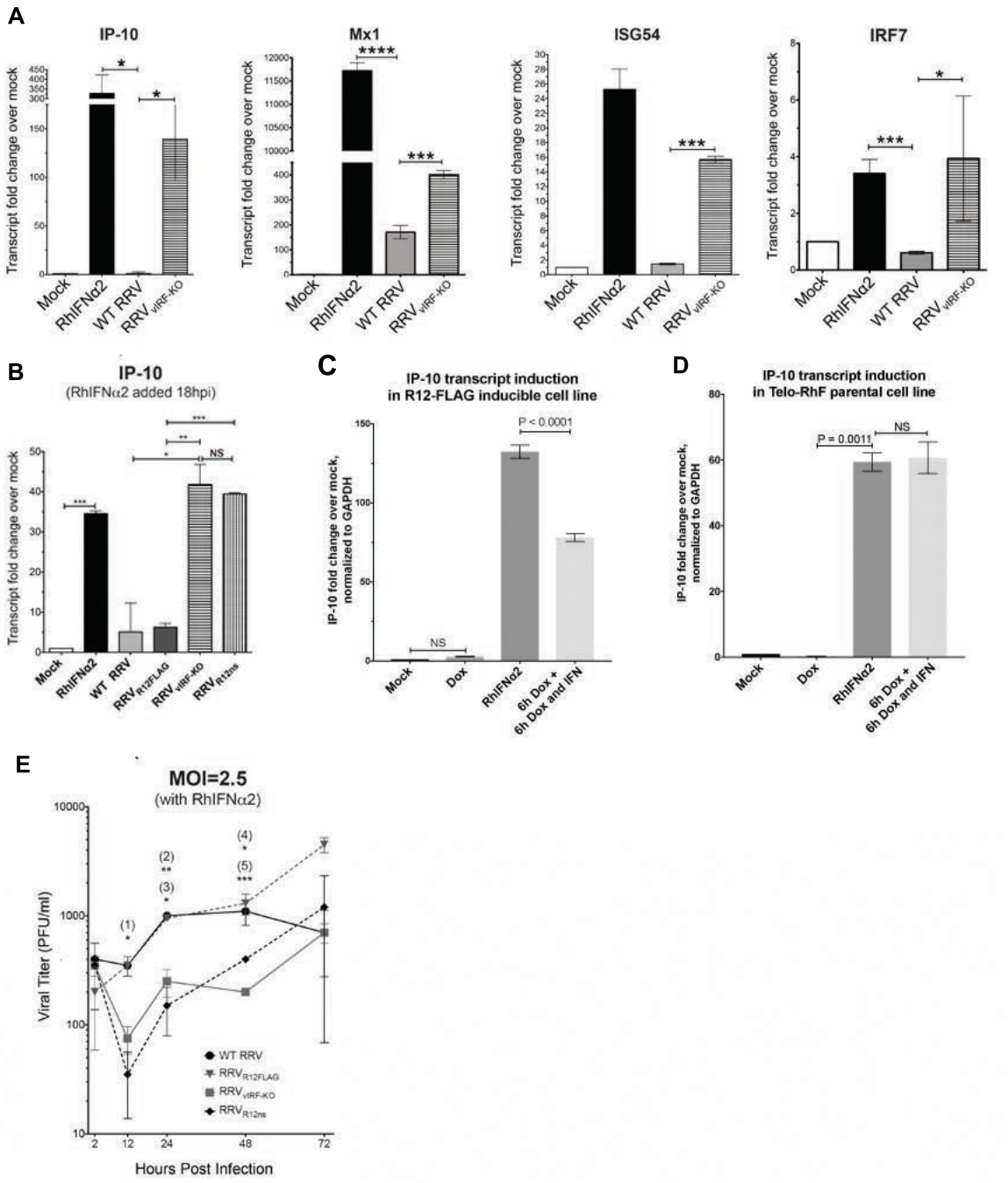

Figure 2.7. R12 expression inhibits ISG induction downstream of IFN signaling and aids viral replication in the presence of type I IFN. A) Telo-RhF cells were

infected with WT RRV-GFP or $RRV_{vIRF\text{-}KO}$-GFP at an MOI of 5 for 24 hours, treated with 100U/ml RhIFNα2 for 18 hours, or left untreated. Infected cells were sorted for GFP signal by flow cytometry. RNA was purified and cDNA was synthesized before the indicated transcripts were measured by qPCR. Data were presented as fold change over mock-infected samples normalized to GAPDH transcript levels and were analyzed by unpaired student t test. P values less than 0.05 were considered significant and asterisks denote the following significant p values: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$). B) Telo-RhF cells were infected with WT RRV, $RRV_{vIRF\text{-}KO}$, $RRV_{R12FLAG}$, or RRV_{R12ns} at an MOI of 5 for 18 hours before 100U/ml RhIFNα2 was added to the infected cell culture media for an additional 8 hours. As a negative control, cells were left untreated, and as a positive control cells were treated with 100U/ml RhIFNα2 for 8 hours. RNA was purified and cDNA was synthesized before IP-10 transcripts were measured by qPCR (cells were not sorted prior to RNA purification). IP-10 transcript levels were normalized to GAPDH transcript levels and presented as fold change over mock. Data were analyzed by unpaired student t test. P values less than 0.05 were considered significant and asterisks denote the following significant p values: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$). C) Telo-RhF-R12FLAG inducible cell line was treated with 2ug/ml dox for 12 hours, treated with 100 units/ml RhIFNα2 for 6 hours, or treated with 2ug/ml dox for 6 hours followed by 6 hours of dox and RhIFNα2 treatment. Total RNA was harvested, purified, and converted to cDNA. Quantitative real-time PCR was performed for IP-10 transcripts and normalized to GAPDH transcript levels. Assay was performed in triplicate and a representative experiment is shown. Data is presented as fold change over mock treated cells. Data were analyzed by unpaired student t-test. P values of less than 0.05 were considered significant. D) Telo-RhF (parental) cells were treated and analyzed as in part (C). Data is

presented as fold change over mock treated cells. Data were analyzed by unpaired student t-test. P values of less than 0.05 were considered significant. E) Primary RhFs were infected with WT RRV, $RRV_{vIRF\text{-}KO}$, $RRV_{R12FLAG}$, or RRV_{R12ns} at an MOI of 2.5 in the presence of 100U/ml RhIFNα2. Viral titers were measured at the indicated times post infection by plaque assay and presented as plaque forming units (PFU) per milliliter (mL). Data were analyzed by unpaired student t test. P values less than 0.05 were considered significant and asterisks denote the following significant p values: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$). (1) WT RRV vs $RRV_{vIRF\text{-}KO}$, WT RRV vs RRV_{R12ns}, RRV_{R12ns} vs $RRV_{R12FLAG}$, and $RRV_{R12FLAG}$ vs $RRV_{vIRF\text{-}KO}$. (2) WT RRV vs $RRV_{vIRF\text{-}KO}$, WT RRV vs RRV_{R12ns}, RRV_{R12ns} vs $RRV_{R12FLAG}$. (3) $RRV_{R12FLAG}$ vs $RRV_{vIRF\text{-}KO}$. (4) WT RRV vs $RRV_{vIRF\text{-}KO,}$ RRV_{R12ns} vs $RRV_{R12FLAG,}$ $RRV_{R12FLAG}$ vs $RRV_{vIRF\text{-}KO}$. (5) RRV_{R12ns} vs $RRV_{vIRF\text{-}KO}$. Experiments were performed twice and representative experiments for parts (A-B) are shown, the average of both experiments for part (C) is graphed.

2.5 Discussion

Viruses that are able to establish a lifelong infection within a host must first be able to circumvent the intrinsic and innate immune defenses. Important for the efficient and robust induction of the innate immune response upon viral infection are the constitutively expressed PML-NBs. It was previously reported that the recombinant mutant $RRV_{vIRF\text{-}KO}$ virus displayed reduced viral titers in whole blood and an increased type I IFN response in serum of infected RMs compared to WT RRV infected RMs (464). However, *in vitro* growth curve analysis of WT RRV and $RRV_{vIRF\text{-}KO}$ viruses showed similar growth kinetics for the two viruses (140). We decided to investigate the role that type I IFN and PML-NBs played in restricting RRV growth and how the vIRFs, specifically the vIRF R12,

counteracted this host defense. We found that R12 is both necessary and sufficient for the RRV-induced disruption of PML-NBs during *de novo* lytic infection. This is important because in the natural host-infection setting, RRV will not only encounter the viral restriction capacity of PML-NBs, but also type I IFN signaling from the type I IFN produced from neighboring uninfected cells. The capacity of RRV to disrupt PML-NBs could have a two-fold effect: (1) overcomes the viral genetic silencing exerted by PML-NB resident proteins and (2) inhibits the type I IFN signaling cascade to prevent ISGs (with their own anti-viral effects) from getting expressed. This second effect, inhibiting type I IFN signaling, would explain why WT RRV could grow to significantly higher titers in the presence of RhIFNα2 compared to $RRV_{vIRF\text{-}KO}$ and RRV_{R12ns} viruses.

While we found that R12 is necessary and sufficient for the disruption of PML-NBs during RRV infection, R12 expression outside the context of RRV infection did not result in the complete loss of PML-NBs. Ectopic R12 protein expression did appear to affect PML-NB organization as there were fewer but larger PML-NBs. Furthermore, ectopic R12 expression resulted in reduction of PML isoform I and II protein levels. R12 may specifically target PML isoforms I/II, preventing the antiviral functions of and nuclear body formation by these two isoforms. A previous publication on PML-NB disruption by RRV implicated the RRV tegument protein encoded by ORF75 and found that while RRV infection of RhF cells resulted in a loss of SP100 protein by 8 hpi, PML protein was not lost until 24 hpi and this could be rescued with cyclohexamide treatment (457). Thus, the inhibition of R12 expression by cyclohexamide treatment could explain why

PML protein levels were rescued. We did observe a loss of SP100 punctate staining pattern with all the viruses we tested. All of our WT and mutant RRVs encode ORF75 and this would explain our SP100 results. The previous publication on RRV ORF75 and PML-NBs did not find ORF75 to affect Daxx protein levels by western blot analysis, however, the authors did not perform IFA to investigate Daxx protein localization. Thus, it remains unknown why Daxx disperses from punctate structures during RRV infection, but the vIRFs do not appear to be involved. The involvement of both ORF75 and a vIRF in the disruption of PML-NBs by RRV is similar to what is observed for KSHV disruption of PML-NBs. KSHV ORF75 induces the loss of ATRX protein and causes the dispersal of Daxx protein from PML-NBs (450). KSHV vIRF3 increases SUMO-modified PML protein levels leading to SUMO-dependent ubiquitination and eventually degradation of all isoforms of PML protein (451). Our data from the dox-inducible R12 stable cell line revealed a possible increase in SUMOylated PML proteins only in the context of RhIFNα2 treatment, but only PML isoforms I/II showed reduced protein levels. Therefore, while RRV may be similar to KSHV in utilizing at least two viral proteins to disrupt PML-NBs, the mechanisms may have diverged. We were able to confirm that transcription of ORF75 occurs during infection with $RRV_{vIRF\text{-}KO}$ and RRV_{R12ns} (data not shown). Thus, hypotheses to explain the involvement of both R12 and ORF75 in PML-NB disruption by RRV include: R12 interacts with ORF75 and this interaction is required for ORF75 to disrupt PML-NBs or induce degradation of PML proteins; the increased SUMOylation of PML (observed during exogenous R12 expression

in Fig. 2.2F) is necessary for ORF75 to disrupt PML-NBs; R12-mediated inhibition of ISG induction is necessary for endogenous levels of ORF75 to disrupt PML-NBs. All of these possibilities are currently being explored.

Our data provides evidence that RRV utilizes R12 to reduce protein levels of PML isoforms I and II. PML isoform II has been implicated in the efficient induction of ISGs that are transcriptionally regulated by the ISGF3 transcription complex. When PML isoform II is absent from cells, ISGF3 targets are not as strongly induced following stimulation with IFNα or poly(I:C) (246). In line with these PML isoform II findings, we discovered that only WT RRV and $RRV_{R12FLAG}$ were able to inhibit IP-10 induction following addition of RhIFNα2 to the infected cell culture media (Fig. 2.7B). Inhibiting ISG induction when faced with type I IFN would maintain a cellular environment more conducive for viral infection and replication. We were able to demonstrate this using viral growth curve analysis in the presence of type I IFN. The viruses which were able to disrupt PML-NBs and inhibit ISG transcription (WT RRV and $RRV_{R12FLAG}$) displayed significantly increased viral titers between 12 and 48 hpi compared to those viruses which were unable to disrupt PML-NBs ($RRV_{vIRF\text{-}KO}$ and RRV_{R12ns}) (Fig. 2.7E). Taken together, we conclude that R12 expression during *de novo* lytic infection reduces protein levels of PML isoforms I/II aiding the disruption of PML-NBs, which prevents the induction of ISGs regulated by the ISGF3 transcription complex even in the presence of type I IFN signaling. This allows RRV to effectively establish infection when the type I IFN response is activated, such as we would expect during in vivo infection (Fig. 2.8).

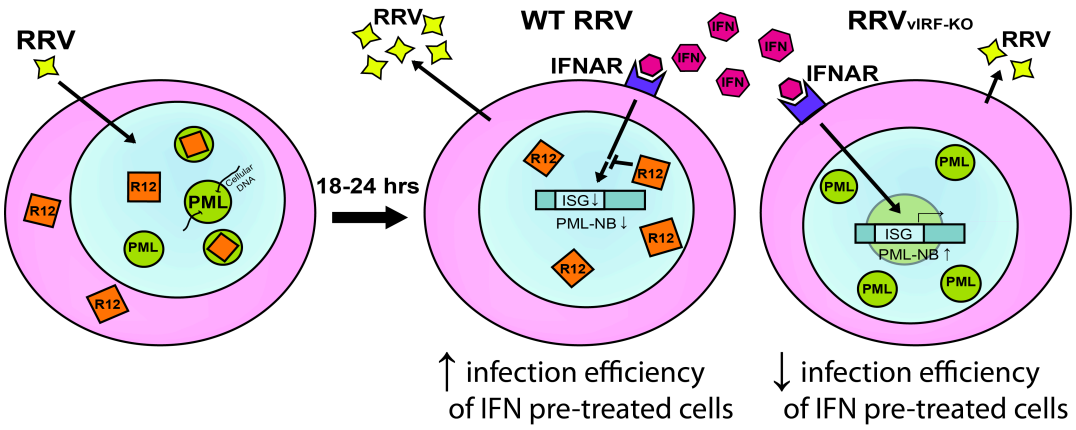

Figure 2.8. Model of R12 involvement in the RRV induced disruption of PML-NB. Following de novo lytic infection with RRV, R12 protein is expressed early and localizes to PML-NBs. 18 to 24 hpi, WT RRV is able to disrupt PML-NBs (dispersing PML, SP100, and Daxx from PML-NBs) so that even in the presence of type I IFN signaling, ISG transcription is inhibited allowing the virus to establish infection and replicate. However, following $RRV_{vIRF\text{-}KO}$ or RRV_{R12ns} infection R12 protein is not expressed, these viruses are unable to disperse PML protein from PML-NBs (although SP100 and Daxx are gone from the PML-NBs), and in the presence of type I IFN signaling these mutant viruses are unable to inhibit ISG transcription leading to an antiviral state within the cell and decreased infection and replication efficiency.

2.6 Materials and Methods

Cells, virus, drugs, and cytokines. Primary rhesus fibroblasts (RhFs) and telomerized RhFs (telo-RhFs) (32) were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Ogden, UT). Telo-RhF-rtTA-R12FLAG inducible cells were grown in DMEM supplemented with 10% tetracycline-free FBS, 3ug/ml puromycin (Sigma-Aldrich, St. Louis, MO), and 300ug/ml hygromycin B

(Invitrogen, Carlsbad, CA). Human BJAB cells were grown in RPMI 1640 (Mediatech) supplemented with 10% FBS. RRV infections were preformed in complete DMEM or RPMI media with 5ug/ml polybrene, following a 2-hour adsorption period cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Mediatech) to remove unbound virus and fresh medium was added. These studies utilized plaque-purified isolates of bacterial artificial chromosome (BAC)-derived RRV_{17577} (WT_{BAC} RRV) (16), WT RRV-GFP, $RRV_{vIRF\text{-}KO}$, and $RRV_{vIRF\text{-}KO}$-GFP. $RRV_{vIRF\text{-}KO}$, WT RRV-GFP and $RRV_{vIRF\text{-}KO}$-GFP viruses were previously reported (140). All RRV stocks were purified through a 30% sorbitol cushion and resuspended in PBS, and viral titers were determined by using a serial dilution plaque assay with RhFs.

Doxycyline hydrochloride (ThermoFisher Scientific, Waltham, MA) was resuspended in dimethyl sulfoxide (DMSO) (ThermoFisher Scientific) and added to cell culture media at 2ug/ml every 24 hours. Rhesus interferon alpha2 (RhIFNα2) (PBL Assay Science, Piscataway, NJ) was used at a final concentration of 100U/ml.

Construction of RRV_{R12ns} and $RRV_{R12FLAG}$. The RRV_{17577}BAC *galK* positive/negative selection system was utilized to create two mutant RRVs; the first was engineered to replace the start codon of R12 with a stop codon to create a non-sense (ns) mutation to prevent the expression of R12 (RRV_{R12ns}). A C terminal FLAG epitope tag was also introduced just before the native termination codon of R12 to ensure no read through expression. The second recombinant replaced the ns mutation with a start codon, to essentially create a revertant of

the RRV_{R12ns} virus. The revertant harbors the C terminal FLAG epitope tag to follow the R12 protein, and we termed this virus $RRV_{R12FLAG}$. This system of generating mutant RRVs was previously described (546). Briefly, R12 and R13 ORFs (nucleotides 87625-90478) in the RRV_{17577}BAC were replaced with *galK*, as described before (R12 5'-flanking primer sequence, 5'-TTTATTGCAGGGACAGGGCAAAAGCAAGCTGTGCACGGTAACAGTATGTGTCAGCACTGTCCTGCTCCT-3'; R13 3' flanking sequence, 5'-TAGGGGAGTGGTGAGGGCTTTTGAGTTAGTTTTCGTGGACCAAGTTCACACCTGTTGACAATTAATCATCGGCA-3'; sequences homologous to the *galK* cassette are underlined). Next we cloned the R12 and R13 ORFs with 250 base pairs of flanking regions into the psP73 vector using EcoRI and HindIII restriction sites engineered into the primers (250bp upstream of R12 primer, 5'-CGGAATTCGCCTAACTATATACGCCCACGGG-3', 250bp downstream of R13 primer, 5'- CGAAGCTTGCTTGGTGCCCTTTAAATTGAACG-3', restriction sites are underlined). Using Quikchange II XL site-directed mutagenesis (Agilent Technologies, La Jolla, CA) according to manufacturers specifications, we inserted a FLAG epitope just before the stop codon of R12 using the following primers: Forward primer 5'-GTATGTGTCACTTGTCATCGTCATCCTTGTAGTCCTGGGCCGCATCC-3' and reverse primer 5'-GGATGCGGCCCAGGACTACAAGGATGACGATGACAAGTGACACATAC-3'. FLAG epitope sequence is underlined. The ns mutation to the R12 ORF was accomplished using the following primers once the R12-FLAG tagged plasmid

was obtained. Forward primer 5'-GCCCGTCCTTCCGCTCACTCTGAGGGTCCGCTCGC-3' and reverse primer 5'-GCGAGCGGACCCTCAGAGTGAGCGGAAGGACGGGC-3'. Mutated start codon is underlined. Finally the R12-mutated R12-R13 repair cassette with flanking regions was digested out of the psP73 plasmid and used to repair the *galK* R12-R13 knock-out RRV_{17577}BAC as described previously (164). After identification of repaired BAC clones by southern blot, PCR, and sequencing, a clone was used to transfect RhFs to make virus and the BAC cassette was removed by CRE recombination as described previously (164). Each virus was plaque purified twice before viral stocks were grown and purified over a 30% sorbitol cushion and resuspended in PBS. Insertion junctions and the R12 ORF were PCR amplified and sequenced from each virus to identify the correct mutations were present and confirm no other alterations to viral genome in these locations.

In vitro growth curves. One-step (MOI = 2.5) and multistep (MOI = 0.1) growth curve analyses were carried out with RhFs, essentially as described previously(140) For growth curves in the presence of RhIFNα2, cells were seeded in culture tubes in the presence of 100U/ml of RhIFNα2, which was kept on the cells throughout the infection time course. Every 24 hours an additional 50U/ml RhIFNα2 was added to the culture tubes to ensure active IFN signaling throughout. Concentrations of type I IFN carried over from the viral growth tubes to the plaque assay plates was undetectable using a pan IFNα ELISA kit (3425-1H-6, Mabtech, Cincinnati, OH), as well as an IFNβ ELISA kit (LumiKine hIFN-

beta, Invivogen, San Diego, CA) according to manufacturers protocols. Additionally, an IFN bioassay to detect biologically active IFN in the viral growth tubes was performed by adding growth curve supernatants to cells harboring a luciferase reporter gene driven by the ISRE promoter. Universal type I IFN dilution series was used as a standard curve. The IFN bioassay measured 2.7 units/ml of type I IFN as the highest concentration (found in only one viral growth curve tube). WT RRV and $RRV_{vIRF\text{-}KO}$ growth was not affected by this concentration IFN (data not shown).

RNA isolation, RT-PCR, and real-time RT-PCR. RNA was isolated from uninfected or infected telo-RhF cells using the Quick-RNA Mini-prep kit and the RNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA). DNA was removed following an in column DNase I enzyme treatment followed by a second out of column DNase I treatment per the protocol included in the kits. Reverse transcription-PCR (RT-PCR) was performed by using Superscript III one-step RT-PCR with Platinum *Taq* (Invitrogen). Transcripts were detected with the following specific oligonucleotide pairs. ORF R13 forward primer: 5'-GGCGGCCCTGGCATATACGG-3'; ORF R13 reverse primer: 5'-CCGAGGTATGAGTGGCATGCAACC-3'. ORF R11 forward primer: 5"-AACCGGTGCACCGACAGTCGC-3'; ORF R11 reverse primer: 5'-CCGTGTCCTCTCGAAAACATC-3'. ORF R12 forward primer: 5'-ATTGTTGCGATAATGATAAGC-3'; ORF R12 reverse primer: 5'-CCGGTGGCATCCGCTTCGTTA-3'. ORF75 forward primer: 5'-GCGGACATGACAGTTTCCCCGTGGG-3'; ORF75 reverse primer: 5'-

TTACTGTCTGTTTCTTATGC-3'.

First-strand cDNA synthesis was carried out using superscript III reverse transcriptase for qRT-PCR (Invitrogen), and cDNA was subsequently amplified using Power SYBR Green master mix (Applied Biosystems, Waltham, MA). Concentrations of target transcripts were determined using a standard curve included on each plate consisting of serial dilutions of cDNA obtained from RhIFNα2-stimulated telo-RhFs. All data were normalized to the levels of GAPDH in each sample, and normalized levels of target transcripts were presented as fold change over mock treated cells.

Virion RNA RT-PCR Assay Purified stocks of $RRV_{vIRF\text{-}KO}$, WT RRV, $RRV_{R12FLAG}$, and RRV_{R12ns} were treated with micrococcal nuclease before purifying RNA that is packaged in the virions. As a positive control, a sample of $RRV_{vIRF\text{-}KO}$ virions treated with micrococcal nuclease had purified total cellular RNA from a WT RRV infected cell culture spiked into the sample after inactivation of the nuclease (by EGTA) but before virion lysis and RNA purification of virion packaged RNA. 40ng of RNA from each sample was reverse transcribed and PCR amplified using R12 specific primers in the presence or absence of reverse transcriptase. Resulting amplified DNA was resolved on a 1% agarose gel.

Immunoprecipitation (IP), PAGE analysis, and immunoblotting. Nuclear and cytoplasmic cell lysates were separated according to kit protocols (NE-PER; ThermoFisher Scientific). Nuclear lysates were immunoprecipitated with an anti-FLAG M2 monoclonal antibody (mAb) (F3165, Sigma-Aldrich) or an anti-PML (H238) polyclonal antibody (pAb) (Santa Cruz Biotechnology, Dallas, TX) in native lysis buffer (50 mM Tris-Cl [pH 8.0], 1% NP-40, and 150 mM NaCl

supplemented with protease inhibitors [100X cocktail; Sigma-Aldrich]), followed by incubation with protein A/G Plus-agarose (Santa Cruz Biotechnology), and lysates were finally collected in radioimmunoprecipitation assay (RIPA) buffer (native lysis buffer with 0.1% SDS and 0.5% sodium deoxycholate). Immunoprecipitation assays to analyze SUMO modifications on PML and R12 proteins were performed by harvesting whole cell lysates in RIPA buffer with 1% SDS and immediately boiling the samples for 5 minutes. RIPA buffer with no SDS was added to the protein lysates to bring the final SDS concentration to 0.1% before PML and R12-FLAG proteins were immunoprecipitated with A/G Plus-agarose. Whole-cell extracts were also collected in RIPA buffer, nuclear and cytoplasmic lysates were collected according to kit protocols (NE-PER; ThermoFisher Scientific), and all samples were analyzed on BOLT 4-12% gradient Bis-Tris Plus protein gels (Invitrogen). Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) via semidry transfer (60 minutes at 15 volts at room temperature). Membranes were probed with anti-human PML mAb (PG-M3, Santa Cruz Biotechnology) anti-FLAG M2 mAb (F3165, Sigma-Aldrich), anti-FLAG M2 (HRP) mAb (A8592, Sigma-Aldrich) anti-human GADPH mAb (51906, Santa Cruz Biotechnology), anti-RRV glycoprotein B mAb (gB, clone 10B5.2, VGTI Monoclonal Antibody Core), anti-RRV ORF52 mAb (clone 3G9.2, VGTI Monoclonal Antibody Core), anti-human SP100 pAb (43151, Abcam, Cambridge, UK), anti-human Daxx pAb (105173, Abcam), anti-laminA/C mAb (E-1) (376248, Santa Cruz Biotechnology), anti-SUMO-1 (Y299) mAb (32058, Abcam), and anti-

SUMO-2/3 (18H8) (Cell Signaling Technology, Danvers, MA). Densitometry was performed using ImageJ software.

Immunofluorescence Analysis (IFA). Cells were grown on glass coverslips in 12-well plates and fixed with methanol (20 minutes at -20°C). Cells were then blocked in 1% bovine serum albumin (BSA) in tris-buffered saline (TBS) (1 hour at room temperature) prior to staining, and all subsequent steps were performed with 1% BSA-TBS. Cells on coverslips were stained with rabbit anti-PML (H238) (Santa Cruz Biotechnology), goat anti-FLAG pAb (1257, Abcam), mouse anti-RRV gB mAb (clone 10B5.2, VGTI Monoclonal Antibody Core), rabbit anti-SP100 (Abcam), or rabbit anti-Daxx (07-471, MilliporeSigma, Burlington, MA) overnight at 4°C and subsequently stained with AlexaFluor-594 anti-mouse IgG (A11020, Invitrogn), AlexaFluor-633 anti-rabbit IgG (A21071, Invitrogen), and AlexaFluor-488 anti-goat IgG (A11055, Invitrogen) (1 hour at room temperature), and nuclei were stained with DAPI. Cells on coverslips were mounted onto slides by using Vectashield (Vector Labs) and examined on a Zeiss Axio Imager.M1 microscope (Zeiss Imaging Solutions, Thornwood, NY) or Leica SP5 AOBS spectral confocal microscope (Leica microsystems, Buffalo Grove, IL). 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).

Generation of a doxycycline (dox)-inducible stable cell line. The pLVX lentiviral vector system (Clontech, Mountain View, CA) was utilized for constructing a stable doxycycline (dox)-inducible cell line as described previously

(463). Briefly, the pLVX-R12FLAG plasmid was constructed by subcloning full-length FLAG-tagged R12 from WT_{BAC} RRV DNA into the pLVX-Tight-Puro retroviral vector. Replication-defective recombinant retrovirus was produced in HEK 293T/17 cells and used to transduce the target cells (telo-RhFs containing a dox- responsive transactivator [tRF-rtTAs]). The dox-inducible R12FLAG cells were maintained in DMEM plus 10% Tet-free FBS containing 3ug/ml puromycin and 300 ug/ml hygromycin B. In order to determine the optimal concentration of dox and duration of dox treatment, cells were experimentally treated with dox at various concentrations and for various lengths of time. We found that 2 ug/ml of dox yielded half-maximal R12FLAG expression at 18 hours post treatment.

Statistical analysis. Data were analyzed by using GraphPad Instat (GraphPad Software, La Jolla, CA), and significant differences were determined by unpaired student *t* test or two-tailed Fisher's exact test, with *P* values of 0.05 being considered significant.

CHAPTER 3

Discussion and Future Directions

Because of the homology of the vIRFs to cellular IRFs it was initially assumed that these viral homologs would have inhibitory functions against the IRFs and IFN pathway. Studies on the vIRFs encoded by KSHV have revealed that these ORFs do inhibit IRFs and the IFN pathway, but have evolved multiple other functions as well. Although some functional redundancy exists between the KSHV vIRFs, it is also evident that these ORFs have diverged to acquire unique functions. Much less is known about the RRV vIRFs. The RRV/RM model affords us the opportunity to investigate the role of specific viral factors in disease progression *in vivo*, as well as perform detailed mechanistic studies *in vitro*. Initial investigations into the functions of the RRV vIRFs compared WT RRV to a mutant RRV lacking all eight vIRFs. Data from these studies showed that while WT RRV and $RRV_{vIRF\text{-}KO}$ displayed similar growth kinetics *in vitro*, $RRV_{vIRF\text{-}KO}$ infection *in vivo* was restricted compared to WT RRV infection. This led us to investigate whether the type I IFN response could account for this discrepancy and whether the RRV vIRFs had a role in inhibiting the IFN/IFN receptor signaling cascade. The focus of this dissertation was to investigate the evasion of type I IFN signaling by RRV vIRFs, and to characterize the specific vIRF(s) responsible for and mechanisms of this evasion.

3.1 RRV disruption of PML-NBs enhances viral infection in the presence of type I IFN.

As a part of cellular intrinsic immunity, PML-NBs present an obstacle to any nuclear replicating virus. Most herpesviruses have now been shown to inhibit the antiviral functions of PML-NBs, albeit by different mechanisms. Importantly, members of the gamma-2 herpesvirus subfamily utilize ORF75 (vFGARAT) and in the case of KSHV, vIRF-3, to inhibit PML-NBs. Chapter 2 of this dissertation characterized the function of the RRV vIRFs during *de novo* lytic infection in the presence of type I IFN signaling. PML-NBs are involved in the robust induction of IFNs and ISGs, and the loss of PML-NBs during RRV infection was found to be dependent on the expression of the vIRFs. Furthermore, of the eight vIRFs missing in the $RRV_{vIRF\text{-}KO}$ virus, we determined that R12 is the vIRF that contributes to the disruption of PML-NBs during *de novo* lytic infection using a recombinant RRV that does not express R12 (RRV_{R12ns}). We showed that R12 expression inhibits ISG induction following RhIFNα2 treatment, both within and outside the context of viral infection. Finally, we demonstrated that R12 enhances viral replication in the presence of type I IFN signaling.

ORF75 of RRV has also been implicated in the disruption of PML-NBs (457). The authors of that study found PML protein levels decreased after 24 hours of RRV infection, even though ORF75 is a tegument protein and can reduce protein levels of SP100 by 8 hours post infection. They also observed two contradictory results: 1) inhibition of protein translation did not rescue PML protein levels in infected human iSLK cells consistent with a virion associated

protein affecting PML, and 2) inhibition of protein translation did rescue PML protein levels in infected rhesus fibroblasts, implying that a tegument protein is not sufficient for PML loss in rhesus fibroblasts. Therefore, the mechanism of PML-NB antagonization during RRV infection may be cell-type specific. We performed our experiments in rhesus fibroblasts and also found reduction in PML protein levels at 24 hours post infection. Our results may be compatible with the fibroblast data in the previous ORF75 study, as the translation of R12 could be required for PML protein loss. Presently, it is not known whether R12 protein is incorporated into RRV virions, or whether virion-associated R12 could affect PML levels. We are currently exploring these possibilities, however, results were not definitive at the time that this thesis was written.

We have also shown that ectopic expression of R12, in the presence of type I IFN signaling but absence of viral infection, results in reduced protein levels of PML isoforms I and II but did not appear to affect the other PML isoforms. Interestingly, ICP27 of HSV-2 alters the mRNA splicing of PML transcripts and switches PML isoform II to PML isoform V, HSV-1 ICP0 null virus replication is inhibited by PML isoforms I and II, and ORF3 of adenovirus type 5 E4 specifically targets PML isoform II (547-550). Therefore, viral targeting of PML isoform II has precedent and due to the role that PML II plays in enhancing ISG induction, may serve as an IFN evasion mechanism. We were able to show that the ectopic expression of RRV R12 could inhibit ISG transcription following RhIFNα2 treatment, supporting the theory that RRV specifically targets PML isoform II to evade the IFN response (Fig. 2.7C).

As stated previously, KSHV vIRF-3 is also able to disrupt PML-NBs (451). Further, an oncogenic downstream effect for PML-NB disruption was investigated and it was demonstrated that knock down of vIRF-3 in KSHV positive PEL cells resulted in reduced proliferation and increased activation of caspase-3 and caspase-7 (506). PML-NBs have many functions and, presumably, all of these functions can be impacted when the PML-NBs are disrupted. While we investigated the impact on IFN signaling, a consequence of the RRV disruption of PML-NBs may also include the development of malignancies or dysregulation of the cell cycle. Importantly, KSHV vIRF-3 is expressed during both lytic and latent infections. As PEL and KS are predominantly composed of latently infected cells, the functions of latently expressed genes (including vIRF-3) could provide insights into the mechanisms of pathogenesis and might also shed light on possible therapeutic targets. Studies on the RRV vIRFs have only characterized expression during lytic infection, and whether any of the RRV vIRFs are expressed during latency remains unknown. One study that examined the transcriptional profile of RRV during latency in HEK293 cells *in vitro* (542, 551). They analyzed RRV ORF transcript levels before and after TPA treatment (to reactivate RRV) and showed moderate-to-low transcript levels of R12 prior to TPA treatment, followed by intermediate transcript levels after TPA treatment. R12 transcript levels mirrored what was observed for ORF73/LANA, suggesting R12 may be capable of being expressed during latency. However, ORF50/RTA (the lytic switch protein) transcript expression followed the same pattern and thus puts into question whether latency was actually achieved in this cell culture (542).

Additionally, the complete characterization of the structure of R12 transcripts has not been performed in any system, and the presence of variably spliced R12 transcripts with unique functions cannot be ruled out.

3.2 Future Directions

Two reports have now implicated RRV ORF75 and R12 in the disruption of PML-NBs during *de novo* lytic infection. It remains to be investigated if ORF75 and R12 work together or interact at all. It is curious that the cellular localization of ORF75 is diffuse throughout the cytoplasm and nucleus while R12 is localized with PML-NBs in the nucleus. One theory is that R12 could physically interact with both ORF75 and PML to bring the effector function of ORF75 to PML-NBs. Co-immunoprecipitation assays would determine whether ORF75 also complexes with PML and R12. An ORF75 over-expression stable cell line displaying abundant ORF75 expression was able to reduce the levels of PML protein in the absence of R12. Whether more physiological levels of ORF75, such as the amount brought in with an infecting virion, would have the same effect is not known. We plan to answer this question by titrating in an ORF75 expression plasmid into the inducible R12 stable cell line to see if low levels of ORF75 require R12 expression to disrupt PML-NBs.

To further confirm the link between IFN signaling, PML-NBs, and virus growth restriction, a PML knockout cell line should be constructed. If ISG induction and PML-NBs are connected, there should be the same lack of (PML isoform II enhanced) ISG induction following infection with WT RRV and $RRV_{vIRF\text{-}KO}$ in the PML knockout cell line. Additionally, we would expect WT RRV and

$RRV_{vIRF\text{-}KO}$ viruses to grow to similar titers in the presence of type I IFN in these PML knockout cell lines. Ectopic expression of individual PML isoforms within the PML knockout cell line could also help determine specific functions of each PML isoform with regard to RRV infection.

Analysis of R12 expression kinetics following RRV infection revealed the unexpected detection of R12 transcripts as early as 2 hours post infection. To follow up on this very rapid appearance of R12 transcripts we investigated whether R12 transcripts were packaged in RRV virions. Surprisingly, R12 transcripts were detected in the WT RRV, $RRV_{R12FLAG}$, and RRV_{R12ns} virions (Fig. 2.6B). The lack of R12 transcript in the $RRV_{vIRF\text{-}KO}$ virions suggests that the RT-PCR primers are specific for R12. RNA packaged inside KSHV virions has been described (552). Some RNA was proposed to be incorporated non-specifically due to the high abundance of those transcripts at the time of capsid formation and virion egress. While at least one transcript, ORF17, was determined to be specifically incorporated into KSHV virions, KSHV vIRF transcripts were not examined in this study. However, DNA array data also obtained in this study examining virion RNA expression across the KSHV genome showed a hit just before ORF58, perhaps in the region of the vIRFs (which sit between ORF57 and ORF58). The authors did not comment on this particular array hit/peak, but this data does suggest that further investigation of KSHV vIRF transcript incorporation into virions may be warranted. At the time of the preparation of this dissertation, no RRV virion-associated RNAs have yet been identified, and RT-PCR can only confirm the short section of R12 transcript that is amplified by the

primers. In order to determine whether full-length R12 transcripts are incorporated into RRV virions, northern blot analysis will need to be performed on RNA purified from virions. Functional assays, such as RRV infection in the presence of transcription inhibitors, to determine whether virion incorporated R12 transcripts could be translated and produce innate immune evasion functions would also be necessary. Additionally, investigation of whether other transcripts or RNAs are packaged inside RRV virions (including the other vIRFs) could be informative. The implications of R12 transcript incorporation into RRV virions are that it would provide a more rapid synthesis of R12 protein following entry into a cell, and would also mean that *de novo* transcription does not need to occur in order for R12 protein expression to occur after infection. Many viruses utilize tegument proteins to disrupt PML-NBs because PML-NBs are present at the moment of infection and could inhibit transcription of viral genes if not dealt with immediately. Adenovirus capsid protein VI targets PML-NBs to counteract Daxx, human papillomavirus (HPV) capsid protein L2 localizes to PML-NBs and is necessary for efficient HPV infection, and several herpesviruses have been shown to utilize tegument proteins to disrupt PML-NBs (ICP0 of HSV-1, pp71 of HCMV, vFGARATs of gamma-2 herpesviruses) (440, 548, 553-555). If R12 transcripts are brought in with the infecting virions, this would effectively result in a similar outcome as a tegument protein, as only translation needs to occur to gain the viral effector necessary for PML-NB disruption.

Another question that remains with regard to R12 is whether it is expressed during latency, as has been found for KSHV vIRF-3 and KSHV vIRF-

4. Latent expression is of interest because RRV does establish latency within infected RMs, and any investigation into the role of R12 in pathogenesis will need to account for the fact that the virus would be mostly latent in the animal. The RM model also affords the ability to investigate *in vivo* expression of R12. While some attempts have been made to establish latent RRV infections *in vitro* in BJAB and HEK293 cells, some amount of lytic replication still occurs, obscuring the results. *Ex vivo* analysis of cells isolated from long-term RRV-infected RMs may provide another model to determine which RRV transcripts are expressed during latency. RRV latency may be better established *in vivo* compared to *in vitro*. T cells, B cells, and other subsets of cells could be separated and analyzed for RRV transcript levels. Because B cells are the major latency reservoir, and engagement of the B cell receptor can reactivate latent virus, a negative selection for B cells should be performed to preserve latency in these cells. Analysis of RRV transcript levels within these sorted cells, before and after TPA or sodium butyrate treatment, would help delineate lytic from latent genes. Before chemically induced reactivation, lack of lytic transcripts such as RTA and ORF52, along with the presence of latent transcripts such as LANA, vCyclin, and vFLIP would confirm the latent infection. Following reactivation, we would expect a large induction of lytic gene transcripts. Meanwhile, latent genes should display no induction, low induction, or decreased transcript levels following reactivation. Deciphering the expression pattern of the vIRFs, including R12, using this method could finally reveal if any of the vIRFs are latently expressed. Latent expression of R12 is not necessarily required for a role of R12 and PML-NB

disruption in the development of pathogenesis. Even PEL and KS tumors, which are predominated by latent infections, still display low levels of lytic infections (556, 557). Additionally, MCD is characterized by lytic infection and while a connection between PML-NBs and MCD has not been made, it may play a role in this malignancy as well. Along this line of thought, the infection of RMs with the RRV_{R12ns} virus (with and without SIV co-infection) and monitoring for malignant progression, compared to WT RRV infection, may be informative. These studies would require a large cohort of RMs and observation for many months or years, and thus these studies may be cost prohibitive. However, *in vivo* studies would help define the specific role for R12 (or other vIRFs) in pathogenesis of RRV and provide insight into the role of KSHV vIRFs in human pathologies.

RRV, like KSHV, has encoded two ORFs (vFGARAT and vIRF) to antagonize PML-NBs, supporting the use of RRV as a model to study KSHV manipulations of these structures. Because PML-NBs have many different functions, the disruption of these sub-nuclear structures by gamma-2 herpersviruses could have far-reaching effects. One such effect is the down-modulation of the type I IFN response, which could further affect adaptive responses. The RRV/RM model provides a means to investigate the effect of PML-NB disruption on pathological outcomes of gamma-2 herpesvirus infections and test therapeutic interventions that could one day treat KSHV infections in humans.

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