Rhesus Rhadinovirus vIRF R12 Disrupts the PML-Nuclear Body via Proteasome-Dependent Protein Degradation Pathway

By

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ABSTRACT

Kaposi's Sarcoma (KS) is one of the most popular complications found in human immunodeficiency virus (HIV)-infected patients, which is caused mainly by the KS-associated herpesvirus (KSHV) infection. However, the scarcity of animal models for studying *de novo* KSHV infection, as well as KSHV-associated diseases greatly hindered these processes, leading to the emergence of using other primate viruses to study the KSHV. Rhesus macaque rhadinovirus (RRV), a monkey γ2 herpesvirus that is closely related to KSHV, shares great genomic and pathogenic similarities with its human counterpart, making it an ideal model to study the KSHV. Both RRV and KSHV encode a cluster of genes with significant homology to cellular interferon (IFN) regulatory factors (IRFs), and these genes were found to be involved in immune signaling, apoptosis, cellular growth and differentiation, resulting in their immune evasion and tumorigenesis. Here we determined that infection of wild type RRV was capable of down-regulating the level of promyelocytic leukemia protein (PML), which is an important intrinsic immune regulatory factor involved in multiple cell signaling pathways. We then further determined that the vIRFs played an important role in RRV-mediated PML down-regulation by comparing the infection of wild type RRV versus vIRF-knock-out RRV. RRV encodes eight vIRFs, making it necessary to figure out which one of the vIRFs is important in the RRV-mediated PML protein down-regulation. We found that, compared to other seven vIRFs, the R12 was the most important factor in interacting with and down-regulating PML protein. Additionally, we found that the RRV- and R12-mediated PML protein down-regulation was proteasome-dependent, and possibly a multi-stage process: the PML protein was aggregated in

the nucleus first and then degraded. Moreover, we constructed a telomerized rhesus fibroblast cells-based Tet-ON/OFF system with R12 expression under the control of doxycycline for future applications. Taken together, our findings further demonstrate the significant regulatory effects of RRV vIRFs on host immune system. If these data could be extrapolated to KSHV-associated infections, our findings would suggest that the vIRFs could be potential drug-targets to enhance host immune responses to KSHV infection.

CHAPTER 1

INTRODUCTION

I. Human Herpesviruses

The *Herpesviridae* are a large family of DNA viruses that cause diseases in animals, including humans. The members of this family are known as herpesviruses. There are eight viruses that naturally infect humans as their primary target, and they are designated Human Herpesvirus-1 (HHV-1) through HHV8. Herpesviruses all share a common virion structure: all herpesviruses are composed of a large, linear, dsDNA genome, encoding an estimated 70-200 open reading frames (ORFs), encased within an icosahedral capsid which is itself wrapped in a tegument containing viral proteins. The whole viral particle is enclosed with an envelope composed of a lipid bilayer (*1*) (Figure 1.1A).

All the human herpesviruses have similar lytic life cycle, which is depicted in Figure 1.1B, and each step will be explained in detail in the following text. The initial step upon herpesvirus infection is binding of the virion to the host cell surface receptors via glycoproteins. The envelope of bonded virions starts to fuse with host cell membrane lipid bilayer and the viral capsid was delivered into the cell, which is later translocated by microfilaments to the nuclear pore. The viral dsDNA genome was then injected into the nucleus, circularized and associated with histones. Transcription of viral genes subsequently occurs in a temporally ordered fashion (*2*) to produce immediate early (IE), early, and late viral proteins. The immediate early genes are expressed immediately following virus entry into host cells, because their expressions are not

dependent on newly synthesized viral proteins. Another important role IE genes play is to antagonize the host innate immune responses (*3*). The early genes are expressed between 12-48 hours post-infection (hpi) and are dependent on the synthesis of IE gene products. These genes are important for viral DNA replication and late viral protein expressions. The late genes encode for the structural proteins that are required for complete virion assembly and maturation (*2*). The replication of herpesvirus DNA genome starts with a rolling circle mechanism, producing circular genome concatamers that are cleaved and packed into preformed capsids in host nucleus (*4*). The virions then are coated with lipid bilayer and other viral structure proteins, followed by maturation process as transmitted from nucleus through nuclear membrane, endoplasmic reticulum, golgi, and cell membrane, and are finally released from the cell by cell lysis (*5, 6*).

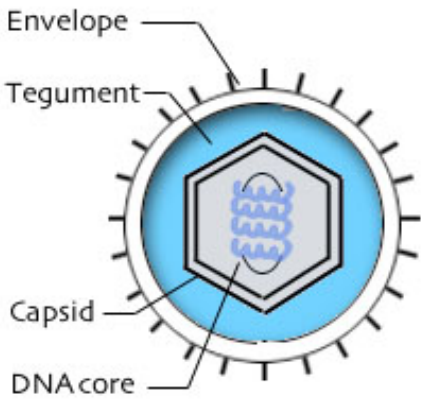
In addition to the lytic replication cycle as described above, herpesviruses also establish latent infection in host. During latent infection, only a small number of viral proteins are expressed to trigger minimal host immune responses, and the viral genome is kept as episomes in infected nucleus (*4*). The latently infected cells usually maintain a normal metabolism, instead of being lysed by lytic virus infection. Latent herpesvirus also has mechanisms to ensure that its genome is efficiently replicated and distributed to daughter cells during cell mitosis. On the other hand, the latent virus can be induced to enter lytic cycle upon certain external treatments or change of cellular environments.

Herpesviruses are known for their ability to establish life long infections, and primary herpesvirus infections can often be asymptomatic. Their primary target cells are usually mucosal epithelial cells, lymphocytes, and neurons. And lytic virus reactivation could result in severe ulcerative and vesicular lesions of the mouth and genitals, especially for HSV-1/2 (*7*). VZV infection usually occurs during childhood, as human immune systems are not mature enough to

defend virus infection, resulting in varicella (chicken pox) (*8*). Moreover, the primary human CMV infection of pregnant women can lead to serious congenital diseases including hearing loss, CNS damage, and mental retardation of the fetus (*9*). EBV and KSHV, the lymphotropic human herpesviruses, are associated with a wide variety of T cell and B cell malignancies (*10*). For example, latent EBV infection was linked with the development of a number of lymphomas, including non-Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, T cell and NK cell lymphomas, and post-transplant lymphoma (*10, 11*). The detailed summary of eight human herpesviruses pathogenesis is shown in Table 1.1.

Figure 1.1. Herpesvirus structure and replication cycle. (A) A generic schematic of herpesvirus virion structure organization. The herpesvirus virions are composed of viral genome, capsid, tegument, and envelope. (B) A diagram of herpesvirus lytic replication life cycle. Upon virus infection, the viral particle is first attached to the host cell extracellular matrix via its surface glycoproteins (1-2). The attached virus particle then fuses its envelope with the host cell and releases its capsid into cytoplasm (4), together with other viral proteins (5b-c). The viral capsid is then travelled to the nuclear pore via the help of cell microfilament (5a), where viral genome is released into nucleus and gets circularized (6-8). The viral genome then expresses immediate early (IE), early, and late proteins in a highly ordered fashion (9-17), followed by new virion assembly (18). The newly synthesized virions then go through nuclear membrane to endoplasmic reticulum and golgi, and get matured and released out of the cell membrane (19-23). *Figure and legend for (B) adapted from (12).*

A


Envelope
Tegument
Capsid
DNA core

B

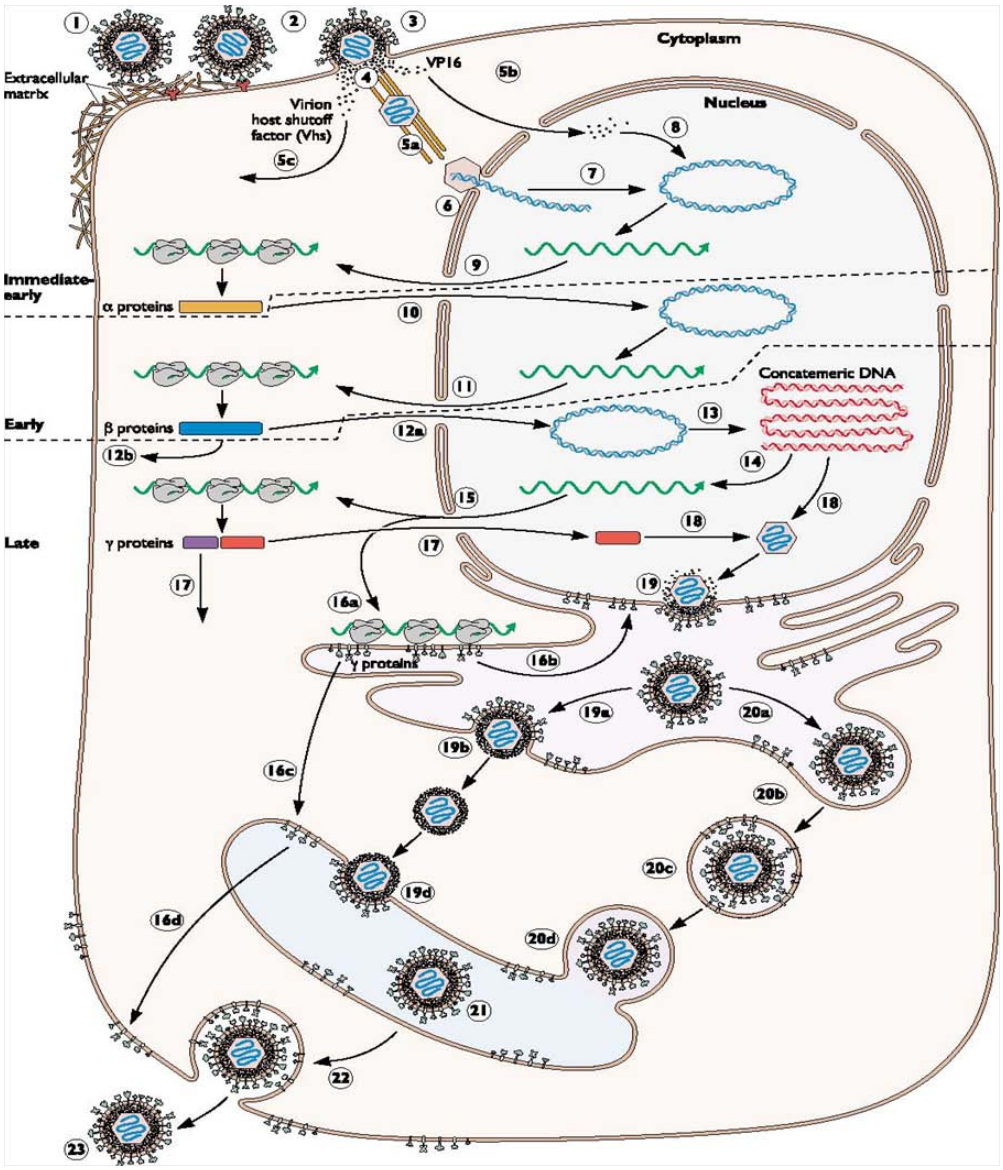

Cytoplasm
Nucleus
Extracellular matrix
Virion host shutoff factor (Vhs)
VP16
Immediate-early
α proteins
Early
β proteins
Late
γ proteins
γ protains
Concatemeric DNA

Table 1.1 Human Herpesvirus (HHV) classification

Type	Synonym	Subfamily	Primary Target Cell	Pathophysiology
HHV-1	Herpes simplex virus-1 (HSV-1)	α	Mucoepithelial	Oral and/or genital herpes(predominantly orofacial), as well as other herpes simplexinfections
HHV-2	Herpes simplex virus-2 (HSV-2)	α	Mucoepithelial	Oral and/or genital herpes (predominantly genital), as well as other herpes simplex infections
HHV-3	Varicella zoster virus (VZV)	α	Mucoepithelial	Chickenpox and shingles
HHV-4	Epstein-Barr virus (EBV), lymphocryptovirus	γ	B cell and epithelial cell	Infectious mononucleosis,Burkitt's lymphoma, CNS lymphoma in AIDS patients,post-transplant lymphoproliferative syndrome(PTLD), nasopharyngeal carcinoma, HIV-associated hairy leukoplakia
HHV-5	Cytomegalovirus (CMV)	β	Monocyte,lymphocyte, and epithelial cell	Infectious mononucleosis-like syndrome, retinitis
HHV-6	Roseolovirus, Herpes lymphotropic virus	β	T cell	Sixth disease (roseola infantumor exanthem subitum)
HHV-7	Pityriasis Rosea	β	T cell	Roseola infantum or exanthem subitum
HHV-8	Kaposi's sarcoma-associated herpesvirus	γ	Lymphocyte	Kaposi's sarcoma, primary effusion lymphoma, some types of multicentric Castleman's disease

II. Kaposi's Sarcoma-associated Herpesvirus

The Herpesviridae family is divided into α-, β-, and γ-herpesvirus subfamilies, according to their biologic behavior and phylogenetic relationship (*1*). The γ-herpesviruses are characterized by *in vitro* and *in vivo* infection of lymphocytes and are further divided into lymphocryptovirus (γ1 herpesviruses) and rhadinovirus (γ2 herpesviruses) genera. Rhadinoviruses have taken on increased importance with the identification of the novel KSHV in association with KS, an inflammatory and neoplastic condition seen in many HIV-infected patients with AIDS (*13*).

KSHV has a dsDNA genome that is approximately 120kb in length. After infection, the virus enters into lymphocytes via macropinosomes where it remains in a latent state expressing the viral latency-associated nuclear antigen (LANA). The virus exists as a naked circular piece of DNA episome and uses the cellular replication machinery to replicate itself (*14*). LANA tethers the viral DNA to cellular chromosomes, inhibits p53, an important cellular tumor suppressor, as well as retinoblastoma protein, and suppresses viral genes needed for virus lytic replication and assembly (*15*). Various signals such as inflammation or compromised host immune system caused by HIV-infection may trigger the virus to enter into lytic cycle. Once this occurs, numerous virus particles could be assembled and released, resulting in death of infected cells, but the rate of lytic replication is different in KSHV-caused diseases as discussed below.

KS is a systemic multi-focal angioproliferative disease that can present with cutaneous lesions with or without internal involvement (*16, 17*). KS lesions are present on the dermis, oral cavity, and visceral organs, and are composed of proliferating spindle cells of endothelia origin, as well as a high number of infiltrating immune cells (*17*). Most of cells in KS lesions are latently infected, with only ~3% showing lytic antigen synthesis (*17*). In addition to KS, KSHV is also

linked to B cell disorders, including primary effusion lymphoma (PEL) (*18*) and multicentric castleman's disease (MCD) (*19*). PEL is a rare disease and only accounts for about 2% of AIDS-associated lymphomas (*20*), but patients with PEL have a poor prognosis, with an average survival time of 2-5 months (*18*). Further analysis of this particular lymphoma characterized PEL cells as differentiated, hyperplastic plasma cells due to their surface expression of CD138, lack of surface immunoglobulin, as well as clonal immunoglobulin gene rearrangements (*18*). MCD, on the other hand, is an aggressive lymphoproliferative disorder involving multiple lymph nodes and extranodal sites (*19*). KSHV is associated with nearly all cases of MCD in HIV-positive patients, and is characterized by enlarged germinal centers within the affected lymph nodes. It was shown that both latent and lytic transcriptional programs present in KSHV-infected cells in MCD (*21*), comparing to a predominantly latent KSHV infection in KS and PEL.

Studying KSHV pathogenesis in molecular level has proved difficult for two main reasons. First, the lytic replication within cultured cells is inefficient. In cultured B cells and endothelial cells, KSHV infection naturally and predominantly results in a latent infection. Use of phorbol esters can induce lytic replication within latently infected PEL cells, but only in 25-30% of these cells (*22*), and only a quarter of those cells complete their lytic cycle (*23*). Since the majority of viral genes are not expressed during latency, examination of specific viral genes has been mostly outside the context of infection. Secondly, it has been difficult to develop an animal model, as the natural host of KSHV is human. Although people utilized immune-compromised mice with or without grafted human tissues to mimicry the infection happened in human, the results were not satisfactory either because the development of KSHV-associated diseases is limited or because it is hard to study the host immune responses in those immune-compromised mice (*24*-

27). Therefore, an approach to study KSHV pathogenesis by utilizing a virus similar to KSHV was established, which will be discussed in the following sections.

III. Rhesus Macaque Rhadinovirus

In order to study the KSHV-associated pathogenesis, two γ-herpesviruses with high homology to KSHV were proposed as models: murine herpesvirus 68 (MHV-68) and rhesus macaque rhadinovirus (RRV). MHV-68 is a natural pathogen within mice and also maintains homology and co-linear genomic organization with KSHV (*28*). The MHV-68 infection of mice resulted in similar pathology as observed in KSHV-infected patients, however, lytic MHV-68 antigens were not detected in these animals (*29*). Another drawback is that MHV-68 does not encode a number of the unique, cellular homologues that are potentially novel and essential players in KSHV-associated disease (*28*, *30*). However, utilizing RRV to infect rhesus macaques successfully overcome these disadvantages and is considered to be the optimal model to study KSHV-associated pathogenesis.

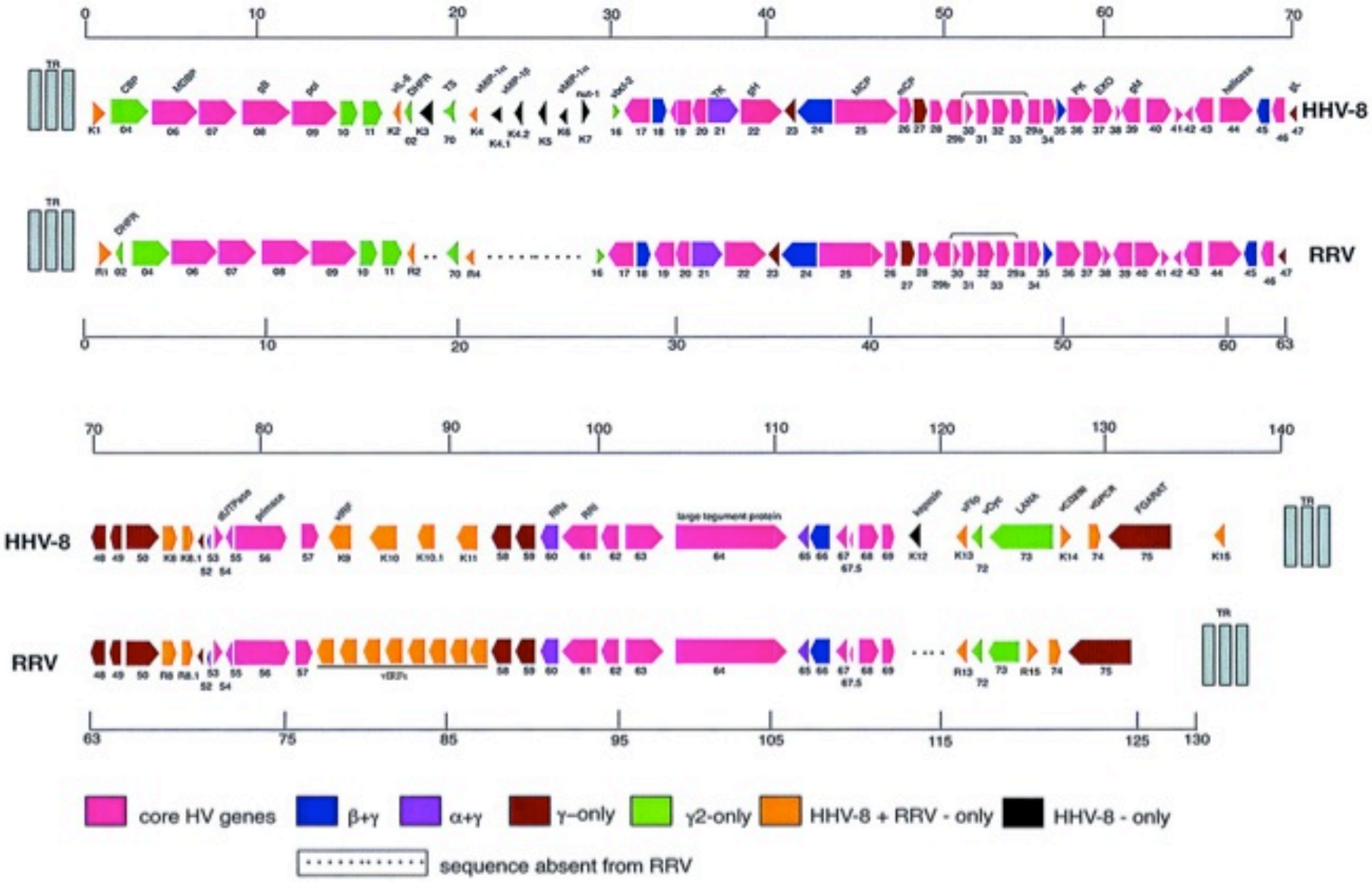
RRV was isolated from rhesus macaques by two separate groups in the late 1990s (*31, 32*), and sequence analysis of the two isolates confirmed their classification as γ2-herpesviruses and demonstrated that the two were highly homologous to each other as well as to their human counterpart, KSHV 1994 (*13, 32, 33*). The first strain, RRV 26-85, was isolated from a healthy rhesus macaque at the New England Primate Research Center (*31*), while the second strain, RRV 17577, was isolated at the Oregon National Primate Research Center from the rhesus macaque infected with simian immunodeficiency virus (SIV) and presenting with a lymphoproliferative disorder (*32*). However, only RRV 17577 strain supported experimental infection of rhesus macaques and was associated with development of specific pathologies (*34-37*).

Analysis of the genomes of RRV 17577 and KSHV demonstrated a high degree of similarity to each other that nearly every one of the genes in KSHV has at least one homologue in RRV

(Figure 1.2) (*32, 33*). RRV has a 131kb dsDNA genome, encoding 79 ORFs, 68 of which align with those encoded in KSHV, including a number of ORFs with homology to cellular genes (*32*). In contrast to KSHV culture systems that are marked by inefficient latent life cycle, *de novo* infection of rhesus fibroblast cells (RFs) with RRV results in highly efficient spontaneous lytic replication and high virus titer, allowing for easy study of lytic viral genes (*38, 39*).

Since the initial isolation of RRV, experimental infection of rhesus macaques with RRV 17577 validated the role of RRV in development of B cell disorders and lymphomas in SIV/RRV dually infected rhesus macaques (*34, 36, 40*). These animals developed a lymphoproliferative disorder (LPD), characterized by splenomegaly, hepatomegaly, angiofollicular lymphadenopathy, and hypergammaglobulinemia, all of which are also clinical manifestations of MCD in humans (*41*). The SIV/RRV co-infection was also associated with non-Hodgkin's lymphoma and RRV-infected rhesus macaques showed latency within B cells (*36, 37*). Moreover, one of these rhesus macaques developed retroperitoneal fibromatosis, which closely resembles KS in humans (*36, 42*). Taken together, RRV 17577 is an ideal model system to study KSHV pathogenesis and its molecular mechanisms.

Figure 1.2. Alignment of KSHV and RRV genomes. ORFs are colored according to their inclusion within specific herpesvirus subfamilies, and are oriented with the pointed end at the 3' end of each ORF. ORF, open reading frame. TR, terminal repeats. *Figure and legend adapted from (43).*


HHV-8
RRV
core HV genes
β+γ
α+γ
γ–only
γ2-only
HHV-8 + RRV - only
HHV-8 - only
sequence absent from RRV

IV. Host Innate and Adaptive Immunity

The innate immune response is the first line of defense against invading pathogens, and is different from the adaptive immunity due to its rapid and non-specific recognition of pathogens, and lack of memory. During innate immune response, phagocytic cells, Natural Killer (NK) cells, and antigen-presenting cells (APCs) will engulf and destroy pathogens, and then initiates production of interferons (IFNs) and other cytokines and chemokines that comprise an immediate inflammatory response (*44*). The innate recognition of non-self antigen is conducted by pattern-recognition receptors (PRRs), aptly named for their capacity to recognize pathogen-associated molecular patterns (PAMPs), unique to invading pathogens (*45-47*). Upon recognition of specific PAMPs, PRRs initiate signaling cascades through specific effector molecules, resulting in activation of IκB kinase (IKK)-related kinases, Tank-binding kinase (TBK), IKK-ε, type I IFN synthesis, and other pro-inflammatory cytokines production (*48*).

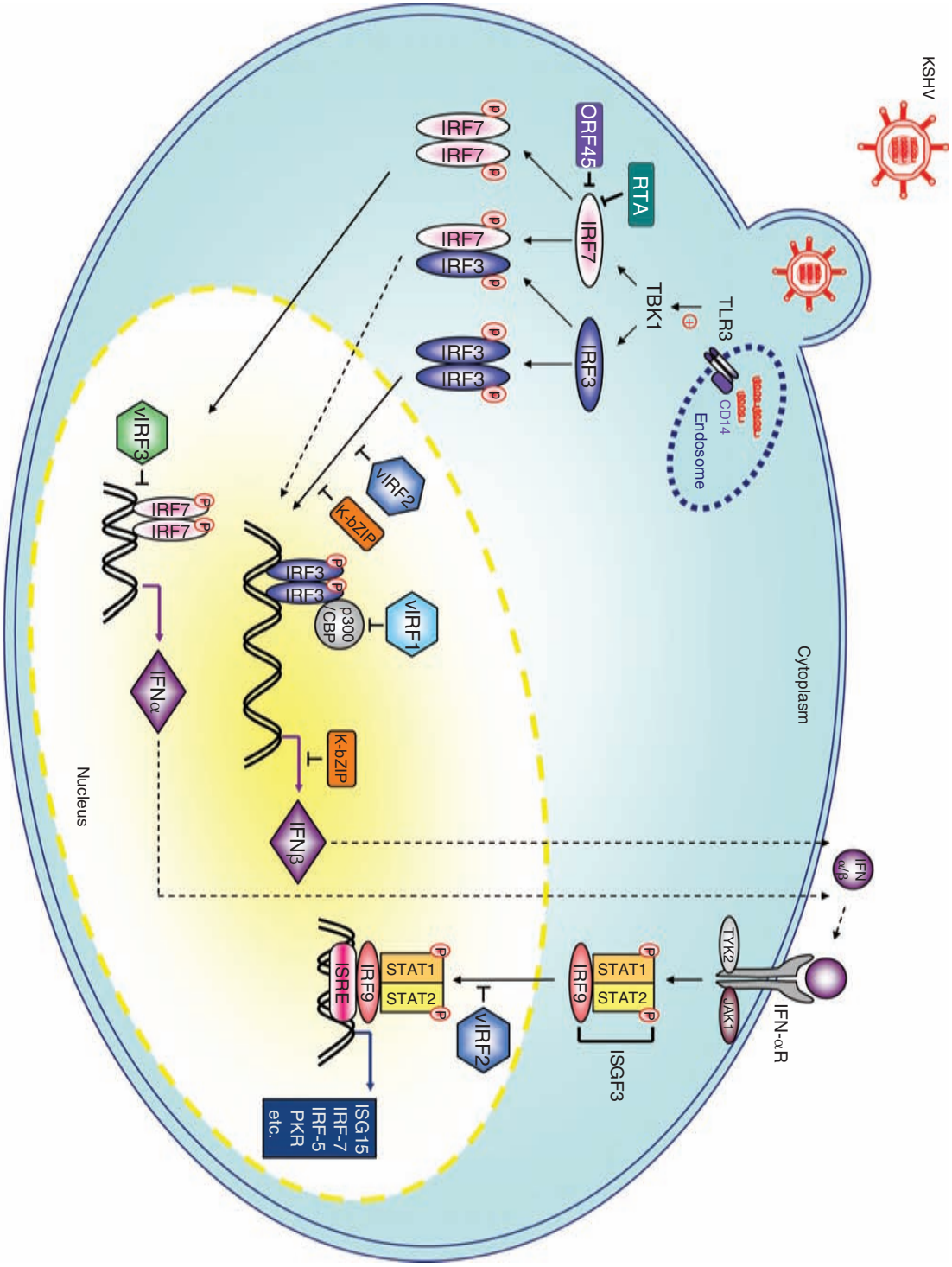
The most thoroughly studied PRRs are the Toll-like receptors (TLRs) that are a family of 9 membrane-associated glycoproteins expressed on a wide range of cells, especially innate immune cells (*45*). TLRs 1, 2, 4, 5, and 6 are expressed on the surface of cell membrane to recognize a wide range of PAMPs found on bacteria, fungi, and viruses; while TLRs 3, 7, 8, and 9 are expressed within endosomal and lysosomal membranes to recognize primarily viruses (*45*). TLR3 is able to detect KSHV infection and activates its down-stream signaling pathways, including TBK1-mediated IFN regulatory factors as shown in Figure 1.3 (*49*).

An adaptive immunity is distinguished from innate immunity due to antigen specificity and memory (*50*). The adaptive immune responses are slower than innate immunity, involves antigen presenting and development of antigen-specific B cell and T cell responses. B cells are the major

cells involved in the creation of antibodies that circulate in blood plasma and lymph, recognizing unique antigens and neutralizing specific pathogens. Upon activation, T lymphocytes will differentiate into either cytotoxic T cells or helper T cells. The cytotoxic T cells are characterized by the cell surface CD8 (cluster of differentiation 8) expression, while helper T cells are characterized by the cell surface CD4 expression. Cytotoxic T cells will induce the death of cells that are infected with viruses (and other pathogens), or are otherwise damaged or dysfunctional. Helper T cells have no cytotoxic or phagocytic activity, and cannot kill infected cells or clear pathogens. However, they manage the immune response by directing other cells to perform those tasks (*50*).

Adaptive immunity relies greatly on innate immunity, especially for type I IFN production, for efficient maturation and activation of dendritic cells (DCs) (*51-53*), which promote antigen presenting required for driving specific T cell response. Additionally, type I IFNs, together with interleukin-2 (IL-2), IL-18, and type II IFN, are important in driving cytokines expression for the balance between the Th1 (T helper 1) T cell response and the Th2 T cell response (*54*).

Figure 1.3. **Innate immune responses upon KSHV infection and the roles of KSHV vIRFs in antagonizing virus infection.** Upon sensing of KSHV infection by TLR3, cellular IRFs, especially IRF3 and IRF7, are dimerized and activated. The activated IRFs move into nucleus to trigger type I IFN production and down-steam antiviral signaling activity. KSHV encodes vIRFs to antagonize these pathways by various mechanisms. TLR, toll like receptor. IFN, interferon. IRF, IFN-regulatory factor. *Figure and legend adapted from (49).*


KSHV
Cytoplasm
Nucleus
TLR3
CD14
Endosome
TBK1
ORF45
RTA
IRF7
IRF3
vIRF3
vIRF2
K-bZIP
vIRF1
p300
CBP
IFNα
IFNβ
IFN α/β
IFN-αR
TYK2
JAK1
STAT1
STAT2
IRF9
ISGF3
ISRE
ISG15
IRF-7
IRF-5
PKR
etc.

V. Viral Interferon Regulatory Factors

Both RRV and KSHV encode a number of genes that have suggested roles in pathogenesis, including several viral homologues of cellular genes likely pirated from the host throughout the evolution of the viruses (*17, 55*). In KSHV, these genes include homologues to IL-6 (vIL-6/ORF K2), CC chemokine ligands (vCCL-1, -2, -3/ORFs K6, K4, K4.1), Bcl-2 (vBcl-2/ORF 16), cyclinD (vCyc/ORF 72), CD200 (vCD200/ORF K14), a G-protein coupled receptor (GPCR) with homology to the IL-8 receptor (vGPCR/ORF 74), a complement control protein (vKCP/ORF4), caspase 8 (FLICE)-like inhibitory protein (vFLIP/ORF K13), and viral interferon regulatory factors (vIRF-1, -2, -3, -4/ORFs K9, K11/11.1, K10.5/10.6, K10/10.1) (*55*). Theses viral homologues of cellular genes have roles in inflammation, the IFN response, cell cycle control, and apoptosis. Specifically, all of the RFs of RRV have a homologue in KSHV, but the slightly shorter rhesus virus genome lacks homologues of K3, K5 (viral modulator of immune recognition 1/2, MIR1/2), K7 (viral inhibitor of apoptosis, vIAP), and K12 (kaposin). In addition, RRV differs from KSHV in the number of ORFs encoding the macrophage inflammatory protein (MIP)-1 (RRV has 3 and KSHV 4) and vIRFs (RRV has 8 and KSHV 4) (*56*). This thesis focuses on the role of vIRFs during RRV infection, and further analyzes key mechanisms involved in vIRF function in hopes of better understanding the role of vIRFs in KSHV-associated diseases.

The vIRFs are viral homologues of cellular IRFs, which are a family of transcription factors that direct transcription of IFN, as well as other cytokines and chemokines (*48*). There are nine characterized IRFs in primate cells, each with specific roles and expression patterns (*48*) (Table 1.2). The N-terminus of IRFs has a DNA binding domain that includes a tryptophan motif, required for binding to ISRE within promoters of IFN-responsive genes (*48*). The C-terminus of

IRFs includes protein-interaction domains that mediate interactions between other IRFs and transcriptional co-factors, and may also serve as a regulatory domain (*48, 57*).

The vIRF was named after KSHV vIRF-1, a 449 amino acid protein encoded by ORF K9 that shared high sequence similarity (~13% amino acid identity) with cellular IRF-8 and IRF-9 (*58*). Despite the similarities with cellular IRFs, vIRF-1 lacks the typical tryptophan cluster within the N-terminal DNA-binding domain that is necessary for cellular IRFs to bind to DNA and control transcriptions (*58*). However, KSHV vIRF-1 was reported to inhibit virus-induced transcription of IFN (*59, 60*), and specifically inhibit IRF-1 and IRF-3 mediated transcription in transient expression assays (*59, 61-63*). Additionally, KSHV vIRF-1 binds to the transcriptional co-activators, p300 and CBP, and inhibits the crucial binding of p300/CBP to IRF-3, as well as inhibits p300 histone acetyltransferase activity (*59, 60, 64, 65*). On the other hand, KSHV vIRF-1 also has oncogenic potential and functions to inhibit apoptosis by a variety of mechanisms, including binding to p53 and inhibiting p53-induced transcription (*66-68*).

KSHV vIRF-2 is encoded by ORF K11/11.1, constitutively expressed in latently infected PEL cells, and is induced further following induction of lytic replication (*69*). KSHV vIRF-2 was reported to inhibit type I IFN-induced transcription, as well as IRF-1 and IRF-3 mediated transcription (*70*). However, whether expression of vIRF-2 occurs naturally during KSHV infection is still under debate and the functions attributed to vIRF-2 may be insignificant in a natural environment (*69, 71-73*).

KSHV vIRF-3 is a 73 kDa protein that shares the most sequence similarity with cellular IRF-4, a lymphoid-specific IRF (*74, 75*). vIRF-3 is constitutively expressed in latently infected B cells, and in almost all KSHV-infected cells in MCD tumors. Thus, vIRF-3 has also been named

latency-associated nuclear antigen 2 (LANA-2). vIRF-3 was shown to inhibit IFN-mediated gene transcription, as well as expression of IRF-5 and p53, demonstrating that vIRF-3 does not only play a role in immune evasion but also in enhancing cellular proliferation and limiting apoptosis (*75*).

KSHV vIRF-4 is strongly induced during lytic replication, but it has not been demonstrated to be important for inhibition of IFN signaling (*69*). However, one study found that vIRF-4 was able to down-regulate p53-induced apoptosis by binding and stabilizing murine double minute 2 (MDM2), a protein that negatively regulates p53, resulting in decreased level of apoptosis (*76, 77*).

Compared with 4 KSHV vIRFs, RRV encodes 8 vIRFs in a cluster between ORFs 57 and 58, namely R6-R13 (*32, 33*). The RRV vIRFs share sequence similarity with KSHV vIRF-1, as well as rhesus cellular IRF-8 and IRF-9 (*58*). Of the 8 RRV vIRFs, R10 shares the highest similarity with rhesus cellular IRF-8 and IRF-9, about 16% and 18% identity, respectively. The other 7 RRV vIRFs share between 11-15% identity with rhesus cellular IRF-8 and IRF-9. Similar to KSHV vIRFs, RRV vIRFs do not have the characteristic tryptophan repeats that are required for DNA binding, with R8 and R12 as only exceptions, which have first 3 tryptophan position aligned with cellular IRFs and a fourth one slightly out of alignment of the fifth tryptophan in cellular IRFs (*32*). Therefore, it is hypothesized that the RRV vIRFs do not function via directly binding to promoter elements of target genes. Further sequence analysis suggested that the first 4 vIRFs (R6-R9) were probably acquired initially, and the later 4 vIRFs (R10-R13) were direct duplication of the first 4 vIRFs, respectively, although it remains a question about whether this phenomena equals to the functional redundancy of these vIRFs (*32*). As we previously reported, the RRV vIRFs are important factors in delaying host innate immune responses (*78*), as well as

the induction of type I and II IFN during RRV infection (*79*). However, the detailed molecular mechanisms the RRV vIRFs utilize still need further clarification. In this thesis, we focused on the interactions between R12, one of the 8 RRV vIRFs, and cellular promyelocytic leukemia protein (PML), which is an important multi-functional signaling protein in host cells.

Table 1.2 IRFs and their functions in the immune response

IRF	Role in Immune Response	Expression	Examples of Target Genes
IRF1	Stimulate expression of IFN-inducible genes; enhances TLR-dependent gene induction in IFN-γ	Constitutive and IFN-inducible in various cell types	iNOS, Caspase-1, TAP1, CIITA, IFN-β, IL-12p35, IL-12p40
IRF2	Antagonize IRF-1 and IRF-9; attenuates type I IFN responses	Constitutive and IFN-inducible in various cell types	Repress IFN-inducible genes, IL-12p40
IRF3	Induces type I IFN and chemokines following virus infection	Constitutive in various cell types	IFN-α1, IFN-β
IRF4	Binds to MyD88 and negatively regulates TLR-dependent induction of pro-inflammatory cytokines	Constitutive in B cells, macrophages, mDCs and pDCs	Indirectly represses IL-12p40, IL-6, TNF-α
IRF5	Binds to MyD88, and induces TLR-dependent induction of pro-inflammatory cytokines	Constitutive in B cells and DCs	Type I IFNs, IL-6, TNF-α
IRF6	Unknown	Constitutive in skin	
IRF7	Binds to MyD88 and induces type I IFNs upon TLR signaling	Constitutive in B cells, pDCs, and monocytes; inducible by type I IFN in various cell types	Type I IFNs
IRF8	Required for TLR-9 signaling in DCs (binds to adaptor, TRAF6); stimulates IFN-γ-inducible genes;promotes type I IFN in DCs	Constitutive in B cells, macrophages, CD8+ DCs, and pDCs	IL-12p40, iNOS, PML, type I IFNs
IRF9	Binds STAT1 and STAT2 to form ISGF3; stimulates transcription of type I IFN-inducible genes		IRF-1, IRF-7, PKR, OAS

Abbreviations: iNOS, inducible nitric oxide synthase; CTIIA, class II major histocompatibility complex transactivator; TAP, transporter associated with antigen processing; TRAF, tumor necrosis factor receptor-associated factor; PML, promyelocytic leukemia protein; PKR, dsRNA-dependent protein kinase; OAS, 2’5’ oligodenylate synthetase.

VI. Promyelocytic leukemia protein

The promyelocytic leukemia protein PML (also called MYL, RNF71, PP8675, or TRIM19) is a tumor suppressor that distinctively localizes to punctate nuclear structures that are interspersed between chromatin (*80*). These structures, which were previously known as nuclear domains-10, Kremer bodies and PML oncogenic domains, are now simply called PML-nuclear bodies (PML-NBs) because PML gene is the scaffold protein recruiting other protein components in nucleus to form the PML-NBs (*81, 82*). The PML protein needs to be sumoylated to be activated, and then it recruits constitutive PML-NB components, including SP100, a protein involved in transcriptional regulation, and Daxx, a transcriptional repressor (*83*). Many other proteins also reside constitutively, but more often transiently, in PML-NBs.

The primate PML gene is located on chromosome 15q22. It spans ~53,000 bases and contains nine exons (*84, 85*) (Figure 1.4). Alternative splicing of PML C-terminal exons leads to the generation of several PML isoforms, most of which localize in the nucleus as mediated by the nuclear localization signal (NLS) within exon 6 (*86*), while some other isoforms localize in the cytoplasm as mediated by the nuclear export signal (NES) within exon 9 (*83*) (Figure 1.4). Different binding interfaces and functional specificity may result from these variable C-terminus sequences in PML isoforms. PML isoform I binds the transcription factor AML1 to enhance AML-1-induced transcription (*87*) and PML isoform III interacts with the centrosome (*88*). PML isoform IV induces premature senescence through p53 binding (*89*) and promotes Myc destabilization and cellular differentiation (*90*). Additionally, a cytoplasmic isoform of PML has been shown to regulate transforming growth factor-β (TGF- β) signaling (*91*). Recently, isoform-specific PML antibodies have been generated, which will be of great use in determining the function of specific PML isoforms as well as the structure of PML-NBs (*92*).

The heterogeneity of PML-NBs enables this nuclear structure to be involved in various cellular functions, including the induction of apoptosis and cellular senescence, inhibition of proliferation, maintenance of genomic stability, and antiviral responses (*83*). To fulfill these diverse functions, PML-NBs utilizes multiple strategies, which can be categorized into three main groups: identification and storage of proteins; post-translational modification of proteins; and regulation of nuclear activities such as transcriptional regulation and chromatin reorganization.

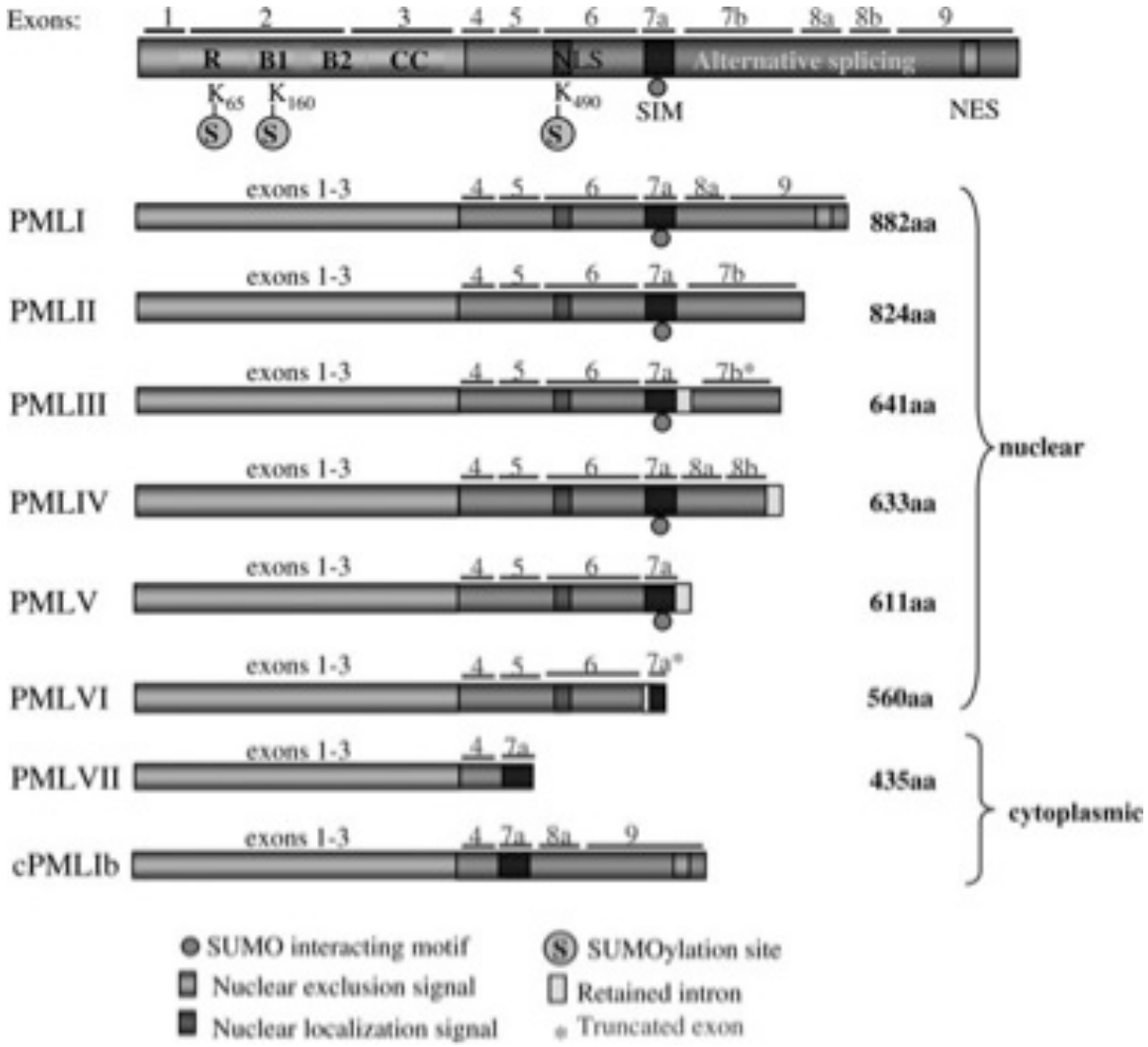
One of the most important functions of PML is to antagonize virus infection, for both DNA and RNA viruses. For instance, during herpes simplex virus-1 (HSV-1) infection, PML sequesters viral ICP0 protein in the cytoplasm, which limits viral protein accumulation and virion replication (*93*). Another example is that by using a PML/Daxx-knock out cell line, it was shown that PML and Daxx protein independently silenced human cytomegalovirus (HCMV) immediate early (IE) gene expression, thus limiting the replication and survival of HCMV in host cells (*94*). PML-NBs has also been shown to confer resistance to RNA viruses. For example, PML protein is found to inhibit transcription of vesicular stomatitis virus (VSV) (*95*) and rabies virus (*96*) genome during infection. And for poliovirus infection, PML was found to activate p53 and p53-mediated apoptosis pathway and cell cycle arresting, enabling host to have a quick response to poliovirus infection (*97*).

However, viruses are capable of counteracting PML-NBs to fire back to the host immune system. For example, the ICP0 protein of HSV-1, the IE1 protein of HCMV, the latency associated nuclear antigen 2 (LANA2) of KSHV, the E4orf3 protein of adenovirus 5, and the BLZF protein of Epstein Barr virus (EBV) all disorganize PML-NBs (*98*). In addition, at least 5 early proteins (E1, E2, E5, E6, and E7) and 3 late proteins (E1-4, L1, and L2) of human papillomavirus are all associated with PML-NBs, suggesting that PML-NBs may be the site for the initiation of viral

infection (*99*). For RNA viruses, LCMV and rabies virus infections result in alteration of PML-NBs mediated by a small nonstructural protein Z and the phosphoprotein P, respectively. In the case of Hepatitis C virus, the protein core interacts with PML protein and abrogates both p53 phosphorylation and acetylation, resulting in inhibition of PML-mediated apoptosis. Recently, it has been shown that EMCV induces PML degradation in proteasome- and sumoylation-dependent pathways (*98, 99*).

Another important function of PML is to activate p53 and increase its level, either by recruiting p53 to PML-NBs and promoting its acetylation and phosphorylation, or by binding and inhibiting MDM2, the main negative regulator of p53 (*100*). In addition, other p53 positive or negative regulators, such as protein inhibitor of activated STAT (PIAS) (*101, 102*) and herpesvirus-associated ubiquitin-specific protease (HAUSP) (*103, 104*), also get recruited to PML-NBs. So all these suggest that PML-NBs provide a scaffold for p53 regulation in a post-translational manner.

Figure 1.4. Structure of PML exons and isoforms. All PML isoforms share the first 3 exons, including the RBCC motif (R), 2 B-box (B1 and B2), and the coil-coil region (CC). PML I to PML VII differ in their C-termini due to an alternative splicing of exons 7 to 9, whereas cPML Ib results from an alternative splicing exon 4-6. PML, promyelocytic leukemia protein. RBCC, RING-B-boxes-coiled-coil. *Figure and legend adapted from(98).*


Exons:
1
2
3
4
5
6
7a
7b
8a
8b
9
R
B1
B2
CC
NLS
Alternative splicing
K65
K160
K490
SIM
NES
PMLI
exons 1-3
4
5
6
7a
8a
9
882aa
PMLII
exons 1-3
4
5
6
7a
7b
824aa
PMLIII
exons 1-3
4
5
6
7a
7b*
641aa
PMLIV
exons 1-3
4
5
6
7a
8a
8b
633aa
PMLV
exons 1-3
4
5
6
7a
611aa
PMLVI
exons 1-3
4
5
6
7a*
560aa
nuclear
PMLVII
exons 1-3
4
7a
435aa
cPMLIb
exons 1-3
4
7a
8a
9
cytoplasmic
SUMO interacting motif
Nuclear exclusion signal
Nuclear localization signal
SUMOylation site
Retained intron
* Truncated exon

VII. Project Summary and Rationale

Although there has been considerable characterization of the molecular functions of the 4 KSHV vIRFs, there has been difficulty studying their role during infection due to poor lytic replication in culture and inadequate animal models. To our knowledge, RRV is the only other virus that encodes vIRFs. Additionally, the high similarity between RRV and KSHV makes RRV infection as an ideal model for further vIRFs study.

As we stated above, PML protein is capable of regulating numerous cell-signaling proteins. In the opposite, the PML protein itself can be regulated by various internal and external treatments. Of particular interest, PML gene is an IFN-inducible gene, as IFN treatment not only increases the number of PML-NBs in nucleus but also enlarges the sizes of each PML-NBs. It has been shown by us that RRV vIRFs decreased the induction of type I and II IFN during *de novo* RRV infection. Therefore, it is important to determine whether RRV infection would further result in the disruption of PML-NBs, and whether vIRFs play important role(s) in this process. Studying the role (s) of vIRFs, and other novel viral genes, within the context of RRV infection is the most relevant way to assess their impact on the pathogenesis and immune response, with the ultimate objective to relate these findings to KSHV-associated pathologies.

VIII. Author's Contributions

All the work in Chapter 2 was performed by the author with the exception of constructing the pLVX-R12-HA plasmid (S.W. Wong).

CHAPTER 2

Viral Interferon Regulatory Factor R12 Disrupts the PML-Nuclear Body via Proteasome-Mediated Protein Degradation during *de novo* Rhesus Rhadinovirus Infection

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ABSTRACT

Rhesus macaque rhadinovirus (RRV), a monkey γ2 herpesvirus that is closely related to Kaposi's Sarcoma-associated herpesvirus (KSHV), shares great genomic and pathogenic similarities with its human counterpart, making it an ideal model to study the KSHV. Both RRV and KSHV encode a cluster of genes with significant homology to cellular interferon (IFN) regulatory factors (IRFs), and these genes were found to be involved in immune signaling, apoptosis, cellular growth and differentiation, resulting in their immune evasion and tumorigenesis. We previously reported that these viral IRFs (vIRFs) significantly hindered the host immune system and decreased type I and II IFN production during *de novo* rhesus macaques infection. Here we further demonstrated that infection of wild type RRV was capable of down-regulating the level of promyelocytic leukemia protein (PML), which is an important intrinsic immune regulatory factor involved in multiple cell signaling pathways. We then further determined that the vIRFs played an important role in this process. Moreover, compared to other seven vIRFs, the R12 was the most important factor in interacting with and down-regulating PML protein. Additionally, we found that the RRV- and R12-mediated PML protein down-regulation was proteasome-dependent, and possibly a multi-stage process: the PML protein was aggregated in the nucleus first and then degraded. We also constructed a telomerized rhesus fibroblast cells-based Tet-ON/OFF system with R12 expression under the control of doxycycline for future applications.

INTRODUCTION

Kaposi's Sarcoma-associated Herpesvirus (KSHV, also known as human herpesvirus-8, HHV-8), the causative agent of Kaposi's sarcoma (KS) in HIV-infected patients (*13*), is one of the seven known human oncoviruses. KS is caused by KSHV infection of endothelial cells, but KSHV also infects B cells permanently (*105, 106*) to suppress the host immune system and causes multiple malignant B cell disorders, including primary effusion lymphoma (PEL), and non-neoplastic lymphadenopathy, as well as a subset of multi-centric Castleman's disease (MCD) (*107, 108*). Although KSHV was isolated more than a decade ago, the difficulty of establishing an *in vivo* model for KSHV greatly slowed down the research of KSHV-host interactions (*27, 109, 110*).

Rhesus Rhadinovirus (RRV) is closely related to the KSHV, and almost every one of the open reading frames (ORFs) in the KSHV genome has a corresponding homologue in the RRV genome (*31-33*). Moreover, after establishing a latent infection in the B cells of rhesus macaques (RM) (*41*), RRV induces an acute hyperproliferation of B cells. In immune-compromised animals this leads to diseases that resemble non-Hodgkin's lymphoma and MCD in immune-compromised animals, which is similar to KSHV/HIV co-infected human patients (*34, 36, 37*). Therefore, using RRV to infect RM provides an ideal in vivo and in vitro model for studying KSHV-associated diseases (*32, 33*).

Both RRV and KSHV dedicate a great portion of their genome to encoding viral homologues of cellular genes to regulate the host immune system (*55*). In particular, both RRV and KSHV encode a cluster of viral interferon regulatory factors (vIRFs) (*32, 33, 58, 111*), the viral homologues of cellular IRFs that are critical in controlling expression of interferon (IFN) and

other pro-inflammatory factors upon virus infection (*48*). RRV encodes eight vIRFs (ORFs R6-R13) and KSHV encodes four vIRFs (vIRF1-4) in the same genomic region (*32, 33*). vIRF1-3 from KSHV have been shown to inhibit IFN expression and secretion, therefore inhibiting IFN-induced downstream anti-viral pathways (*59, 61, 63, 70, 72, 74, 112*). On the other hand, KSHV vIRF1, 3, and 4 have been shown to disrupt p53-mediated apoptosis, creating a favorable environment for virus survival (*67, 68, 75, 77*). Recently, it was reported that RRV vIRFs are capable of decreasing the induction of type I and II IFN in rhesus macaques during de novo RRV infection (*78, 79*).

Promyelocytic leukemia protein (PML) is a cellular protein with seven major isoforms (PML I-VII) that share the same N-terminus, but differ in their C-termini (*84, 85*). PML isoforms I-VI localize mainly in the nucleus, while isoform VII localizes mainly in the cytoplasm (*84, 92*). The nuclear PML isoforms, after sumoylation, function as scaffold proteins to recruit other protein components in the nucleus to form the PML nuclear bodies (PML-NBs) (*113*). Two constitutive components found to associate with PML permanently are Daxx and SP100, both of which are transcriptional regulators (*81, 113*). The PML protein is a multi-functional protein and is involved in the DNA-damage response, cell apoptosis, senescence, angiogenesis, and the host innate immune system (*83*). PML proteins maintain a steady level in normal cells; however, many viruses encode viral proteins to antagonize PML function during infection, including ICP0 protein from HSV-1 (*114*), IE1 protein from HCMV (*115*), and vIRF3 (or latency-associated nuclear antigen 2, LANA2) from KSHV (*116, 117*).

Since RRV vIRFs are capable of down-regulating IFN induction during virus infection, and IFN is capable of inducing PML protein expression (*118*), we hypothesized that RRV vIRFs are capable of down-regulating PML levels. We tested this hypothesis by infecting primary rhesus

fibroblast cells (RFs) with wild type RRV (WT-RRV) or vIRF-knock out RRV (vIRF-KO-RRV). We proved this hypothesis by showing that WT-RRV is capable of down-regulating PML protein levels in a time-dependent manner, while vIRF-KO-RRV loses this capability. We next wanted to know which one of the vIRF(s) was responsible for regulating PML protein levels, and addressed this question by transiently expressing each of the eight vIRFs individually in telomerized rhesus fibroblast (tRF) cells. We hypothesized that there should be a close interaction between PML protein and any vIRFs involved in in regulating PML protein levels. By using an immunofluorescence assay (IFA), we found that only R12 was able to co-localize with PML in transfected tRF cells, while the other 7 RRV vIRFs displayed distinctly different cellular localizations compared to PML protein. The R12-PML interaction was also confirmed by immunoprecipitation assays (IP). Furthermore, we found that R12-mediated PML protein down-regulation was both time-dependent and proteasome-dependent. At the same time, we constructed a tRF cell line that expresses R12-HA protein under the control of doxycycline (dox) treatment (the Tet-ON/OFF system). This cell line enabled us to examine the effects of R12 on PML more precisely. The work described herein is the first report to show that RRV is capable of regulating PML protein levels, and that R12 promotes the PML down-regulation via the proteasome-mediated protein degradation pathway.

MATERIALS AND METHODS

Cells, Virus, Plasmids, and Drugs

HEK 293T cells, primary rhesus fibroblasts (RF) cells and telomerized rhesus fibroblast (tRF) cells were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Ogden, UT). All cell culture incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2. Viruses used in these studies include wild-type GFP-RRV17577 (WT-RRV), and vIRF-KO GFP-RRV17577 (vIRF-KO-RRV), both of which were generated from the WT-BAC recombinant virus. All virus stocks were purified through a 30% sorbitol cushion and re-suspended in PBS, and titers were determined using standard plaque assay in RFs. Construction of plasmids pcDNA-vIRF-HA were described previously (35). All plasmids were purified from *E.coli* using the PerfectPrep EndoFree Plasmid Maxi Kit (5 Prime, Gaithersburg, MD). The cyclohexamide (CHX; Sigma-Aldrich, St. Louis, MO) stock concentration (in ethanol) was 50mg/ml, and the working concentration was 75μg/ml. The MG132 (Sigma-Aldrich, St. Louis, MO) stock concentration (in DMSO) was 10mM, and the working concentration was 10μM. The ganciclovir (GCV; Sigma-Aldrich, St. Louis, MO) stock concentration (in H_2O) was 10mM, and the working concentration was 10 μM. The cells were pre-treated with the drugs two hours before infection or transfection, and the drug remained in the medium as the infection or transfection took place, except as otherwise indicated.

Plasmid Transfections and Virus Infections of Cell Cultures

For cell culture experiments, plasmid transfections in either 6-well plates or 12-well plates were employed, with or without pre-situated coverslips. The appropriate amount of plasmids were

transfected using the TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI). For virus infection assays, a virus multiplicity of infection (MOI) of 3 was used for both WT-RRV and vIRF-KO-RRV infections. The viral stocks were diluted in complete cell culture medium, and the appropriate amount of virus was calculated to insure the correct MOI was applied. Infected cells or coverslips were collected at different time points post-infection for further analysis.

Immunoprecipitation (IP), SDS-PAGE analysis, and Western Blot

For IP, cell lysates were immunoprecipitated with a mouse anti-hemagglutinin (HA) monoclonal antibody (mAb) (H9658, Sigma-Aldrich, St. Louis, MO) or a rabbit anti-PML polyclonal antibody (pAb) (H-238; Santa Cruz Biotechnology, Santa Cruz, CA) in native lysis buffer (50 mM Tris-Cl [pH 8.0], 1% NP-40, and 150 mM NaCl supplemented with phosphatase inhibitors [100X cocktail; Sigma-Aldrich, St. Louis, MO] and protease inhibitors [100X cocktail; Sigma-Aldrich, St. Louis, MO]), followed by incubation with protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and lysates were washed extensively in radioimmunoprecipitation assay (RIPA) buffer (native lysis buffer with 0.1% SDS and 0.5% sodium deoxycholate) to remove non-specific binding proteins. The samples were then boiled in 2X sample buffer (Life Technologies Inc, Carlsbad, CA) For western blot, whole-cell extracts were collected in radio-immune-precipitation assay (RIPA) buffer, and the nuclear and cytoplasmic lysates were collected according to commercial kit protocols (NE-PER; Thermo-Scientific, Waltham, MA). The samples were analyzed by Novex 4%-12% Tris-Bis Mini Gels (Life Technologies Inc, Carlsbad, CA), and then transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA). Membranes were blocked for 1 hour in TBST (Tris-

buffered saline with 0.1% Tween-20) containing 5% milk, after which primary antibodies were added to detect specific proteins (used at a 1:1000 dilution), for overnight treatment. After a 3X 5 minutes rinse in TBST, secondary antibodies consisting of horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, 1:5000 dilution) or anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, 1:5000 dilution) or donkey anti-goat IgG (Promega, Fitchburg, WI, 1:5000 dilution) were added in TBST and 5% milk for 1 hour before TBST rinsing as described above and detection with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). The primary antibodies used in this study are: mouse anti-HA mAb (H9658, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PML pAb (H-238, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GAPDH mAb (SC-51906, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-LDH pAb (SC-33781, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-SP100 pAb (SC-16328, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Daxx pAb (D7810-.2ML, Sigma-Aldrich, St. Louis, MO), and mouse anti-RRV major capsid protein mAb (Monoclonal antibody core, Vaccine & Gene Therapy Institute, Beaverton, OR)

Immunofluorescence

Cells were grown on glass coverslips in 12-well plates and fixed with 4% paraformaldehyde in PBS (phosphate-buffered saline, 20 min at RT). Cells were then permeabilized and blocked in 5% normal goat serum (NGS)– 0.1% Triton X100 in PBS (PBST) (1 h at room temperature [RT]) prior to staining, and all subsequent steps were performed with 1% NGS–PBST. Cells on coverslips were stained with rabbit anti-human PML pAb (H-238; Santa Cruz Biotechnology,

Santa Cruz, CA) overnight at 4°C and subsequently stained with anti-rabbit IgG-Texas Red (Vector Labs, Burlingame, CA). Subsequently, cells were stained with anti-HA-fluorescein isothiocyanate (FITC) (HA-7) (Sigma-Aldrich, St. Louis, MO) (2 h at RT, 1:500 dilution), and nuclei and/or DNA was detected by using Hoechst 33258 dye (1:1000 dilution). Cells on coverslips were mounted onto slides using Vectashield (Vector Labs, Burlingame, CA) and examined on a Zeiss Axio Imager.M1 microscope (Zeiss Imaging Solutions, Thornwood, NY). Images were acquired by using a Zeiss Axiocam camera (MRm) with Axiovision software (version 4.6) and subsequently processed by using Adobe Photoshop (Adobe Systems, San Jose, CA).

RNA isolation and RT-PCR

RNA was isolated from RFs and tRF by using the RNeasy kit (Qiagen, Valencia, CA) and DNA endonuclease, RQ1, was used to remove DNA from RNA preparations (Promega, Fitchburg, WI) according to commercial kit protocols. Reverse transcription-PCR (RT-PCR) was performed by using Superscript III one-step RT-PCR with Platinum Taq (Life Technologies Inc, Carlsbad, CA). Transcripts were detected with the specific oligonucleotide pairs listed in Table 2.1.

Tet-ON/OFF System Construction

The pLVX lentivirus vector system was utilized for constructing the stable Tet-ON/OFF system. The cell lines and vectors used in this assay were obtained from Victor DeFilippis (Vaccine & Gene Therapy Institute, Beaverton, OR). The pLVX-R12-HA plasmid was constructed by

cleaving the R12-HA fragment out of the pcDNA-R12-HA plasmid with EcoR I/Not I double digestion (Life Technologies Inc, Carlsbad, CA). Next, the R12-HA fragment was ligated into the pLVX vector that was also cleaved by EcoR I/Not I double digestion. The positive clone was selected, sequenced, and verified for expressing of R12-HA. HEK 293T cells were seeded into a 6-CM dish the day before packaging. In order to produce lentiviral particles that expressed R12-HA, the pLVX-R12-HA, together with two accessory plasmids, VSVG-pMD2.G and pSPAX2, were transfected together into pre-seeded 293T cells. The cell culture media was replaced with fresh complete culture media 18 hours post transfection, and target cells were plated at the same time in a 6-well plate. The tRF cells containing the dox-responsive trans-activator were used as target cells in this assay. 24 hours after the media change, the viral supernatant from packaging 293T cells was harvested and purified by centrifugation and passing through a 0.22 μm filter. The purified supernatant was seeded on target cells together with an appropriate concentration of polybrene to facilitate infection. At 8 hours post-infection, the target cell media was replaced with fresh complete media. After the target cells were 90%-100% confluent, the packaging cells were discarded, and several wells of target cells were combined into one T-75 flask, and a low concentration of puromycin (Sigma-Aldrich, St. Louis, MO, 1 μg/ml), the selecting reagent, was added. When the selected cells acheived 90%-100% confluence, the target cells were expanded again to a T-175 flask and a high concentration of puromycin (5 μg/ml) was added to further select the positive target cells. After the target cells grew stably in a high concentration of selecting reagent, the expression of R12-HA was tested to confirm it was under dox control. The optimal concentration and length of dox treatment was also determined.

RESULTS

WT-RRV is capable of down-regulating PML protein level during virus infection

As previously reported, we found that WT-RRV infection of the RMs would decrease the induction of type I and II IFNs (*78, 79*), and that IFNs were capable of inducing PML protein expression (*118*). Therefore, we hypothesized that WT-RRV infection would down-regulate PML levels. We tested this hypothesis by infecting RF cells with WT-RRV at MOI=3, an MOI that should theoretically infect all of the cells. At different time points post-infection (2, 4, 8, 12, and 24 hours), cells were harvested and whole cell lysates were subjected to SDS-PAGE analysis, followed by western blot (Figure 1, upper panel). It was shown that PML protein level was down-regulated as the infection continued, at least to 24 hours post-infection (lane 1-6). The reason why we only infected RF cells up to 24 hours is that, at this high MOI, a high percentage (>70%) of cells start to die and detach on/after this time. The down-regulation of PML was also be observed using IFA (Figure 2). The levels of Daxx and SP100 proteins, two well-known resident proteins in PML-NBs, were also down-regulated by WT-RRV infection (Figure 1A, lane 1-6).

To further clarify whether structural or non-structural viral proteins are critical for regulating PML-NBs, we infected RF cells with WT-RRV, with or without cycloheximide (CHX) present. CHX blocks both cellular and viral protein biosynthesis, thus any intracellular proteins would be structural proteins, while non-structural proteins, including the vIRFs, would not be synthesized. At different time points post-infection (2, 4, 8, 12, and 24 hours), cells were harvested and lysed before SDS-PAGE analysis and western blot (Figure 3A). As observed, the CHX treatment selectively abolished the down-regulation of the PML protein level in WT-RRV-infected cells.

Therefore, it must be newly synthesized viral proteins, not the incoming viral structural proteins, that are essential in down-regulating PML protein levels.

vIRFs play an essential role in down-regulating PML protein levels

Previously, it has been shown that vIRFs are essential factors in down-regulating IFN induction (*78, 79*), so we hypothesized that the vIRFs are key factors in the down-regulation of PML levels during WT-RRV infection. To test this hypothesis, we infected RF cells with vIRF-KO-RRV, which was generated by the BAC-RRV system as we reported previously (*78, 79*). We used the same MOI of the mutant virus because the growth curve of the vIRF-KO-RRV is similar to that of the WT-RRV (*78, 79*). It was shown that vIRF-KO-RRV lost the capability to down-regulate PML protein levels (Figure 1A, lane 7-12). This was also true for Daxx and SP100, the two constitutive components of the PML-NBs (Figure 1, lower panels). We also used CHX to treat vIRF-KO-RRV-infected cells, and as expected, vIRF-KO-RRV lost the capacity to down-regulate the PML protein levels, regardless of CHX treatment (Figure 3B). Moreover, we used ganciclovir (GCV) to block virus replication and tested whether the defect in viral late gene expression would affect WT-RRV-mediated PML down-regulation. GCV was used to treat RF cells 2 hours pre-infection, and cells were harvested and lysed at different time points post-infection (Figure 3C-D). As seen in the western blot, PML protein levels were still down-regulated even in the presence of GCV. GCV inhibits late viral gene expression, making the viruses replication-defective; however, the initial virus infection and early viral gene expression are not affected. Thus, experiments with GCV suggested that the late viral genes are not critical for controlling PML protein levels, and that it is immediate early (IE) or early viral protein that

play an important role in PML protein down-regulation. Taken together, the newly synthesized early viral protein, the vIRFs, are important factors in down-regulating the level of PML protein as well as Daxx and SP100.

vIRF R12 interacts with and down-regulates the level of PML protein

RRV encodes eight vIRFs, so it is important to know which one of vIRFs is responsible for the PML down-regulation observed above. To clarify this, we transiently over-expressed each individual vIRF by transfecting tRF cells with eight vIRF-expressing plasmids. The expression of each of the eight vIRFs was probed via the HA tag in the C-terminus by western blots. Compared to the mock-transfected or empty vector-transfected cells, the R12-transfected cells showed a down-regulation of the PML protein levels at 48 hours post transfection (Figure 4A). However, this down-regulation of PML protein was not observed in the other seven samples of vIRF-transfected cells, indicating that it was R12 that was responsible for regulating PML protein levels. Moreover, only R12 was shown to co-localize with PML protein in transfected nuclei (Figure 4B). Next, we transiently expressed the R12 protein in tRF cells, and found that down-regulation of PML levels depended on the amount of R12 expressed and the time post-transfection (Figure 5A). This was also true for the two PML-NBs accessory proteins, Daxx and SP100. We further proved this by showing that R12 expression was capable of down-regulating nuclear PML protein isoforms, but not cytoplasmic PML isoforms (Figure 5B).

To test whether there is any interaction between the PML protein and R12, pcDNA-R12 or empty vector was transfected into tRF cells, and 24 hours post-transfection, the cells were harvested and lysed. This time point was chosen because insufficient R12-HA was expressed by

12 hours post-transfection and the PML protein level was sub-optimal by 48 hours post-transfection (Figure 5A). Next, an IP assay was performed to attempt to pull down the HA-tagged R12 protein with a polyclonal anti-PML antibody (Figure 6A). It was observed that the anti-PML antibody could pull down the R12-HA in R12 positive cells group, as compared with R12 negative groups and normal IgG groups. Moreover, we performed the IP assay in the opposite way using the anti-HA antibody to pull down PML isoforms (Figure 6B). We found that only some isoforms of PML could be pulled down by the anti-HA antibody, indicating that different isoforms of PML may have a different binding capacity or interaction with R12-HA. The interaction between the PML protein and the R12 was also verified by the IFA assay as shown in Figure 6C, as the two proteins co-localized very well in R12-positive nuclei. Therefore, we concluded that R12 was able to interact with the PML protein and was capable of down-regulating PML, Daxx, as well as SP100 protein levels in vitro.

R12-mediated PML down-regulation is proteasome-dependent

As reported previously, vIRF3 from KSHV is capable of disrupting PML-NBs, and this is dependent on the proteasome-mediated protein degradation pathway. Moreover, the RRV vIRFs are widely considered to be homologous to the vIRFs in KSHV. Therefore, we hypothesized that R12-induced PML down-regulation is also proteasome-dependent. To verify this assumption, we first tested whether the mRNA levels of the PML transcripts would be affected by WT-RRV infection. RF cells were infected with WT-RRV or vIRF-KO-RRV, and after different time points post-infection (2, 4, 8, 12, and 24 hours), we collected cells, extracted total RNA from the cells, and performed RT-PCR using gene-specific primers as shown in Table 2.1 (Figure 7). By

using the same amount of total RNA as a template, the mRNA level of each specific gene can be quantified. As the PML protein has several isoforms, we selected a primer set that would amplify a common region in all PML isoforms. Therefore, we could detect the overall level of PML transcripts, instead of just a few specific isoforms. As shown, the PML mRNA level was kept steady, despite an infection that continued up to 24 hours. Therefore, we concluded that the mRNA levels of the PML gene are not down-regulated by WT-RV infection, while the amount of R12 transcripts increased steadily over time.

We then used the proteasome inhibitor, MG132, to treat the infected cells to see whether the down-regulation of PML protein is dependent on the proteasome. The RF cells were pre-treated with an optimized concentration of MG132 for 2 hours before infection, while another group of cells were treated with DMSO in parallel as a control group. The MG132-treated or the DMSO-only-treated cells were infected by either WT-RRV or vIRF-KO-RRV for different durations (2, 4, 8, 12, and 24 hours). The MG132 or DMSO remained in the culture medium as the infection continued. Then cells were collected and whole cell lysates were subjected to SDS-PAGE analysis, followed by western blot to detect the PML protein levels (Figure 8A-B). In cells treated with DMSO, WT-RRV infection caused PML protein down-regulation, consistent with our previous observations; however, this down-regulation was not observed in cells treated with MG132. Instead, the PML protein level was slightly increased with MG132 treatment, and this was also true for MG132-treated, vIRF-KO-RRV-infected cells. Virus infection and replication, as well as cell viability, was not significantly affected by DMSO or MG132 treatment, as indicated by the steady increment of RRV major capsid protein expression and stable GAPDH expression, respectively. Therefore, we concluded that the WT-RRV induced PML protein degradation was mediated by the proteasome-dependent pathway.

To verify that it was R12 that was responsible for this proteasome-dependent PML degradation, we pre-treated the tRF cells with MG132 or DMSO as control for 2 hours before transfecting them with pcDNA-R12 or empty vector. The MG132 or DMSO stayed in the culture medium as the transfection continued. At different time points post-transfection (12, 24, and 48 hours), cells were collected and the whole cell lysates were subjected to SDS-PAGE, followed by western blot analysis (Figure 8C). We observed that in cells treated with DMSO, the presence of R12 caused PML protein levels to go down; however, the PML protein level stayed at a stable, or slightly increased, level in cells treated with MG132. Thus, we concluded that WT-RRV-mediated PML down-regulation was proteasome-mediated, and R12 played an essential role in this process.

Construction of a stable tRF cell-based Tet-ON/OFF system

To further examine the interactions between R12 and PML, and to monitor the effect of R12 expression on PML protein, we constructed a Tet-ON/OFF system, in which the expression of R12-HA is the under control of doxycycline (dox) treatment. The advantage of using this stable gene expression system is that, compared to transient gene expression, the Tet-ON/OFF system allows a more accurate control over how much and for how long the gene of interest is expressed. At the same time, a cell line that stably expresses R12 would give a much higher R12 expression efficiency, e.g., more than 90% of R12 positive cells compared to 30% of R12 positive cells in traditional transient-overexpression assays. Therefore, we could monitor the change of PML protein as the expression of R12 changed in a real time way. In order to construct this system, we first inserted the R12-HA cDNA fragment into the pLVX lentivirus vector, which is known for

its high delivery rate and minimal cell toxicity. Nexxt the pLVX-R12-HA plasmid was transfected into packaging HEK 293-T cells together with two accessory plasmids: VSVG-pMD2.G and pSPAX2, which provide necessary viral proteins to generate lentiviral virions. Then medium containing the infectious lentiviral virions was used to infect tRF cells containing stable dox-responsive trans-activators. After selecting with puromycin, the stable tRF cell line that expresses R12-HA upon dox induction was kept alive. The cell line was tested to ensure good dox responsiveness. In dox-negative cells, the R12-HA protein level was kept at a minimal level. However, in dox-positive cells, the R12-HA protein expression was increased significantly (Figure 9, lanes 1 and 2). On the other hand, with more R12 expression, the level of PML protein was significantly down-regulated, which is consistent with what was shown above. Moreover, we found that 1μg/ml of dox was sufficient to induce strong R12-HA expression (Figure 9, lanes 2-4).

DISCUSSION

KSHV and RRV dedicate a great portion of their genome to encoding immuno-regulatory proteins (*55*). Among them, vIRFs are of particular interest because they make the two viruses unique compared to other herpesviruses, which do not encode any similar genes. The KSHV vIRFs were named because of their relatively high similarity with cellular IRFs, as well as their dominant-negative effects on the cellular IRFs (*32, 33, 58, 111*). Similarly, RRV encodes 8 vIRFs that also share high homology with their cellular homologues, and R6, R7, R8, R10, and R11 vIRFs share between 19% and 26% amino acid identity with KSHV vIRF-1 (*32, 33*). Moreover, RRV has a natural infection system for *in vivo* infection, which is not the case for KSHV. Therefore, RRV becomes an ideal model to study its human counterpart.

The multi-functional PML protein, although identified decades ago, has not been paid enough attention until recently. It was found to be involved in multiple signaling pathways, especially p53-related cell cycle regulation and host anti-virus responses (*83*). As reported previously, the RRV vIRFs are important in decreasing the induction of type I and II IFN during *in vivo* rhesus macaque infection (*78, 79*). At the same time, the PML is an IFN-inducible gene (*118*), leading us to hypothesize that RRV vIRFs are also important in controlling PML protein levels. By comparing the infection of RF cells with WT-RRV and vIRF-KO-RRV, we found that WT-RRV was capable of down-regulating the levels of PML protein, while this capacity was lost in vIRF-KO-RRV. Therefore, vIRFs play an important role in down-regulating PML protein levels. To further prove this, CHX and GCV were applied to cells 2 hours prior to infection. CHX treatment was used to block the new protein expression, thus the only protein in the host cells would be inoculum-derived viral structural proteins and cellular proteins. The CHX treatment blocked the PML down-regulation caused by WT-RRV infection, suggesting that the newly synthesized viral

proteins were important factors in down-regulating PML protein levels. GCV blocks the late viral protein synthesis; thus, GCV treatment allowed for only IE and early viral proteins expression. However, PML protein levels were not affected by treating with GCV, suggesting that the late viral proteins are not as critical as IE or early viral proteins. Taken together, it is sufficient to prove that the vIRFs, the IE viral proteins, were responsible for down-regulating PML protein levels.

As there are eight vIRFs in the RRV genome, it was of interest to determine which one of vIRF(s) are indispensible for down-regulating PML protein levels. We hypothesized that, in order to down-regulate PML protein levels, the vIRFs should be able to interact with PML protein. In order to test this, we transiently expressed each of the eight vIRFs in tRF cells independently. To probe each vIRF, all of them were tagged with a C-terminus HA tag. By using the IFA assay, we clearly showed the relative localization between each vIRF and PML protein (Figure 4B). R6 expressed evenly all over the cells, but the PML protein expressed mainly in the nucleus. R8-R11, together with R13, expressed mainly in the cytoplasm, making it hard for them to regulate the nuclear PML protein. R7 and R12 expressed predominantly in the nuclear compartment; however, R7 dispersed evenly in the nucleus, while R12 formed big aggregates that were similar to PML-NBs. Additionally, IFA pictures showed clearly that the PML protein co-localized very well with R12, rather than R7. Therefore, we concluded that R12 is the most likely factor that interacts with PML protein and mediates its down-regulation.

We then transiently expressed the R12-HA again in tRF cells and examined PML levels at different time points post-transfection. It was shown that R12 itself was enough to efficiently down-regulate PML protein levels, as well as Daxx and SP100 protein levels. By separating the nucleus from cytoplasm, we confirmed that the R12-mediated PML down-regulation occurs

mostly with the nuclear PML isoforms, while the cytosol PML isoforms were not significantly affected. Furthermore, the interaction between PML protein and R12 was confirmed by IP assay. However, it was shown that only some of the PML isoforms could be pulled down by the R12-HA, suggesting that different isoforms of PML protein may have different binding capacity towards R12. Isoform-specific PML antibodies could be employed to further determine which isoforms of PML can be pulled down and which cannot. We would then be able to tell which domains of the PML protein are more important for interaction with R12.

Although we confirmed that R12 interacts with PML protein and was able to efficiently down-regulate PML protein levels, we wanted to further clarify the mechanism of down-regulation. According to previous reports, the PML down-regulation and PML-NB disruption is associated with an unusual sumoylation level (*81, 113, 119-121*). The PML itself must be properly sumoylated before being functional. However, in many cases, the PML protein level changes are accompanied by a mal-sumoylation, leading to the proteasome-dependent protein degradation pathway (*83*). Therefore, we hypothesized that the RRV- or R12-mediated PML down-regulation happens at the protein level and was mediated by proteasome-dependent pathways. We first looked at the transcripts levels of PML to see whether the WT-RRV infection would affect the PML gene transcripts. The RT-PCR assays showed that PML gene transcripts were not affected by the WT-RRV infection. Thus, we moved next to examine the protein stability level. In order to do this, we used MG132 to disrupt proteasome-mediated protein degradation. Upon MG132 treatment, the WT-RRV-mediated PML down-regulation was abrupt, and this was also true for Daxx and SP100, suggesting that the proteasome plays a critical part in the WT-RRV-induced PML protein down-regulation. Since we previously observed that R12 is the protein in WT-RRV that plays the most important role in down-regulating PML protein levels, we hypothesized that

PML protein down-regulation mediated by R12 was also proteasome-dependent. Thus, we treated R12-HA-expressing tRF cells with or without MG132, and found that MG132 blocked the R12-mediated PML down-regulation, which is consistent with the virus infection assays. Therefore, we concluded that R12 in WT-RRV plays an important role in down-regulating PML protein via the proteasome-dependent protein degradation pathway.

There are many pathways that could lead to proteasome-mediated protein degradation. In order to further determine which pathway RRV or R12 utilizes, we constructed a stable tRF cell line that expresses R12-HA under the control of doxycycline, namely the Tet-ON/OFF system. By using this system, we could control the expression of R12 more accurately and have more than 90% of cells under control, if not all, rather than relying on transient overexpression assays that have less than 50% transfection efficiency. Moreover, a low concentration of dox is enough to efficiently induce R12-HA expression, and a higher concentration of dox is not required, making the system high efficient and low toxic.

Interestingly, by taking a closer look at the IFA pictures and comparing the R12-positive cells with R12-negative cells, we observed that PML protein tended to aggregate more in R12-positive cells than in the R12-negative cells. For example, in Figure 6C, the PML protein in R12-positive cells formed several big structures, while the PML protein in R12-negative cells formed small punctuate structures that were diffused evenly in the nucleus. Therefore, it will be interesting to figure out whether the R12-mediated PML degradation is a multi-step process: PML first aggregated and then degraded. Our tRF cell-based Tet-ON/OFF system is a great tool to verify this, as the expression of R12 can be controlled accurately in this system. Moreover, it will be interesting to use this system to track a single live cell to monitor the real time interaction between R12 and PML protein in the future.

In conclusion, we showed that RRV is capable of down-regulating PML protein levels, and this is accomplished by R12, one of the eight vIRFs. Moreover, the RRV- and R12-mediated PML protein degradation is via the proteasome-mediated protein degradation pathway and is probably a multi-stage process. The innovative tRF cell-based Tet-ON/OFF system may give us a clearer way to determine exact interactions between R12 and PML proteins, both *in vitro* and *in vivo*.

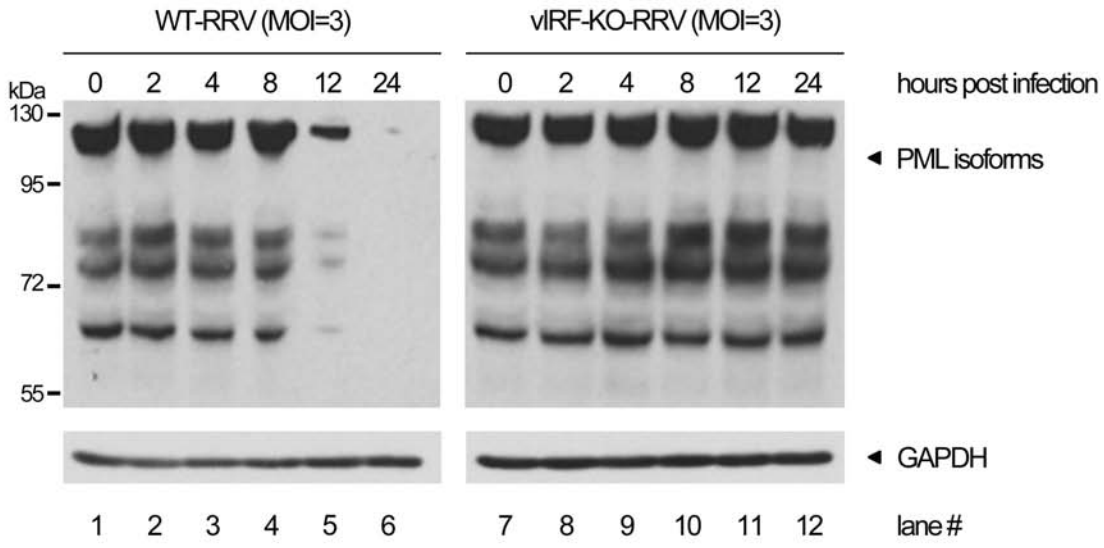
Table 2.1. Oligonucleotide sets used for RT-PCR

ORF	Oligonucleotide	Oligonucleotide Sequence 5'-3'
R3(vMIP)	R3 forward	CCT ATG GGC TCC ATG AGC
	R3 backward	ATC GTC AAT CAG GCT GCG
R12	R12 forward	ATT GTT GCG ATA ATG ATA AGC
	R12 backward	CCG GTG GCA TCC GCT TCG TTA
PML	PML forward	CTG TGC TGC TCG TGC GCT CT
	PML backward	TCC TGC GCC TGC AAG TGC GC
GAPDH	GAPDH forward	GTG GAT ATT GTT GCC ATC AAT
	GAPDH backward	ATA CTT CTC ATG GTT CAC ACC

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; ORF, open reading frame; vMIP, viral macrophage inflammatory protein; PML, promyelocytic leukemia protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 2.1. Western blots showed the difference in WT-RRV and vIRF-KO-RRV infections on PML protein levels. The same amount of WT-RRV or vIRF-KO-RRV (MOI=3) were used to infect RF cells, and cells were harvested or fixed after different time points post-infection. The harvested cells were then lysed and proteins were separated by SDS-PAGE. Western blots were used to detect the protein level of A) PML and B) Daxx and SP100. WT-RRV was shown to down-regulate PML protein level in a time-dependent manner, while vIRF-KO-RRV lost this capability.

A


WT-RRV (MOI=3)
vIRF-KO-RRV (MOI=3)
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
130
95
72
55
PML isoforms
GAPDH
1 2 3 4 5 6
7 8 9 10 11 12
lane #

B

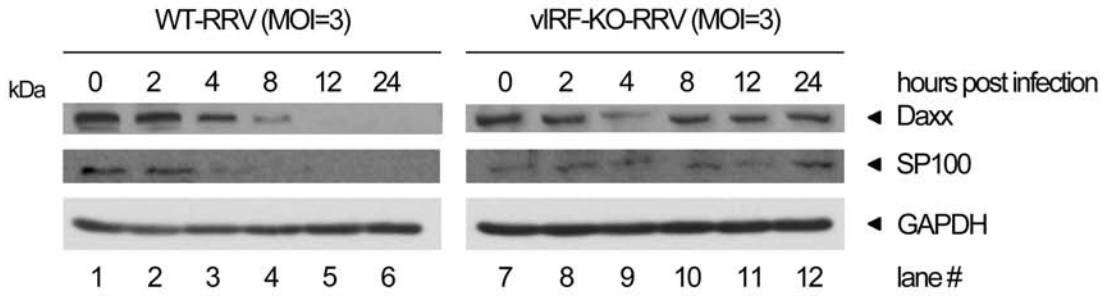
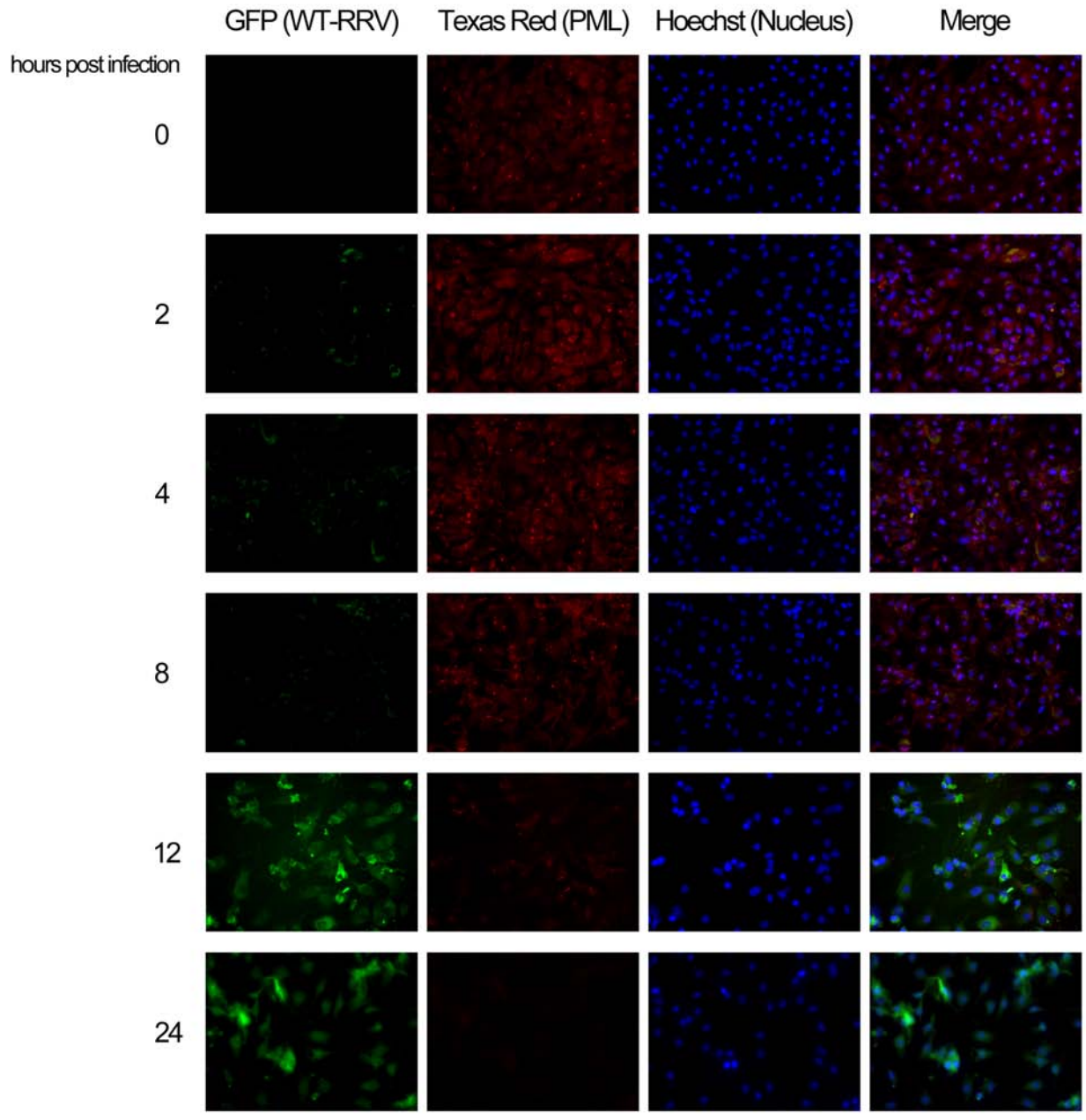

WT-RRV (MOI=3)
vIRF-KO-RRV (MOI=3)
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
Daxx
SP100
GAPDH
1 2 3 4 5 6
7 8 9 10 11 12
lane #

Figure 2.2. Immunofluorescence assays showed the difference in A) WT-RRV-GFP and B) vIRF-KO-RRV-GFP infection on PML protein levels. After plating RF cells on cover slips, the same amount of WT-RRV or vIRF-KO-RRV (MOI=3) was used to infect RF cells, and cells were fixed and penetrated following the standard IFA protocol. Then the PML protein was visualized by anti-PML protein, while the presence of viruses was visualized by the GFP tag carried by viruses. Hoechst dye was used to indicate the location of cell nuclei and the merged pictures were shown to compare the relative amount of viruses and PML protein. The pictures shown in A and B have a magnification of 20x.

A


GFP (WT-RRV)
Texas Red (PML)
Hoechst (Nucleus)
Merge
hours post infection
0
2
4
8
12
24

B

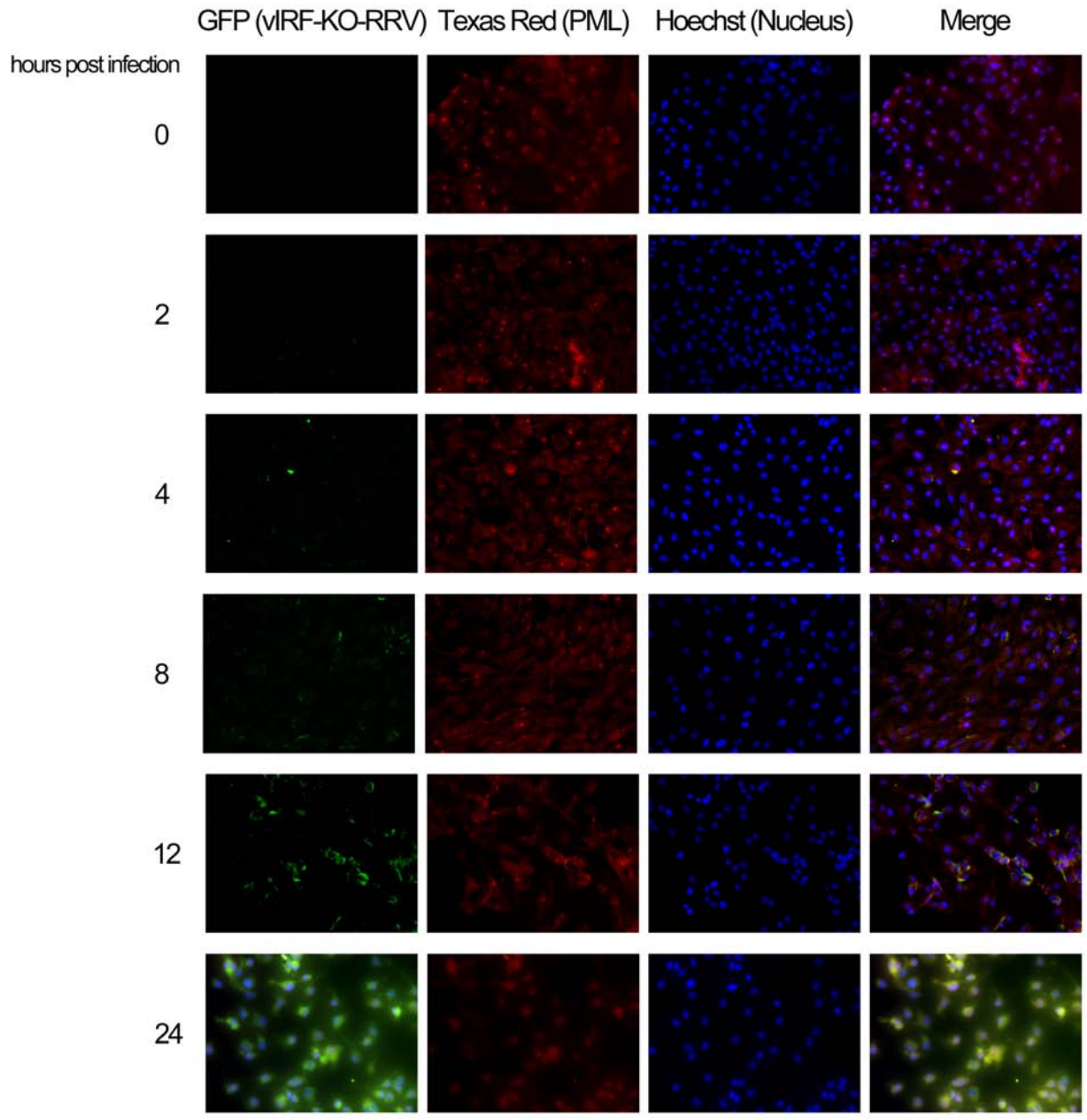
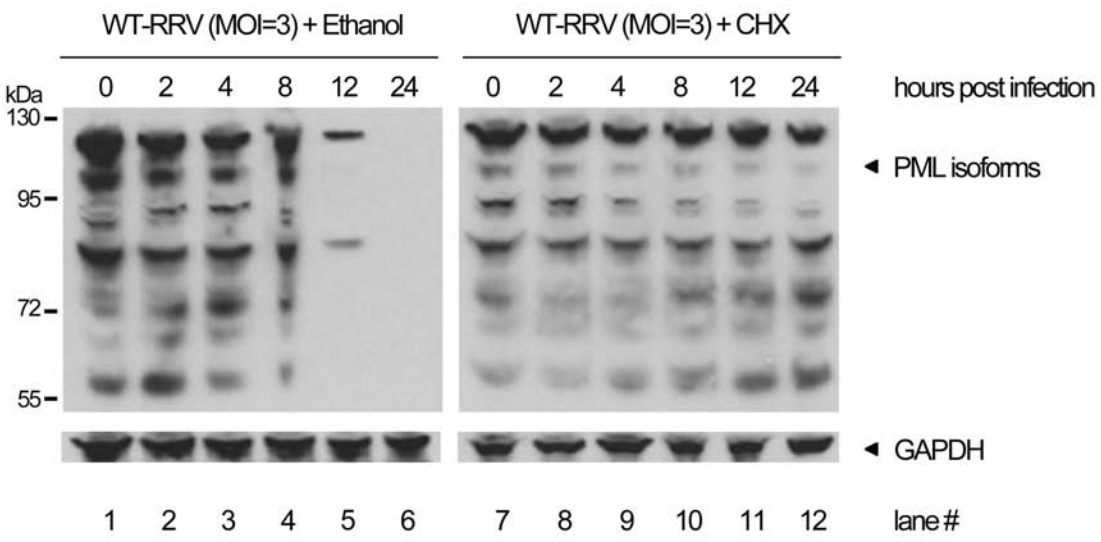
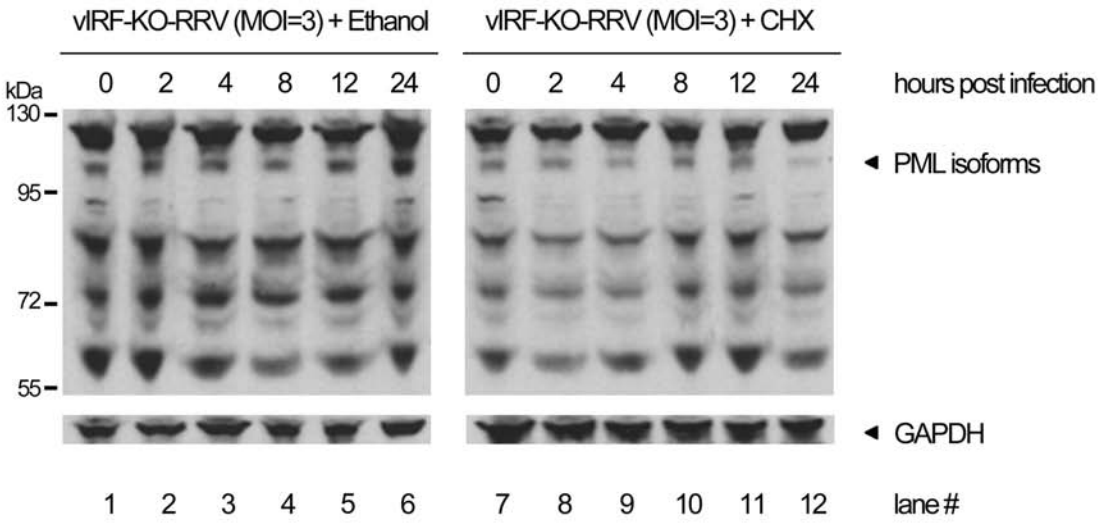

GFP (vIRF-KO-RRV)
Texas Red (PML)
Hoechst (Nucleus)
Merge
hours post infection
0
2
4
8
12
24

Figure 2.3. Cycloheximide (CHX), not ganciclovir (GCV), was shown to block the WT-RRV-mediated PML protein down-regulation. RF cells were pretreated for 2 hours with CHX/ethanol (A and B) or GCV/H_2O (C and D) before infection. The same amount of WT-RRV or vIRF-KO-RRV (MOI=3) was used to infect RF cells. The drugs were present in cell culture medium as the infection continued, and cells were harvested or fixed after different time points post-infection. The harvested cells were then lysed and proteins were separated by SDS-PAGE. Then western blots were used to detect the protein level of PML in WT-RRV-infected cells (A and C) and vIRF-KO-RRV-infected cells (B and D). The WT-RRV-mediated PML down-regulation was blocked by the presence of CHX, but not by GCV.

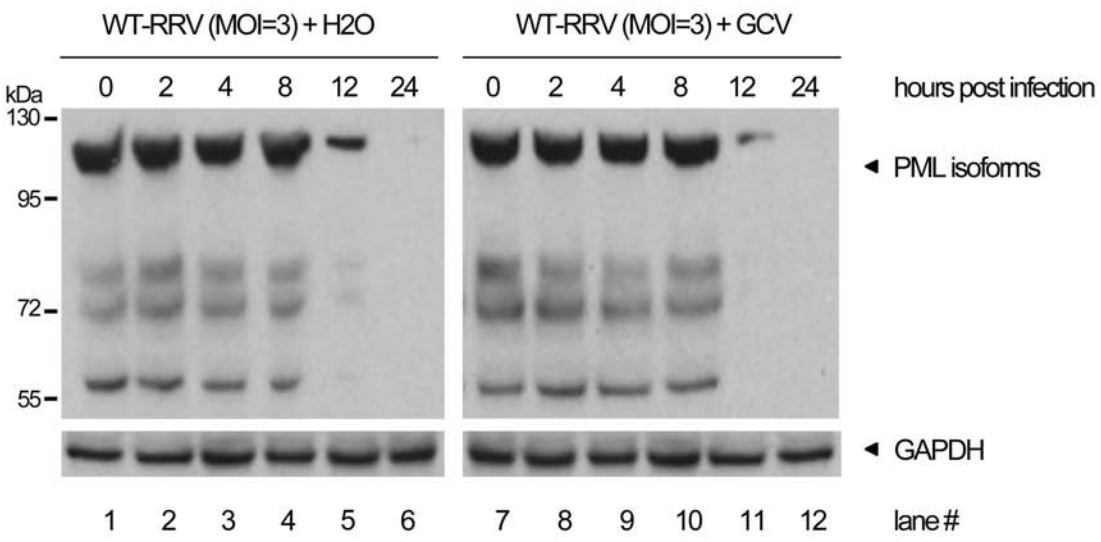
A


WT-RRV (MOI=3) + Ethanol
WT-RRV (MOI=3) + CHX
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
kDa
130
95
72
55
PML isoforms
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12
lane #

B


vIRF-KO-RRV (MOI=3) + Ethanol
vIRF-KO-RRV (MOI=3) + CHX
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
kDa
130
95
72
55
PML isoforms
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12
lane #

C


WT-RRV (MOI=3) + H2O
WT-RRV (MOI=3) + GCV
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
130
95
72
55
PML isoforms
GAPDH
1 2 3 4 5 6
7 8 9 10 11 12
lane #

D

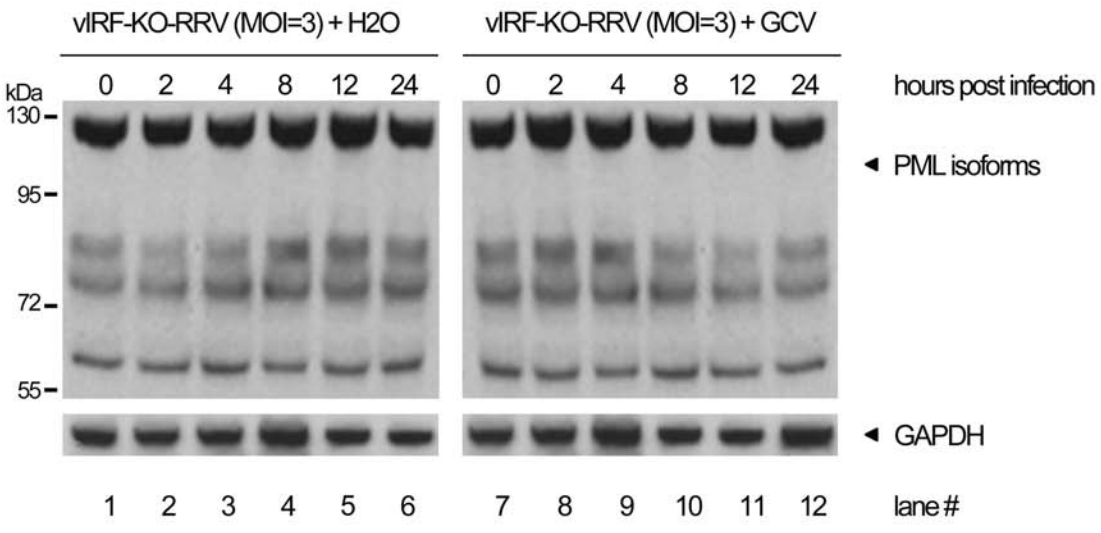
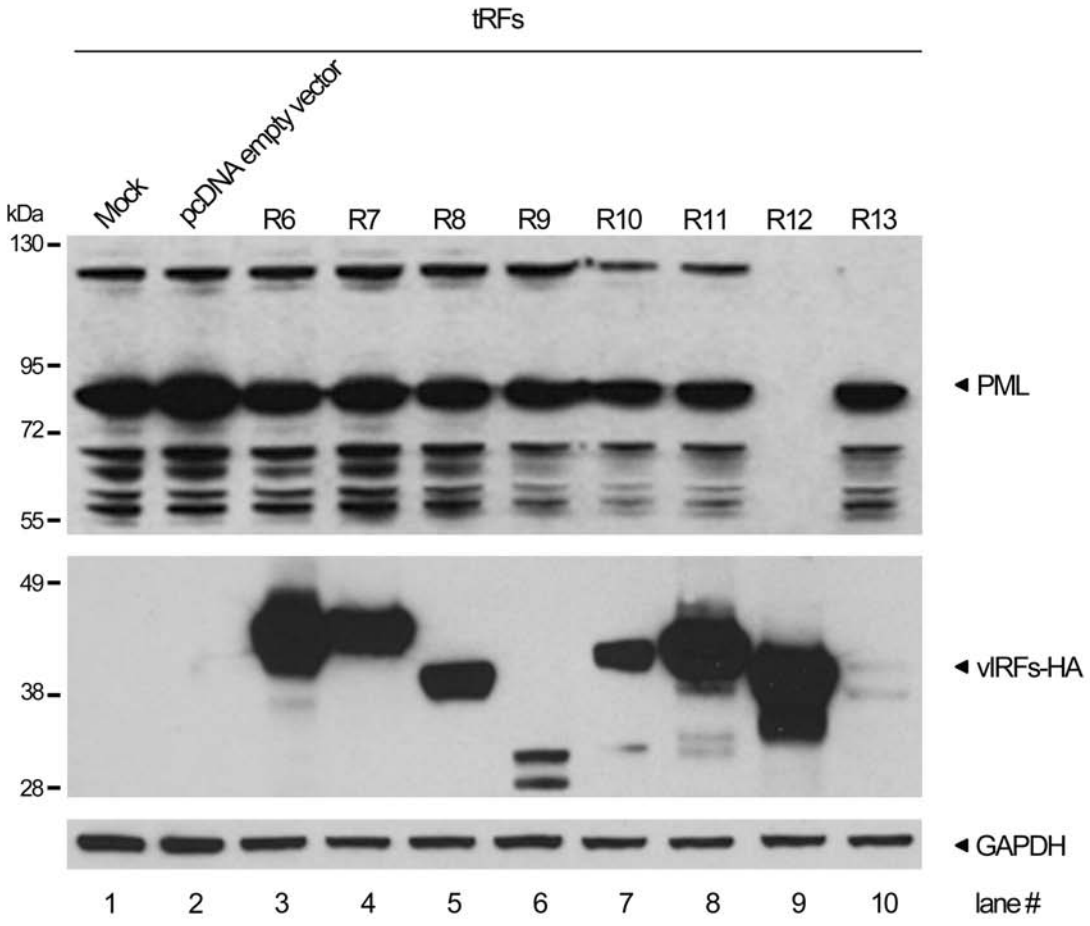

vIRF-KO-RRV (MOI=3) + H2O
vIRF-KO-RRV (MOI=3) + GCV
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
130
95
72
55
PML isoforms
GAPDH
1 2 3 4 5 6
7 8 9 10 11 12
lane #

Figure 2.4. Only R12 was shown to down-regulate PML protein level and co-localize with PML protein in transfected nucleus. A) tRF cells were mock-transfected or transfected with pcDNA-empty vector or each of eight pcDNA-vIRFs-HA. 48 hours post-transfection, cells were harvested for western blots to detect PML protein and each of eight vIRFs. B) tRF cells were plated on coverslips in 12-well plates for 24 hours before being transfected with each of eight pcDNA-vIRF-HA plasmids. The coverslips were collected 48 hours post-transfection and processed for IFA assays to visualize PML protein and each of eight vIRFs. The pictures shown in B have a magnification of 63x under oil lens. Only R12 was shown to significantly down-regulate PML protein levels, and efficiently co-localize with PML protein in transfected nucleus.

A


tRFs
Mock
pcDNA empty vector
R6
R7
R8
R9
R10
R11
R12
R13
kDa
130
95
72
55
49
38
28
PML
vIRFs-HA
GAPDH
1
2
3
4
5
6
7
8
9
10
lane #

B

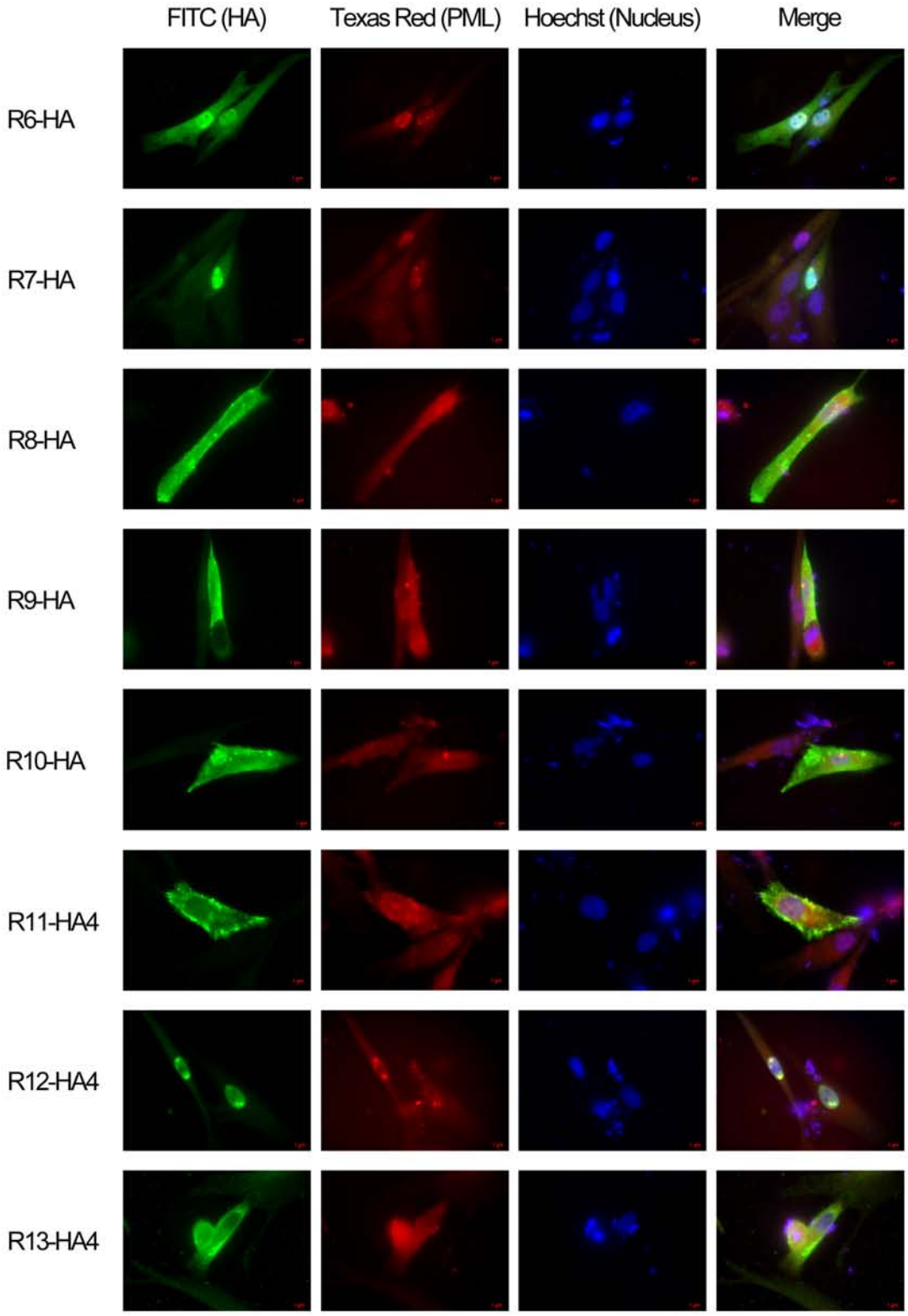
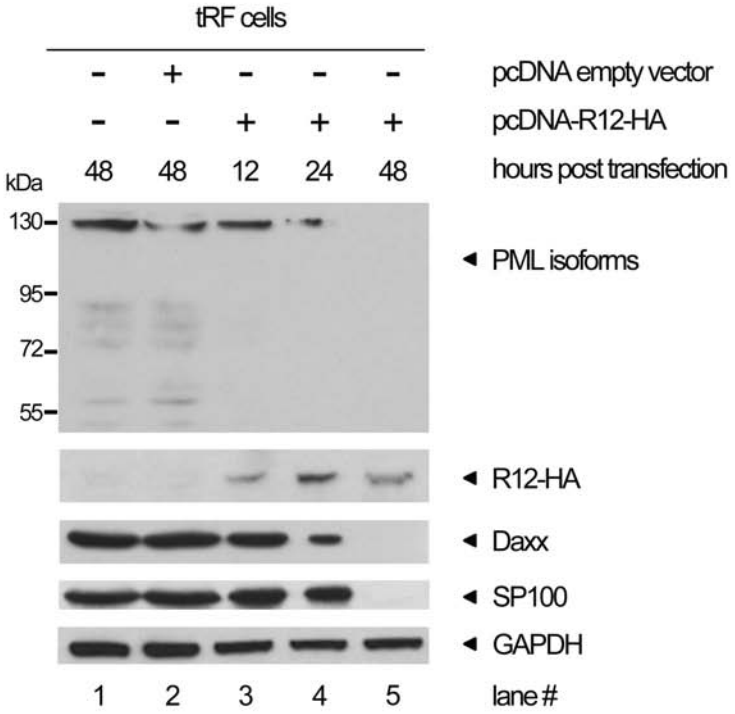

FITC (HA)
Texas Red (PML)
Hoechst (Nucleus)
Merge
R6-HA
R7-HA
R8-HA
R9-HA
R10-HA
R11-HA4
R12-HA4
R13-HA4

Figure 2.5. R12-mediated PML protein down-regulation was time-dependent and happened only to nuclear PML protein. A) tRF cells were mock-transfected (lane 1), or transfected with pcDNA-3.1 empty vector (lane 2), or transfected with pcDNA-R12-HA plasmid (lane 3-5). Mock- and empty vector-transfected cells were harvested 48 hours post-transfection, while pcDNA-R12-HA transfected cells were harvested 12, 24, or 48 hours post-transfection. The cells were then lysed and analyzed by SDS-PAGE, followed by western blots. B) pcDNA-3.1 empty vector- or pcDNA-R12-HA-transfected cells were harvested 48 hours post-transfection. Then the cell nucleus and cytoplasm were separated as described, followed by SDS-PAGE and western blot analysis. Figure A showed that R12-mediated PML down-regulation was time-dependent, and figure B showed that R12 only down-regulated nuclear PML protein level, not the PML isoforms in cytoplasm.

A


tRF cells
- + - - -
- - + + +
48 48 12 24 48
kDa
130
95
72
55
pcDNA empty vector
pcDNA-R12-HA
hours post transfection
PML isoforms
R12-HA
Daxx
SP100
GAPDH
1 2 3 4 5
lane #

B

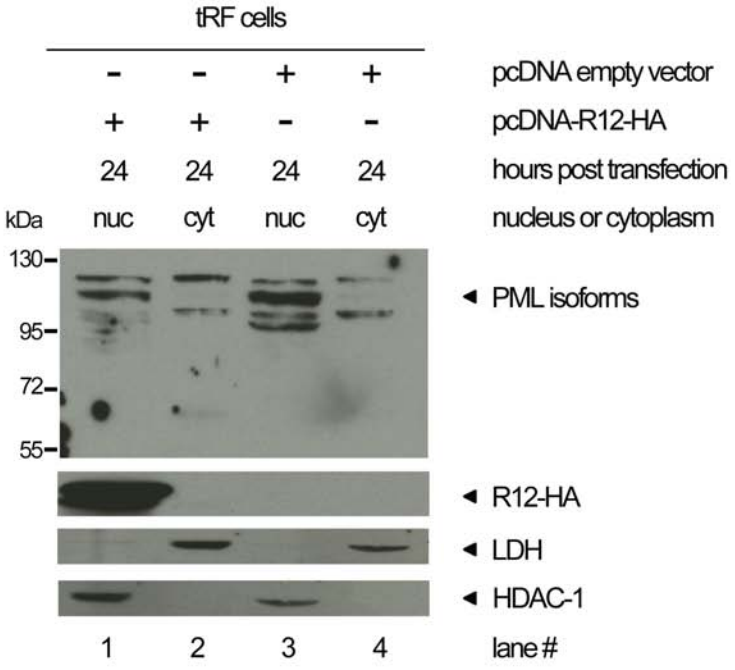
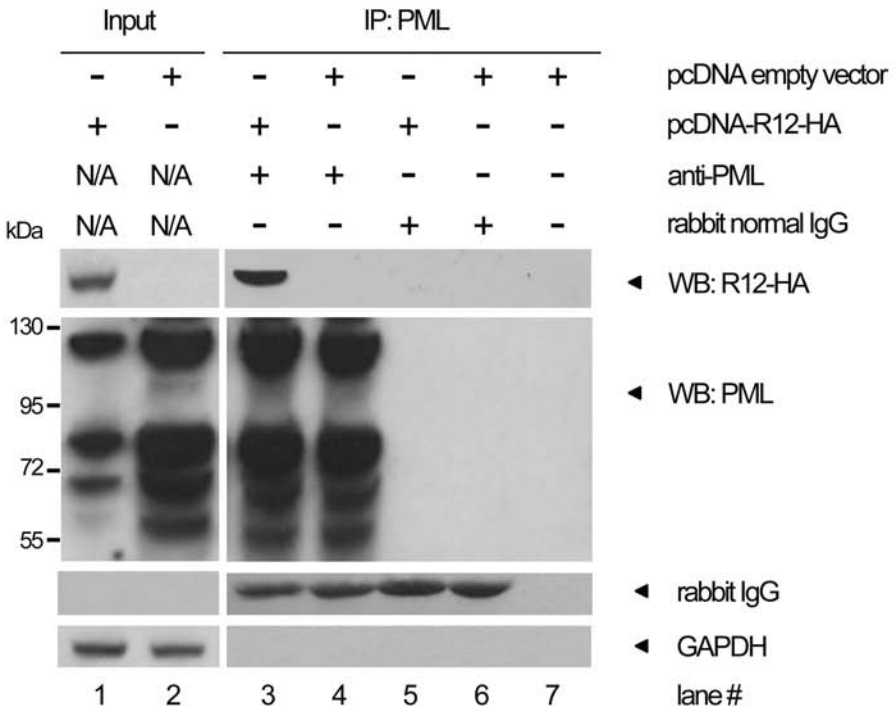
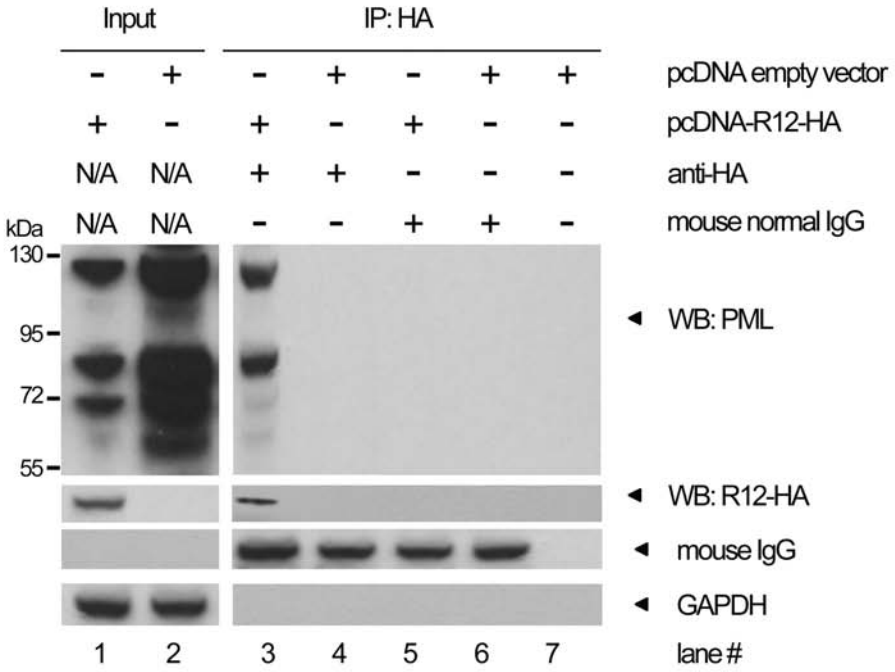

tRF cells
- - + +
+ + - -
24 24 24 24
kDa
nuc cyt nuc cyt
130
95
72
55
pcDNA empty vector
pcDNA-R12-HA
hours post transfection
nucleus or cytoplasm
PML isoforms
R12-HA
LDH
HDAC-1
1 2 3 4
lane #

Figure 2.6. R12 was shown to interact and co-localize with PML protein in transfected nucleus. pcDNA-3.1 empty vector- or pcDNA-R12-HA-transfected tRF cells were harvested 24 hours post-transfection. The same amount of proteins were applied to the immunoprecipitation assays by either anti-PML antibody (A) or anti-HA antibody (B). R12 was shown to interact with PML protein in transfected tRF cells. C) tRF cells were plated on coverslips in 12-well plates for 24 hours before being transfected with pcDNA-R12-HA plasmids. The coverslips were collected 24 hours post transfection and processed by IFA assays to visualize PML protein and R12-HA. The pictures shown in C have a magnification of 63x under oil lens. R12 was shown to co-localize with PML protein in transfected nucleus of tRF cells.

A


Input
IP: PML
pcDNA empty vector
pcDNA-R12-HA
anti-PML
rabbit normal IgG
kDa
WB: R12-HA
130
95
72
55
WB: PML
rabbit IgG
GAPDH
lane #

B


Input
IP: HA
pcDNA empty vector
pcDNA-R12-HA
anti-HA
mouse normal IgG
kDa
130
95
72
55
WB: PML
WB: R12-HA
mouse IgG
GAPDH
lane #

C

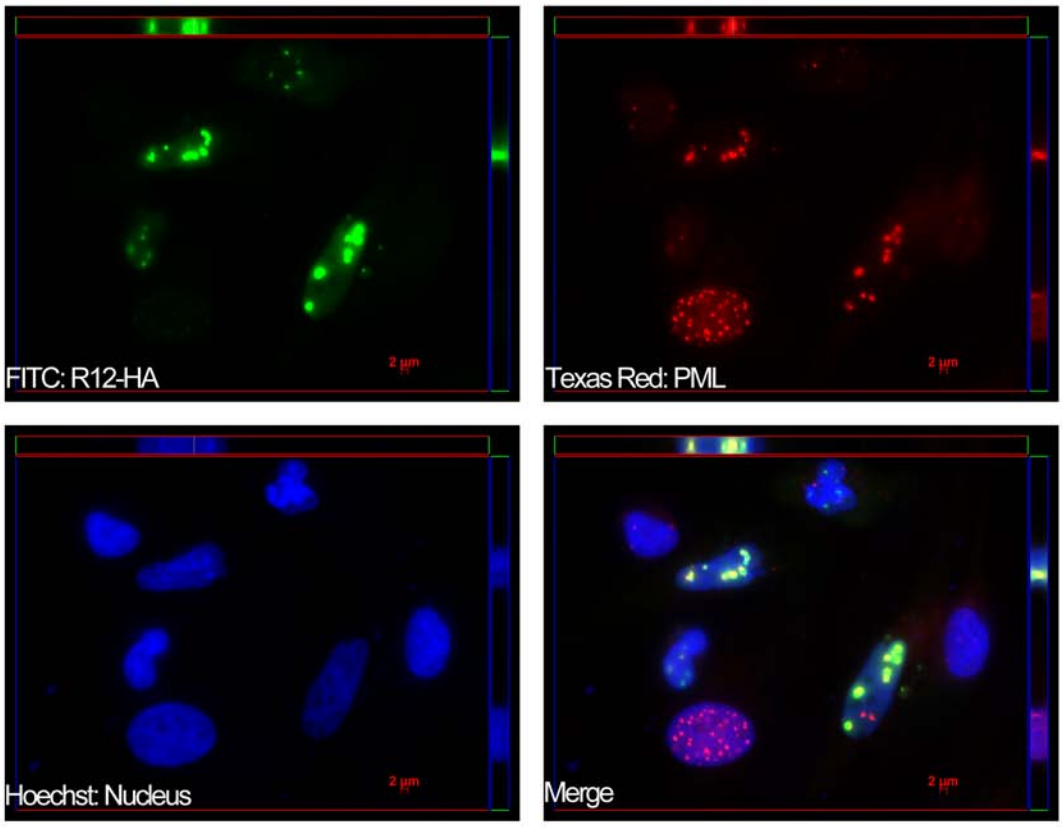

FITC: R12-HA
2 µm
Texas Red: PML
2 µm
Hoechst: Nucleus
2 µm
Merge
2 µm

Figure 2.7. WT-RRV infection did not alter PML transcript level. The same amount of WT-RRV or vIRF-KO-RRV (MOI=3) was used to infect RF cells, and cells were harvested after different time points post-infection. The harvested cells were then lysed and total RNA was isolated. The same amount of total RNA from each group was used as templates to do the reverse transcriptase PCR (RT-PCR) with primers shown in Table 2.1. Then the RT-PCR products were analyzed by agarose electrophoresis. It was shown that PML transcript level was not altered by WT-RRV or vIRF-KO-RRV infection.

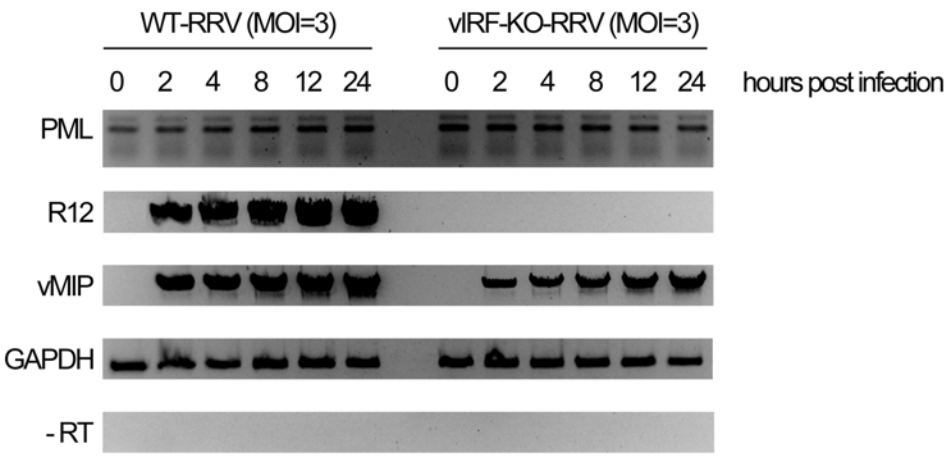
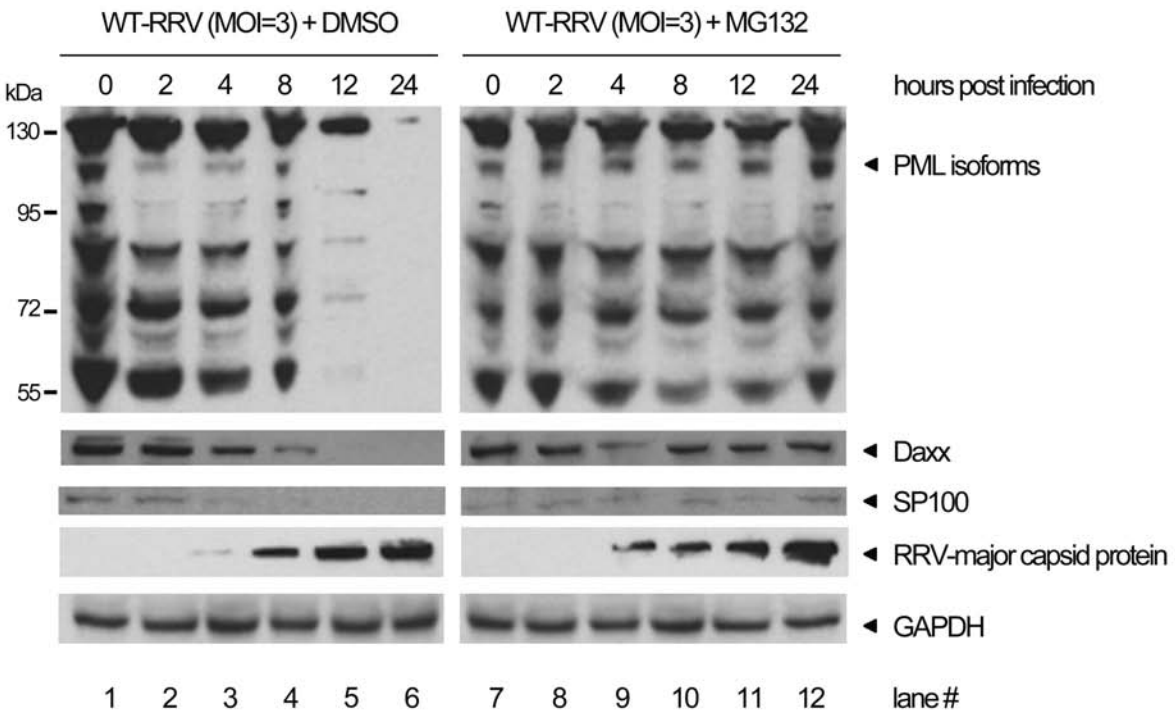
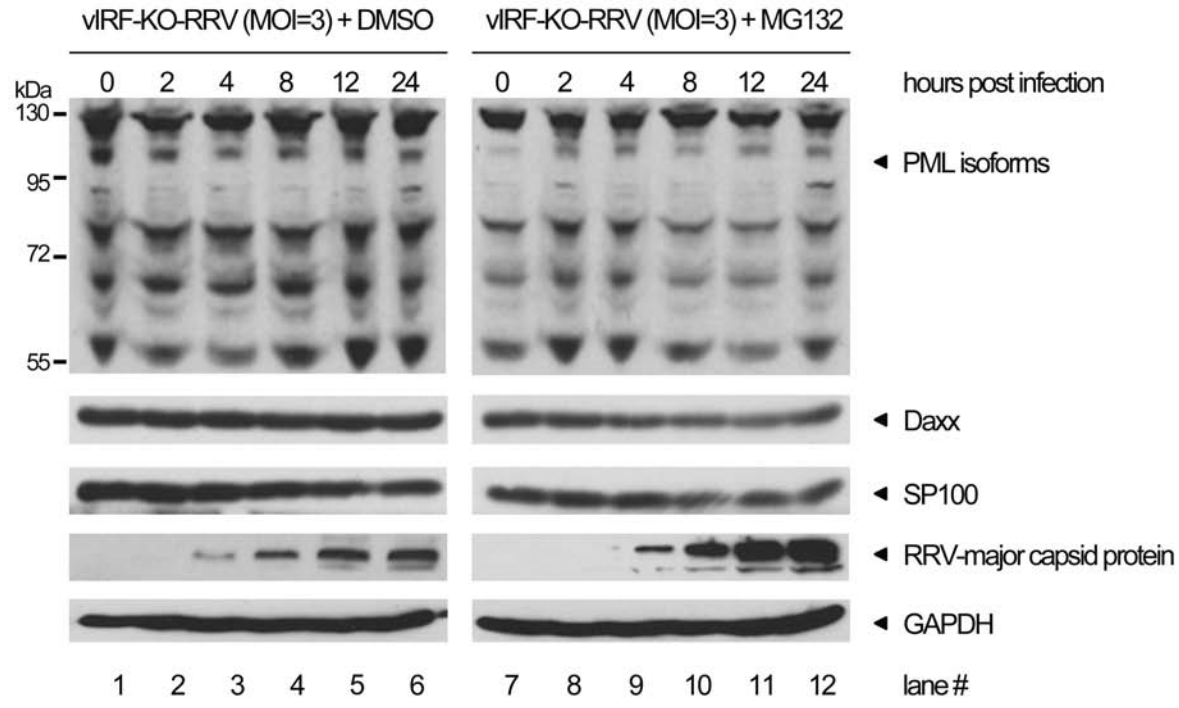

WT-RRV (MOI=3)
vIRF-KO-RRV (MOI=3)
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
PML
R12
vMIP
GAPDH
- RT

Figure 2.8. R12-mediated PML protein down-regulation was proteasome-dependent. RF cells were pretreated with DMSO or MG132 2 hours before infection. Then the same amount of WT-RRV (A) or vIRF-KO-RRV (B) (MOI=3) was used to infect RF cells in the presence of DMSO or MG132. After different time points post-infection, cells were harvested and lysed. Cell lysis was applied to SDS-PAGE followed by western blot analysis. WT-RRV-mediated PML down-regulation was blocked by the presence of MG132. C) tRF cells were non-treated or treated with DMSO or treated with MG132 2 hours before transfection. Then cells were either mock-transfected or transfected with pcDNA-3.1 empty vector or pcDNA-R12-HA. The cells were harvested 12, 24, or 48 hours post-transfection and cell lysis was obtained, which was then applied to the SDS-PAGE and western blot analysis. Inhibiting the proteasome by MG132 would disrupt the R12-mediated PML degradation.

A


WT-RRV (MOI=3) + DMSO
WT-RRV (MOI=3) + MG132
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
130
95
72
55
PML isoforms
Daxx
SP100
RRV-major capsid protein
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12
lane #

B


vIRF-KO-RRV (MOI=3) + DMSO
vIRF-KO-RRV (MOI=3) + MG132
kDa
0 2 4 8 12 24
0 2 4 8 12 24
hours post infection
130
95
72
55
PML isoforms
Daxx
SP100
RRV-major capsid protein
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12
lane #

C

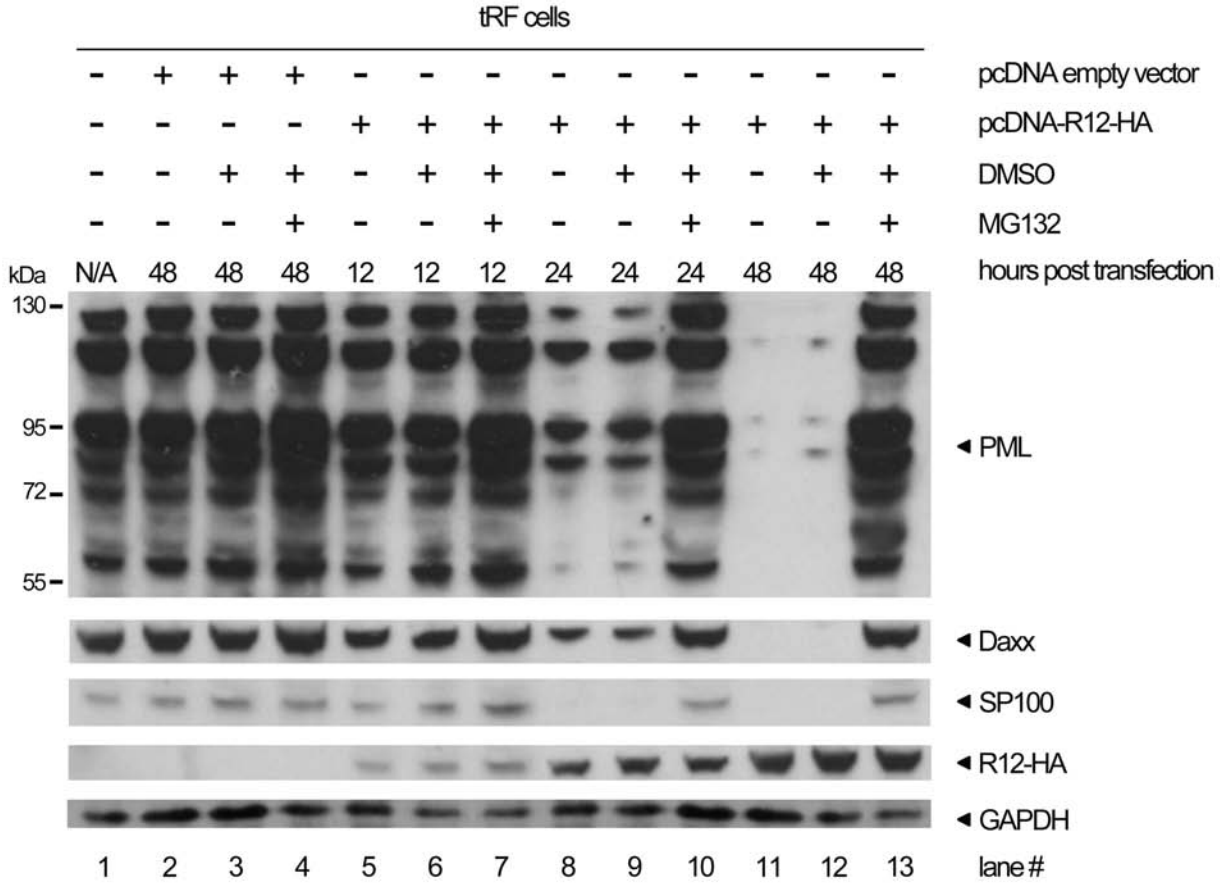
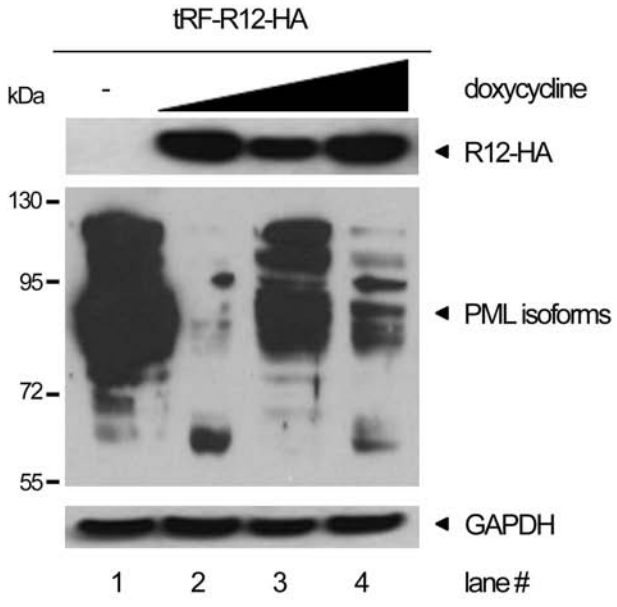

tRF cells
pcDNA empty vector
pcDNA-R12-HA
DMSO
MG132
kDa
N/A 48 48 48 12 12 12 24 24 24 48 48 48
hours post transfection
130
95
72
55
PML
Daxx
SP100
R12-HA
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13
lane #

Figure 2.9. Doxycycline (dox)-induced R12 expression in tRF cell-based Tet-ON/OFF system.

tRF-R12-HA cells were plated 24 hours in complete medium together with different concentrations of dox: 0 in lane 1, 1μg/ml in lane 2, 2μg/ml in lane 3, and 4μg/ml in lane 4. Then cells were harvested and lysed for western blot to detect R12-HA expression and PML protein levels. 1μg/ml of dox was sufficient to induce R12-HA expression, while higher concentrations of dox did not give better R12-HA induction.


tRF-R12-HA
kDa
-
doxycycline
R12-HA
130
95
72
55
PML isoforms
GAPDH
1
2
3
4
lane #

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

I. The role of R12 and other vIRFs during RRV infection

In chapter 2 of this thesis, we compared the infection of primary rhesus fibroblast (RF) cells with wild type RRV (WT-RRV) or with vIRF-knock out RRV (vIRF-KO-RRV). The WT-RRV infection down-regulated the cellular PML protein levels, which was not observed in the vIRF-KO-RRV infected RF cells, suggesting that vIRFs play an important role in mediating the PML protein levels. To strengthen this conclusion, we treated the RF cells with cycloheximide (CHX), which blocked the viral and host gene synthesis, and it was shown that CHX blocked the WT-RRV-induced PML protein down-regulation, suggesting that the newly synthesized viral proteins are of great importance. However, ganciclovir (GCV) treatment did not block this down-regulation, suggesting that the late viral genes are not key parts in this process. Therefore, the vIRFs, as the IE gene, play an important role in down-regulating PML protein level during WT-RRV infection.

In order to further clarify which one of the 8 RRV vIRFs is the most important in down-regulating PML protein levels, we transiently expressed each of the 8 vIRFs in telomerized rhesus fibroblast (tRF) cells, each of which was tagged with a C-terminus HA tag. It was shown that R12 itself was sufficient enough to down-regulate PML protein levels, while other 7 vIRFs expression was not coupled with PML down-regulation. And this down-regulation was time-dependent. Moreover, the immunofluorescence assay (IFA) was used to show that R12 was most

likely to co-localize with PML protein in cell nucleus, and immunoprecipitation assay (IP) confirmed the biochemical interaction between R12 and PML protein.

In order to further determine how PML protein level was down-regulated, we then compared the PML transcripts level upon WT-RRV and vIRF-KO-RRV infections. As shown, the WT-RRV or vIRF-KO-RRV infection did not alter the PML transcripts level, suggesting that the WT-RRV-induced PML protein down-regulation does not happen in the transcription level. We then hypothesized that it was protein stability that caused the PML protein down-regulation. This hypothesis was confirmed by the fact that MG132, a proteasome inhibitor, blocked the WT-RRV-induced PML protein down-regulation, and suggested that this is a proteasome-dependent process. Furthermore, the MG132 also blocked the R12-mediated PML protein down-regulation, which happened in a transient overexpression context. Taken together, the RRV-induced PML protein down-regulation is mediated by R12, one of the 8 vIRFs, and this is dependent on the proteasome-mediated protein degradation pathway.

We then observed that PML-NBs become aggregated in R12-expressing nucleus, indicating the PML degradation may be a two-step process: PML was aggregated and then degraded. To test this possibility and to overcome the low transfection efficiency problem caused by transiently expressing R12 in tRF cells, we constructed a tRF-based Tet-ON/OFF system that would express R12-HA upon doxycycline (dox) induction. The system was proved to work by showing that R12-HA was significantly induced by low concentration of dox and that PML protein levels was down-regulated upon R12-HA expression. We will discuss the usage of this powerful Tet-ON/OFF system in the next section.

II. Future Directions

The findings presented in this thesis clearly demonstrate that the RRV vIRFs, especially R12, play an important role in promoting the cellular PML protein into proteasome-dependent protein degradation pathways. These data offer important strides in understanding the roles of vIRFs during virus infection and diseases, but also propose new questions to investigate.

First, it will be important to determine which one of the proteasome-dependent pathways does the PML go into, as there are numerous E3 ligases encoded by cellular and viral genes. Interestingly, KSHV encodes at least 2 viral E3 ligases, K3 and K5, whose homologue were not present in RRV genome. Thus, it will be important to figure out whether RRV- or R12-induced PML degradation is mediated by a cellular E3 ligase or by an unknown RRV viral E3 ligase.

Secondly, as we stated above, PML aggregation was observed in R12-positive cells, thus, it will be interesting to determine whether PML protein degradation is a 2-step process: aggregation and degradation. We have already constructed a tRF-based Tet-ON/OFF system, and we could utilize this system to monitor the interaction between R12 and PML in a single live cell, which will give us direct evidence as how R12-mediated PML protein degradation happens. We are currently working on refining the Tet-ON/OFF system to generate a more homogeneous one: turning it into a monoclonal system with all cells synchronized into the same life cycle phase, which will give us more convincing data on the interaction between R12 and PML protein.

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