Characterization of Host Responses to Vaccinia Virus Infection

by

Trung Phuoc Huynh

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2013 by the committee
Graduate Supervisory Committee:

Bertram Jacobs, Chair
Brenda Hogue
Yung Chang
Tatiana Ugarova

ARIZONA STATE UNIVERSITY

May 2013

ABSTRACT

Vaccinia virus (VACV) is the current vaccine for the highly infectious smallpox disease. Since the eradication of smallpox, VACV has been developed extensively as a heterologous vaccine vector for several pathogens. However, due to the complications associated with this replication competent virus, the safety and efficacy of VACV vaccine vector has been reevaluated. To evaluate the safety and efficacy of VACV, we study the interactions between VACV and the host innate immune system, especially the type I interferon (IFN) signaling pathways. In this work, we evaluated the role of protein kinase R (PKR) and Adenosine Deaminase Acting on RNA 1(ADAR1), which are induced by IFN, in VACV infection. We found that PKR is necessary but is not sufficient to activate interferon regulatory factor 3 (IRF3) in the induction of type I IFN; and the activation of the stress-activated protein kinase/ c-Jun NH_2-terminal kinase is required for the PKR-dependent activation of IRF3 during VACV infection. Even though PKR was found to have an antiviral effect in VACV, ADAR1 was found to have a pro-viral effect by destabilizing double stranded RNA (dsRNA), rescuing VACVΔE3L, VACV deleted of the virulence factor E3L, when provided in trans. With the lessons we learned from VACV and host cells interaction, we have developed and evaluated a safe replication-competent VACV vaccine vector for HIV. Our preliminary results indicate that our VACV vaccine vector can still induce the IFN pathway while maintaining the ability to replicate and to express the HIV antigen efficiently. This suggests that this VACV vector can be used as a safe and efficient vaccine vector for HIV.

DEDICATION

To my parents.

Thank you for everything. Without your never-failing love, support and encouragement

this would never happened.

ACKNOWLEDGEMENTS

and giving me the opportunity to do research in his lab. I learned how to ask scientific question, to do science and to teach science from him.

Finally, I would also like to thank my committee members, Dr. Brenda Hogue, Dr. Yung Chang, and Dr. Tatiana Ugarova for all their advice, questions, constructive recommendations and comments on my projects.

TABLE OF CONTENT

LIST OF FIGURES

OVERALL INTRODUCTION

Infectious diseases are caused by pathogens such as bacteria, viruses, parasites or fungi. Fortunately, many diseases can be prevented through vaccination, one of the most effective public health interventions. Vaccination was first introduced by Edward Jenner in the 1700s (164), and was the foundation of the eradication of smallpox, a highly dangerous and contagious pathogen, in the 1970s. In our lab, we study and develop vaccinia virus (VACV) as a vaccine vector for several infectious pathogens such as the Human Immunodeficiency Virus (HIV), and the African Swine Virus (ASV). In order to develop a safe and efficient VACV vaccine vector, we study the interactions between VACV proteins and host cellular proteins. These studies give us the insight into genetically engineering VACV as a safe and efficient vaccine vector.

Viruses are obligate intracellular pathogens that require the host cellular machinery for their survival. In contrast the host cells have developed several mechanisms to prevent viruses from establishing successful infection. One of these mechanisms is the interferon (IFN) system of the innate immune system. IFNs were the first cytokine discovered to have the ability to interfere with virus survival (80). The IFN system is classified into three types (24) in which type I IFNs (IFN-α/β) are the most well studied and are known primarily to inhibit viral replication (9). The innate immune system relies primarily on type I IFNs as its first line of defense against viral infection (171, 176).

The IFN signal transduction pathway consists of two signaling cascades: a virus-induced IFN production signal and an IFN receptor-mediated secondary signal to establish an antiviral state (78, 206). The virus-induced IFN production signal is initiated

1

by the detection of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs). PAMPs are components or byproducts of microorganisms during theirs life cycle inside the host. The most common PAMP in viral infection is viral nucleic acids, including viral genomic deoxy-ribose nucleic acid (DNA), single stranded ribose nucleic acid (ssRNA), and double stranded RNA (dsRNA) (2). These PAMPs can be recognized by four classes of pathogen recognition proteins (PRPs): toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytoplasmic DNA sensors (89). TLRs and RLRs have been shown to be important in regulating the induction of type I IFNs (221), which is a critical cytokine of the antiviral response, while NLRs have been shown to regulate the production of interleukin-1 (IL-1) (86, 153).

TLRs are found on the cell surface or within the lumen of endosomes or lysosomes. TLRs are type I transmembrane proteins in which the N-terminal is outside of the membrane. They consist of an extracellular ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain, known as the Toll-interleukin 1 receptor homology domain (TIR) (21). Currently, there are 10 members of mammalian TLR (1) in which TLR3, TLR7, TLR8 and TLR9 have been shown to recognize only viral nucleic acids (27), thus suggesting its role in prevention of viral infection. These TLRs are found within the lumen of endosome, lysosome and ER (101, 139). TLR3 can recognize various form of dsRNA such as synthetic polyinosinic-polycytidylic acid (poly IC), genomic dsRNA such as reovirus (RSV) (7, 118), or byproduct dsRNA made during the course of VACV, encephalomyocarditis virus (EMCV) infection and West Nile virus (WSV) infection (7, 45, 208). TLR7 and TLR8

are phylogenetically similar, both recognize ssRNA and are mostly activated during the course of RNA virus's infections (52, 74). In contrast, TLR9 recognizes unmethylated 2′-deoxyribo cytidine–phosphate–guanosine (CpG) DNA motifs presenting in several DNA viruses such as Herpes simplex virus 1 and 2 (HSV1/HSV2) (94, 113) and mouse cytomegalovirus (MCMV) (94). All known TLRs, with the exception of TLR3, signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88) leading to the activation of nuclear factor kappa B (NF-κB), interferon regulatory factor 7 (IRF7) and mitogen-activated protein kinases (MAPKs), which induce inflammatory cytokines (1, 83). TLR3 signals through the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) (220) lead to the activation of NF-κB, MAPKs, and especially interferon regulatory factor 3 (IRF3) to induce expression of IFN-β (90). With the inductions of IFN-β and various inflammatory cytokines, the infected cells can induce an antiviral state against the incoming viral infection.

RLRs are cytoplasmic proteins that can detect viral RNA (221) in various cell types (87). Three members of this family have been identified: RIG-I (223), melanoma differentiation-associated gene 5 (MDA5) (85) and laboratory of genetics and physiology 2 (LGP2) (167). These RLRs consist of two tandem N-terminal caspase-recruitment domains (CARDs), a DExD/H box RNA helicase domain, and a well conserved C-terminal repressor domain; in contrast, LGP2 only consist of one CARD domain (196). LGP2 has been suggested to be a negative regulator of the RLRs signaling (167, 222). Although both RIG-I and MDA-5 are interferon inducible proteins, RIG-I detects short dsRNA and 5'-triphosphate ssRNA while MDA5 detects long dsRNA. Upon detection of viral RNA, the CARDs of RIG-I/MDA5 associate with the CARD of the mitochondrial

adapter protein IFN-β promoter stimulator-1 (IPS-1) (155), which is also known as mitochondrial antiviral signaling (MAVS), virus-induced signaling adapter (VISA), or CARD adapter inducing IFN-β (CARDIF) (91, 128, 178). In turn, activated IPS-1 associates with tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) (169) which recruits and activates TANK-binding kinase (TBK-1), and inhibitor of NF-κB kinase ε (IKKε) (75, 179). Activated IKKε subsequently phosphorylates IRF3 (60) leading to the formation of IRF3 homodimer, which translocates into the nucleus (157) and bind to positive regulatory domain (PRD) regions of the IFN promoter, resulting in the expression of type I IFNs.

Once the expressed IFN-α/β is secreted outside of the infected cells, it can bind to the IFN-α/β receptor (IFN-α/βR) of the same cell for an autocrine effect or they can bind to the IFRNs of an adjacent cell for a paracrine effect. The IFN-α/βR composes of two subunits: IFNAR-1 and IFNAR-2 (140, 190, 205). Biding of IFNs to its receptor activates the downstream Janus kinase (JAK) pathway (190). These kinases in turn phosphorylate their downstream substrates which are the signal transducer and activator of transcription (STAT) (105, 190). This leads to the formation of IFN-stimulated gene factor 3 (ISGF3) (23, 48), which consists of STAT1, STAT2 and IRF9. This complex translocates into the nucleus, binds to the IFN-stimulated response element (ISRE) and induces the transcriptions of several IFN-stimulated genes (ISGs) (50, 190). Most of ISGs serves as antiviral component during the course of viral infection.

Protein kinase R (PKR) is among those ISGs that have antiviral effects. It is expressed constitutively and ubiquitously in most cells and is highly expressed upon IFN induction (168). It is composed of two N-terminal dsRNA binding motifs (dsRNA-BM)

4

and a C-terminal serine/threonine kinase domain (127). Activation of PKR is through

binding to dsRNA, followed by homodimerization and autophosphorylation in the kinase

domain (41). In turn, activated PKR phosphorylates its target, the α subunit of eukaryotic

initiation factor 2 (eIF2α), at serine 51 leading to the inhibition of global protein synthesis

(112, 142). Thus, PKR can inhibit virus survival by inhibiting viral protein synthesis. It

has been suggested that IRF3 can be activated in a PKR-dependent manner (228).

Chapter 1 of this dissertation will confirm this finding in the vaccinia virus system.

PKR has also been reported to be involved in MAPK signaling, especially p38

and stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK) (8, 69, 79,

195). P38 and SAPK/JNK are among the stress-associated kinases that are activated by

UV radiation, detection of bacteria liposaccharides (LPS), or dsRNA during viral

infection (11, 217). They are also key components of the innate immune system,

especially the interferon pathway, due to their roles in activation of AP-1 family

transcription factors c-Jun and ATF-2, which lead to the induction of several pro-

inflammatory cytokine genes (88, 110). Since there was evidence linking PKR, p38,

SAPK/JNK to the activation of IRF3 (226, 228), we asked if these kinases are required

for the activation of IRF3. This work is described in chapter 1.

Another important antiviral ISGs is the 2'-5'-oligoadenylate synthetase

(OAS)/RNase L, which can also be activated by dsRNA (162, 185). Upon binding to

dsRNA, OAS uses Adenosine Triphosphate (ATP) to synthesize 2'-5'adenylate

molecules, called 2-5A, that subsequently activate the latent endonuclease RNase L

resulting in the degradation of viral messenger RNA (mRNA) and cellular mRNA and

ribosomal RNA (rRNA) (61, 218) thus leading to an arrest of protein synthesis (216).

RNase L has been reported to have antiviral effects in several viruses (184). RNase L can also induce the production of IFNs by activation of RIG-I/MDA5 to furthermore induce the antiviral state during viral infection (115). By working in synergy, PKR and OAS ensure the inhibition of viral protein synthesis.

Although the main function of ISGs is to establish the antiviral state, there are ISGs that can be either antiviral or proviral, dependent upon virus and host combination. The human adenosine deaminase acting on RNA (ADAR) protein is an example. ADAR was first discovered in *Xenopus laevis* to have the ability to destabilize dsRNA structure (15, 161). It belongs to a family of RNA editing enzymes that catalyze the C-6 deamination of adenosine (A) to yield inosine (I) in RNA substrates with dsRNA character (13, 138, 202). Because inosines are read as guanosines (G) during translation, ADAR editing activity can lead to codon changes in mRNA that subsequently alters protein function (109, 175). ADAR has two types of A-to-I editing processes: the first one is highly site-selective with the adenosine deamination occurring at one or very few specific A's in an RNA while the second one can occur at multiple adenosines in RNA substrate with stable duplex structure (13, 70, 138, 202).There are three members of the ADAR gene, ADAR1, ADAR2, and ADAR3 (13, 14, 65, 114, 138, 202) of which ADAR3 lacks the deaminase enzymatic activity (40, 123). Although ADAR2 is ubiquitously expressed in most tissues (123), it is most abundant in brain tissue (122). Its editing activity is primarily isolated to cellular pre-mRNA because of its nuclear localization (51, 173). Furthermore, ADAR2 editing activity has not been reported for viral RNA.

ADAR1 is the most studied member of the ADAR family. There are two forms of ADAR1 due to alternative promoters (148), the constitutively and ubiquitously expressed p110 and the IFN inducible p150 (66, 149). Both forms of ADAR1 consist of a C-terminal deaminase catalytic domain, three dsRNA binding motifs, RI, RII and RIII, in the central region (148, 170). However, the p150 form possesses two Z nucleic acid binding domain (Z-NA-BD), Zα and Zβ, while the p110 only has a Zβ domain (76, 148, 174) (Figure 1). The p150 form has been found shuttling between the cytoplasm and the nucleus (56, 62, 156, 191) whereas the p110 form is found predominantly in the nucleus (148); therefore, the A-to-I editing by p150 might occur in both cytoplasm and the nucleus while p110's editing activity might occur only in the nucleus. The A-to-I editing activity of ADAR1 has been reported in various RNA viruses such as measles virus (35), human parainfluenza virus (136), respiratory syncytial virus(117), influenza virus (200), lymphocytic choriomeningitis virus (LCMV) (225), Riff Valley virus (192), mumps virus (36), hepatitis C virus (198), and HIV (54) and a DNA virus, mouse polyoma virus (95). A-to-I viral RNA editing has been shown to have a proviral effect in certain RNA viruses such as measles virus (201) and HIV (42, 54, 154), and an antiviral effect in some RNA viruses such as influenza (200, 209), LCMV (225), and hepatitis C virus (198). Our previous data suggested that there is an IFN-inducible protein that can inhibit late eIF2α phosphorylation in vaccinia virus infection (99); we hypothesized that ADAR1 is the responsible protein for this phenomenon. We explored the role of ADAR1 in VACV, an *Orthopox* DNA virus, in Chapter 2.

The genus *Orthopoxvirus* is one of the 8 genera of the *Poxviridade* family (131). The *Orthopoxvirus* genus is the most studied of the poxvirus family due to the variola

virus (VARV), the causative agent of smallpox, being a member of this genus. Smallpox

has been eradicated since 1980 (212) due to vaccination, a concept that was first

introduced by Edward Jenner who discovered that cowpox virus could be used

successfully to protect humans against smallpox (164). The vaccination concept is based

on the foundation that members of this genus are antigenically related, so exposure with

any orthopoxvirus can protect against exposure with any other orthopoxvirus. Since the

19th century, another member of this genus has replaced cowpox as a vaccine for

smallpox, vaccinia virus. VACV is now the most widely studied and prototypic poxvirus.

Poxviruses are a family of large brick-shaped, enveloped, double stranded DNA

viruses that have a wide host-range and can infect both vertebrates and invertebrates

(131, 132). Unlike other DNA viruses, poxviruses replicate entirely in the cytoplasm of

the infected cells (131). Poxviruses' genome can vary from about 134kb to more than 300

kb and has a hair pin loop of inverted terminal repetitions that connect the two DNA

strands (12, 64). The genome encodes for about 200 proteins in which nearly 100 genes

are conserved among the members (204). These genes are involved in viral replication

and are general located in the center of the genome (204). In contrast, genes that locate in

the termini of the genomes are less conserved, but play a critical role in virus-host

interactions such as the ability to evade host immune responses. Since different

poxviruses have different immune evasion genes, it has been suggested that these genes

were acquired during poxvirus-host adaptation (177).

Like any other poxvirus, VACV encodes several immune evasion genes in order

to suppress the host innate immune system, especially the IFN system. Thus, VACV has

an IFN resisting phenotype. These proteins can inhibit the action of IFN by preventing

IFN from binding to its natural receptor, or blocking the virus-induced IFN production signaling cascade. Two VACV proteins have been identified to prevent the binding of IFNs to their receptors: B19R and B8R (3, 6, 187). The B19R gene, or B18R in Western Reserve (WR) strain, encodes for an early expressed 60-65 kilo Dalton (kDa) IFN-α/βR-like glycoprotein that shares significant homology to cellular IFN-α/βRs (44) and can bind and inhibit type I IFN, specifically (3). The protein can bind to human type I IFN with high affinity (108) but only binds to mouse type I IFN with low affinity (193). The protein can be secreted off the cells or can be embedded on the cell surface of infected cells (3, 203). There are VACV strains that do not express B19R such as VACV strains Lister and modified vaccinia virus Ankara (MVA), and some strains express a truncated version of B19R with a greatly reduced affinity for type I IFN, such as Wyeth strain or ACAMBIS2000® strain (ACAM2000®) (193). B19R has been categorized as a virulent gene because virus lacking B19R is attenuated in both intranasally and intracranially infected mice despite the fact that it binds to mouse IFN with low affinity (44, 193).

The B8R gene encodes an early expressed 43 kDa glycoprotein that share homology to the lunar domain of the type II IFNR and can bind and inhibit type II IFN (4). Unlike B19R protein, B8R protein is only secreted from infection cells as a homodimer (4). Even though it has a broad specificity to type II IFNs from human, cow, rat, rabbit and horse, it cannot bind and inhibit mouse type II IFN (5, 133, 134, 194). The virulent factor of B8R has yet to be determined due to conflicting data. Originally, deletion of B8R resulted in an attenuated virus in the rabbit model (188). However, deletion of B8R showed no difference in virulence in the mouse model (194) but another report has shown that deletion of B8R resulted in an attenuated virus in an intranasally

infected mouse model (207). Due to their functions, both B19R and B8R have been deleted out in several VACV vaccine vectors as a way to improve their immunogenicity (92).

In addition to blocking the IFN signaling by preventing the binding of IFNs to their receptors, VACV can inhibit the IFN signaling cascade of TLRs and RLRs which induce the production of IFNs upon detection of viral PAMP. The most common PAMP produced by VACV is dsRNA (45). Like any other DNA viruses, VACV makes dsRNA as a consequence of transcription of genes from both strands of the genome (55). In VACV, termination of intermediate and late transcription lacks control, resulting in several long run-on intermediate/late transcripts that have heterogeneous 3' ends and are longer than the expected transcript (16). These transcripts can hybridize with early transcripts or other intermediate/late run-on transcripts from genes encoded on the opposite strand of the DNA to form dsRNA which leads to signaling through TLR and RLR. VACV genes have been identified to encode proteins that block the upstream and downstream pathways of TLR/RLR signaling such as A46R (189), A52 (26), and K7 (84). These proteins have been shown to target the MyD88/TRIF/TRAF pathway (26, 73, 84, 189) and TBK1/IKKε pathway (84).

VACV also encodes a set of immune evasion genes that can inhibit the intracellular action of IFN to induce an antiviral state. These proteins, in particular, are the products of the E3L, H1L, and K3L genes. The H1L gene encodes the phosphatase VH1, which has been shown to dephosphorylate STAT1 and STAT2 (116), key components of the ISGF3 which induce transcription of several antiviral ISGs. The E3 and K3 proteins are intracellular proteins that block the action of the IFN-induced PKR

and OAS activated by dsRNA (49). Activation of these proteins leads to inhibition of

both viral and cellular protein synthesis in infected cells as described above. K3L encodes

for an early 10.5 kDa protein that act as a pseudo-substrate of PKR to inhibit

phosphorylation of eIF2α (20, 32), hence allowing viral protein synthesis. K3L is

responsible for the IFN^R phenotype of VACV and expression of K3L in

encephalomyocarditis virus (ECMV), an IFN sensitive virus (IFN^S), infected cells can

rescues the virus from the antiviral effect of IFN (181). VACV deleted of K3L (VACV

ΔK3L) is IFN^S (20).

In contrast to K3L, VACV E3 protein blocks IFN actions by sequestering dsRNA

and prevents the activation of PKR and OAS (39, 166) and possibly the TLR and RLR

signaling cascade. The virulent E3L gene encodes for two early proteins, p25 and p20.

These proteins share homology to the family of dsRNA binding proteins. The full length

p25 form of E3L is a 190 amino-acid-long protein that consists of two separate domains,

an N-terminal Z-NA-BD and a C-terminal dsRNA-BM (29) that resemble those of

ADAR1 as described above. The truncated p20 form of E3L is missing the first 37 amino

acids from the N-terminus due to leaky scanning and translation initiation at a second

downstream start codon (39). E3L has been conferred to the IFN^R phenotype of VACV.

Expression of E3 in infected cells with vesicular stomatitis virus (VSV), an IFN^S virus,

can rescue the virus from the antiviral effect of IFN (181). E3L is also necessary for the

broad host-range phenotype of VACV and contributes to VACV pathogenesis. VACV

deleted of E3L (VACVΔE3L), is IFN sensitive in both human and rabbit cell lines (10,

17, 38), has a restricted host range (17, 19, 38), and is greatly reduced in pathogenesis

(29). In mouse models, VACVΔE3L is apathogenic in both intranasal and intracranial

models (29). In cell in culture system, VACVΔE3L results in the activation of PKR and

OAS by dsRNA, hence inhibiting viral and cellular protein synthesis in HeLa cells. It can

also lead to cellular induced apoptosis in HeLa cells while wild type VACV (VACV wt)

does not (103).

Several studies have been done to map the IFN^R ability, PKR inhibition, and

pathogenesis to the two domains of E3 (Figure 2). VACV pathogenesis in C57BL/6

mouse model has been mapped to both N-terminal and C-terminal regions of the gene

(28, 29). Even though the C-terminal dsRNA-BD of E3 was originally thought to be

responsible for inhibition of PKR and IFN resistance (10, 38), new evidence has

suggested that the N-terminal Z-NA-BD is also necessary for inhibition of PKR and IFN

resistance both in mice and in mouse embryonic fibroblasts (MEFs) (99, 215). The host-

range phenotype of E3L has been mapped to the C-terminal also (17, 19, 38), but recent

studies in our lab have shown that deletion of the Z-NA binding domain, or any mutation

of the N-terminal, can result in a narrow host range phenotype in JC murine

adenocarcinoma cell line (Trainor and Jacobs, in preparation). More domains have been

identified within E3 besides the Z-NA-BD and dsRNA-BD. Within the dsRNA-BD of E3

there are certain amino acids, which are not required for dsRNA binding, can bind

directly to PKR (166, 180), suggesting that E3 can inhibit PKR directly. There is also a

domain from amino acid 117 to 164 that can inhibit IRF3 phosphorylation independent of

dsRNA-BD (Blattman and Jacobs, in preparation). Taken together, VACV E3L protein

plays a critical role in VACV' evasion of the innate immune system, especially the IFN pathway,

by sequestering dsRNA and inhibiting PKR, OAS and IRF3 pathway (Figure 3).

Following the eradication of smallpox, VACV has been developed as a vaccine vector for several pathogens. This is feasible due to the fact that it is relatively easy to generate recombinant VACVs expressing heterologous genes (130, 145). VACV has been proven to be an efficient vaccine vector due to its successful use as a rabies vaccine to eliminate rabies in wild animals in parts of the United States and Europe (31, 146, 147). Therefore, it is logical to develop VACV as a vaccine vector for human pathogens, such as HIV.

The human immunodeficiency virus is a *lentivirus* in the *retroviridae* family, and is the cause of the acquired immunodeficiency syndrome (43, 213), a condition in which an individual's immune system is progressively weakened to the extent that common opportunistic infections (O.Is) can thrive, and the individual succumbs to these O.Is. Since its emergence in 1981, HIV has become a pandemic affecting human life in several aspects. Even though anti-retroviral therapy has successfully improved HIV patients' lives, according to the World Health Organization, as of 2011, there are 34.0 million people living with AIDS. Thus, the need of an HIV vaccine is unquestionable.

VACV virus has substantial support to be used as an HIV vaccine candidate since the HIV trial in Thailand (163). Although VACV has great potential as a vaccine vector, safety issues have been the universal concern in VACV-based vaccine development due to complications associated with VACV vaccination such as eczema vaccinatum (in patients with a history of eczema or atopic dermatitis), generalized vaccinia, and progressive vaccinia (in immunocompromised individuals) (96, 97, 124, 160). Several VACV strains have been developed by attenuation to be used as a vaccine such as ACAM2000®, the second generation and current smallpox vaccine (71), MVA (120),

and NYVAC (197). Both ACAM2000® and MVA were attenuated through serial passage in Vero cells (71) and chicken embryonic fibroblast cells (120) in culture respectedly. In contrast, NYVAC is attenuated through genetically deletion of 18 open reading frames (ORFs) from the VACV Copenhagen (VACV COP) strain (197). The deleted genes include a cassette of 12 ORFs spanning C7L through K1L, in which C7L and K1L are host range genes. Thus, NYVAC is highly attenuated in human cell lines. Therefore, NYVAC is one of the best candidates for a safer VACV vaccine vector for HIV.

NYVAC attenuation is due to its inability to replicate in human cell lines. In non-permissive cells, NYVAC stops at early stages of its replication cycle (137). Thus, it is likely that the amount of antigen accumulation during virus infection may not be enough to elicit a high immune response. Thus, it is likely that the efficacy of these viruses is limited. To improve the NYVAC vaccine vector, our lab, in collaboration with the Poxvirus T Cells Discovery Consortium (PTVDC), designed a replication-competent NYVAC expressing the HIV antigen. In addition, we genetically deleted out VACV B19R, the IFNα/βR like protein, to further improve the efficacy of NYVAC vaccine vector. This work will be discussed in Chapter 3.
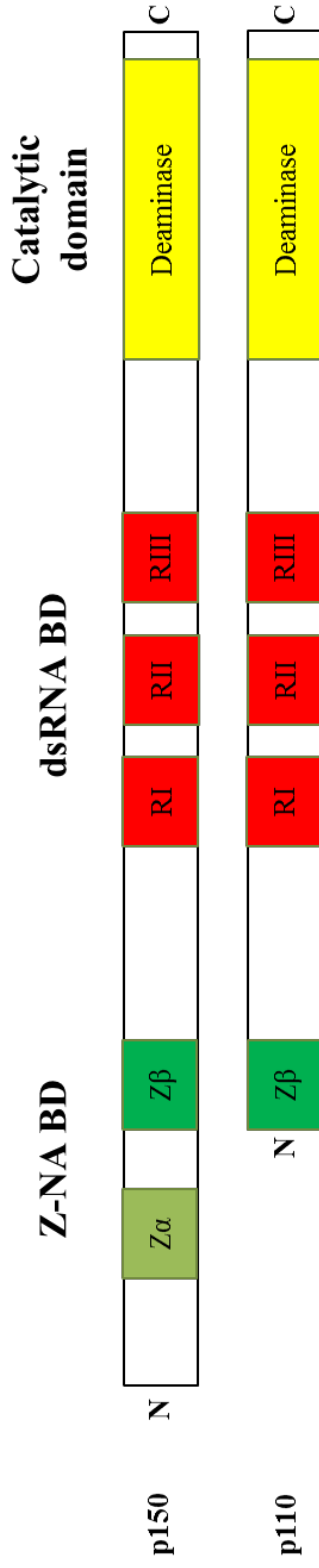
Figure 1 – Domains organization of human ADAR1. Alternative promoters result in two different forms of ADAR1, the IFN-induced p150 and the constitutive and ubiquitous p110. Both forms share the same dsRNA binding domains and the catalytic domains. P150 has two Z-DNA binding domain, Zα and Zβ, while p110 only possesses a Zβ domain
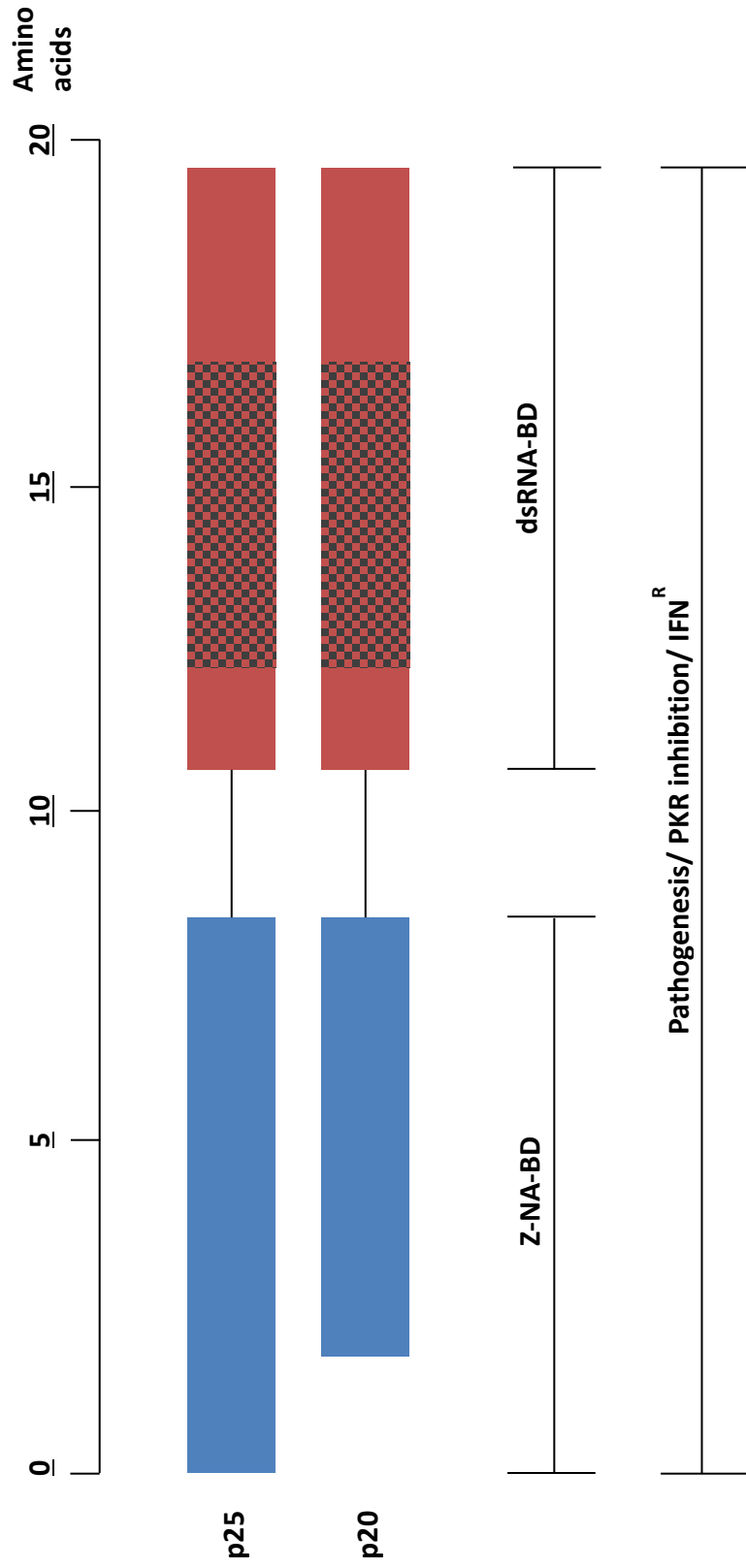
Figure 2 – Domains of E3 protein. E3L gene encodes two proteins, the full length p25 and the truncated p20 which is missing the first 37 amino acids of the N terminal. The N-terminus contains a Z-NA binding domain (BD), while the C-terminus contains the dsRNA BD. Both full length domains are required for pathogenesis within an animal model, as well as, for complete inhibition of PKR and IFN resistance. There are also a domain resides between amino acid 117 and 164 that can inhibit IRF3 phosphorylation independent from dsRNA binding.
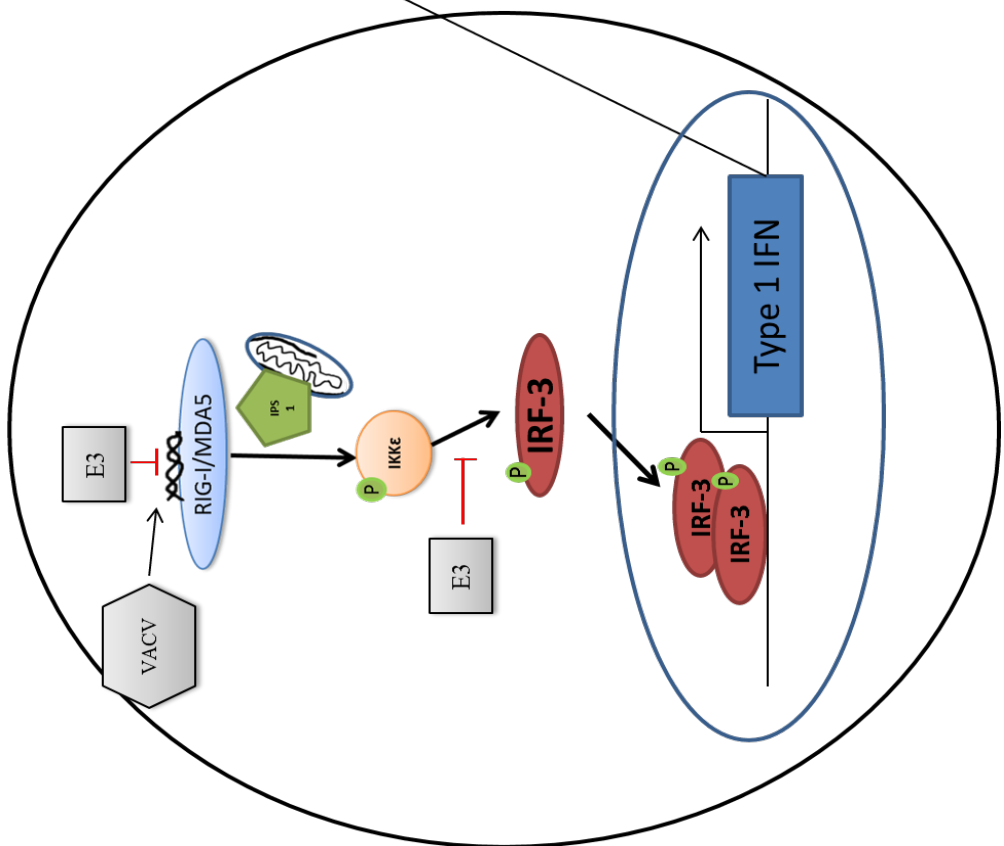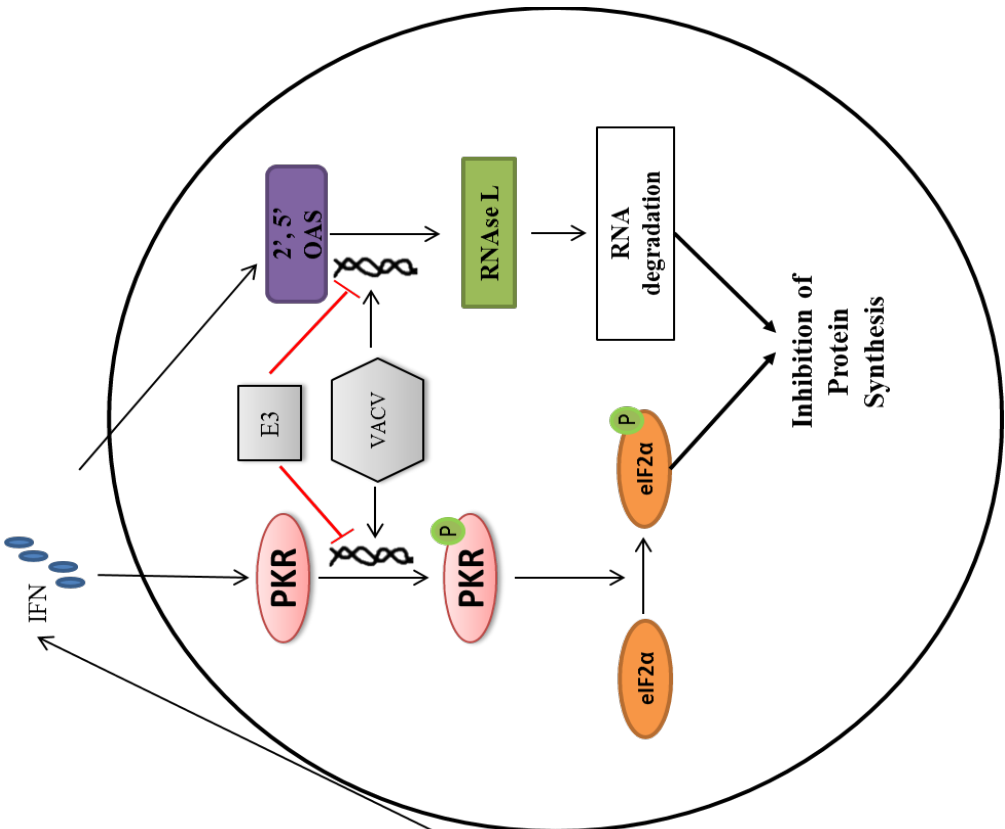
Figure 3 - E3 protein inhibits the IFN Inducible PKR and 2',5' OAS Pathways.

dsRNA generated by VACV during its life cycle can be detected by PRPs such as RIG-I/MDA5 which in turns phosphorylate IKKε and subsequently IRF3. Activated IRF3 homodimerizes and translocates to the nucleus to turn on transcription of INFα/β. Newly synthesized INFα/β induces numerous ISGs such as PKR and 2',5' OAS. PKR and 2',5' OAS become activated upon binding to dsRNA. Activated PKR induces the phosphorylation of the α-subunit of eIF2, which leads to an inhibition of protein synthesis. 2',5' OAS synthesizes 2'5' oligoadenylate that activates RNase L, leading to a degradation of cellular and viral RNA. Both pathways eventually lead to an inhibition of global protein synthesis. E3 sequesters dsRNA that eventually blocks RIG/MDA5 pathway. It also inhibits the activation of PKR and 2'5' OAS. Another region of E3 can inhibit the activation of IRF3 independent of the ability to bind to dsRNA.

18

CHAPTER 1

THE ROLE OF PROTEIN KINASE R AND THE STRESS-ACTIVATED PROTEIN

KINASE/C-JUN NH$_2$-TERMINAL KINASE IN ACTIVATION OF INTERFERON

REGULATORY FACTOR 3

ABSTRACT

The interferon regulatory factor 3 (IRF3) and the cellular antiviral protein kinase R (PKR) are both activated by double stranded RNA (dsRNA) and play important roles in the host innate immune response. Activation of PKR leads to the phosphorylation of eIF2α, inhibiting protein synthesis. Activation of IRF3 increases the production of type I interferons (IFNs), potent antiviral cytokines. Vaccinia virus (VACV) dsRNA binding protein, E3, can prevent both activation of IRF3 and PKR. It has been suggested that PKR also plays a role in activation of IRF3. Here, we confirmed the role of PKR in activation of IRF3 during VACVΔE3L infection. We then further investigated if activation of IRF3 by PKR is signaled through the mitogen activated protein kinases (MAPKs). The inhibition of p38 activation did not inhibit the activation of IRF3 while the inhibition of the stress-activated protein kinase/ c-Jun NH$_2$-terminal kinase (SAPK/JNK) activation inhibited the activation of IRF3. Further characterization data showed that PKR-dependent activation of IRF3 in VACVΔE3L requires the phosphorylation of IRF3 at Serine 173 by SAPK/JNK. This suggests that PKR is necessary but not sufficient to lead to activation of IRF3, and that PKR activates IRF3 via SAPK/JNK.

19

INTRODUCTION

Type I IFNs (IFNα/β) are cytokines of the innate immune system known to inhibit virus replication. They also modulate the adaptive immune response in addition to establishing an innate antiviral state (25, 102). IFNs induction occurs when infected cells are alerted of viral infection by the presence of dsRNA, a byproduct of many viruses (81). The induction of IFNs can occur within hours of virus infection unless protein synthesis is inhibited (9). DsRNA can be detected by RIG-I/MDA5, pathogen recognition proteins of the RLR system, which trigger the activation of TBK-1 and IKKε through TRAF3 and mitochondrial adapter protein IPS1 (75, 169, 179). Activated IKKε subsequently phosphorylates IRF3 at the C-terminal serine/threonine cluster between amino acids 396 and 405 (60). Following these phosphorylations, IRF3 is associated with the cAMP-response element-binding (CREB) protein, resulting in the second phosphorylation at serines 385 and 386, leading to the formation of IRF3 homodimer (144). Ultimately, activated IRF3 translocates into the nucleus (157) and binds to the positive regulatory domain of the IFN promoter, resulting in the expression of type I IFNs.

IFNs can stimulate production of hundreds of ISGs upon binding to their specific receptors (IFNα/β-R) located on the cells' surface via the signaling cascade of the JAK/STAT pathway (23, 25, 48, 50, 105, 190). Some of these ISGs, such as PKR, serve a function to prime the cells into an antiviral state (171). The PKR protein, which is also constitutively expressed in cells, consists of two N-terminal regulatory domains that bind to dsRNA and a C-terminal kinase catalytic domain (127). Upon binding to dsRNA, PKR undergoes dimerization and autophosphorylation to become an active protein kinase (41,

119, 121). Activated PKR subsequently phosphorylates the α subunit of eIF2 complex, a key component of the translational machinery (112). In its normal state, the eIF2 complex is inactive in its complex with GDP. With the help of the eIF2B, the guanine nucleotide exchange factor, eIF2 exchanges GDP to GTP, resulting in the binding of methionine transfer RNA to the 40S ribosomal subunit, hence initiation of protein synthesis (77). However, phosphorylation of eIF2α blocks the exchange of GDP to GTP, thus inhibiting cellular protein synthesis. Ultimately, virus induced activation of PKR can lead to global protein synthesis shut off in order to inhibit virus survival.

In addition to eIF2α, PKR has also been reported to interact with other stress signaling pathways in response to viral infection such as the p38 and SAPK/JNK pathway (8, 69, 79, 195). Both p38 and SAPK/JNK belongs to the MAPK family that responds to several cellular stress stimuli such as UV, osmosis stress, viral infection and mitogens. These MAPK proteins regulate several cellular function such as cellular proliferation, differentiation, apoptosis, etc (151). Both p38 and SAPK/JNK can lead to induction of several pro-inflammatory cytokines via the activation of their downstream target ATF2 and c-Jun respectively (88, 110). Both ATF2 and c-Jun with IRF3 and NF-κB can form an enhanceosome complex that activates IFN-β gene transcription (143). This implicates that PKR, in conjunction with p38 and SAPK/JNK, might play a role in the IFN-induction signaling cascade.

Several viruses have evolved mechanisms to evade PKR activation and the interferon system (63, 98). VACV is a DNA virus that produces dsRNA in the infected cells due to overlapping of convergent intermediate and late transcriptions (81, 211). To counter the effect of dsRNA, VACV encodes the E3L gene for an early expressed

dsRNA-binding protein that can sequester dsRNA made by VACV, hence inhibiting activation of PKR (17, 39, 210). The E3 protein also interferes with the innate immune system by blocking the activation of IRF3 and IRF7 (100, 186, 219).VACVΔE3L, VACV deleted of E3L gene, is IFN sensitive and has a narrow host range in cell in culture (10, 17-19, 38). This is a result of inhibition of protein synthesis induction of apoptosis in infected cells (93) and the activation of IRF3, resulting in the activation of the innate immune response.

PKR has been reported to play a role in regulating IFN-β production in response to dsRNA (53, 67). By using PKR-stably-knocked-down HeLa cell lines, it was suggested that activation of PKR can lead to activation of IRF3 through IPS1 (228). To confirm this finding, we utilized VACVΔE3L expressing a pseudo-substrate of PKR, the *Ambystoma tigrinium* virus' vIF2 homolog that shares homology to eIF2 (58, 224) and we transiently overexpressed a dominant negative form of PKR in VACVΔE3L infection. Our results agreed with the previous finding that PKR is necessary for virus induced activation of IRF3. Since PKR has been reported to activate p38 and SAPK/JNK (227), this led us to examine the role of p38 and SAPK/JNK in the PKR-depdendent-activation of IRF3. Our results indicate that SAPK/JNK, not p38, is involved in PKR-dependent-activation of IRF3. It was reported that SAPK/JNK phosphorylates the N terminal of IRF3 at serine 173 (226); thus, we hypothesized that the phosphorylation of IRF3 at serine 173 is necessary for the PKR-dependent-activation of IRF3 in VACVΔE3L infection.

MATERIALS AND METHODS

Cells and viruses.   Rabbit kidney cells stably expressing VACV E3 protein (RK-E3L) were maintained in Eagle's Minimum Essential Medium (MEM) (Cellgro) supplemented with Tetracycline-free 5% fetal bovine serum (FBS) (Thermo Fisher). Baby hamster kidney (BHK-21), Rabbit Kidney (RK)-13 cells were maintained in Eagle's MEM (Cellgro) supplemented with 5% FBS (HyClone). HeLa and BSC40 cells were maintained in Dulbecco's Modified-Minimal Essential Medium (DMEM) (Cellgro) supplemented with 5% FBS. All cells were incubated at 37°C in the presence of 5% CO_2. The VACV Copenhagen strain herein is referred to as wild type VACV (VACV wt). VACV Copenhagen strain deleted of the E3L gene, VACVΔE3L was generated as previously described (38). VACV Copenhagen strain expressing the vIF2αh in the E3L locus, VACVΔE3L::viF2αh was generated as previously described (Tripuraneni and Jacobs, in preparation).

Expression of dominant negative hPKR.   pDeNyMCS (empty vector) and the dominant negative (DN) pDeNY-hPKR plasmid, which expresses human PKR lacking 6 amino acids (361-366) between catalytic domains IV and V which render DN-hPKR unable to autophosphorylate or activate substrate proteins, were purchased from Invivogen. The plasmids were transformed into OneShot®TOP10 chemically competent *E. Coli* cells (Invitrogen). Transformed bacteria were cultured in Terrific Broth containing Zeocin at 50 μg/ml. Plasmids were isolated from bacteria using the Maxi Prep Kit (Qiagen). Plasmids were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. After 48 hours post transfection, the transfected cells were infected with VACV wt and VACVΔE3L at multiplicity of

infection (MOI) of 5. The cells were lysed with 1X SDS buffer after 6 hours post infection.

Plasmid construction. pcDNA-V5-IRF3 plasmids were constructed by Dr. Saumen Sarkar, and were acquired through Addgene. pcDNA-V5-IRF3 were then used in whole plasmid PCR using IRF3S173A F primer (5' CTGCGGGCTCCCAGCTTGGAC 3'), IRF3S173A R primer (5' GCTGGGAGCCCGCAGGGGCTGAG 3'), dNTPs (Promega) and *Platinum Pfx Polymerase* (Invitrogen) to generate pcDNA-V5-IRF3-S173A which prevents the phosphorylation of IRF3 at the N-terminal. Mutated DNA product was transformed into One Shot® TOP 10 chemically competent *E. Coli* (Invitrogen) according to manufacturer's instructions and incubated on ampicillin coated LB agar plates at 37°C overnight. Isolated colonies were selected; plasmid DNA was extracted using the PureYield™ Plasmid MiniPrep System (Promega) and the DNA was resuspended in 50 µL nuclease free water (Invitrogen). Plasmid pcDNA-V5-IRF3-S173A was sequenced with T7 promoter sequence primer (5' TAATACGACTCACTATAGGG 4') and IRF3 seq primer (5' ATTTCCGCTCTGCCCTCAAC 3'); and sequence results were blasted with the protein sequence of human IRF3 (Uniprot Q14653).

Immunoprecipitation assay. Sub-confluent HeLa was transfected with either pcDNA-V5-IRF3 or pcDNA-V5-IRF3-S173A plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. After 48 hours post transfection, the transfected cells were infected with VACV wt and VACVΔE3L at multiplicity of infection (MOI) of 5. The cells were lysed with RIPA buffer (1% NP40, 0.1%SDS, 0.5% Sodium deoxycholate, 100 mM Sodium fluoride, 2 mM Sodium orthovanadate) with 1X protease inhibitor (Sigma). Cell lysates were pre-cleared with ProteinA/G bead slurry

(Santa Cruz) and normal mouse IgG (Sigma) by rotating for 1 hour at 4°C. The lysates were spun down and the supernatants were collected and immunoprecipitated with anti-V5 antibody (Thermo Fisher) by rotating over night at 4°C. Parts of the supernatants were saved for input analysis. The precipitations were pulled down with Protein A/G bead slurry and were washed with ice cold RIPA buffer 3 times at 4°C. The immunoprecipitation was collected and re-suspended in 30 μL of 2X SDS buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol, 200 mM β-Mercaptoethanol).

Virus infection. The virus was diluted in MEM containing 2% FBS. Cell monolayers in a 60 mm dishes were infected with 100 μL of virus after aspirating the media off the dishes. Cells were incubated at 37°C, 5% CO_2 for 1 hour, with rocking every 10 minutes. Following infection, the appropriate cell culture media, containing DMSO (Sigma), p38 inhibitor SB239063 (Sigma) in DMSO, or JNK inhibitor SP600125 (Sigma) where indicated, was added.

Protein extraction and Western Blotting. 50% confluent HeLa cell monolayers in 60 mm dishes were infected with viruses at a MOI of 5. Infected cells were incubated with DMSO (Sigma), p38 inhibitor SB239063 (Sigma) in DMSO, or JNK inhibitor SP600125 (Sigma) where indicated. At 6 hours post infection, cells were scraped into 150 μL of 1X SDS (62.5 mM Tris-Cl, 10% glycerol, 2% SDS, 0.0005% bromophenol blue, 0.1% 2-mercaptoethanol, 1X Halt Protease and Phosphotase Inhibitor Cocktail (Pierce Thermo Scientific)). Lysates were transferred into QIAshredder columns (Qiagen), spun at 16,000xg for 2 minutes at 4°C, and stored at -80°C. The samples were boiled for 5 min and ran on 10% and 12% SDS-PAGE gels at 150V. Proteins were

25

transferred to nitrocellulose membranes at 100 volts for 60 min in 10 mM CAPS, pH 11

with 20% methanol. The membranes were blocked with 3% milk (Carnation® Non-fat

Dry Milk) TTBS (20 mM Tris-HCl, pH 7.8, 180 mM NaCl, 0.05% Tween-20) for 1hr.

The membranes were probed with primary antibodies overnight. Secondary goat anti-

rabbit IgG conjugated to horseradish peroxidase (1:10,000, Santa Cruz) or anti-mose IgG

conjugated to horseradish peroxidase (1:10,000, Santa Cruz) was added followed by

chemiluminescence (Pierce Thermo Scientific). SAPK/JNK antibody, phospho-

SAPK/JNK antibody, p38 antibody, phosphor-p38 antibody, eIF2α antibody, phosphor-

eIF2α antibody, PKR, and IRF3 antibody were purchased from Cell Signaling. GAPDH

antibody and 6X-His tag antibody were purchased from Abcam. Phospho-PKR antibody,

phospho-IRF3 at S386 antibody was purchased from Epitomics.

     Protein synthesis shut off assay.   50% confluent HeLa cell monolayers in 60

mm dishes were infected with VACV wt or VACVΔE3L at a MOI of 5. Infected cells

were incubated with DMSO (Sigma), p38 inhibitor SB239063 (Sigma) in DMSO, or JNK

inhibitor SP600125 (Sigma) where indicated. At 5.5 hours post infection, cells were

washed twice with PBS and were incubated with methionine free DMEM (CellGro) for

30 minutes. Following starvation for methionine, the cells were incubated for 30 minutes

with labeling media containing [^{35}S] methionine (50 μCi/mL) (Perkin Elmer). Cells were

scraped into 150 μL of 1X SDS (62.5 mM Tris-Cl, 10% glycerol, 2% SDS, 0.0005%

bromophenol blue, 0.1% 2-mercaptoethanol, 1X Halt Protease and Phosphotase Inhibitor

Cocktail (Pierce Thermo Scientific)). Lysates were transferred into QIAshredder columns

(Qiagen), spun at 16,000xg for 2 minutes at 4°C, and stored at -80°C. The samples were

boiled for 5 min and ran on 10% and 12% SDS-PAGE gels at 150V. The gels were

26

stained with staining solution (0.1% Coomassie Brilliant Blue R-250, 40% methanol and

10% glacial acetic acid) for 15 minutes, then were destained with destaining solution

(40% methanol and 10% glacial acetic acid) three times, each 15 minutes, and were

ultimately destained overnight in 10% glacial acetic acid. The gels were then dried and

exposed to blue film.

RESULTS

Overexpression of dominant negative PKR inhibits IRF3 phosphorylation at S386. To investigate if PKR is necessary for virus induced activation of IRF3 in VACVΔE3L, we utilized the overexpression system of dominant negative PKR (DN-PKR). This dominant negative form lacks 6 amino acids in the kinase domains (361-366) resulting in the inability of autophosphorylation of PKR (Figure 4). HeLa cells were mock transfected or transfected with either empty vector (pDeNy-MCS) or with vector expressing the DN-PKR (pDeNy-hPKR). After 48 hours post transfection, cells were mock infected or infected with either VACV wt or VACVΔE3L at a MOI of 5. Cellular extracts were obtained at 6 hours post infection and equal volumes of protein were analyzed by Western blot probed by specified antibodies. VACV wt, possessing full length E3, inhibited both PKR and IRF3 activation among untransfected and transfected cells (Figure 5, lane 2, 5, 8). In the untransfected cells and empty vector transfected cells, VACVΔE3L led to activation of PKR and IRF3 (Figure 5, lane 3,6), indicated by phosphorylation at S386, the phosphorylation site required for activation of IRF3 (144). Cells transfected with the DN-PKR leads to an overexpression of PKR that inhibits activation of PKR and activation of IRF3 in VACVΔE3L infection (Figure 5, lane 9). This suggests that PKR is necessary in activation of IRF3 during VACVΔE3L infection.

Recombinant VACV expressing the viral initiation factor 2 α homologue inhibits both PKR and IRF3 activation. To further confirm that PKR is necessary in activation of IRF3, we utilized the recombinant VACV expressing the viral initiation factor 2α homologue (vIF2αh) from the E3L locus. VACV wt was first deleted of E3L gene by replacing it with the LacZ gene (38). Then the A57R of *Ambystoma Tigrinium*

28

virus that encodes the vIF2αh was put into E3L locus, replacing the LacZ gene (Figure 6A). The vIF2αh protein shares homology to eIF2α in the N-terminal (58, 224) and can act as a pseudo-substrate for PKR and specifically lead to degradation of PKR. To ensure that we have successfully made VACVΔE3L::vIF2αh, we tagged both E3 and vIF2αh proteins with 6X-His tag at the amino terminus of the protein. HeLa cells mock infected or infected with VACV wt-His, VACVΔE3L and VACVΔE3L::His-vIF2αh at a MOI of 5. Cellular extracts were obtained at 3 hours post infection and equal volumes of protein were analyzed by Western blot probed with anti-6X His antibody. Expression of E3 was observed around 25 kDa while expression of vIF2αh was observed around 35 kDa (Figure 6B).

To determine if PKR is required for IRF3 activation, HeLa cells were mock infected or either infected with VACV wt, VACVΔE3L or VACVΔE3L::vIF2αh. Cellular extracts were obtained at 6 hours post infection and equal volumes of protein were analyzed by Western blot probed by specified antibodies. As expected, VACV wt inhibited both PKR and IRF3 activation (Figure 7, lane 2) while VACVΔE3L led to activation of both PKR and IRF3 (Figure 7, lane 3). As a pseudo-substrate for PKR, the vIF2αh led to degradation of PKR in the VACVΔE3L::vIF2αh infection. Degradation of PKR by vIF2αh prevented the activation of IRF3 (Figure 7, lane 4); thus, combining with the above data (Figure 5), PKR is necessary in IRF3 activation during VACVΔE3L infection.

The specific SAPK/JNK inhibitor, SP600125, but not the specific p38 inhibitor, SB203580, inhibits PKR-dependent activation of IRF3. It has been reported that VACVΔE3L can lead to activation of the MAPK kinase pathway, especially

the p38 and SAPK/JNK pathways (227). To determine if p38 or SAPK/JNK is involved

in PKR-depdent-IRF3 activation, we utilized the specific inhibitor of p38 and

SAPK/JNK. SB2035880 is known and widely used as a specific p38 inhibitor (57) while

SP600125 is known and widely used as a specific SAPK/JNK inhibitor (22). HeLa cells

were mock infected or either infected with VACV wt or VACVΔE3L. Cells were then

incubated with media containing either DMSO (0.1%), 10μM p38 inhibitor (in DMSO),

or 10μM SAPK/JNK inhibitor (in DMSO). Cellular extracts were obtained at 6 hours

post infection and equal volumes of protein were analyzed by Western blot probed by

specified antibodies. As expected, VACV wt inhibited activation of PKR, p38,

SAPK/JNK, c-Jun, ATF2 and IRF3 among treated cells (Figure 8, lane 2, 5, 8) while

VACVΔE3L led to activation of all of these pathways in DMSO treated cells (Figure 8,

lane 3). In cells treated with p38 inhibitor, VACVΔE3L still led to PKR activation and

IRF3 activation but failed to fully activate p38 indicating by the low amount of

phosphorylation of p38 and ATF2 (Figure 8, lane 6). In cells treated with SAPK/JNK

inhibitor, VACVΔE3L still led to PKR activation but failed to activate either SAPK/JNK

or its downstream target, c-Jun (Figure 8, lane 9). Interestingly, the SAPK/JNK inhibitor

led to inhibition of IRF3 activation in VACVΔE3L regardless of the activation of PKR

(Figure 8, lane 9). This suggests that PKR is necessary to activate IRF3 in VACVΔE3L

infection but it is not sufficient to activate IRF3 and it requires SAPK/JNK, but not p38,

activation in order to activate IRF3.

The specific SAPK/JNK inhibitor, SP600125, and the specific p38 inhibitor,

SB203580, do not inhibit virus activity. To determine that the specific p38 and

SAPK/JNK inhibitor do not affect virus activity, we mock infected or infected HeLa cells

30

with either VACV wt or VACVΔE3L at an MOI of 5 and infected cells incubated with media containing either DMSO (0.1%), 10μM p38 inhibitor (in DMSO), or 10μM SAPK/JNK inhibitor (in DMSO). Cells were then assayed for protein synthesis by labeling with ^{35}S-methione at 6 hours post infection. Either p38 or SAPK/JNK inhibitor affected cellular protein synthesis (Figure 9, lane 1, 3, 6). VACV wt led to viral protein synthesis among all treated cells (Figure 9, lane 2, 5, 8) while VACVΔE3L led to global protein synthesis shut off (Figure 9, lane 3, 6, 9). This suggests that p38 and SAPK/JNK inhibitors do not affect virus activity.

SAPK/JNK activation is also required for the PKR-dependent activation of IRF3 in primary human Keratinocyte. Since keratinocyte is the first layer of cells in the human integument system that interacts with VACV during vaccination, we set out to determine if the SAPK/JNK is needed for PKR-dependent activation of IRF3 in this cell line. Primary human keratinocyte were mock infected or either infected with VACV wt or VACVΔE3L. Cells were then incubated with media containing either DMSO (0.1%), or 10μM SAPK/JNK inhibitor (in DMSO). Cellular extracts were obtained at 6 hours post infection and equal volumes of protein were analyzed by Western blot probed by specified antibodies. As expected, VACV wt inhibited activation of PKR, SAPK/JNK, c-Jun and IRF3 among treated cells (Figure 10, lane 2, 5) while VACVΔE3L led to activation of all of these pathways in DMSO treated cells (Figure 10, lane 3). In cells treated with SAPK/JNK inhibitor, VACVΔE3L still led to PKR activation but failed to activate either SAPK/JNK, c-Jun, or IRF3 (Figure 10, lane 6). This confirms the above data (Figure 8) that SAPK/JNK is necessary for PKR-dependent activation of IRF3 in VACVΔE3L.

31

Phosphorylation of IRF3 at serine 173 is necessary for activation of IRF3 in

VACVΔE3L. It has been reported that SAPK/JNK can phosphorylate IRF3 at serine 173 (S173) at the N terminal (125). To determine if the VACVΔE3L induced activation of IRF3 is mediated by SAPK/JNK through phosphorylation of IRF3 at position S173, HeLa cells were transfected with pcDNA expressing N-terminal V5-tagged IRF3 (pcDNA-V5-IRF3) or pcDNA expressing the mutated IRF3 which cannot be phosphorylated at position S173 (pcDNA-V5-IRF3-S173A). After 48 hours post transfection, cells were mock infected or infected with either VACV wt or VACVΔE3L. Cells lysates were prepared after 6 hours post infections and were subjected to immunoprecipitation using V5 antibody. The precipitated proteins were analyzed by Western blot probed by specified antibodies. VACV wt inhibited IRF3 phosphorylation at position S386 in pcDNA-V5-IRF3 transfected cells (Figure 11 Lane 2) and in pcDNA-V5-IRF3-S173A transfected cells (Figure 11 Lane 5). VACVΔE3L led to large amount of IRF3 phosphorylation in pcDNA-V5-IRF3 transfected cells (Figure 11 Lane 3). This VACVΔE3L induced IRF3 phosphorylation is subverted in pcDNA-V5-IRF3-S173A transfected cells. This suggests that serine 173 of IRF3 is necessary for activation of IRF3 in VACVΔE3L infection and the PKR-dependent IRF3 phosphorylation is mediated by SAPK/JNK phosphorylating IRF3 at S173.

DISCUSSION

The activation of IRF3 by dsRNA has been well established to be a result of the signaling cascade of RIG-I/MDA5 and TBK1/IKKε (60, 179). However, more kinases have been shown to involve in virus-induced phosphorylation of IRF3 such as phosphatidylinositol 3-kinase (107), PKR (228) and the mitogen-associated-protein-kinase SAPK/JNK (226). In this study, we utilized two methods to confirm the role of PKR in the activation of IRF3 in VACVΔE3L infection. We demonstrated that PKR is necessary in mediating IRF3 activation; however, it is not sufficient to activate IRF3. We found out that SAPK/JNK activation is required for PKR-dependent activation of IRF3. It has been reported that the SAPK/JNK kinase phosphorylate IRF3 at the amino terminal at serine 173 (226). Using our immunoprecipitation result, we suggest our hypothesized working model in which PKR activation leads to SAPK/JNK activation that will phosphorylate the amino terminal of IRF3. This will allow confirmation change so that IKKε can phosphorylate the carboxyl terminal of IRF3; thus leading to activation of this transcription factor (Figure 12).

Two methods were used to inhibit the PKR pathway: overexpression of the dominant negative of PKR and degradation of PKR by *Ambystoma Tigrenium* viral pseudo-substrate of PKR, the viral initiation factor 2α homologue. Overexpression of dominant negative of PKR led to inhibition of IRF3 activation, indicated by phosphorylation of IRF3 at serine 386, in VACVΔE3L (Figure 5) while VACVΔE3L::vIF2αh also inhibited activation of IRF3 because of PKR degradation in this viral infection (Figure 7). Thus, it suggests that PKR is necessary for activation of

IRF3 in VACVΔE3L infection. This data is in agreement with what was previously reported using stably-PKR-knocked-down HeLa (228).

Since VACVΔE3L can lead to activation of the MAPK kinase pathway, we thought that PKR-depdenent-IRF3 activation could be signaled through this pathway. Using small molecule inhibitors, we demonstrated that the SAPK/JNK kinase, not the p38 kinase, is required for VACVΔE3L induced PKR-dependent activation of IRF3 in both HeLa cells (Figure 8) and the physiological relevant human keratinocyte cells (Figure 10). Even though we only use small molecule inhibitors to prove our hypothesis, these inhibitors are known to be specific only to p38 and SAPK/JNK, and they has been widely used and accepted in literature (22, 57). In addition, our data showed that the inhibitor did not affect cellular or viral protein synthesis (Figure9). We also attempted to transiently knock down SAPK/JNK using siRNA. However, when SAPK/JNK was completely knocked down, VACVΔE3L led to more IRF3 phosphorylation compared to that of normal cells. Utilizing stably-SAPK/JNK-knocked-out-MEF, we observed the same phenomenon. Because SAPK/JNK plays roles in multiple signaling pathways in cells, we thought that knock down of SAPK/JNK can result in complementing pathways that can induce IRF3 activation in VACVΔE3L.

Thus, to confirm that SAPK/JNK is necessary for VACVΔE3L induced IRF3 activation, we utilized immunoprecipitation of V5 tagged IRF3. It has been reported that SAPK/JNK phosphorylated IRF3 at the amino terminal at serine 173 (226). Thus, we asked whether or not VACVΔE3L can induce activation of IRF3 when S173 cannot be phosphorylated. Our results showed that VACVΔE3L can phosphorylate V5-IRF3 but not V5-IRF3S173A (Figure 11). This suggests that phosphorylation of IRF3 at serine 173

34

is necessary for IRF3 activation and SAPK/JNK kinase is necessary for VACVΔE3L induced PKR-dependent activation of IRF3. Our hypothesized working model is that during VACVΔE3L infection, dsRNA will activate PKR which in turn activates SAPK/JNK that subsequently phosphorylates IRF3 at serine 173. This will allow conformational change of the protein. On the other hand, dsRNA will activate RIG-I/MDA5 pathway which leads to phosphorylation of IKKε. When IRF3 changes its conformation, IKKε will phosphorylate IRF3 at the cluster of threonine 395 and serine 396. This will allow CREB to phosphorylate IRF3 at serine 386 and IRF3 is activated.
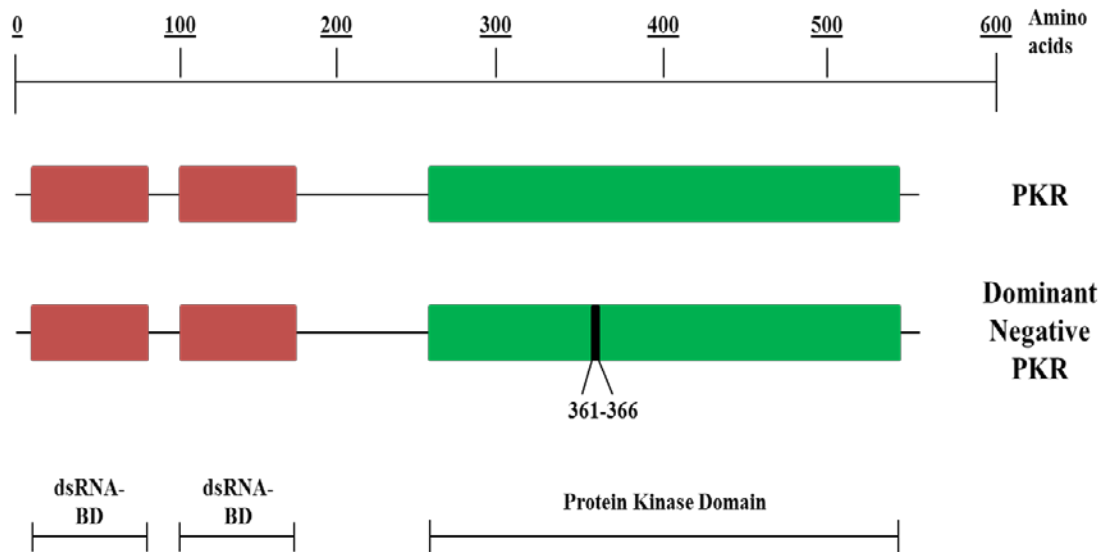
Figure 4 – Schematic representation of dominant negative PKR.
The human protein kinase R is 551 amino acid long and consists of 2
dsRNA binding domains ((9a.a – 77 a.a) and (100a.a – 167a.a)) and a
carboxyl terminal kinase domain (267a.a – 538a.a). Dominant negative
PKR lacks 6 amino acids in the kinase domain (361a.a – 366a.a) that
results in the DN-PKR's inability to autophosphorylate and inhibit
phosphorylation of endogenous PKR.
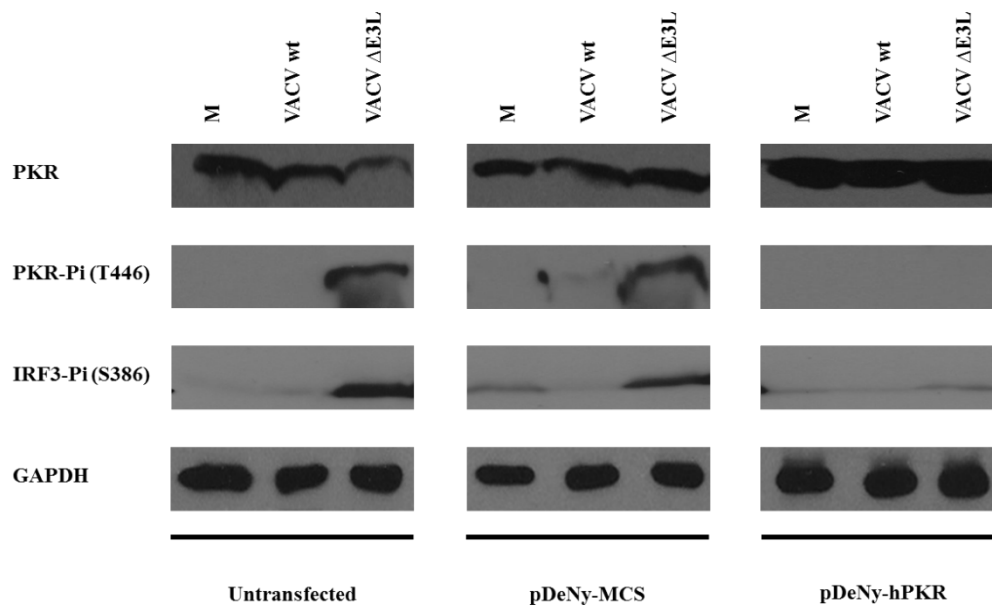
Figure 5 – Overexpression of dominant negative PKR leads to inhibition of IRF3 phosphorylation in VACVΔE3L infection. HeLa cells were either untransfected or transfected with empty vector (pDeNY-MCS) or vector expressing dominant negative PKR (pDeNy-hPKR). After 48 hours post transfection, cells were either mock infected or infected with either VACV wt or VACVΔE3L at an M.O.I of 5. Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using above specified antibodies to proteins.

A.

E3L

VACV wt — | Z-NA-BD | dsRNA-BD |

VACV ΔE3L — | LacZ |

VACV ΔE3L::vIF2αh — | vIF2-h |

B.

Mock | VACV His-wt | VACVΔE3L | VACVΔE3L::His-vIF2αh

→ vIF2αh
→ E3L p25
→ GAPDH

Figure 6 – VACV expressing the viral eIF2α homologue. A. Schematic representation of virus construction. VACV wt represents wild type VACV which encodes a full length E3L protein (p25) with a Z-NA binding domain in the amino and a dsRNA binding domain in the carboxyl terminal. VACVΔE3L represents virus deleted of the E3L gene, which expresses *LacZ* from the E3L locus. VACVΔE3L::vIF2αh represents a VACV construct which encodes a viral eIF2α homologue off *Ambystoma Tigrinium Virus* from the E3L locus. **B.** HeLa cells were either mock infected or infected with VACV wt with a N-terminal His-tagged E3L (VACV wt-His), VACVΔE3L, or recombinant VACV expressing a N-terminal His-tagged viral eIF2α homologue from the E3L locus (VACVΔE3L::vIF2αh-His). Cell lysates were prepared at 3 hours post infection. The proteins were resolved on a 12% SDS PAGE gel followed by western blotting using antibody against His-tag and GAPDH.
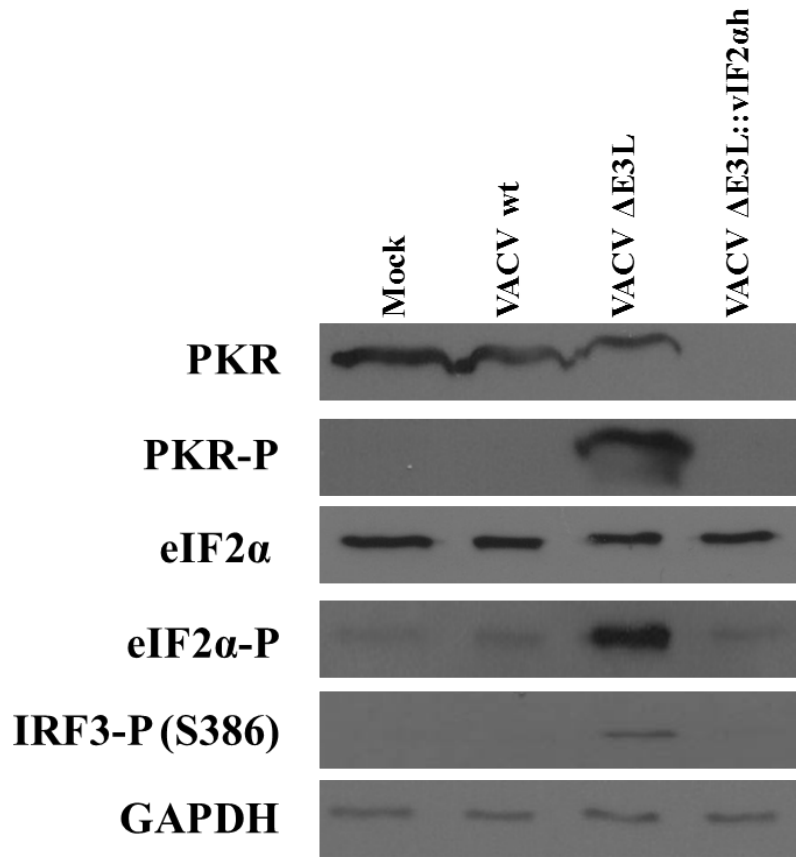
Figure 7 – VACV expressing the viral eIF2α homologue inhibits IRF3 phosphorylation. HeLa cells were either mock infected or infected with VACV wt, VACVΔE3L, or recombinant VACV expressing the viral eIF2α homologue from the E3L locus (VACVΔE3L::vIF2αh). Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 12% SDS PAGE gel followed by western blotting using above specified antibodies.
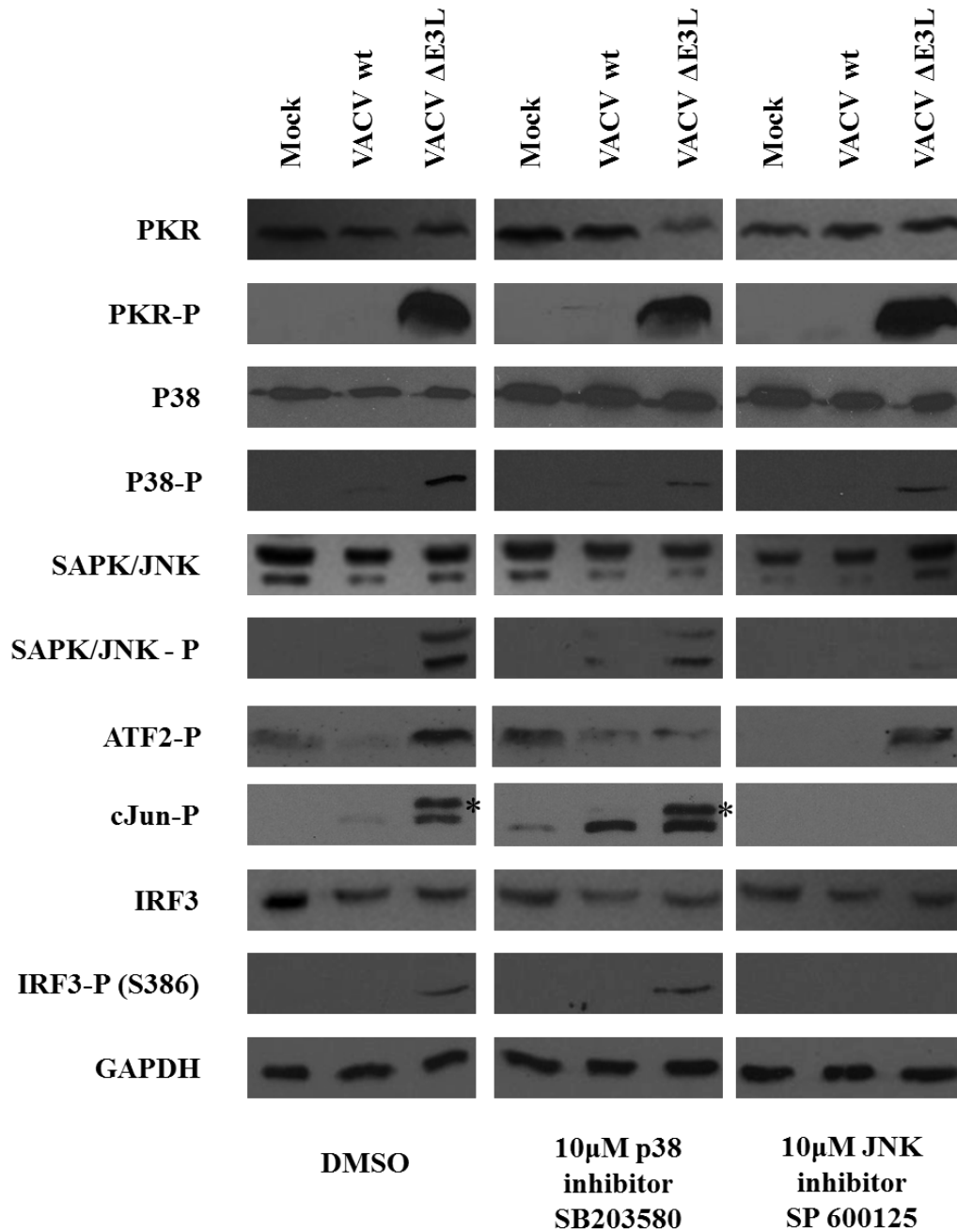
Figure 8 – Inhibition of SAPK/JNK phosphorylation prevent PKR-dependent-IRF3 phosphorylation. HeLa cells were mock infected or either infected with VACV wt or VACVΔE3L. Cells were mocked incubated with DMSO or incubated with either p38 inhibitor (SB203580) or SAPK/JNK inhibitor (SP600125). Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using above specified antibodies.
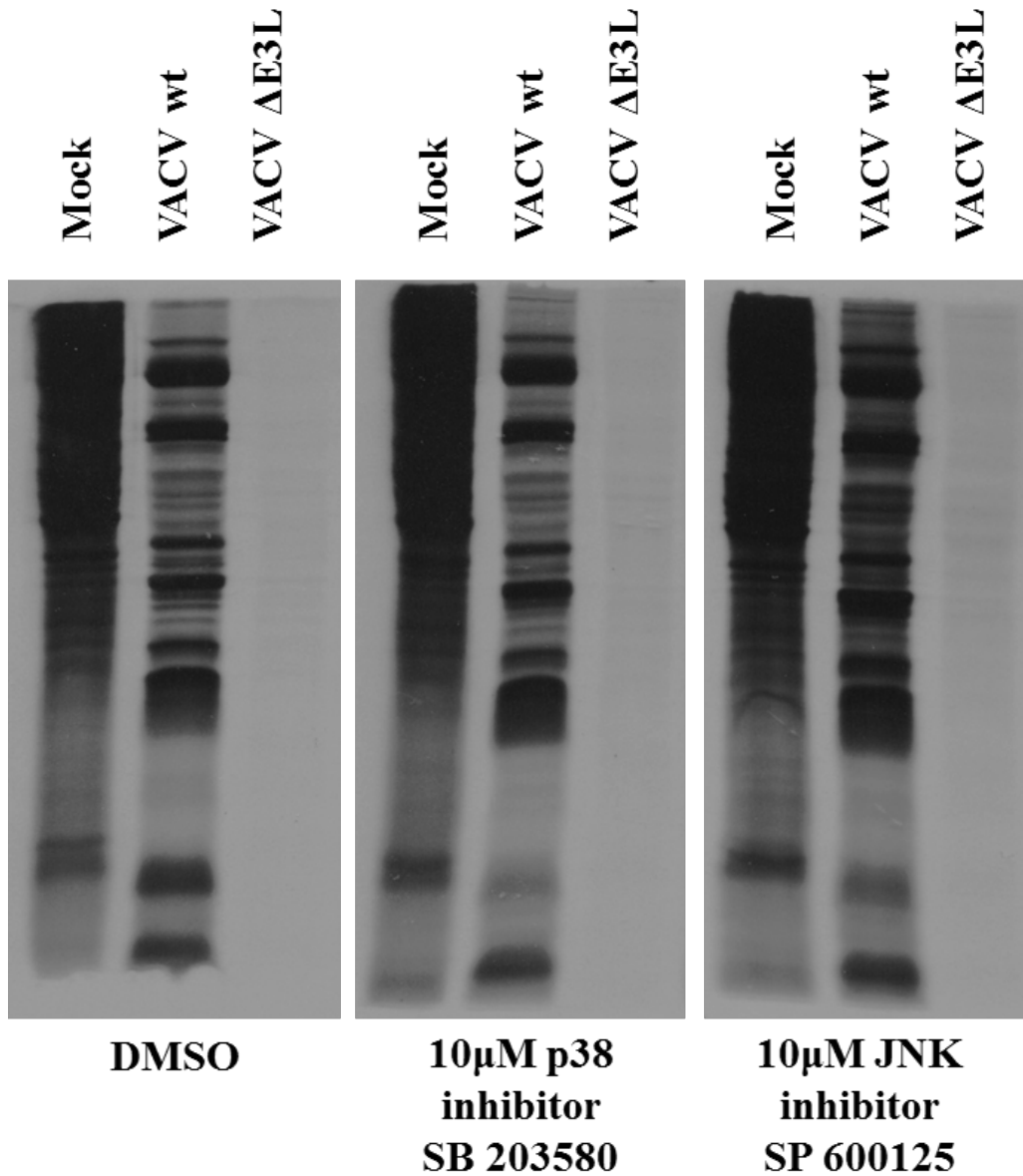
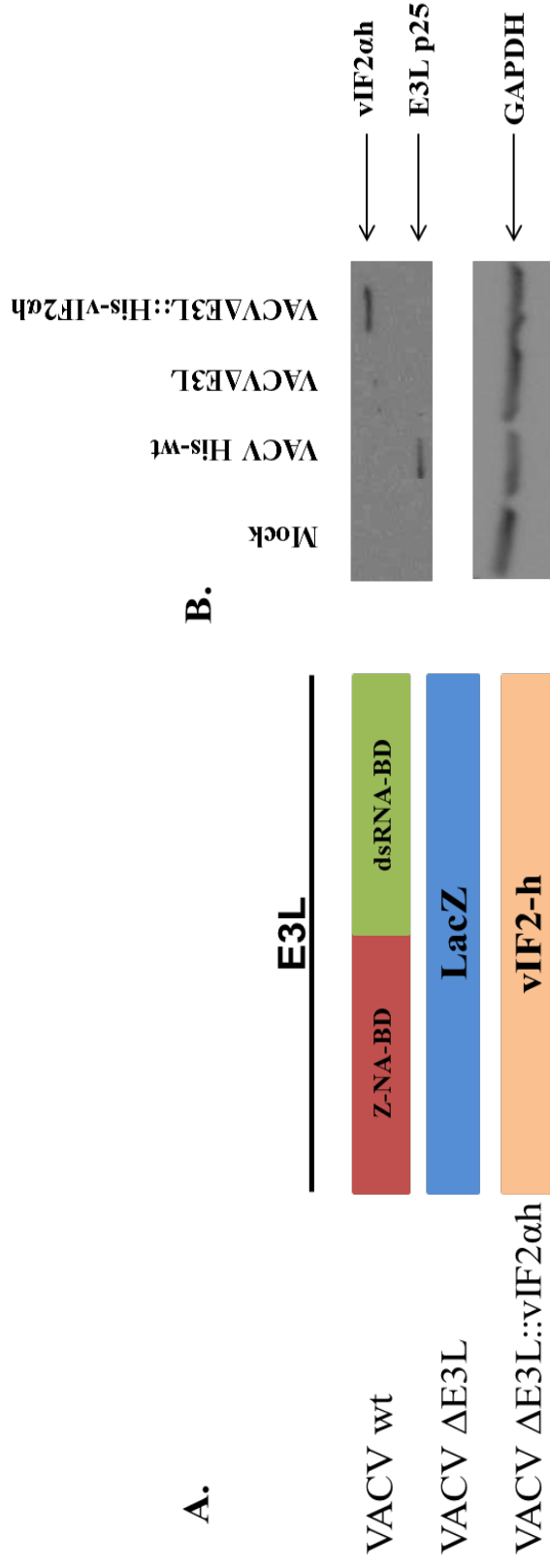**Figure 9 – Both p38 and SAPK/JNK inhibitors do not affect viral protein synthesis.** HeLa cells were mock infected or either infected with VACV wt or VACVΔE3L. Cells were mocked incubated with DMSO or incubated with either p38 inhibitor (SB203580) or SAPK/JNK inhibitor (SP600125). Cells were radio-labeled with [^{35}S] methionine at 6 hours post infection. After 30 minutes of radio-labeled, cell lysates were prepared. The lysates were resolved on a 12% SDS-PAGE gel and subjected to autoradiography
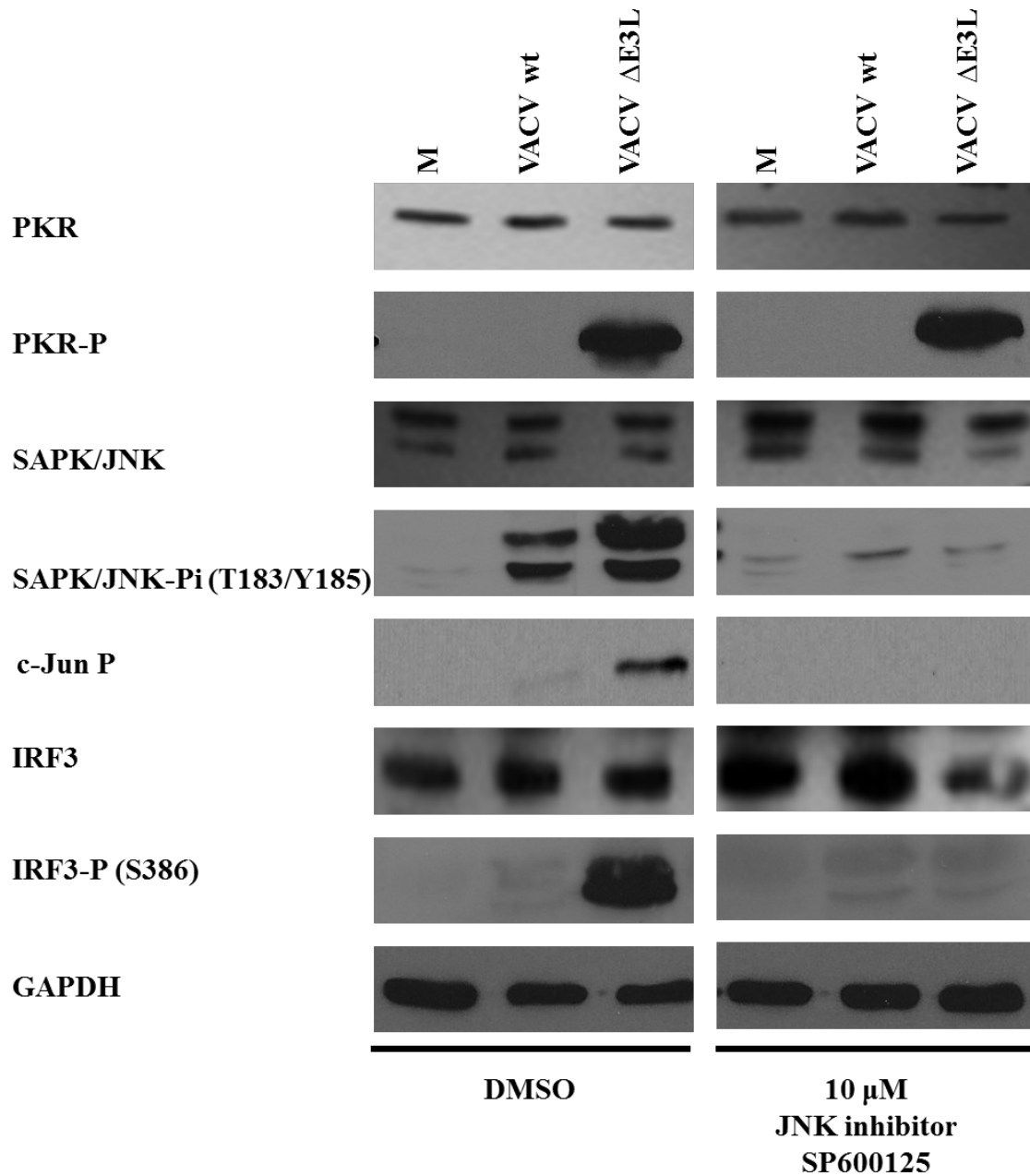
Figure 10 – Inhibition of SAPK/JNK phosphorylation prevent PKR-dependent-IRF3 phosphorylation in primary human Keratinocyte.
Primary human Keratinocytes cells were mock infected or either infected with VACV wt or VACVΔE3L. Cells were mocked incubated with DMSO or incubated SAPK/JNK inhibitor (SP600125). Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using above specified antibodies.
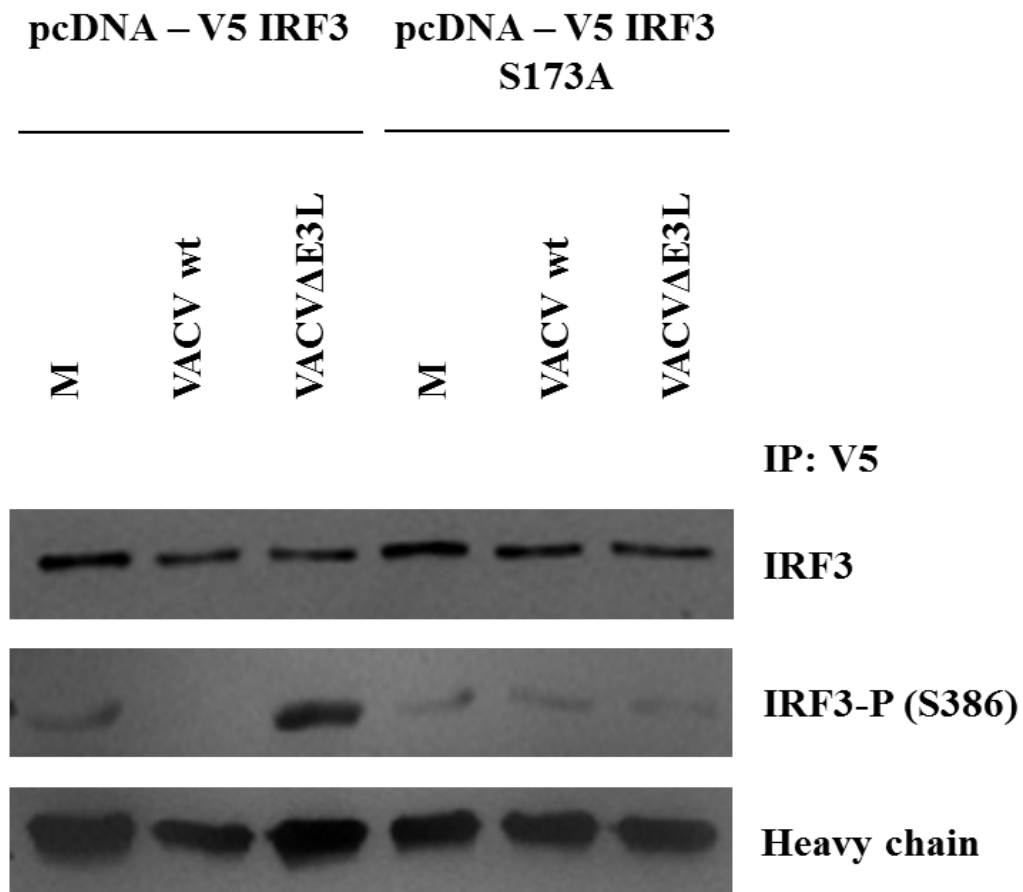
Figure 11 – VACVΔE3L induced IRF-3 phosphorylation at Serine 386 requires phosphorylation of the N terminal at Serine 173A. HeLa cells were transfected with either pcDNA-V5 IRF3 or pcDNA-V5 IRF3S173A. After 48 hours post transfection, cells were either mock infected or infected with either VACV wt or VACVΔE3L at an M.O.I of 5. Cell lysates were prepared at 6 hours post infection. Cell lysates were then immunoprecipitated with V5 antibody. Proteins were resolved on a 10% SDS PAGE gel and probed with specified antibodies.
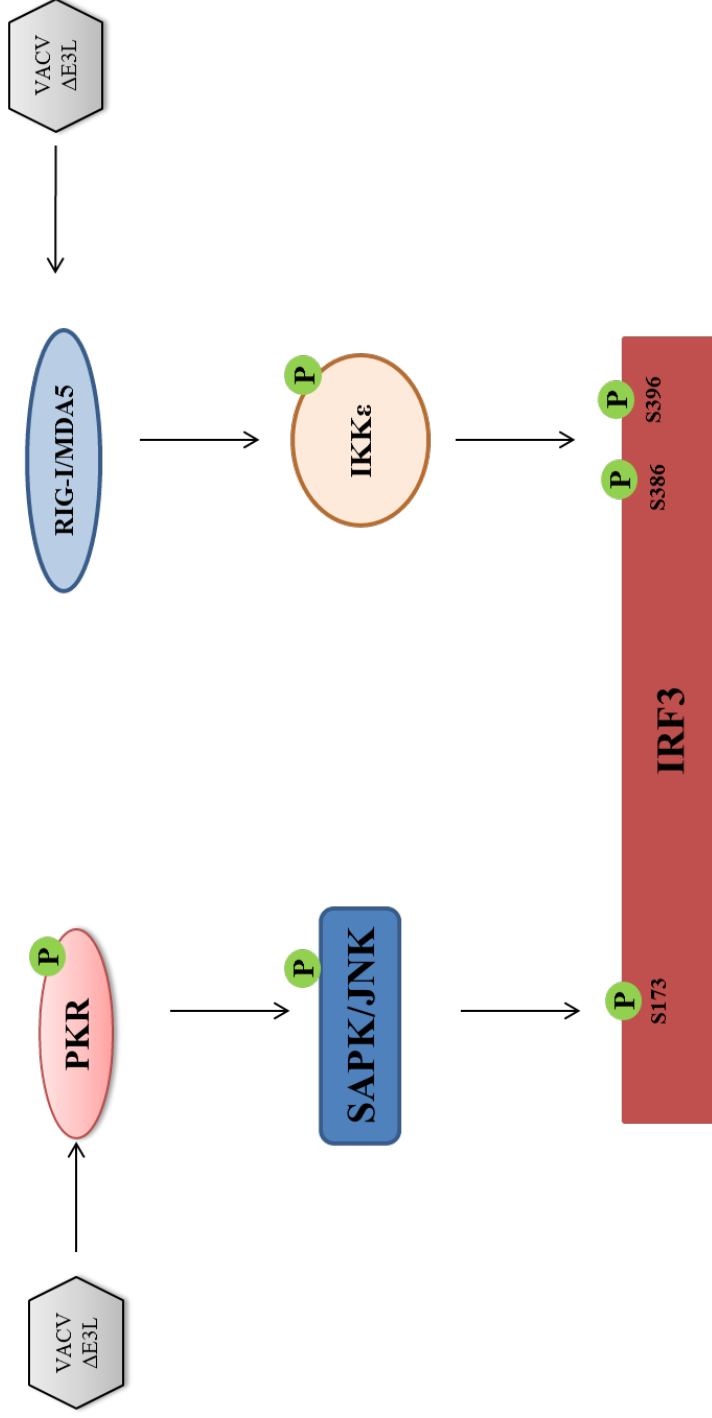
Figure 12 – Hypothesized working model for PKR-dependent activation of IRF3 in VACVΔE3L infection. VACVΔE3L leads to activation of PKR which results in activation of the MAPK pathway. Meanwhile, VACVΔE3L leads to activation of RIG-I/MDA5 which subsequently phosphorylate IKKε through IPS1. Activated SAPK/JNK phosphorylates the N-terminal of IRF3 at S173 and changes the conformation of the protein allowing activated IKKε phosphorylates IRF3 at S396 which will lead to the activation of IRF3 through phosphorylation at S396 by CREB protein.

44

CHAPTER 2

THE ROLE OF ADENOSINE DEAMINASE ACTING ON DSRNA 1 (ADAR1) IN

VACCINIA VIRUS INFECTION

ABSTRACT

Adenosine Deaminases Acting on RNA 1 (ADAR1) catalyzes the deamination reaction of adenosine to produce inosine in double stranded RNA (dsRNA). ADAR1 has been extensively studied during viral infection. The Vaccinia virus (VACV) virulence factor E3L and ADAR1 contain both a Z-NA binding domain and a dsRNA binding domain that are functionally homologous. ADAR1 is presented in cells both in the constitutively expressed form, p110, and the interferon (IFN) induced form, p150. Our data showed that when cells were treated with IFN, there was less dsRNA detected in the VACVΔE3L infected cells, suggesting this phenomenon might be due to the action of the IFN-induced ADAR1 protein. This is correlated with the reduction of eIF2α phosphorylation in IFN treated, VACVΔE3L infection. Using a vaccinia virus expressing the IFN-induced ADAR1 in VACVΔE3L infection, we demonstrated that ADAR1 rescued viral protein synthesis and replication of VACVΔE3L. Thus, ADAR1 plays a proviral role in VACV infection. This is the first time that the pro-viral role of ADAR1 is described.

INTRODUCTION

Viruses are obligate intracellular parasites that hijack cellular machinery for their survival. The interferon (IFN) system of the innate immune response can prime the cells into an antiviral state to resist viral infections (80). This antiviral state is a condition in which IFNs induce expression of several antiviral proteins such as the dsRNA-activated protein kinase R (PKR) and the 2'-5'-oligo adenylate synthetase (OAS) (106, 162, 168, 185). Both proteins are expressed in inactive forms and are activated upon detection of dsRNA, a byproduct made by most viruses during their intracellular life cycles (33, 46, 104). Activation of PKR leads to phosphorylation of the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) which results in the inhibition of protein translation initiation, thereby resulting in protein synthesis shut off in infected cells (77, 112). Activation of the OAS system leads to activation of RNase L, a latent endoribonuclease. Active RNase L can cleave both ribosomal RNA and messenger RNA in the infected cells (61), thereby leading to an arrest of protein synthesis (216).

Several viruses have developed mechanisms to evade the interferon system (63). VACV has been shown to resist the antiviral state established by IFNs in most cell lines (10, 19). The ability of VACV to resist the antiviral state relies of its virulence factor, the E3L gene (39, 219). This virulent gene is necessary for the broad-host-range phenotype of VACV (18) and the IFN resistant phenotype (19), and contributes greatly to VACV pathogenesis (29). E3L encodes a 25 kDa protein and an N-terminal-37-amino-acid-truncated p20 kDa protein, both contains a Z-nucleic acid (Z-NA) (Z-formed nucleic acid) binding domain (ZNA-BD) in its amino terminus and a dsRNA binding motif (dsRNA-BM) in its carboxyl terminus (29, 39). E3 proteins inhibit both PKR and OAS

46

activation by sequestering dsRNA made during its life cycle in infected cells (37-39, 45, 165, 182). These inhibitions allow protein synthesis to occur in the infected cells and the virus continues to replicate. VACV deleted of E3L (VACVΔE3L) is IFN sensitive (IFN^S) (10, 17, 38) and has a restricted host range (17, 19, 38) in cells in culture. These phenotypes are due to the activations of PKR and OAS. VACVΔE3L is greatly reduced in pathogenesis (29). Both the ZNA-BD and the dsRNA-BM have been shown to contribute to the broad host range phenotype, the IFN resistance (IFN^R) through PKR inhibition (99, 215) and neurovirulence in mice (28, 29). Sequence comparison reveals that E3L shares homology to several IFN induced dsRNA binding proteins, such as the Adenosine Deaminase Acting on dsRNA 1 (ADAR1) protein.

ADAR1 is a cellular, IFN inducible protein that belongs to a protein family of RNA editing protein that catalyzes the C-6 deamination of adenosine (A) to yield inosine (I), in RNA substrates with dsRNA character (13, 138, 202). Because inosine is read as guanosine (G) during translation, this A to I editing activity lead to codon change in mRNA that, in turn, alters protein function (109, 175). ADAR1 exists in two forms, the constitutively and ubiquitously expressed p110 and the IFN inducible p150 which is driven by an IFN inducible promoter (66, 148, 149). Both p110 and p150 consist of a C-terminal deaminase catalytic domain, and three dsRNA-BMs in the central region (148, 170); however, the p150 consists of two ZNA-BDs, Zα and Zβ, while the p110 only has the Zβ domain (76, 174). Interestingly, the IFN induced p150 of ADAR1 and E3L is very similar in the organization of their domains in that both proteins possess the Zα ZNA-BD and dsRNA-BMs (111).

47

The role of ADAR1 has been reported to be both antiviral and proviral in several RNA viruses system (170). Here, we investigated the role of ADAR1 in VACV, a DNA virus. It has been reported that VACV deleted of the Z-NA binding domain (VACVΔ83N) leads to eIF2α phosphorylation during late time infection. However, this phenomenon can be inhibited by pre-treatment of IFN (99). Due to the fact that ADAR1 is IFN inducible and portrays deaminase activity, we hypothesized that ADAR1 deaminates dsRNA made by VACV leading to the inhibition of late eiF2α phosphorylation. Our results indicate that pretreatment of IFN leads to the reduction of dsRNA in VACVΔ83N and VACV deleted of E3L (VACVΔE3L) infections. By replacing VACV E3L gene with the p150 form of ADAR1, we studied the role of ADAR1 in VACVΔE3L infection. Our results indicate that ADAR1 can rescue protein synthesis and replication of VACVΔE3L in HeLa cells. However, these phenotypes require the deaminase activity of ADAR1. In summary, our data suggests that ADAR1 plays a proviral role in VACV infection.

Cells and viruses.   Rabbit kidney cells stably expressing VACV E3 protein (RK-E3L) were maintained in Eagle's Minimum Essential Medium (MEM) (Cellgro) supplemented with Tetracycline-free 5% fetal bovine serum (FBS) (Thermo Fisher). Baby hamster kidney (BHK-21), and Rabbit Kidney (RK-13) cells were maintained in Eagle's MEM (Cellgro) supplemented with 5% FBS (HyClone). HeLa and BSC40 cells were maintained in Dulbecco's Modified-Minimal Essential Medium (DMEM) (Cellgro) supplemented with 5% FBS. All cells were incubated at 37°C in the presence of 5% CO_2. The VACV Copenhagen strain and the VACV Western Reserve (WR) strain herein are referred to as wild type VACV (VACV wt). VACV Copenhagen strain or VACV WR strain deleted of the E3L gene, VACVΔE3L was generated as previously described (38). VACV Copenhagen strain or VACV WR strain deleted of the ZNA-BD of E3L gene, VACVΔ83N was generated as previously described (183). VACV WR strain expressing the p150 ADAR1 (VACVΔE3L::ADAR1) and the catalytic inactive p150 ADAR1 (VACVΔE3L::ADAR1m) were generated as previously described (135).

Reconstruction of pmpADAR1 and pmpADAR1m.   The ADAR1 gene was amplified out of the WR strain VACVΔE3L::ADAR1 genome using E3L F (5' CGAAACACCAGAGGATG 3') and E3L R (5' TAGTCGCGTTAAATAGTACTA 3') primers, dNTPs (Promega) and *Platinum Pfx Polymerase* (Invitrogen). The PCR product was column purified using the Wizard® SV Gel and PCR Clean up Kit (Promega) according to manufacturer's instructions. The PCR product and pmpMCS plasmid were digested with *HindIII* (NEB) according to manufacturer's instructions. The digested pmpMCS vector was then treated with *Alkyline Phosphotase* (NEB) to remove 5'

phosphate end according to manufacturer's instruction and then column purified as above. Digested ADAR1 product was cloned into digested pmpMCS using *T4 DNA ligase* (NEB) according to manufacturer's instructions. Ligated DNA was transformed into One Shot® TOP 10 chemically competent *E. Coli* (Invitrogen) according to manufacturer's instructions and incubated on ampicillin coated LB agar plates at 37°C overnight. Isolated colonies were selected; plasmid DNA was extracted using the PureYield^{TM} Plasmid MiniPrep System (Promega) and the DNA was resuspended in 50 μL nuclease free water (Invitrogen). Plasmid pmpADAR1 was screened for proper orientation of the cloned genes using *BamHI* (NEB) enzymes. Corrected orientation plasmids of pmpADAR1 were sequenced with E3L F primer, ADAR 1F primer (5' AGACAGAAACTCCACATCTGTCTC 3'), ADAR 2F primer (5' CTCCTTCTACAGTCATGGCTTG), ADAR 3F primer (5' GATGCCCTCCTTCTACAGTC 3'), ADAR 4F primer (5' CCTTTTGGAGTACGCCCG 3'), and ADAR 5F primer (5' TATGGAAAGCACAGAATCCCG 3'); and sequence results were blasted with the protein sequence of ADAR1 (Uniprot P55265). pmpADAR1 was then used as DNA template in whole plasmid PCR using ADARHQEA F primer (5' GTCAATGACTGCCAGGCAGCAATAATC 3'), ADARHQEA R primer (5' GGGAGATTATTGCTGCCTGGCAGTC 3'), dNTPs (Promega) and *Platinum Pfx Polymerase* (Invitrogen) to generate pmpADAR1m which has two point mutations: H910Q and E912A. Mutated DNA product was transformed into One Shot® TOP 10 chemically competent *E. Coli* (Invitrogen) according to manufacturer's instructions and incubated on ampicillin coated LB agar plates at 37°C overnight. Isolated colonies were

selected; plasmid DNA was extracted using the PureYield^{TM} Plasmid MiniPrep System (Promega) and the DNA was resuspended in 50 μL nuclease free water (Invitrogen). Plasmid pmpADAR1m were sequenced with E3L F primer, ADAR 1F primer (5' AGACAGAAACTCCACATCTGTCTC 3'), ADAR 2F primer (5' CTCCTTCTACAGTCATGGCTTG), ADAR 3F primer (5' GATGCCCTCCTTCTACAGTC 3'), ADAR 4F primer (5' CCTTTTGGAGTACGCCCG 3'), and ADAR 5F primer (5' TATGGAAAGCACAGAATCCCG 3'); and sequence results were blasted with the protein sequence of ADAR1 (Uniprot P55265).

Generation of Copenhagen VACVΔE3L::ADAR1 and VACVΔE3L::ADAR1m by Coumermycin selection. pmpADAR1 and pmpADAR1m were used in the *in vivo* recombination assay with VACVΔE3L::Neo^R-GFP-GyrB-PKR and recombinant viruses were selected under Coumermycin selection as previously described (214). Briefly, sub confluent BHK21 cell monolayers were with VACVΔE3L::Neo^R-GFP-GyrB-PKR at a multiplicity of infection of 0.05 and simultaneously transfected with 1 μg of plasmids using Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen). At 48 hours post infection, infected cells were harvested in MEM containing 2% FBS. This was allowed by 3 rounds of freezing at -80°C and thawing on ice for 30 minutes to release the virus. The recombinant viruses were selected by coumermycin at 10ng/ml concentration in RK-13 cells for 3 rounds. The recombinant viruses were sequenced to determine the correct sequence of ADAR1 and ADAR1m containing the H910Q and E912A mutation in the E3L locus of VACV. Expression of ADAR1 and ADAR1m were confirmed by Western Blotting.

Sequencing of the ADAR1 gene from the recombinant virus. 100 μL of virus

stock with a titer of $1x10^9$ pfu/mL was used for DNA extraction. Virus DNA was

extracted by treating the virus with a 100 μL of phenol equilibrated with 10 mM Tris

HCl, pH 8.0, 1 mM EDTA (Sigma). The aqueous phase from the above step was re-

extracted with 100 μL phenol:isoamylalcohol:chloroform (25:24:1) (Sigma). The aqueous

phase from the above step was re-extracted with 100 μL volume

chloroform:isoamylalcohol (24:1) (Sigma), followed by precipitation with 2.5 volumes of

95% ethanol, 1/10 volume of 7.5M ammonium acetate, and 10μL glycogen (Fermentas).

The DNA was washed in 70% ethanol, dried and resuspended in 50 μL of distilled water.

PCR was performed using E3L flanking primers to amplify the gene for

sequencing. Briefly, 100 ng of virus DNA template, 500 μM of E3L F, 500 μM of E3L

R, 500 μM dNTPs, 2 mM $MgSO_4$, 1X Pfx buffer, 1X Enhancer and *Platinum pfx*

polymerase enzyme (Invitrogen) were mixed in a 50 μL reaction volume. PCR

amplifications were performed with 95ºC for 5 minutes, followed by 30 cycles of

amplification (95ºC for 1 minute, 55ºC for 1 minute, 68ºC for 5 minutes). The PCR

product was subjected to agarose gel electrophoresis (0.8%, GTG grade) at 100 volts for

40 minutes, and the DNA band was cut from the gel. DNA was extracted from the gel

using Wizard® SV Gel and PCR Clean up Kit (Promega) according to the

manufacturer's instructions, and sequenced using ADAR 1F primer (5'

AGACAGAAACTCCACATCTGTCTC 3'), ADAR 2F primer (5'

CTCCTTCTACAGTCATGGCTTG), ADAR 3F primer (5'

GATGCCCTCCTTCTACAGTC 3'), ADAR 4F primer (5'

CCTTTTGGAGTACGCCCG 3'), and ADAR 5F primer (5'

52

TATGGAAAGCACAGAATCCCG 3'); and sequence results were blasted with the protein sequence of ADAR1 (Uniprot P55265).

Virus infections and stocks. BHK-21 cells were used to amplify the recombinant viruses. Stocks of recombinant viruses were generated by amplification of a single corrected plaque in 60 mm dish of BHK-21 cells. At 100% cytopathicity, the infected cells were harvested by scraping and centrifuging at 1000 rpm for 10 minutes at 4^oC. The cell pellet was resuspended in 10 mM Tris HCl pH 8.8. Three round of alternate freezing (-80^oC) and thawing on ice for 30 minutes, followed by vortexing for 30 seconds (3 times) was performed to lyse the cells and release the virus. The prep was then spun at 1000 rpm for 10 minutes at 4^oC and the virus in the supernatant was transferred into a fresh tube and stored at -80^oC. The virus was titered in RK-E3L cells.

For infections, the virus was diluted in MEM containing 2% FBS. Cell monolayers in a 60 mm dishes were infected with 100 μL of virus after aspirating the media off the dishes. Cells were incubated at 37^oC, 5% CO_2 for 1 hour, rocking every 10 minutes. Following infection, the appropriate cell culture media was replaced.

Protein extraction and Western Blotting. 50% confluent HeLa cell monolayers, pretreated with 1,000 IU/ml of human α A/D interferon (PBL) for 18 hours where indicated, were infected with viruses at a MOI of 5. At the indicated times post infection, cells were scraped into 150 μL of 1X SDS (62.5 mM Tris-Cl, 10% glycerol, 2% SDS, 0.0005% bromphenol blue, 0.1% 2-mercaptoethanol, 1X Halt Protease and Phosphotase Inhibitor Cocktail (Pierce Thermo Scientific)). Lysates were transferred into QIAshredder columns (Qiagen), spun at 16,000xg for 2 minutes at 4^oC, and stored at -80^oC. The samples were boiled for 5 min and ran on 10% and 12% SDS-PAGE gels at

150V. Proteins were transferred to nitrocellulose membranes at 100 volts for 60 min in 10 mM CAPS, pH 11 with 20% methanol. The membranes were blocked with 3% milk (Carnation® Non-fat Dry Milk) TTBS (20 mM Tris-HCl, pH 7.8, 180 mM NaCl, 0.05% Tween-20) for 1hr. The membranes were probed with primary antibodies overnight. Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000, Santa Cruz) or anti-mose IgG conjugated to horseradish peroxidase (1:10,000, Santa Cruz) were added followed by chemiluminescence (Pierce Thermo Scientific). ADAR1 antibody was purchased from Santa Cruz, eIF2α-P antibody was purchased from Cell Signaling, dsRNA antibody was purchased from Engscions, and GAPDH antibody was purchased from Abcam.

Immunofluorescence Microscopy. HeLa cells were seeded on poly L- lysine treated coverslips in 6 well dishes. HeLa cell monolayers, pretreated with 1,000 IU/ml of human α A/D interferon (PBL) for 18 hours where indicated, were infected with viruses at a MOI of 5. At indicated time points, the cells were rinsed twice with cold PBS. Subsequently, ice cold methanol was added and the cells were placed at -20ºC for 20 minutes. The cells were then washed twice with ice cold PBS and blocked with blocking buffer (0.3% gelatin, 0.1% triton X-100 in PBS) at room temperature for 30 minutes. The cells was incubated with prepared primary antibody: mouse anti-dsRNA (Engscions) and rabbit anti-VACV, diluted in 0.1% triton x-100 in PBS, overnight at 4°C. The cells were then washed five times with blocking buffer for 10 minutes per wash. The cells was incubated with prepared secondary antibodies:anti-rabbit IgG Alexa Fluor 488 and anti-mouse IgG Alexa Fluor 594 (Invitrogen), diluted in blocking buffer, for 1 hour at room temperature in the dark. After incubation, the cells were washed 3 times with blocking

buffer for 10 minutes per wash. 4'6diamino-2-phenylindole (DAPI) (Invitrogen) was added at a concentration of 5 µg/mL for 5 minutes at room temperature in the dark. The cells were rinsed with $^{dd}H_2O$ and then mounted onto slides with ProLong Gold antifade mounting reagent (Invitrogen). The samples were allowed to cure overnight. Samples were analyzed using the EVO-FL microscope (AMG).

Slot Blot for dsRNA. 50% confluent HeLa cell monolayers, pretreated with 1,000 IU/ml of human α A/D interferon (PBL) for 18 hours where indicated, were infected with viruses at a MOI of 5. At time indicated, total RNA was extracted from using the RNeasy Mini Kit (Qiagen). 100 ng to 1 µg of total RNA was diluted in 1mM EDTA, pH 8.0 to a final volume of 50 µL. Hybond-N+ (Amersham Pharmacia Biotech) positively charged membrane was soaked in $^{dd}H_2O$ and then in 10X SSC (Amersham Pharmacia Biotech). Diluted RNA was applied through a VacuSlot VS manifold and transferred onto membrane under vacuum. Membrane was washed twice with 10X SSC buffer. The membrane was UV crosslinked with a Stratagene Stratalinker 1800 (Stratagene) for 90 seconds. Western blot analysis was performed with J2 monoclonal dsRNA antibody (Engscions) for detection of the dsRNA.

Single-step growth assay. HeLa cells in 6 well plates were infected with MOI of 5 with VACV wt, VACVΔE3L, VACVΔE3L::ADAR, VACVΔE3L::ADARm. Plates were incubated at 4^oC for 30 minutes and rocked every 10 minutes. Plates were washed thrice with warm media. Half of the infected cells were harvested for 0 hour time point. Plates were incubated at 37^oC, 5%CO_2 for 24 hours. Infected cells were then harvested for 24 hour time point. Samples were frozen and thawed 3 times (frozen at -80^oC for 30 minutes, and then thawed on ice for 30 minutes, followed by 3 minutes thaw at 37^oC) to

release viruses. Ultimately, samples were spun at 1,000xg for 10 minutes at 4°C and the supernatants were collected. Viruses were titered using RK-E3L. The experiment was done in triplicate.

Multi-step growth assay. HeLa cells in 6 well plates were infected with MOI of 0.01 with VACV wt, VACVΔE3L, VACVΔE3L::ADAR1, VACVΔE3L::ADAR1m. Plates were incubated at 4°C for 30 minutes and rocked every 10 minutes. Plates were washed thrice with warm media. Half of the infected cells were harvested for 0 hour time point. Plates were incubated at 37°C, 5%CO_2 for 72 hours. Infected cells were then harvested for 72 hour time point. Samples were frozen and thawed 3 times (frozen at -80°C for 30 minutes, and then thawed on ice for 30 minutes, followed by 3 minutes thaw at 37°C) to release viruses. Ultimately, samples were spun at 1,000xg for 10 minutes at 4°C and the supernatants were collected. Viruses were titered using RK-E3L. The experiment was done in triplicate.

RESULTS

Pre-treatment of IFN reduces delayed-late time eIF2α phosphorylation.

It was reported that VACV deleted of the N-terminal of E3L can inhibit eIF2α phosphorylation at delayed-late time post infection when the infected cells were pre-treated with IFN (99). To confirm this, HeLa cells were mock treated or treated with 1000 U/ml of IFNα/β for 18 hours. Cells were then either mock infected or infected with either VACV wt, VACV deleted of the N-terminal of E3L, VACVΔ83N or VACVΔE3L at an MOI of 5. As expected mock and VACV wt infected cells showed no detectable eIF2α phosphorylation (Figure 13, lane 1, 3 and 9). VACVΔE3L infection led to eIF2α phosphorylation at 6 hours post infection (initial-late phase of the infection) and eIF2α phosphorylation increased at 9 hours post infection (delayed-late phase of the infection) (Figure 13, lane 5 and 11). Infection with VACVΔ83N did not lead to eIF2α phosphorylation until 9 hours post infection even though the amount of eIF2α phosphorylation was not as high as there were in VACVΔE3L infection (Figure 13, lane 4 and 10). When cells were pretreated with IFN, VACV wt was still able to inhibit eIF2α phosphorylation both at 6 and 9 hours post infection (Figure 13, lane 6 and 12). At 9 hours post infection, IFN-treated cells infected with VACVΔ83N and VACVΔE3L showed decreases of eIF2α phosphorylation compared to those without IFN treatment (Figure 13, lane 13 and 14). These results raised the question that if inhibition of the delayed-late time eIF2α phosphorylation in VACVΔE3L and VACVΔ83N infection is due to decrease in dsRNA made by these viruses.

Pre-treatment of IFN reduces dsRNA made by VACV. Since eIF2α phosphorylation is a result of activation of PKR by dsRNA made during VACV

57

infection, we hypothesized that reduction in eIF2α phosphorylation in IFN-treated cells infected with VACVΔ83N and VACVΔE3L is due to the decrease level of dsRNA made during VACV infection. To test this hypothesis, we employed the use of immunofluorescence and slot blotting probing with anti-dsRNA. HeLa cells were mock treated or treated with 1000U/ml of IFNs. After 18 hours post treatment, cells were mock infected or infected with either VACV wt, VACVΔE3L or VACVΔ83N. The infected cells were either fixed for immunofluorescence or harvested for total RNA. For immunofluorescence, cells were incubated with antibodies against total VACV proteins (anti VACV) and dsRNA.

The immunofluorescence data showed that dsRNA was detected within infection of VACV wt, VACVΔ83N and VACVΔ3L (Figure 14). When the cells were pre-treated with IFNs, the amount of dsRNA was not changed in VACV wt infection. Lower amount of dsRNA was detected in VACVΔ83N and VACVΔE3L infection when the cells were pre-treated with IFN (Figure 14).

To confirm our immunofluorescence data, a slot blot was performed. 100 ng of total RNA were applied onto a positive membrane via vacuumed slots. The RNA was then UV-hybridized and blotted with antibody against dsRNA. As expected, mock infected cells did not produce any dsRNA (Figure 15, lane 1). VACV wt, VACVΔ83N and VACVΔE3L virtually made the same amount of dsRNA (Figure 19, lane 2, 3 and 4). When the cells were treated with IFN, the amount of dsRNA in VACV wt did not decrease significantly (Figure 19, lane 5). On the other hand, the amount of dsRNA in VACVΔ83N decreased about 2 folds while it decreased about 4 folds in VACVΔE3L infection (Figure 19, lane 6 and 7). Taken together, both our immunofluorescence data

and slot blot data suggested that there are other factors that can decrease the amount of dsRNA in VACV infection; thus resulting in inhibition of delayed-late time eIF2α phosphorylation in VACVΔ83N and VACVΔE3L.

Virus construction. Literature searches to explain the above phenomenon focused our attention to the IFN inducible ADAR1 protein. Domain-wise, ADAR1 has both ZNA-BD and dsRNA-BMs as VACV E3 protein. Both Zα domain of E3 and ADAR1 shares close homology as well as the dsRNA-BMs (Figure 16). In addition, ADAR1 possesses a deaminase domain that can denature RNA with double stranded character. Thus, we hypothesized that expressing ADAR1 in VACVΔE3L can lead to reduction in the amount of dsRNA made by VACVΔE3L and it will rescue VACVΔE3L infection in non-permissive cell. To test the hypothesis, we put the IFN-induced full length ADAR1 gene into the E3L locus of VACV. We also put a mutant ADAR1 gene (ADAR1m) where the protein still retains its ability to bind to dsRNA but it is incapable of carrying out the deaminase function because of two point mutations (H910Q and E912A) in the catalytic domain (125) (Figure 17A). In order to confirm ADAR1 expression, sub-confluent HeLa cells were mock infected or infected at MOI of 5 with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cell lysates were prepared at 6 hours post infection and the proteins were visualized by western blotting using antibodies against ADAR1 protein. The full length ADAR1 (a 150 kDa protein) were detected from the infection of VACVΔE3L::ADAR1 and VACVΔE3L::ADAR1m compared to uninfected cells or cell infected with either VACV wt or VACVΔE3L. The constitutive form of ADAR1 (a 110 kDa protein) was detected

59

from all lysates (Figure 17B). These results indicated that IFN-induced ADAR1 protein was successfully expressed from the E3L locus of VACV.

ADAR1 deaminates dsRNA made by VACVΔE3L. To test whether or not the IFN-induced ADAR1 protein expressed by VACV can deaminate dsRNA, we employed the use of immunofluorescence and slot blotting. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. The infected cells were either fixed for immunofluorescence or harvested for total RNA. For immunofluorescence, cells were incubated with antibodies against total VACV proteins (anti VACV) and dsRNA. Both VACVwt (data not shown) and VACVΔE3L led to production of dsRNA in the infected cells. However, when IFN-induced ADAR1 was expressed from the E3L locus, dsRNA was undetectable using immunofluorescence. The inactive catalytic mutant of ADAR1 which cannot deaminate dsRNA led to production of dsRNA in the infected cells even though the level of dsRNA was not as high as in the VACVΔE3L infection (Figure 18). This low level detection of dsRNA might be due to the interaction of the ADAR1 dsRNA-BMs or other cellular dsRNA binding proteins where dsRNA blocks the binding affinity of the detection antibody against dsRNA.

To confirm our immunofluorescence data, a slot blot was performed. 100 ng of total RNA were applied onto a positive membrane via vacuumed slots. The RNA was then UV-hybridized and blotted with antibody against dsRNA. As expected, mock infected cells did not produce any dsRNA (Figure 19, lane 1). Both VACV wt and VACVΔE3L made dsRNA (Figure 19, lane 2 and 3). VACVΔE3L::ADAR1 infection led to a 7 fold decrease in dsRNA (Figure 19, lane 4). The decrease in dsRNA correlated

with the ability to deaminate dsRNA because VACV expressing the inactive catalytic ADAR1 still led to formation of dsRNA detected by slot blot (Figure 19, lane 5). Taken together VACV expressing ADAR1 protein in E3L locus can lead to a significant decrease in dsRNA and this reduction is correlated with the ability to deaminate dsRNA.

Expression of ADAR1 in E3L locus rescues VACVΔE3L protein expression.

Since dsRNA made by VACV can lead activation of PKR and ultimately lead to protein translation shut off, we asked if expression of ADAR1 in E3L locus can rescue VACVΔE3L protein expression. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using specified antibodies. We looked at phosphorylation of eIF2α as an indication of protein translation shut off. VACV wt has E3 that can sequester dsRNA thus it inhibited eIF2α phosphorylation and allowed for viral protein expression (Figure 20, lane 2). VACVΔE3L led to phosphorylation of eIF2α, thus viral protein expression was inhibited (Figure 20, lane 3). Expression of IFN-induced ADAR1 in E3L locus inhibited eIF2α phosphorylation and allowed viral protein synthesis (Figure 20, lane 4). However, this rescue can only happen when ADAR1 retains its deaminase activity since VACVΔE3L::ADAR1m infection leads to eIF2α phosphorylation and inhibition of viral protein synthesis (Figure 20, lane 5). Thus, VACV expressing IFN-induced ADAR1 protein in E3L locus can rescue VACVΔE3L protein expression and this reduction is correlated with the ability to deaminate dsRNA.

Expression of ADAR1 in E3L locus rescues VACVΔE3L replication in HeLa.

Since IFN-induced ADAR1 could rescue viral protein synthesis in VACVΔE3L,

61

we asked if it could lead to rescue of replication in a VACVΔE3L-restricted cell line such as HeLa. In order to study the replication of recombinant VACV, HeLa cells were infected at MOI of 1 with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1, or VACVΔE3L::ADAR1m. Cells were washed 3 times with warm PBS and harvested at 30 minutes post infection (0 hour post infection) and 24 hours post infection. VACV wt showed approximately a 100-fold-increase in replication 24 hours post infection while VACVΔE3L did not show an increase in replication (Figure 21). VACVΔE3L::ADAR1 showed approximately a 10-fold-increase in replication after 24 hours while VACVΔE3L::ADAR1m showed no changes in replication within 24 hours post infection (Figure 21).

To ask if ADAR1 can rescue VACVΔE3L replication in a multi-step condition, HeLa cells were infected at MOI of 0.01 with VACV wt, VACVΔE3L, VACVΔE3L::ADAR1, or VACVΔE3L::ADAR1m. Cells were washed 3 times with warm PBS and harvested at 30 minutes post infection (0 hour post infection) and 72 hours post infection. VACV wt showed approximately an 800-fold-increase in replication 72 hours post infection while VACVΔE3L did not show an increase in replication (Figure 22). VACVΔE3L::ADAR1 showed approximately a 400-fold-increase in replication after 72 hours while VACVΔE3L::ADAR1m showed no change in replication within 24 hours post infection (Figure 22). Taken together, this data demonstrate the ability of ADAR1 to partially rescue replication of VACVΔE3L in HeLa cells, and this requires the catalytic deaminase activity of ADAR1.

Expression of ADAR1 in E3L locus rescues restricted host range phenotype of VACVΔE3L. VACV host range is dependent on the presence of a functional

E3L dsRNA-binding domain. To ask if IFN-induced ADAR1 can rescue VACVΔE3L host range phenotype, VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 and VACVΔE3L::ADAR1m were titered in the permissive cell line RK13, or non-permissive cell line HeLa, BSC40 (African green monkey kidney cell) and JC (murine mammary carcinoma cell). VACV wt formed plaques virtually in all cell types even though small plaque phenotypes were observed in both HeLa and JC (Figure 23). VACVΔE3L did not plaque in non-permissive cell line (Figure 23). VACVΔE3L::ADAR1 formed plaques in all cell types but the plaque morphology is even smaller than VACV wt in JC (Figure 23). And the ability to rescue VACVΔE3L restricted host range requires the ability to deaminate dsRNA because VACVΔE3L::ADAR1m did not form plaques in non-permissive cell lines (Figure 23).

Expression of ADAR1 in E3L locus cannot restore the IFN resistance phenotype of VACVΔE3L. Since ADAR1 can restore the broad host range phenotype of VACVΔE3L, we asked if it can also restore the IFN resistance phenotype of VACVΔE3L. To determine this, the plaque reduction assays were performed. RK13 cells were treated with varying doses of recombinant IFNα/β 18 hours post infection. Treated cells were then infected with approximately 100 plaque-forming-units of VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cells were stained with crystal violet by 48 hours post infections. As expected, VACV wt is resistant to the antiviral effect of IFN but VACVΔE3L failed to inhibit this effect (Figure 24). Unexpectedly, both VACVΔE3L::ADAR1 and VACVΔE3L::ADAR1m viruses followed a similar trend as the VACVΔE3L infection of IFN sensitivity (Figure 24). As a result, the expression of ADAR1 could not rescue VACVΔE3L IFN sensitivity.

63

DISCUSSION

In this study, we initially set out to investigate the phenomenon of inhibition of delayed-late time eIF2α phosphorylation during VACVΔ83N infection in IFN primed cells. Our results agreed with those previously reported (99) that eIF2α phosphorylation was inhibited during VACVΔ83N infection in IFN primed cells. There was also a significant decrease in eIF2α phosphorylation during VACVΔE3L infection in IFN primed cells (Figure 13). This was surprising to us since IFN would increase expression of PKR which would lead to more activation of eIF2α. We thus hypothesized that this inhibition of eIF2α phosphorylation is correlated with the level of dsRNA present in the infected cells.

To test the above hypothesis, we utilized slot blot and immunofluorescence methods. Our IF data showed that there was a decrease in dsRNA detected in both VACVΔE3L and VACVΔ83N infection when the cells were treated with IFN. Our slot blot data showed that in VACVΔE3L infection, there was a significant decrease in the amount of dsRNA when the cells were treated with IFN while there was a modest decrease in dsRNA in IFN-treated cells infected with VACVΔ83N (Figure 15). Taken together, the hypothesis is correct since there was a decrease in the level of dsRNA when the cells were treated with IFNs in both VACVΔ83N and VACVΔE3L. Previously published research led us to an IFN-induced enzyme that portrays a deaminase activity that has been shown to destabilize dsRNA structure (138).

Due to its close homology to E3 (Figure 16), The IFN-induced form of ADAR1 was put into the E3L locus of VACVΔE3L in order to study its function during VACV infection and its ability to rescue VACVΔE3L. In addition to binding Z-NA and dsRNA,

64

the ADAR1 proteins differs from E3 in that it has deaminase activity that allows it to deaminate adenosines to inosines in dsRNA (13, 148). To study if this deaminase activity is necessary for rescuing VACVΔE3L, a catalytically inactive mutant of ADAR1 was also put into the E3L locus of VACVΔE3L. Our data suggests that binding to dsRNA alone is not sufficient to allow rescuing of VACVΔE3L but rather a synergistic affect between binding to dsRNA and the deaminase activity can partially recue replication of VACVΔE3L.

Our results indicate that IFN-induced ADAR1 exhibited deaminase activity in VACVΔE3L infection since VACVΔE3L expressing IFN-induced ADAR1 showed a significant decrease in dsRNA (Figure 18 and 19). And the ability to reduce the level of dsRNA requires the catalytic domain of ADAR1 since VACVΔE3L expressing the catalytically inactive mutant of ADAR1 showed no reduction in dsRNA (Figure 18 and 19). Rescue experiments in HeLa cells demonstrate that the IFN-induced ADAR1 protein could rescue protein synthesis of VACVΔE3L and this ability also requires the catalytic domain of ADAR1 since VACVΔE3L::ADAR1m could not rescue protein synthesis of VACVΔE3L (Figure 20). This rescue of protein synthesis is also consistent with the ability of ADAR1 to inhibit the phosphorylation of eIF2α in VACVΔE3L infection (Figure 20). Thus, this data suggests that our phenomenon of inhibition of eIF2α phosphorylation during VACVΔ83N and VACVΔE3L in IFN treated cells is due to the activity of the IFN-induced ADAR1.

ADAR1 also has the ability to rescue replication of VACVΔE3L in HeLa cells. Our data shows that ADAR1 can only partially rescue VACVΔE3L. The partial rescue of VACVΔE3L by ADAR1 in HeLa cells correlates with the intermediate levels of

replication of VACVΔE3L::ADAR1 in both single-step (Figure 21) and multi-step (Figure 22) growth assay. And this ability of ADAR1 to rescue replication of VACVΔE3L requires its deaminase activity since VACVΔE3L::ADAR1m could not replicate at all in HeLa cells as well as VACVΔE3L. We further investigated if ADAR1 can rescue VACVΔE3L in other cell lines. Our results showed that ADAR1 can rescue VACVΔE3L in all tested non-permissive cell lines (Figure 23) and this ability does require the deaminase activity. Taken together, ADAR1 can partially rescue VACVΔE3L' protein synthesis and replication in non-permissive cell lines; however, this rescue does require the deaminase activity of ADAR1.

Although ADAR1 can partially rescue the replication of VACVΔE3L in non-permissive cell lines, the IFN resistance assays conducted with the recombinant ADAR1 virus in RK13 cell gave different results. To our surprise, both VACVΔE3L::ADAR1 andVACVΔE3L::ADAR1m showed an IFN sensitivity trend similar to one in VACVΔE3L (Figure 24). This may be due to the differences in affinity between E3 and ADAR1 for the viral activators of the IFN response in RK13 cells. This is consistent with the demonstrated differences in specificity between PKR and ADAR1 dsRNA binding domain (150).

We have attempted to show deaminase activity in VACVΔE3L::ADAR1's RNA transcript. However, our attempt has not been successful. Previously published data indicated that the Tudor staphylococcus nuclease (Tudor-SN) domain of the RISC complex can bind to hyper-edited dsRNA and promotes its cleavage (34). The cleavage sequence for Tudor-SN has been shown to be IIUI stretch base pair. This sequence has a normal DNA sequence of AATA which presents multiple time in VACV genome since

poxvirus is an AT rich virus. Since ADAR1 can exhibit hyper-editing activity, we hypothesized that our deaminated RNA transcript had been cleaved by Tudor-SN resulting in our inability to retrieve deaminated RNA sequence. In order to test this hypothesis, we are currently attempting to knock down the Tudor-SN protein or overexpressing a dsRNA that does not have IIUI base pair in VACVΔE3L::ADAR1 infection.

In conclusion, here we showed that the IFN-induced gene, ADAR1, can rescue VACVΔE3L while provided in trans by deaminating dsRNA made by VACVΔE3L. We also thought that the IFN-induced form of ADAR1 is responsible for deaminating dsRNA made by VACVΔ83N and VACVΔE3L. VACVΔ83N still possess a dsRNA binding domain of E3 thus it can inhibit eIF2α phosphorylation at 6 hours post infection. However, we thought the amino-terminal of E3 is required to stabilize the sequestering of dsRNA made by VACVΔ83N; thus, VACVΔ83N failed to inhibit eIF2α phosphorylation at 9 hours post infection. When the cells were treated with IFNs to mount the antiviral state, we thought that at 9 hours post infection, dsRNA made by VACVΔ83N were exposed and bound to the IFN-induced ADAR1 and subsequently got deaminated by ADAR1 and hypothetically degraded by Tudor-SN. We also tried to transiently knock down ADAR1 during VACV wt and VACVΔ83N infection. Our knock down data showed that ADAR1 is necessary for VACV E3 protein synthesis when the cells were treated with IFNs. This might be due to over activation of PKR in primed cells when induced PKR binds to dsRNA (Supplementary figure 1). This suggests that ADAR1 is dampening the effect of PKR during the antiviral state and VACV possibly uses ADAR1 to its advantage to overcome the antiviral state. Thus, ADAR1 might play a pro-viral role

67

during VACV infection. The role of ADAR1 has been studied and described in several RNA virus systems, this is the first time that ADAR1 is shown to have a proviral effect in VACV, a DNA virus.
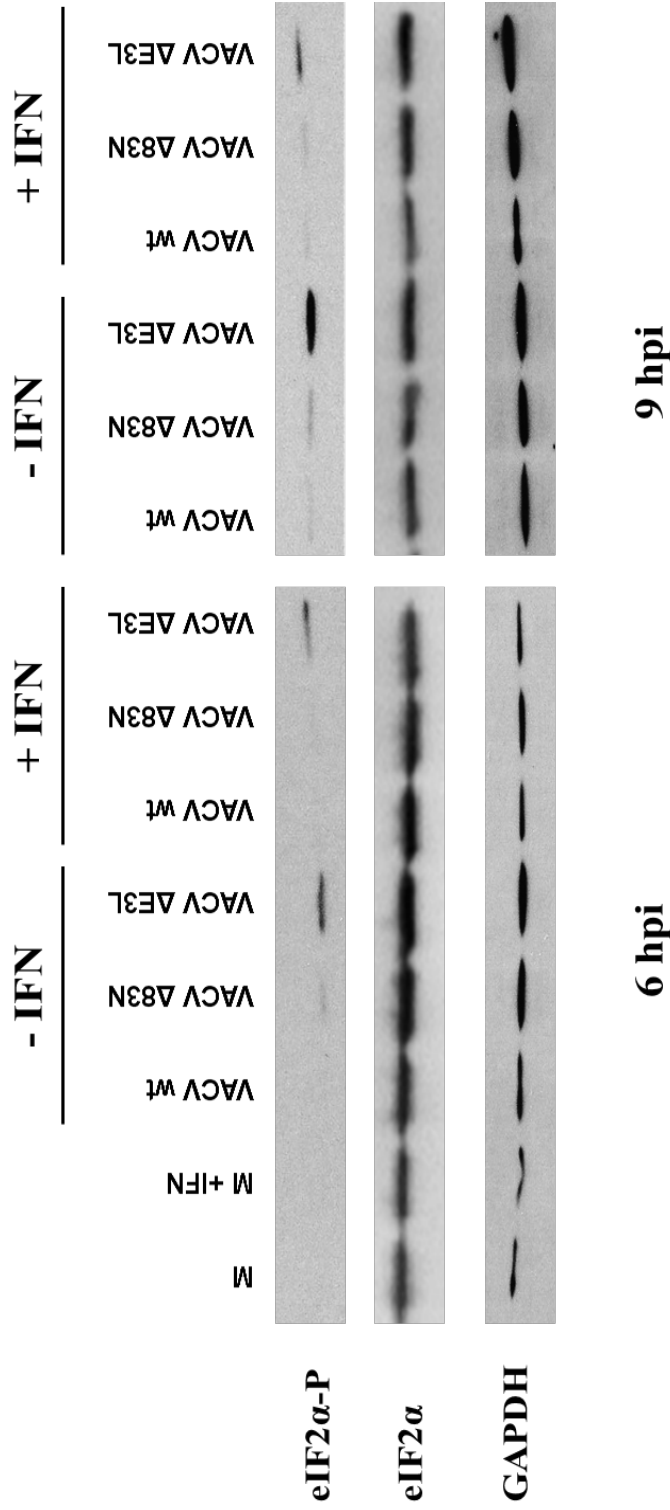
Figure 13 – Pre-treatment of IFN reduces late time eIF2α phosphorylation. HeLa cells were mock treated or treated with 1000 U/ml of IFNα/β for 18 hours. Cells were then either mock infected or infected with either VACV wt, VACV deleted of the N-terminal of E3L, VACVΔ83N or VACVΔE3L at an M.O.I of 5. Cell lysates were prepared at the above time indicated. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using above specified antibodies
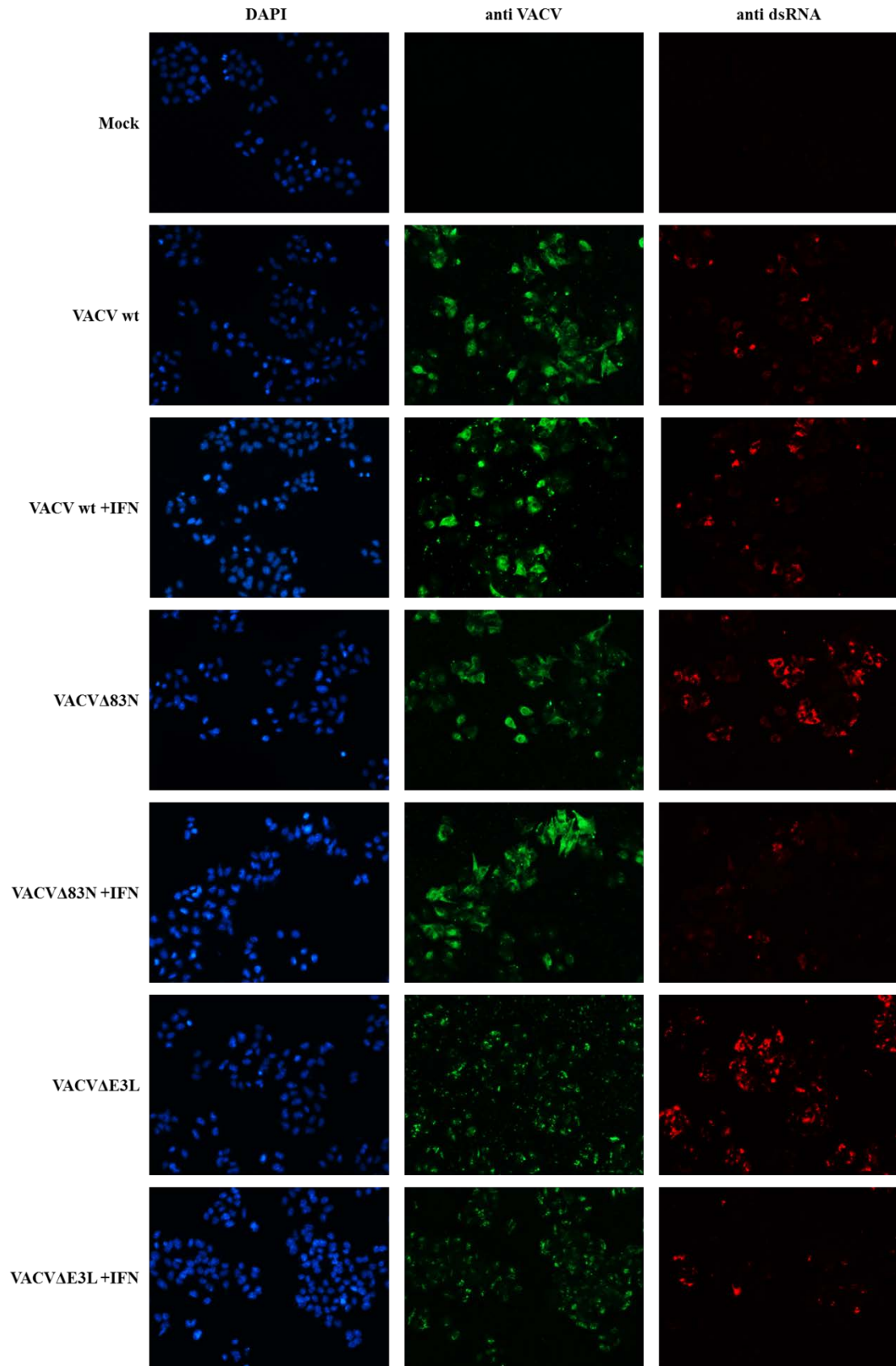
69

	DAPI	anti VACV	anti dsRNA
Mock			
VACV wt			
VACV wt +IFN			
VACVΔ83N			
VACVΔ83N +IFN			
VACVΔE3L			
VACVΔE3L +IFN			

70

Figure 14 – Pretreatment of IFN reduced level of dsRNA in VACV infection. HeLa cells were mock treated or treated with 1000 U/ml of IFN. After 18 hours post treatment, cells were mock infected or infected with either VACV wt, VACVΔ83N or VACVΔE3L. Cells were fixed with ice cold methanol after 9 hours post infection. Cells were incubated with antibodies against total VACV proteins (VACV) and dsRNA. Cells were subjected to immunofluorescence.
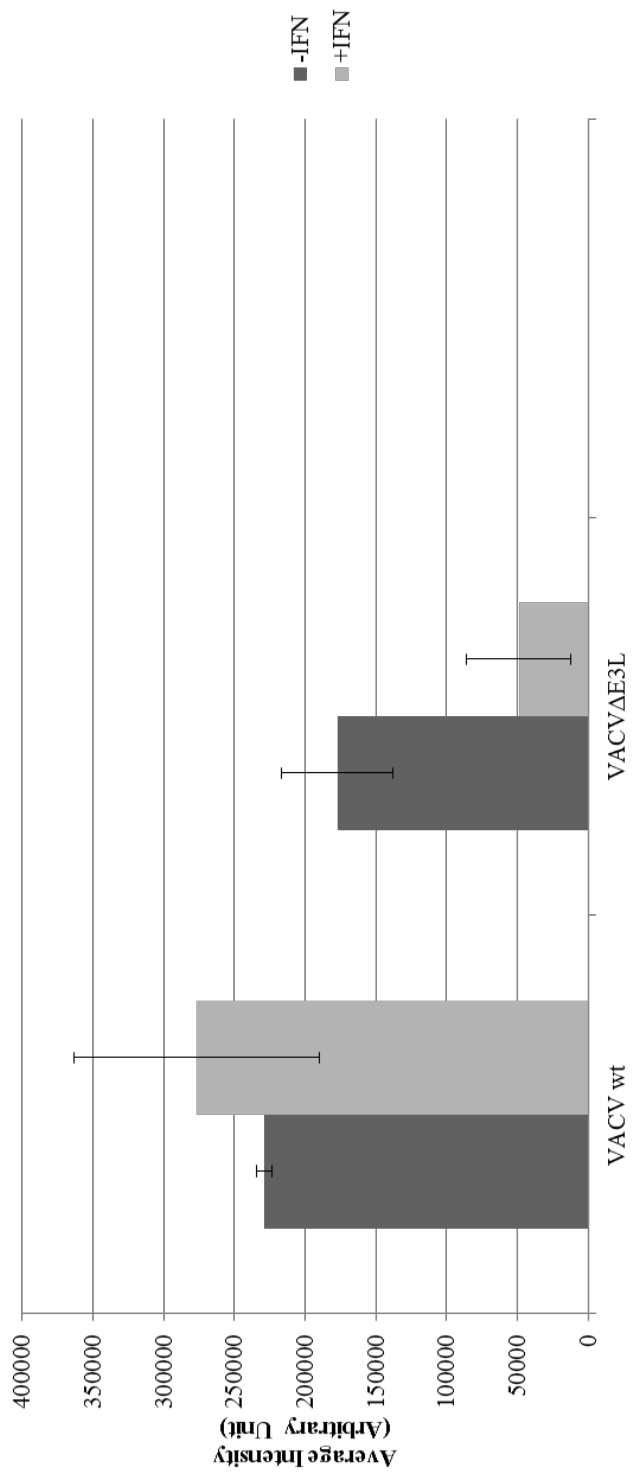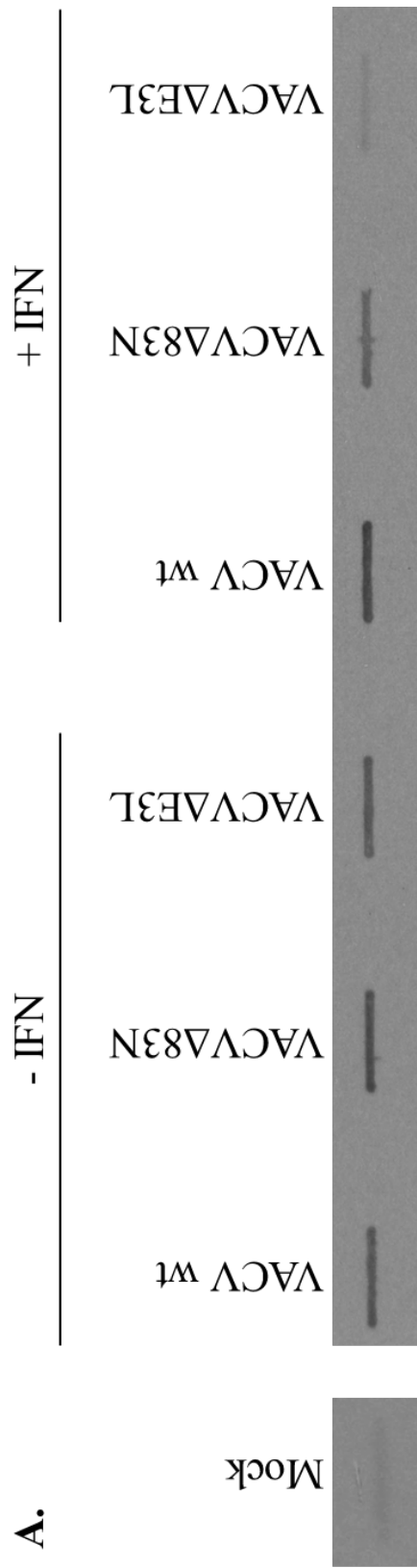
Figure 15 – Pretreatment of IFN reduced level of dsRNA in VACV infection. A. Representative slot blot of total RNA of infected cells. HeLa cells were mock treated or treated with 1000 U/ml of IFN. After 18 hours post treatment, cells were mock infected or infected with either VACV wt, VACVΔ83N or VACVΔE3L. Total RNA were extracted at 6 hours post infection. 100 Nano gram of total RNA were applied onto a positive membrane via vacuumed slots. The RNA were then UV-hybridized and blotted with antibody against dsRNA. **B.** Quantification of slot blots. Three slot blots experiments (A) were done. The radiography films were scanned and analyzed by TOTAL LAB software
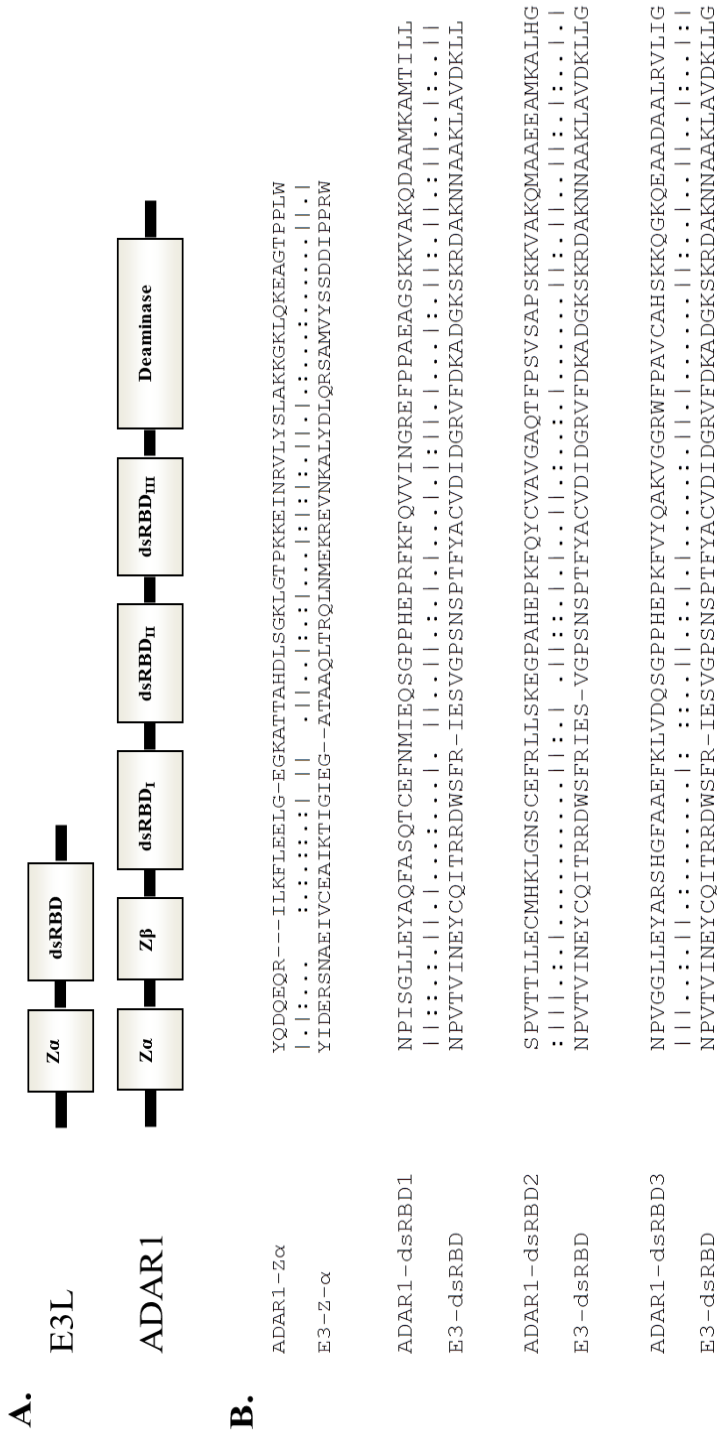
73

Figure 16 – Homology between E3 and the IFN-induced ADAR1. A. Schematic representation of homology between E3 and the IFN-induced ADAR1. Both E3 and the IFN-induced ADAR1 possess a Z-NA binding domain (Zα) that can bind to Z form nucleic acid at the N-terminal. E3 has a dsRNA binding domain (dsRBD) at the C-terminal while ADAR1 possesses 3 dsRBD in the center region. **B.** Sequence homology of each domain between ADAR1 and E3
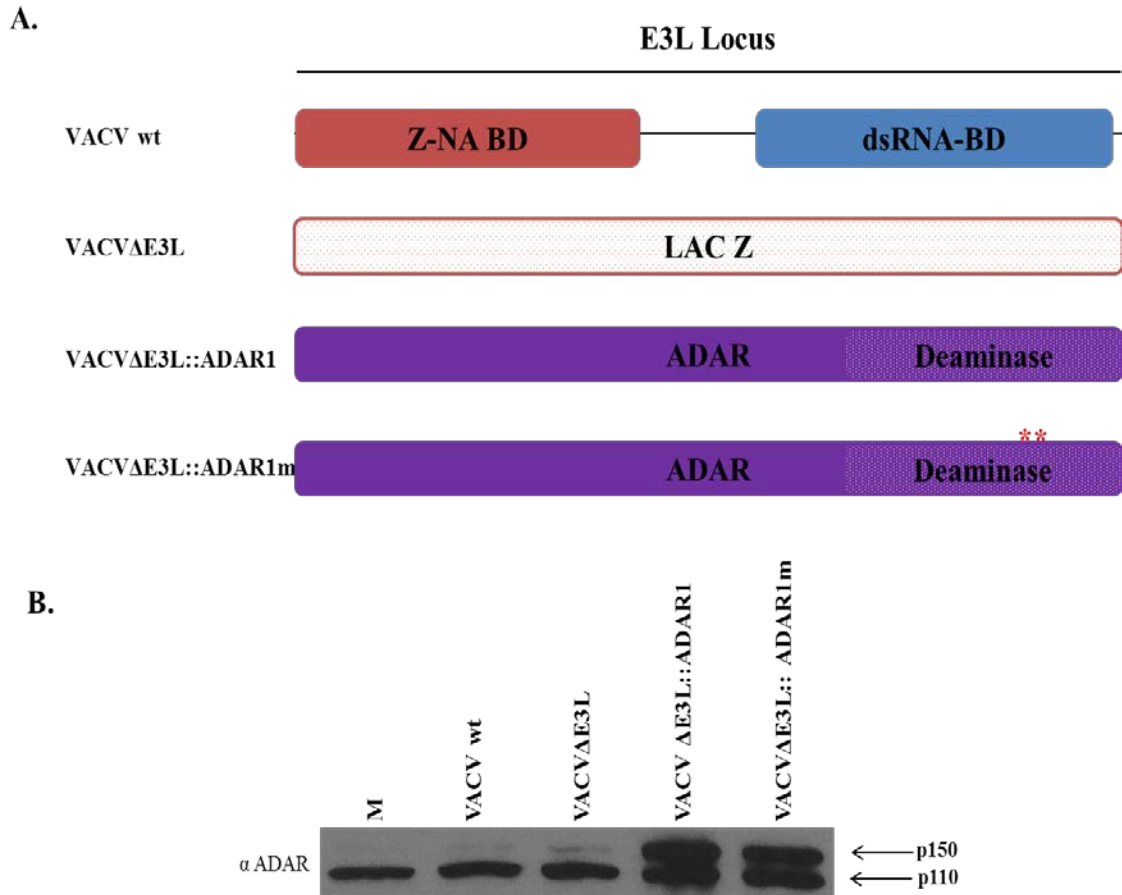
74

Figure 17 – Virus construction and protein expression of ADAR1.

A. Schematic representation of virus constructs**.** VACV wt represents wild type VACV, which encodes a full length E3 protein with a Z-NA binding domain in the amino terminus and a dsRNA binding domain in the carboxy terminus. VACVΔE3L represents virus deleted of the E3L gene, which expresses *LazZ* from the E3L locus. VACVΔE3L::ADAR1 represents VACV expressing the wild type IFN-induced ADAR1. VACVΔE3L::ADAR1m represents VACV expressing the catalytically inactive mutant of ADAR1. This mutant contains two point mutations (asterisks) (H910Q, E912A) in the catalytic domain that render it catalytically inactive. These mutations are denoted by an asterisk in the figure. **B. Protein expression of ADAR.** HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, recombinant VACV expressing the IFN inducible ADAR from the E3L locus (VACVΔE3L::ADAR) or recombinant VACV expressing the inactive catalytic ADAR from the E3L locus (VACVΔE3L::ADARm). . Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using antibodies to ADAR1 protein.

VACVΔE3L::ADAR1m
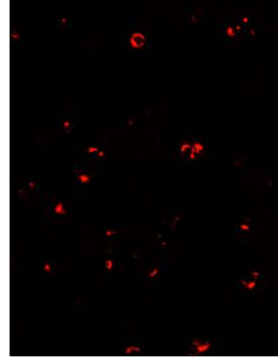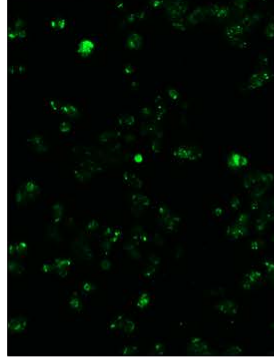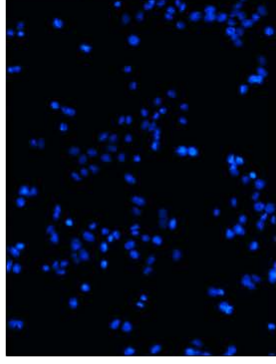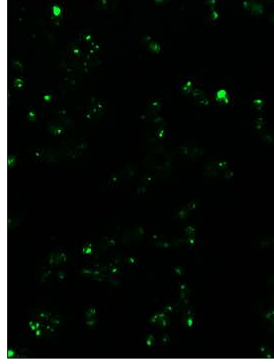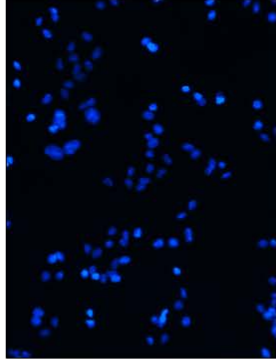
VACVΔE3L::ADAR1

VACVΔE3L
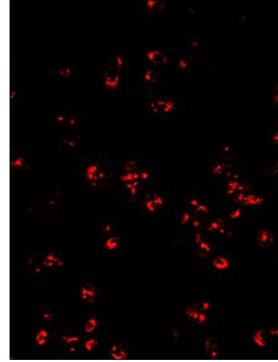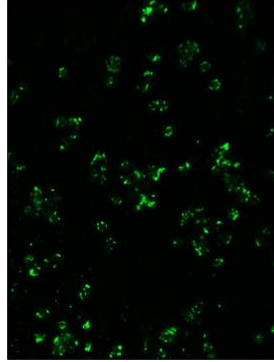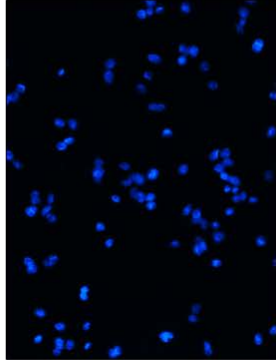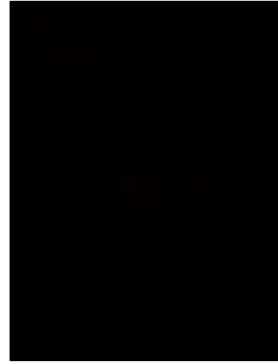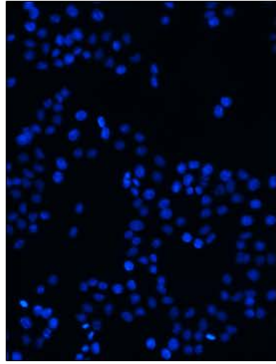
MOCK

DAPI

anti VACV

anti dsRNA

Figure 18 – VACV expressing the full length ADAR1 deaminates dsRNA. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cells were fixed with ice cold methanol after 6 hours post infection. Cells were incubated with antibodies against total VACV proteins (VACV) and dsRNA. Cells were subjected to immunofluorescence.
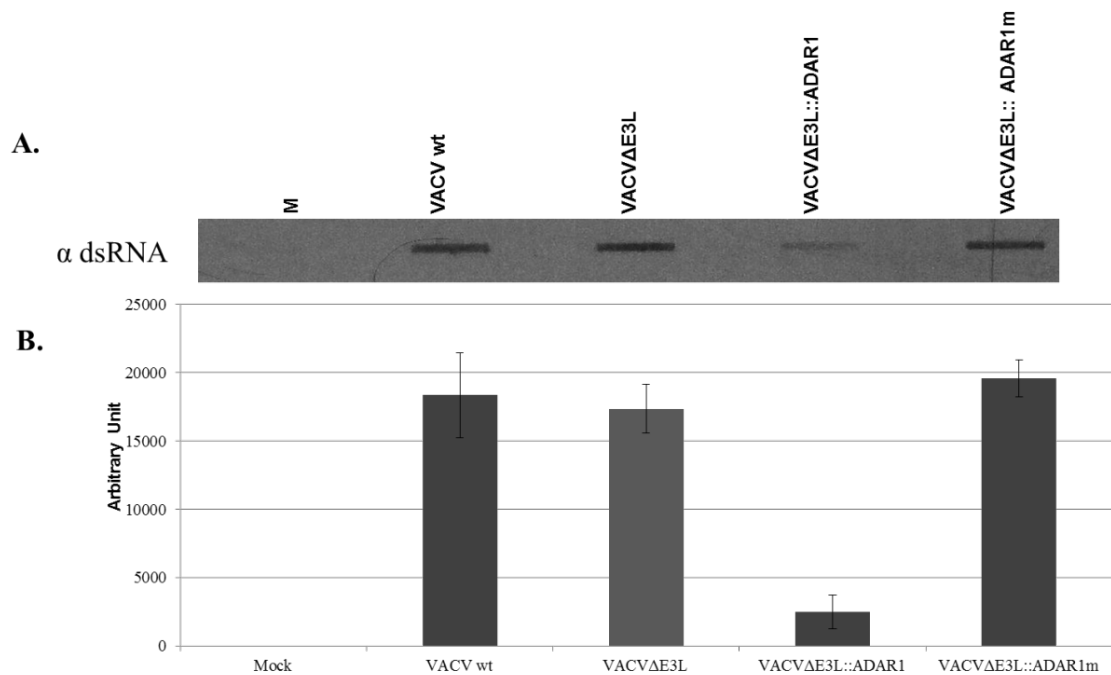
Figure 19 – VACV expressing the full length ADAR1 deaminates dsRNA.

A. Slot blot of total RNA of infected cells. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Total RNA were extracted at 6 hours post infection. 100 Nano gram of total RNA were applied onto a positive membrane via vacuumed slots. The RNA were then UV-hybridized and blotted with antibody against dsRNA. **B.** Quantification of slot blots. Three slot blots experiments (A) were done. The radiography films were scanned and analyzed by TOTAL LAB software.
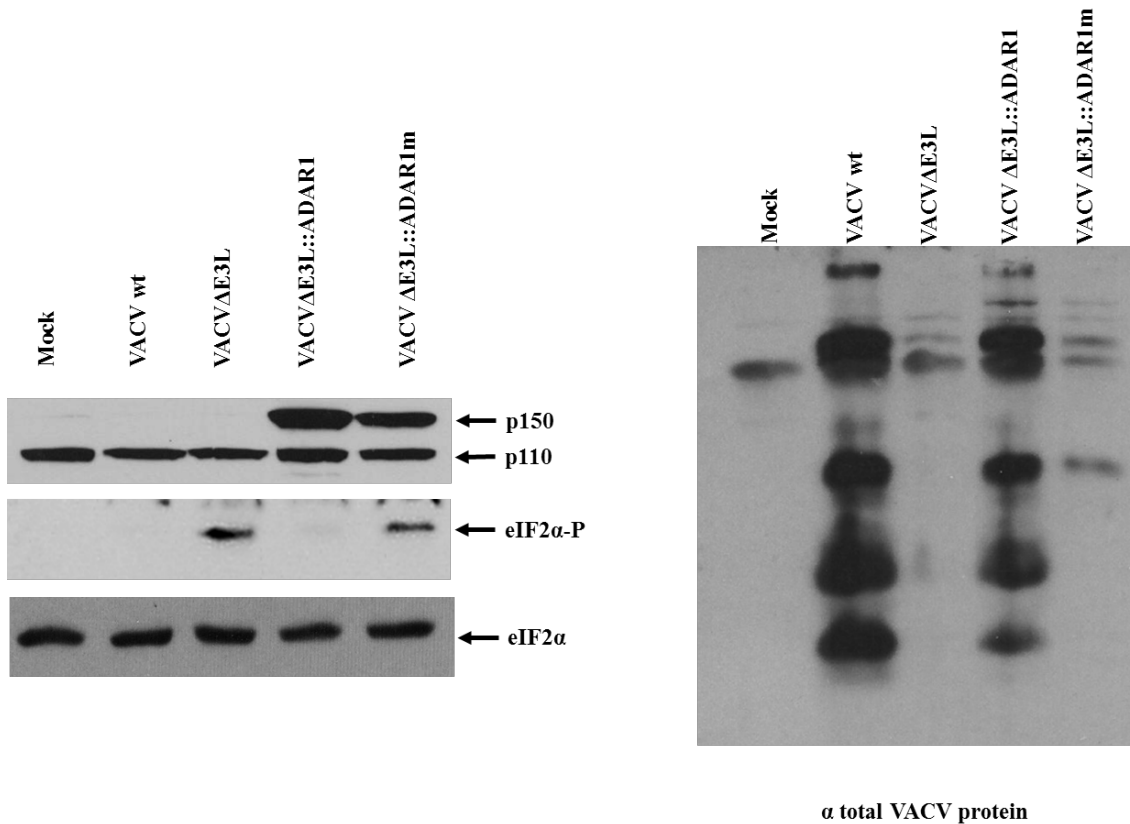
Figure 20 – VACV expressing the full length ADAR1 rescues VACVΔE3L late protein synthesis. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cell lysates were prepared at 6 hours post infection. The proteins were resolved on a 10% SDS PAGE gel followed by western blotting using specified antibodies.
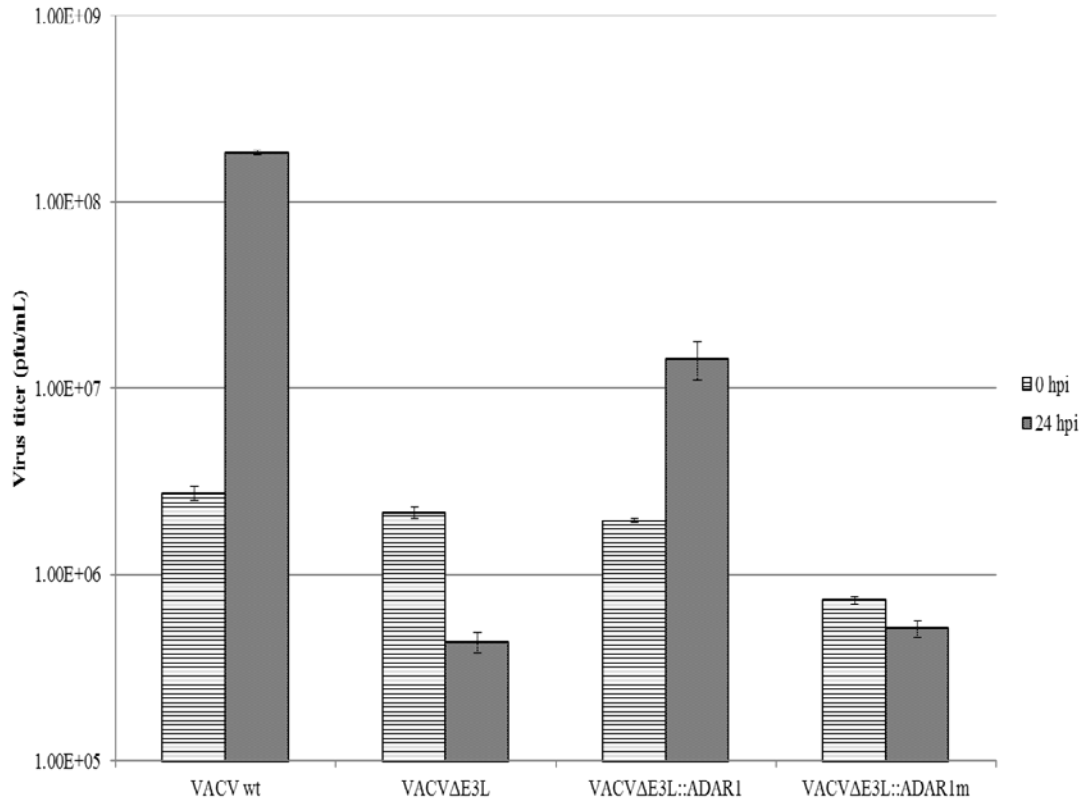
Figure 21 – Single step growth of VACV recombinant virus in HeLa. HeLa cells were infected at M.O.I of 1 with VACV wt, VACVΔE3L, VACVΔE3L::ADAR1, VACVΔE3L::ADAR1m. Cells were washed 3 times with warm PBS and harvested at 30 minutes post infection (0 hour post infection) and 24 hours post infection. Collected viruses were titered in RK13 cells. Experiments were done in duplicate
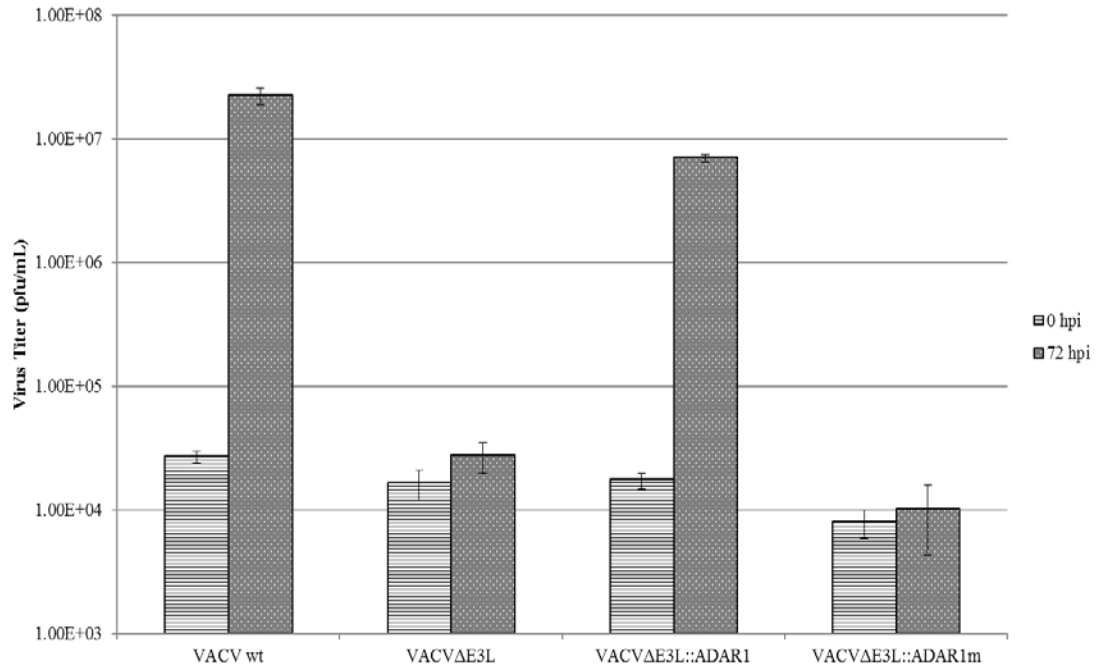
Figure 22 – Multi growth of VACV recombinant virus in HeLa.
HeLa cells were infected at M.O.I of 0.1 with VACV wt, VACVΔE3L, VACVΔE3L::ADAR1, VACVΔE3L::ADAR1m. Cells were washed 3 times with warm PBS and harvested at 30 minutes post infection (0 hour post infection) and 72 hours post infection. . Collected viruses were titered in RK13 cells. Experiments were done in duplicate

JC

HeLa

BSC40

RK13

VACV wt

VACVΔE3L

VACVΔE3L::ADAR1
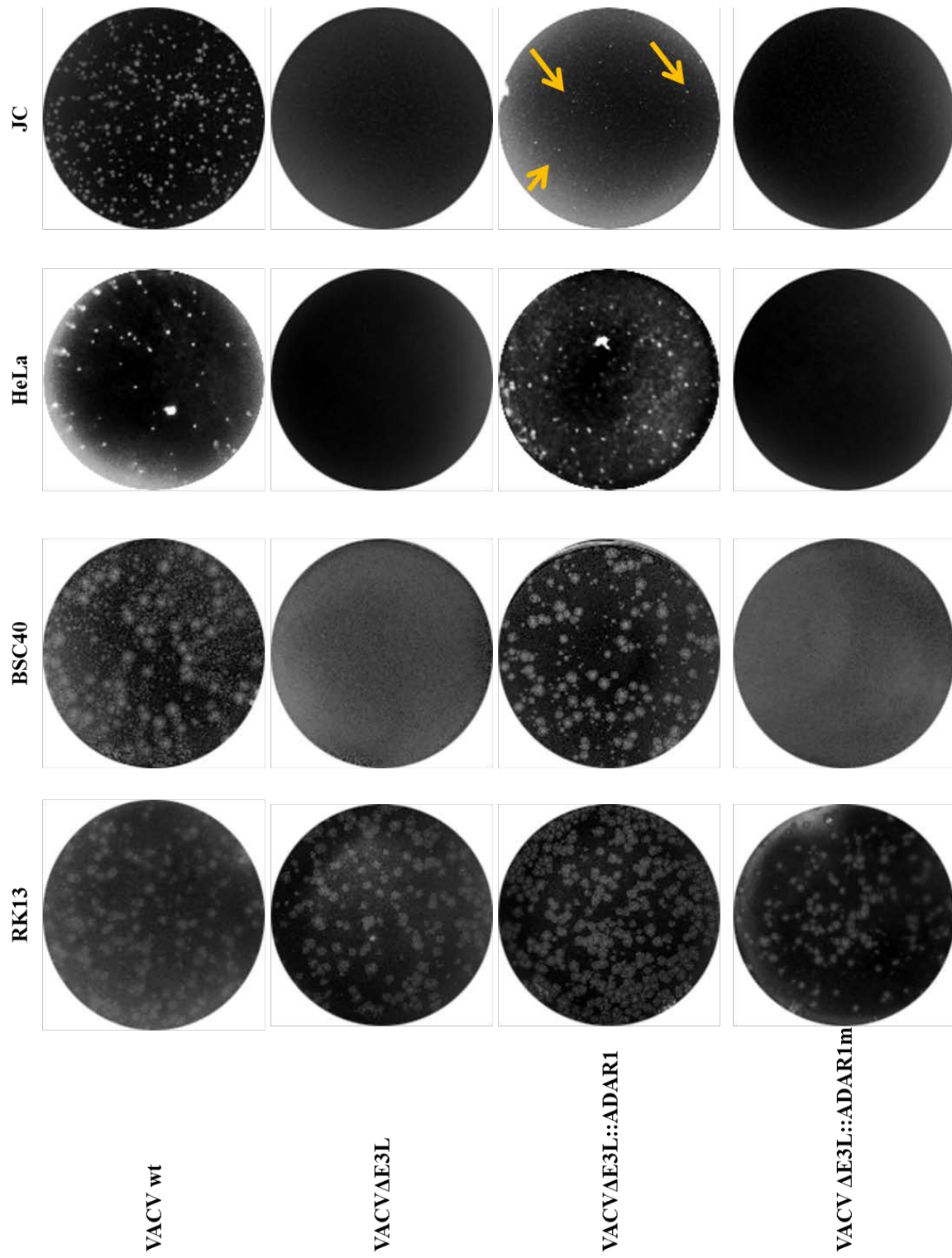
VACV ΔE3L::ADAR1m

Figure 23 – VACV expressing the full length ADAR1 rescues VACVΔE3L restricted host range. VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 and VACVΔE3L::ADAR1m were titered in permissive cell line RK13, or non-permissive cell line HeLa, BSC40 (African green monkey kidney cell) and JC (murine mammary carcinoma cell). Representative well was shown. The experiments were done in duplicate.
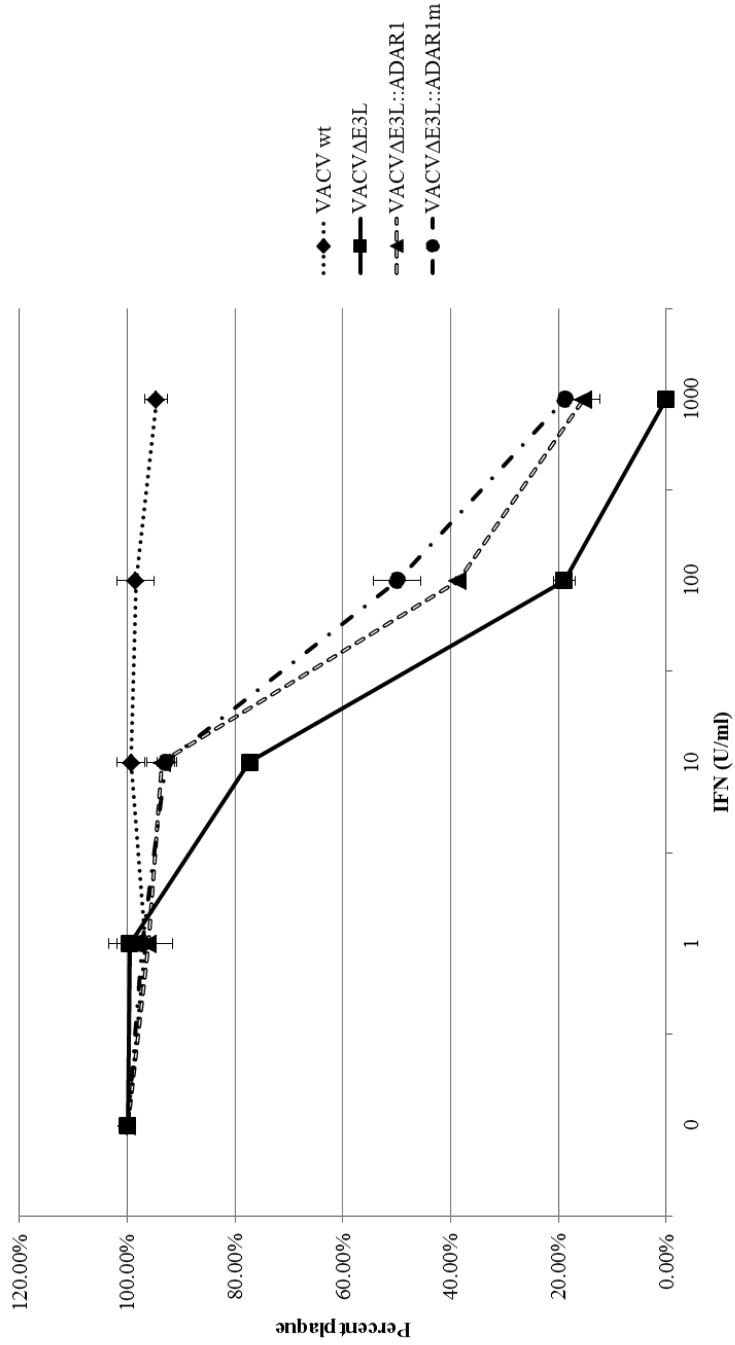
Figure 24 – IFN sensitivity assay. RK13 cells were treated with various dose of recombinant IFNα/β 18 hours post infections. Treated cells were then infected with approximately 100 plaque-forming-units of VACV wt, VACVΔE3L, VACVΔE3L::ADAR1 or VACVΔE3L::ADAR1m. Cells were stained with crystal violet by 48 hours post infections.

CHAPTER 3

CHARACTERIZATIONS OF REPLICATION COMPETENT NYVAC STRAIN

VACCINIA VIRUS AS A VACCINE VECTOR FOR HUMAN

IMMUNODEFICIENCY VIRUS 1

ABSTRACT

In 2011, there were 34 million people living with HIV/AIDS, though there is still no current vaccine for this pandemic. Vaccinia virus (VACV), a member of the poxvirus family, was successfully used in the eradication of smallpox. Since then, it has been studied and developed as heterologous vaccine vector for several animal diseases. In 2009, using ALVAC®, a member of poxvirus family, as a vaccine vector for HIV in the "Thai trial," known as RV144, researchers for the first time showed that the vaccine was modestly effective in the reduction of HIV infection. Hence, poxvirus has been studied and developed for a safer and more effective HIV vaccine vector. Here we constructed and characterized a replication competent and immunogenic poxvirus HIV vaccine vector using the vaccinia virus (VACV) strain NYVAC. To achieve a replication competent virus, we reinserted the two host range genes, K1L and C7L, into NYVAC. The VACV interferon α/β receptor like protein, B19R, was removed to improve the immunogenicity of the vector. Our results shows that the replication competent NYVAC vaccine vectors, NYVAC-C-KC and NYVAC-C-KC ΔB19R, are fully replicative in human keratinocyte and human dermal fibroblast, express high level of HIV antigens in infected cells, and activate the innate signal transduction pathways while being highly attenuated. These preliminary data indicate that these viruses have a potential as an improved vaccine vectors for HIV.

INTRODUCTION

Since it was first reported in the 1980s, HIV/AIDS has become one of the greatest pandemics of our time, effecting human personally, socially and economically. As of 2011, there are 34.0 million people living with AIDS. Thus, the need of a HIV vaccine is unquestionable. Using a combination of a recombinant canarypoxvirus ALVAC® and the protein gp120, the phase III Thai clinical trial in 2009 observed a limited protection against HIV-1 infection, about 31% (163). This suggests that poxvirus vector can be used as one of the future HIV/AIDS vaccine candidate vectors; however, further development is required for a safer and effective vaccine vector.

Vaccinia virus (VACV) is a large DNA virus that can replicate entirely in the cytoplasm (131). It has been used as a mean of vaccination for the smallpox pathogen since the method was first introduced by Edward Jenner in the 1700s (164). After the eradication of smallpox in 1970s, VACV has been studied and developed as vaccine vectors for several pathogens. VACV is considered to be a good candidate for heterologous vaccine vector because its ease to generate recombinant VACVs to express heterologous genes (130, 145). Two strains of VACV have been extensively developed as HIV vaccine vectors, modified vaccinia virus Ankara (MVA) and New York Vaccinia virus (NYVAC), which have been evaluated in both non-human primate (30, 47, 129) and human trials (72, 159, 172). These two vectors are safe vaccine vectors due to them being highly attenuated (59). The fact that they cannot replicate in human cells, thus preventing production of viral progeny and HIV-1 antigens, makes these two vectors not be able to induce strong immune response by themselves. Thus our goal is to develop a

86

replication competent HIV-1 vaccine vector in order to increase the amount of HIV-1 antigens for better immune response while maintaining the attenuation of the vector.

NYVAC is a derivative of the Copenhagen (COP) VACV strain. NYVAC was constructed by precisely deleting 18 open reading frames (ORFs) of COP's genome (197). Among those 18 ORFs are 12 continuous ORFs spanning from K1L to C7L, which are host range genes allowing for VACV to replicate in human cell lines as well as rabbit kidney cell line (68, 152). Several virulent genes were also removed in NYVAC such as: J2R, encoding thymidine kinase gene (TK); B13R and B14R, encoding serine protease inhibitor; A26L, encoding A-type inclusion body gene; A56R, encoding hemagglutinin; and ORF14L, encoding large subunit of ribonucleotide reductase gene. Deletion of J2R leads to more attenuated virus (199) and this locus was used to insert the HIV-1 clade C antigens. In order to make replication competent NYVAC in human cell lines, both K1L and C7L was put back into NYVAC genomes. In addition, to improve the immunogenicity of the vaccine, an additional deletion of B19R was carried out.

The B19R gene encodes an early expressed 60-65 kilo Dalton (kDa) interferon (IFN) α/β receptor (IFNα/β-R) like glycoprotein. It has significant homology to cellular IFN-α/βRs (44) and can bind to IFNα/β and inhibit the IFN antiviral action (3). This protein can bind to human type I IFN with high affinity (108). It can be secreted into solution or can be embedded on the cellular surface of infected cells (3, 203). Deletion of B19R leads to attenuation of VACV in both intranasally and intracranially infected mice despite the fact that it binds to mouse IFN with low affinity (44, 193).

Our findings showed that insertion of K1L and C7L restored host range of NYVAC and allowed NYVAC to replicate in various human cell lines including HeLa,

human keratinocyte and human dermal fibroblast. Replication competent NYVAC also led to an increased expression of HIV-1 antigens compared to the original NYVAC. This can provide significant improvement in the cross-presentation, thus, making the vector more immunogenic. In addition, insertion of K1L and C7L did not alter the attenuation of NYVAC in the intracranial mouse model, ensuring the safety of NYVAC vaccine vector. Finally, deletion of B19R in NYVAC leads to innate immune pro-inflammatory response signaling, particularly in the IRF3 pathway. Taken together, by restoring replication competence and deleting immune-modulating gene in NYVAC, we have developed a safer and more immunogenic VACV vaccine vector candidate for HIV.

MATERIALS AND METHODS

Cells and viruses.   Baby hamster kidney (BHK-21), Rabbit Kidney (RK-13)

cells were maintained in Eagle's MEM (Cellgro) supplemented with 5% FBS (HyClone).

HeLa and BSC40 (African green monkey kidney cells) were maintained in Dulbecco's

Modified-Minimal Essential Medium (DMEM) (Cellgro) supplemented with 5% FBS.

Human Keratinocyte (Invitrogen) were maintained in EpiLife media (Invitrogen) with

addition of Human Keratinocyte Growth Supplement (Invitrogen). Adult skin human

dermal fibroblast (Lonza) was maintained in FGM^{TM}-2 Bullet kit media (Lonza). All

cells were incubated at 37°C in the presence of 5% CO_2. The VACV Copenhagen strain

herein is referred to as wild type VACV (VACV wt). VACVΔE3L was generated as

previously described (38). NYVAC-C was obtained from Sanofi-Pasteur. NYVAC-C

ΔB19R was obtained from Dr. Mariano Esteban's lab.

Construction of the replication competent NYVAC-C KC and NYVAC-C

KC ΔB19R.   This work was done by Dr. Karen Kibler in our lab and was first described

in (92). Briefly, The C7L and K1L genes were amplified from the Copenhagen genome

using overlapping PCR with the following primers NY1 (5'

GTTTGCATCGTGCTTTAACATCAATGG 3'), NY2 (5'

GTCTTACTCATTGCATCGTACGGTTGGCTTATTCCATAGTAGCTTGTG 3'), NY3

(5' CTACTATGGAATAAGCCAACCGTACGATGCAATGAGTAAGACAATAGG 3')

and NY4 (5' GTACCTGGCAATAGGTGATAATATGAC 3'). The "KC" fragment was

then inserted into the NYVAC or NYVAC-C genome by in vivo recombination: 150 ng

DNA was used to transfect MRC-5 cells according to the manufacturer's protocol

(Lipofectamine, Invitrogen), in 35 mm dishes; cells were infected with the parental virus,

either NYVAC or NYVAC-C, at a multiplicity of infection (MOI) of 0.05 by adding the virus to the transfection mix. After 30 minutes of incubation, 1 ml of OptiMEM with 1% serum was added to each dish. Cells were scraped into the medium at 36 hours post-infection. Following three rounds of freeze/thaw, the IVR scrape was used to infect Vero cells to select for viruses competent for large plaque formation in Vero cells. The same method was used to insert KC into NYVAC-C-ΔB19R.

Virus infection.   The virus was diluted in MEM containing 2% FBS. Cell monolayers in a 60 mm dishes were infected with 100 µL of virus after aspirating the media off the dishes. Cells were incubated at $37^{o}C$, 5% CO_2 for 1 hour, with rocking every 10 minutes. BHK-21 cells were used to amplify the recombinant viruses used in this study. Stocks of recombinant viruses were generated by amplification of a single corrected plaque in 60 mm dish of BHK-21 cells. At 100% cytopathicity, the infected cells were harvested by scraping and centrifuging at 1000 rpm for 10 minutes at $4^{o}C$. The cell pellet was resuspended in 10 mM Tris HCl pH 8.8. Three rounds of alternate freezing ($-80^{o}C$) and thawing on ice for 30 minutes, followed by vortexing for 30 seconds (3 times) was performed to lyse the cells and release the virus. The prep was then spun at 1000 rpm for 10 minutes at $4^{o}C$ and the virus in the supernatant was transferred into a fresh tube and stored at $-80^{o}C$. The virus was titered in BHK and BSC40 cells.

PCR confirming deletion of B19R. 100 µL of virus stock with a titer of $1x10^{9}$ pfu/mL was used for DNA extraction. Virus DNA was extracted by treating the virus with a 100 µL of phenol equilibrated with 10 mM Tris HCl, pH 8.0, 1 mM EDTA (Sigma). The aqueous phase from the above step was re-extracted with 100 µL phenol:isoamylalcohol:chloroform (25:24:1) (Sigma). The aqueous phase from the above

step was re-extracted with 100 μL volume chloroform:isoamyl alcohol (24:1) (Sigma), followed by precipitation with 2.5 volumes of 95% ethanol, 1/10 volume of 7.5M ammonium acetate, and 10μL glycogen (Fermentas). The DNA was washed in 70% ethanol, dried and resuspended in 50 μL of distilled water.

PCR was performed using B19R flanking primers, B19RF F (5' TCACGACGAAACAATGTTAC 3') and B19RF R (5' ATGTTCTCTATCGGTGAGATAC 3'). Briefly, 100 ng of virus DNA template, 500 μM of B19RF F, 500 μM of B19RF R, 500 μM dNTPs, 2 mM $MgCl_2$, 1X $MgCl_2$ buffer, and Platinum *Taq* polymerase enzyme (Invitrogen) were mixed in a 50 μL reaction volume. PCR amplifications were performed with 95ºC for 5 minutes, followed by 30 cycles of amplification (95ºC for 1 minute, 55ºC for 1 minute, 68ºC for 2 minutes). The PCR product was subjected to agarose gel electrophoresis (1%, GTG grade) at 100 volts for 1 hour.

Protein extraction and Western Blotting. 50% confluent HeLa cell or human Keratinocyte monolayers were infected with viruses at a MOI of 5. At the indicated times post infection, cells were scraped into 150 μL of 1X SDS (62.5 mM Tris-Cl, 10% glycerol, 2% SDS, 0.0005% bromphenol blue, 0.1% 2-mercaptoethanol, 1X Halt Protease and Phosphotase Inhibitor Cocktail (Pierce Thermo Scientific)). Lysates were transferred into QIAshredder columns (Qiagen), spun at 16,000xg for 2 minutes at 4ºC, and stored at -80ºC. The samples were boiled for 5 min and ran on 10% and 12% SDS-PAGE gels at 150V. Proteins were transferred to nitrocellulose membranes at 100 volts for 60 min in 10 mM CAPS, pH 11 with 20% methanol. The membranes were blocked with 3% milk (Carnation® Non-fat Dry Milk) TTBS (20 mM Tris-HCl, pH 7.8, 180 mM NaCl, 0.05%

Tween-20) for 1hr. The membranes were probed with primary antibodies overnight. Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000, Santa Cruz) or anti-mose IgG conjugated to horseradish peroxidase (1:10,000, Santa Cruz) was added followed by chemiluminescence (Pierce Thermo Scientific). Rabbit polyclonal anti-gp120 antibody was from Centro Nacional de Biotecnología; polyclonal anti-gag p24 serum was from ARP NIBSC, Centralised Facility for AIDS reagent, UK; IRF3-P antibody was purchased from Epitomics; PKR-P and eIF2α-P antibodies was purchased from Cell Signaling, and GAPDH antibody was purchased from Abcam.

Multi step growth assay.   Human Keratinocyte or Dermal Fibroblast cells in 6 well plates were infected with MOI of 0.01 with specified viruses. IFN treatment, as indicated, was added at 1000 units/ml for 24 hours prior to infection. Plates were incubated at 4°C for 30 minutes and rocked every 10 minutes. Plates were washed thrice with warm media. Half of the infected cells were harvested for 0 hour time point. Plates were incubated at 37°C, 5%CO_2 for 72 hours. Infected cells were then harvested for 72 hour time point. Samples were frozen and thawed 3 times (frozen at -80°C for 30 minutes, and then thawed on ice for 30 minutes, followed by 3 minutes thaw at 37°C) to release viruses. Ultimately, samples were spun at 1,000xg for 10 minutes at 4°C and the supernatants were collected. Viruses were titered using BHK cells. The experiment was done in triplicate.

Pathogenicity in newborn mice.   Pregnant CD1 mice were purchased from Charles River at 10 days gestation. The animals were housed one animal per cage. Intracranial infections with the indicated viruses, using a total volume of 10 μL, were conducted at 48 to 72 hours post-birth of the pups (at least 10 pups per virus), using a 27-

gauge needle, as previously described (125). Animals were monitored twice daily for 14

days for morbidity and mortality.

RESULTS

Construction of recombinant virus. HIV-1 clade C genes were inserted

into the empty J2R locus of NYVAC by our collaborator at Sanofi-Pasteur to generate

recombinant NYVAC expressing HIV protein, herein referred to as NYVAC-C. Clade C

gp120 and the poly proteins gag-pol-nef were expressed by a divergent synthetic

early/late promoter (Figure 25). This NYVAC-C was then further attenuated by deleting

its IFNα/β-R-like-protein gene (B19R), herein referred to as NYVAC-C ΔB19R (Figure

25). To restore replication competency of these recombinant viruses, a cassette of K1L

and C7L genes were inserted into these viruses (Figure 25). These replication competent

recombinant viruses herein are referred to as NYVAC-C KC and NYVAC-C KC ΔB19R.

To test for the loss of B19R genes, viral genomic DNAs were extracted using

standard phenol/chloroform extraction. PCR was done with flanking primers (300 bp

outward of B19R gene) and the products were run on a 1% Agarose gel. As expected,

NYVAC-C and NYVAC-C-ΔB19R still had the B19R gene intact as a 1.5 kb band were

observed in these two (Figure 26). However, NYVAC-C-ΔB19R and NYVAC0C-KC-

ΔB19R did not possess the B19R gene as the PCR only showed a 600 bp flanking arms

product (Figure 26).

To test if the insertion of K1L and C7L restored the broad host range phenotype

of NYVAC recombinant viruses, 100-200 pfu of NYVAC recombinant viruses were

plaqued in BSC40 and RK13 cells for 48 hours and were then stained with crystal violet.

BSC40 is a permissive cell line for NYVAC thus, all the recombinant viruses plaqued

NYVAC-C-KC ΔB19R did not out in BSC40 (Figure 27). NYVAC-C and NYVAC-C

does not contain their host range genes thus they couldn't plaque out in RK13 cells

(Figure 27). Only NYVAC-C KC and NYVAC-C KC ΔB19R which have their host range gene reinserted could plaque out in Rk13 cells (Figure 27).

Restoring replication competence of recombinant viruses increases expression of HIV-1 antigens in infected cells. To test if restoring replication competence of NYVAC recombinant viruses would lead to increase expressing of HIV-1 antigen, HeLa cells were infected with VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C ΔB19R, NYVAC-C KC, or NYVAC-C KC ΔB19R. Cells lysates were prepared after 6 hours post infections and were resolved on a 7.5% poly-acrylamide gel followed by western blotting using rabbit polyclonal anti-gp120 antibody and mouse polyclonal anti-gag p24 serum. As expected, VACV wt and VACVΔE3L do not possess any recombinant gene thus did not express HIV-1 proteins (Figure 28, lane 2 and 3). Since NYVAC-C and NYVAC-C ΔB19R cannot replicate in HeLa (data not shown), the expression level of HIV-1 gp120 and the poly-protein gag-pol-nef was low, especially in the NYVAC-C ΔB19R infection (Figure 28, lane 4 and 6). Reinsertion of K1L and C7L into NYVAC recombinant viruses increases expression of HIV-1 antigens because both NYVAC-C KC and NYVAC-C KCΔB19R expressed a high amount of gp120 and gag-pol-nef poly-protein (Figure 28, lane 5 and 7).

Restoring replication competence of recombinant viruses restores virus replication in primary human cell lines. To test if the addition of the K1L and C7L genes would restore replication in primary human cell types that are physiologically relevant to poxvirus infection, we analyzed multi-step growth curves in human keratinocytes and human dermal fibroblasts since these cells are the first layer of the skin cells get infected with vaccinia virus during a vaccination. Primary human keratinocytes

95

and human dermal fibroblast were mock treated or treated with IFN as specified for 18

hours before they were infected with either VACV wt, NYVAC-C, NYVAC-C KC or

NYVAC-C KC ΔB19R. As expected, NYVAC-C did not replicate in any of the primary

cells due to the loss of host range genes (Figure29). In both cell lines, NYVAC-C-KC

and NYVAC-C KC ΔB19R replicated to titers comparable to that of the VACV wt (as wt

Cop VACV in figure) (Figure 29). When the cells were pre-treated with IFN, VACV wt

is fully resistant to treatment with IFN thus it replicated to high titer. However NYVAC-

C-KC and NYVAC-C-KC ΔB19R did not replicate to the extent of VACV wt in IFN-

treated cells, especially in dermal fibroblasts (Figure 29). Although, this indicates that

NYVAC-C KC and NYVAC-C-KC ΔB19R are IFN sensitive, the addition of the K1L

and C7L genes to recombinant NYVAC did restore replication competence in human

cells.

Replication competence recombinant NYVAC still have a safety profile.

To analyze the safety profile of our recombinant viruses, we utilized a newborn

mouse model, the most sensitive mouse model available for detecting pox virus

pathogenesis (125). Newborn mice were inoculated intracranially (IC) with different

doses of VACV wt (Cop as in figure), VACV wt expressing HIV-1 antigen (Cop-C),

MVA expressing HIV-1 antigen (MVA-C), NYVAC-C, NYVAC-C KC, NYVAC-C KC

ΔB19R and the current vaccine strain of VACV, New York City Border of Health

(NYCBH). As expected, VACV wt was highly pathogenic in this model, with an LD50

of about 10 pfu. VACV expressing HIV-1 antigen from the J2R locus is further

attenuated by about one log due to the loss of the thymidine kinase gene, and had an

LD50 of about 100 pfu, comparable to that of the NYCBH. MVA-C and NYVAC-C

were apathogenic, having LD50 of about 10^7 pfu. Reinsertion of K1L and C7L into

NYVAC-C did not restore pathogenesis of NYVAC-C KC since it was attenuated by

approximately four logs compared to NYCBH and had a LD50 of 10^6 pfu. Deletion of

B19R from NYVAC-C-KC further attenuated the virus by approximately one log,

approaching the attenuation of MVA and NYVAC-C (Figure 30). Thus, even though

NYVAC-C KC and NYVAC-C KC ΔB19R replication competences were restored, they

still acquired a safety profile because they were apathogenic in the mouse model.

Deletion of B19R in recombinant NYVAC led to pro-inflammatory signaling

cascade. To determine if our recombinant NYVAC can lead to immune signaling

cascade in infected cells, HeLa and primary human keratinocyte were mock infected or

infected at M.O.I of 5 with either VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C KC,

NYVAC-C ΔB19R, or NYVAC-C KC ΔB19R. Cell lysates were prepared at 6 hours post

infection. Cell lysates were resolved on a 10% poly-acrylamide gel followed by Western

blotting using specified antibodies. As expected, VACV wt inhibited all signaling

cascade while VACVΔE3L led to signaling cascade of PKR, eiF2α and IRF3 in both

HeLa and human keratinocyte (Figure 31). NYVAC-C led to a minimal amount of

phosphorylation of PKR and eIF2α but not IRF3 in both cell types whereas deletion of

B19R from NYVAC-C led to activation of IRF3 in HeLa (Figure 31A) but not in human

Keratinocyte (Figure 31C). Reinsertion of K1L and C7L into NYVAC-C led to inhibition

of all signaling cascade in both HeLa and Keratinocyte (figure 31) as observed in VACV

wt infection. Even though NYVAC-C KC ΔB19R inhibited PKR and eIF2α signaling

cascade, thus its replication competence phenotype, it still led to IRF3 signaling cascade

in HeLa (Figure 31A). In human keratinocyte, it was observed that it was dependent on

97

the donor whether or not NYVAC-C KC ΔB19R led to IRF3 signaling cascade. Our results showed NYVAC-C KC ΔB19R could lead to IRF3 phosphorylation in one donor's cells while it couldn't in other donor's cells (Figure 31B and 31C). In the donor 2's keratinocyte, there was a light shifted band of IRF3 in the NYVAC-C KC ΔB19R infection (Figure 31C). However, this band was not observed in the positive control VACVΔE3L but observed in the VACVwt and NYVAC-C KC, we deduced this band was not the band indicating the activation of IRF3.

In an attempt to compare our replication competent vaccine vector NYVAC-C KC ΔB19R to the Thai trial vaccine vector ALVAC®, HeLa were mock infected or infected at M.O.I of 5 with either VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C KC, NYVAC-C ΔB19R, NYVAC-C KC ΔB19R or ALVAC®. Cell lysates were prepared at 6 hours post infection. Cell lysates were resolved on a 10% poly-acrylamide gel followed by Western blotting using specified antibodies. ALVAC® led to PKR and eIF2α phosphorylation thus it indicated that ALVAC® is not replication competent in human cells. In addition, ALVAC® still did not lead to IRF3 signaling cascade while NYVAC-C KC ΔB19R can (Figure 32). This showed that NYVAC-C KC ΔB19R can be considered as a vaccine vector that is fully replication competent while still maintaining its safety profile and its immunogenicity by signaling the IRF3 pathways which has been shown to be a key transcription factor in the IFN pathway.

DISCUSSION

The results of the phase III Thai clinical study with the use of ALVAC® showed modest success (163); it proves that poxviruses can be considered as a vaccine vector for HIV. Since then, MVA and NYVAC, attenuated strain of VACV, have been considered as promising HIV vaccine candidates due to their high safety profile (126). However, both MVA and NYVAC, like ALVAC®, are replication incompetent in human cells; thus reducing the amount of antigen able to be expressed in the cells, limiting their ability to induce strong immune response on their own. Therefore, to improve these vaccine vectors, the method of restoring replication competence in these vectors while maintaining their safety profile has been considered.

In this investigation, we have constructed a new vector with replication competence in human cultured cells. This was done by the reinsertion of the host restriction genes K1L and C7L (68, 152) into the genome of NYVAC-C, a vector that expresses gp120- and gag-pol-nef antigens of HIV-1 from clade C. To further improve the immunogenicity of this vector, we also deleted the IFN-α/β-R like protein gene, B19R. This gene was selected for deletion due to the high affinity of its protein to bind to human type I IFN (6, 108), thus interfering with the immune signaling. Here, we evaluated the replication competence, the safety profile and the ability to elicit immune signaling cascade of the following vaccine vector constructs: NYVAC-C, NYVAC-C KC, NYVAC-C ΔB19R and NYVAC-C KC ΔB19R

Reinsertion of the K1L and C7L genes restored viral replication in human cultured cells, including physiologically relevant keratinocytes and dermal fibroblasts (Figure 23). Furthermore, we observed that the presence of K1L and C7L in NYVAC-C

99

and NYVAC-C ΔB19R improved the level of expression of HIV-1 antigens, especially in the latter (Figure 28). It has been shown that the K1L gene is necessary to prevent activation of PKR in the infected cells (125). Therefore, restoring the host range genes in NYVAC-C KC and NYVAC-C KC ΔB19R led to inhibiting PKR and eIF2α activation while NYVAC-C and NYVAC- ΔB19R led to these signaling cascades (Figure 31). Activation of these cascades leads to inhibition of protein synthesis in the infected cells (112). Therefore, HIV antigen expression by the viral vector is enhanced by the presence of K1L. Thus, we have achieved our first goal of restoring replication competence of our vaccine vectors and increasing the amount of HIV-1 antigens in the infected cells.

A concern exists that the replication competent vaccine vector maybe too pathogenic to use as a vaccine. To evaluate the safety profile of our replication competence vector, we utilized the intracranially inoculated newborn mouse model. Our result showed that the vector NYVAC-C-KC is highly attenuated compared to the Copenhagen and NYCBH strains, 10^5 and 10^4 less pathogenic respectively (Figure 30). The deletion of B19R has been shown to decrease pathogenicity in the mouse model (44, 193). Thus, NYVAC-C KC ΔB19R exhibited an attenuated profile as much as MVA as indicated in our result. Taking this into account, we have achieved our goal to develop a replication competence vaccine vector for HIV while still maintaining its safety profile.

We also looked at the ability of our construct to induce the pro-inflammatory signaling pathway. To investigate this, we looked at the ability of our vector to induce IRF3 activation, a key modulator in the IFN pathway. Our results showed that even though possessing the host range genes K1L and C7L that can inhibit the activation of PKR, NYVAC-C KC ΔB19R still led to activation of IRF3 (Figure 31). IRF3 is the key

transcription factor required for the induction of type I IFN, which has been known as a key modulator of the innate and adaptive immune responses. Compared to ALVAC® which did not lead to activation of IRF3, our data suggests that the immunogenicity of NYVAC-C KC ΔB19R may have improved due to its ability to signal the IRF3 pathway (Figure 32). Even though there was no immunological assay performed in this work, our collaborator, using microarray analysis, has showed that NYVAC-C KC ΔB19R induced IFN gene expression and the activation of IFN induced transcription factors that necessary to activate the innate and adaptive immune system (158). Unpublished data from our collaborator showed that the replication competent vaccine vector is capable of inducing strong T cell response against HIV-1 antigen in non-human primate model while the replication deficient vaccine vector failed to induce T cell response. This suggests that we have successfully developed a safe, replication competent, and immunogenic vaccine vector for HIV-1. In conclusion, our work demonstrated that we have developed a replication competent vaccine vector that still maintains its safety profile and its immunogenicity. Our work continues in determining the ability of these vaccine vectors as an HIV vaccine. Phase 1 clinical trial for these vaccine vectors are scheduled to be undertaken in the near future in order to access the ability of these replication competence vaccine vectors.
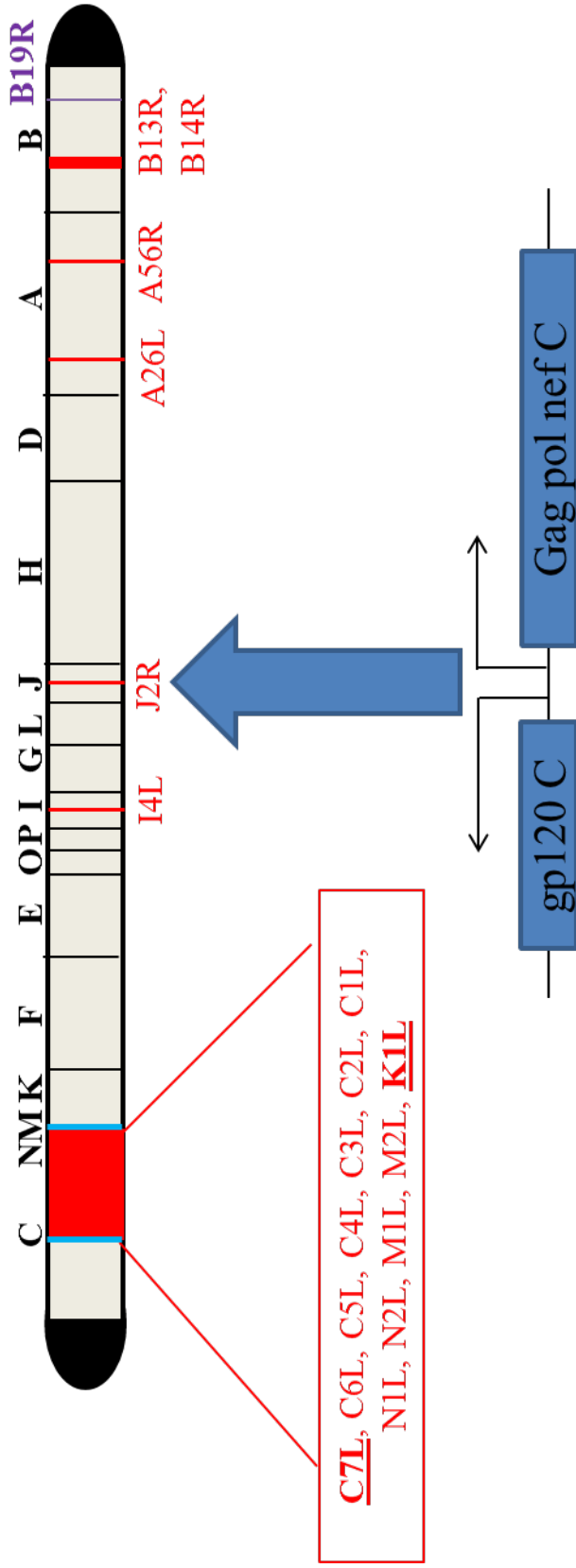
Figure 25 – Schematic representation of construction of NYVAC recombinant virus. NYVAC was deleted of 18 open reading frames (in red). HIV-1 Clade C antigens were inserted into the empty J2R locus of NYVAC yielding NYVAC-C. The B19R gene of NYVAC (in purple) was deleted in NYVAC-C to generate NYVAC-C ΔB19R Both K1L and C7L (blue and red underlined) were reinserted into NYVAC-C and NYVAC-C ΔB19R to generate the replication competence NYVAC-C KC and NYVAC-C KC ΔB19R
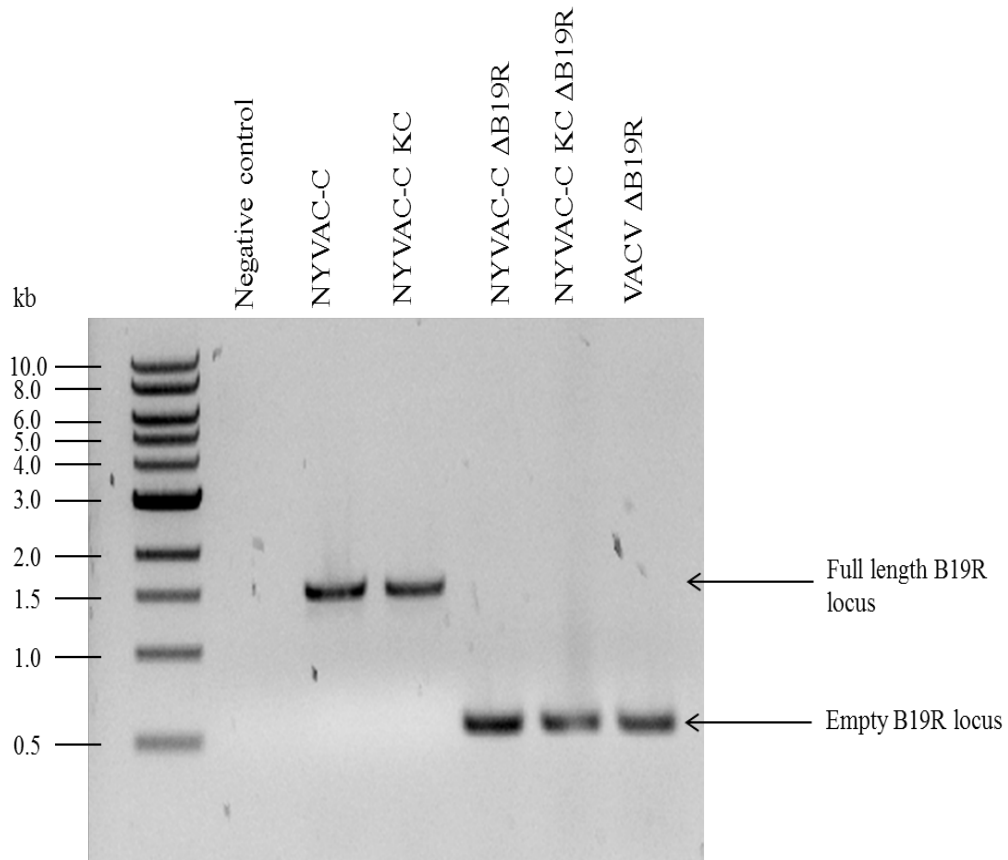
Figure 26 – Confirmation of the loss of B19R gene. Viral genomic DNAs of NYVAC-C, NYVAC-C-KC, NYVAC-C-ΔB19R, NYVAC-C-KC-ΔB19R and the positive control VACV ΔB19R were extracted using standard phenol/chloroform extraction. PCR was done with flanking primers (300bp outward of B19R gene) and the products were run on a 1% Agarose gel. B19R
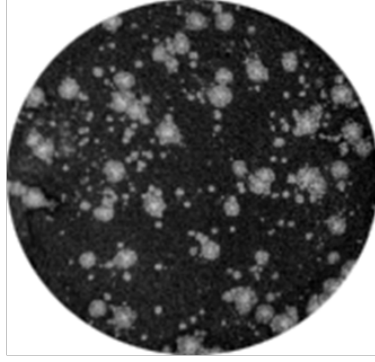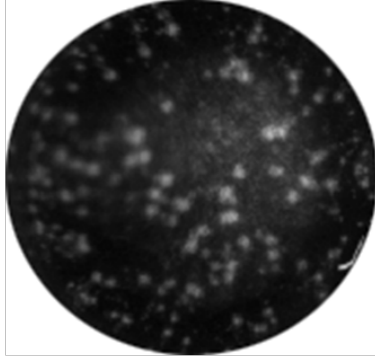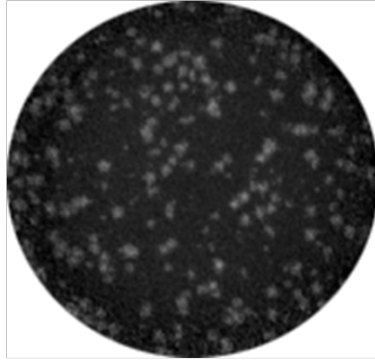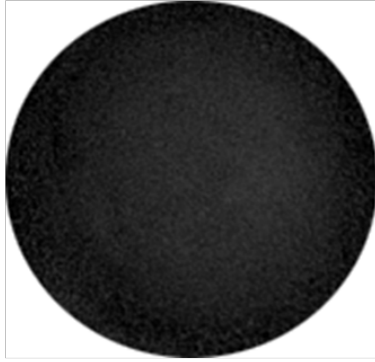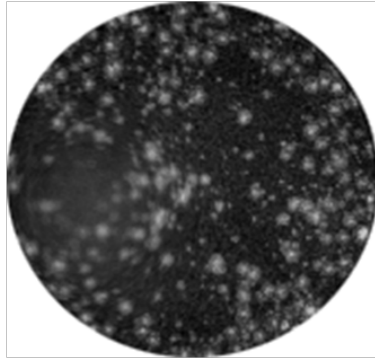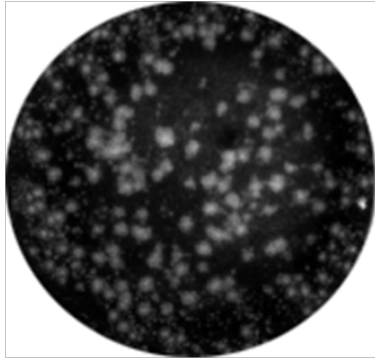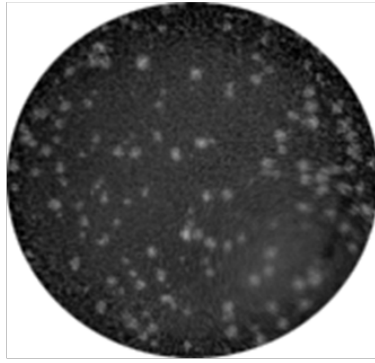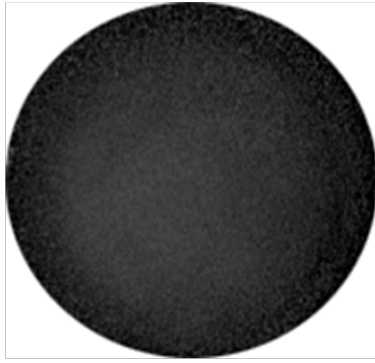
Figure 27 – Insertion of KC rescues host range phenotype of recombinant NYVAC. About 200 plaque forming units of NYVAC-C, NYVAC-C KC, NYVAC-C KC, NYVAC-C ΔB19R and NYVAC-C KC ΔB19R were used to infect 100% confluent RK13 cells and BSC40 cells. 48 hours post infection, infected cells were stained with crystal violet.
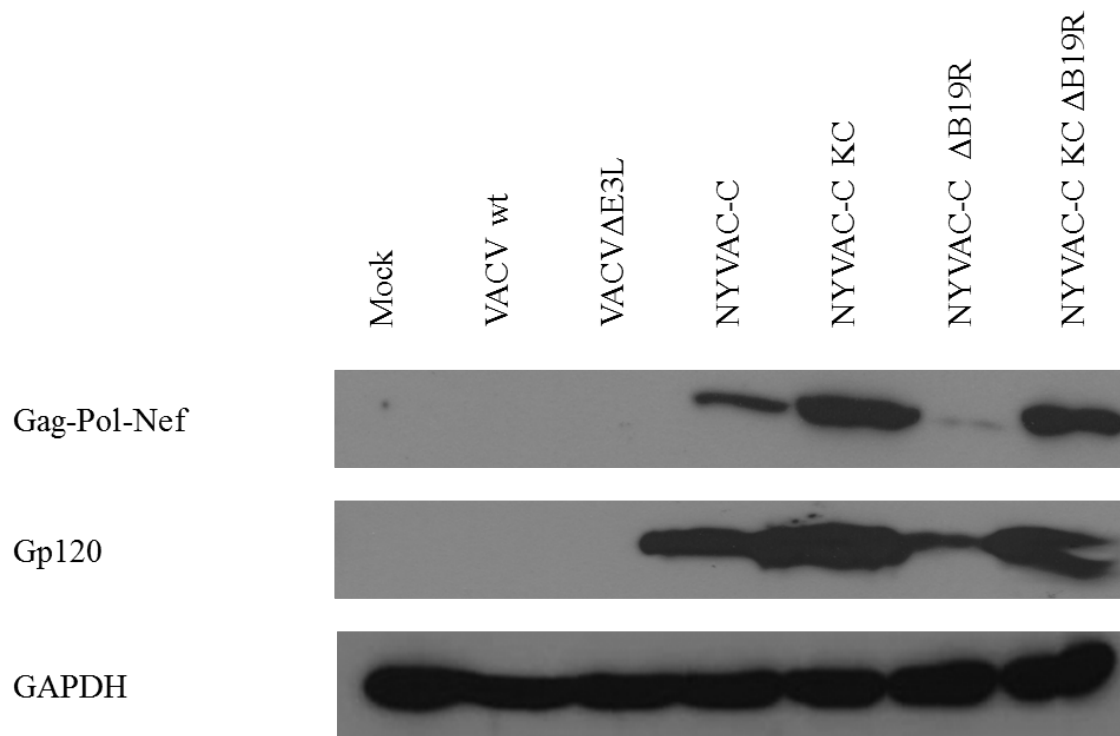
Figure 28 – Restoring replication competency of recombinant NYVAC viruses lead to increased expression of HIV-1 antigen. HeLa cells were mock infected or infected with either VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C ΔB19R, NYVAC-C KC, or NYVAC-C KC ΔB19R. Cells lysates were prepared after 6 hours post infections and were resolved on a 7.5% poly-acrylamide gel followed by western blotting using rabbit polyclonal anti-gp120 antibody and mouse polyclonal anti-gag p24 serum.
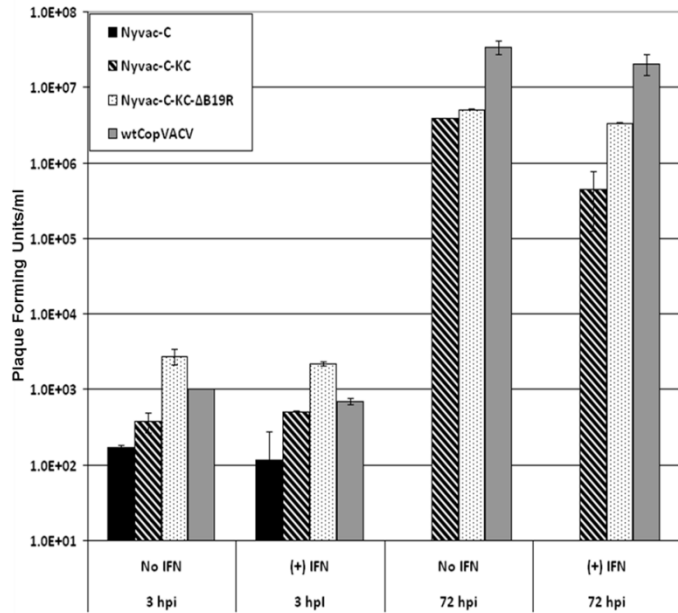
A. Human keratinocyte
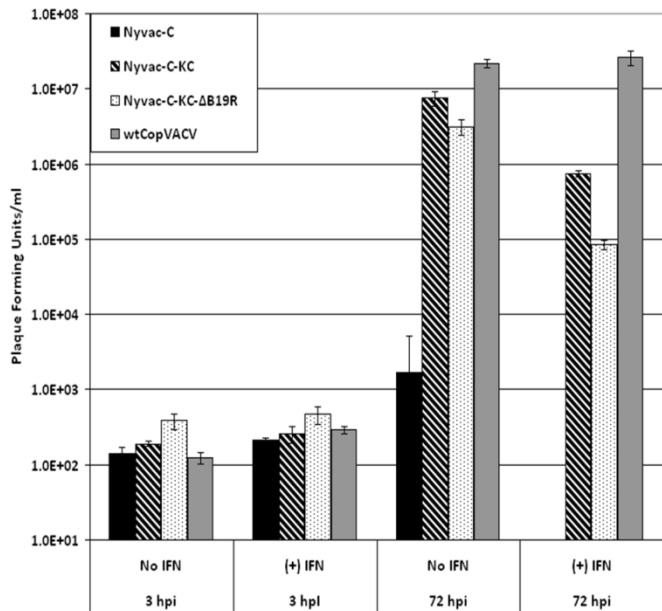
B. Human Dermal fibroblast

Figure 29 – Multi step growth of recombinant NYVAC[1]. Human keratinocytes (A) and human dermal fibroblast (B) cells were mock treated and treated with 1000 U/ml of IFN for 18 hours before they were infected at M.O.I of 0.1 with either VACV wt, NYVAC-C, NYVAC-C KC or NYVAC-C KC ΔB19R. Cells were washed 3 times with warm PBS and harvested at 3 hours post infection) and 72 hours post infection. . Collected viruses were titered in BHK cells. Experiments were done in duplicate
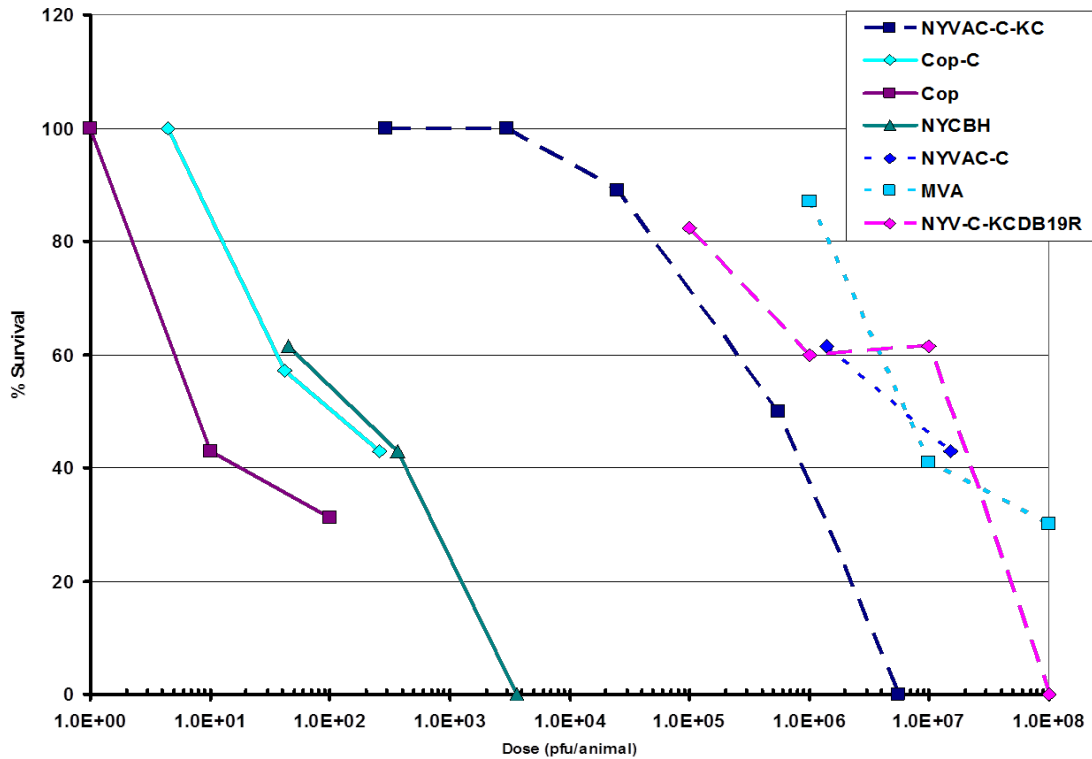
Figure 30 – Pathogenesis of recombinant NYVAC[1]. 1 day old mice were inoculated intracranially with indicated doses of VACV wt (Cop), VACV wt expressing HIV-1 antigen (Cop-C), Modified Vaccinia virus Ankara (MVA), NYVAC-C, NYVAC-C KC, NYVAC-C KC ΔB19R and the current vaccine strain of VACV, New York City Border of Health (NYCBH). Mice were counted daily and observed for 14 days post infection.
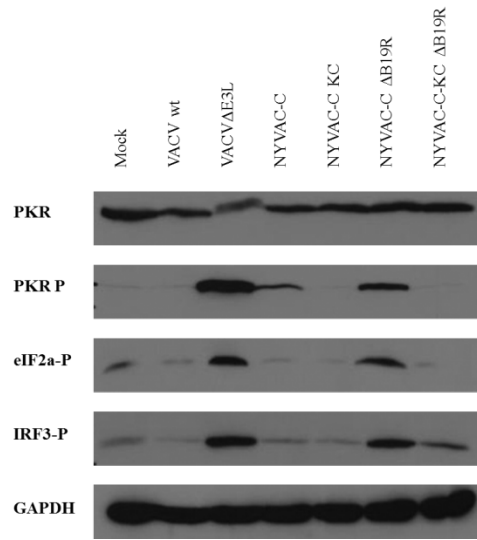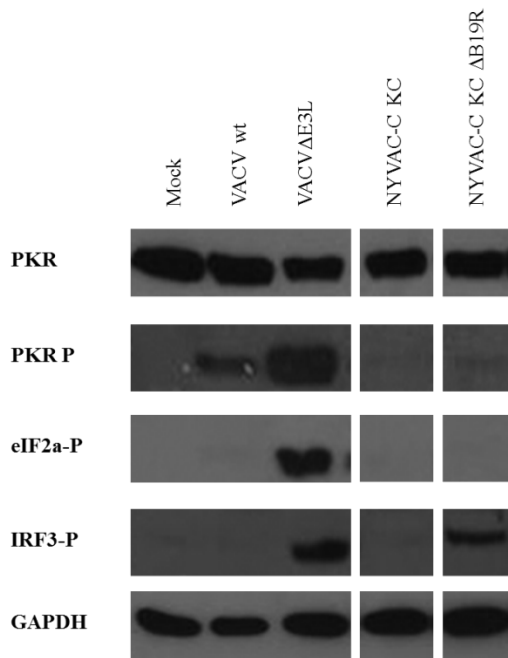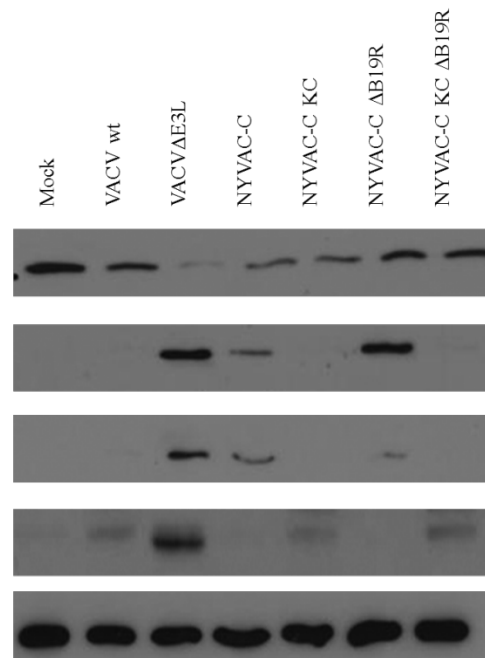
Figure 31 – Signal transduction induced by NYVAC recombinant virus[1]. HeLa (A) and primary human Keratinocyte (B and C) were mock infected or infected at M.O.I of 5 with either VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C KC, NYVAC-C ΔB19R, or NYVAC-C KC ΔB19R. Cell lysates were prepared at 6 hours post infection. Cell lysates were resolved on a 10% poly-acrylamide gel followed by Western blotting using specified antibodies.

109
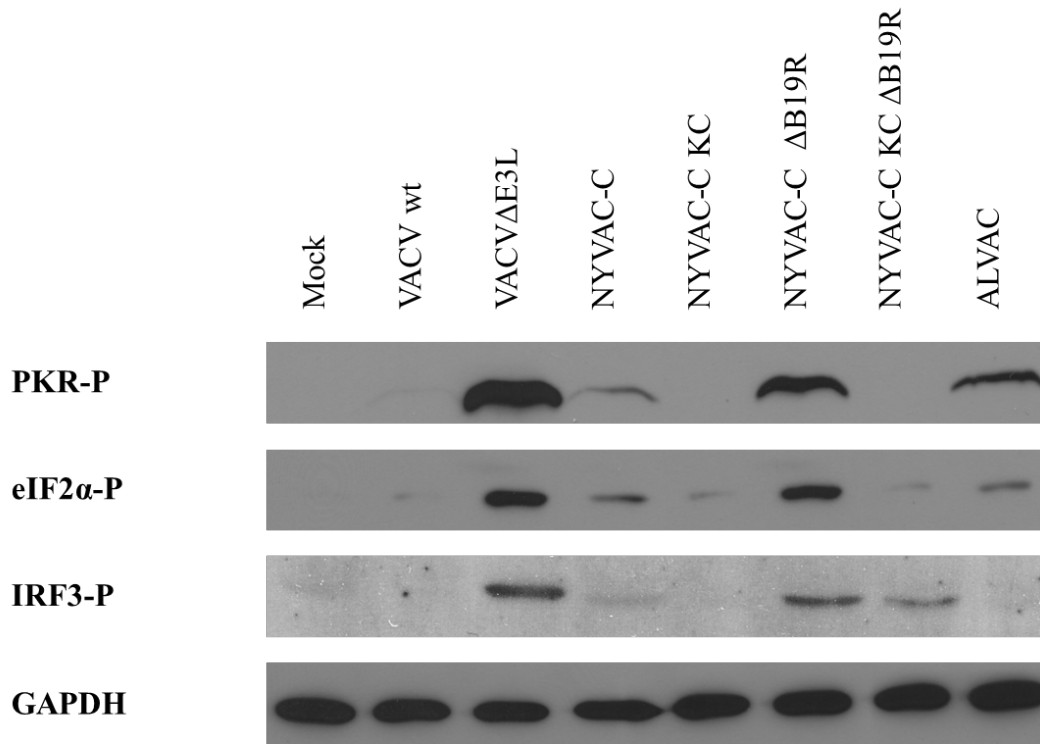
Figure 32 – NYVAC-C KC ΔB19R can lead to IRF3 activation but not ALVAC®. HeLa were mock infected or infected at M.O.I of 5 with either VACV wt, VACVΔE3L, NYVAC-C, NYVAC-C KC, NYVAC-C ΔB19R, NYVAC-C KC ΔB19R or ALVAC®. Cell lysates were prepared at 6 hours post infection. Cell lysates were resolved on a 10% poly-acrylamide gel followed by Western blotting using specified antibodies.

OVERALL DISCUSSION

Type I IFNs are the primary first line of defense against viral infection (171). The IFN signal transduction pathway consists of two signaling cascades: a virus-induced IFN productionsignal and an IFN receptor-mediated secondary signal to establish an antiviral state (78, 206). The virus-induced IFN productionsignaling is initiated by the detection of PAMPs by host pattern recognition receptors. The most common PAMP produced by VACV is dsRNA (45) as a consequence of convergent transcription of late genes from opposite strands of the genome (55). It is thought that dsRNA can lead to signal transduction cascade of the RIG-I/MDA-5 pathway (223) to establish the virus-induced IFN production signaling via activation of IRF3 (60, 179). To overcome this, VACV encodes the E3L gene which expresses a dsRNA binding protein that can sequester dsRNA (39) from cellular PRRs; thus, inhibiting the virus-induced IFN production signaling. Therefore, VACVΔE3L, VACV deleted of the virulent E3L gene, can lead to virus-induced IFN production signaling via activation of IRF3. In the first study presented here, we investigated the cellular components that lead to VACVΔE3L induced activation of IRF3. Our first aim of the study was to examine the role of protein kinase R in activation of IRF3. This was accomplished by using two different methods: the overexpression of the dominant negative form of PKR, and virus expressing the PKR pseudo-substrate, vIF2αh, that can degrade PKR; we showed that PKR is necessary for VACVΔE3L induced activation of IRF3. Our data agreed with one reported by our collaborator using stably-PKR-knocked-down HeLa (228). Since PKR was reported to be involved in MAPK kinases signaling pathway (8, 79, 195), we hypothesized that either the p38 kinase or SAPK/JNK kinase are involved in PKR-dependent activation of IRF3.

Using pharmacological small molecule inhibitor, we found that not the p38 kinase, but the SAPK/JNK is required for PKR-dependent activation of IRF3. Thus, PKR is necessary for virus-induced activation of IRF3, but it is not sufficient. During the time of this study, it was reported that SAPK/JNK phosphorylates IRF3 at serine173 at the N-terminal (226). Using an immunoprecipitation assay of overexpressed V5-tagged IRF3 and V5-tagged mutant IRF3, we found that serine 173 of IRF3 is necessary for PKR-dependent activation of IRF3. Taking this into consideration, we proposed a mechanism of VACVΔE3L induced activation of IRF3 as shown in figure 12. During VACVΔE3L infection, dsRNA leads to the activation of both SAPK/JNK and IKKε. Both these kinases are involved in the activation of IRF3. SAPK/JNK phosphorylates the N-terminal of IRF3 at serine 173 in order for the phosphorylation of IRF3 at the C-terminal by IKKε to occur. The phosphorylation of IRF3 at the C-terminal leads to the activation of this transcription factor. This is the first time that SAPK/JNK is shown to participate in PKR-dependent-activation of IRF3 via phosphorylation of serine 173 in VACV infection.

The second cascade in the IFN signaling pathway is the up-regulation of IFN-induced genes. This cascade is first initiated once IFNs bind to their receptors (190) on the same infected cells for an autocrine effect or the neighboring cells for a paracrine effect. This will activate the JAK/STAT pathway (190) which results in the activation of multiple IFN stimulated genes which are mostly antiviral genes such as PKR, RIG-I, MDA5, or the OAS system. In the second study, we investigated the role of the IFN-induced gene, ADAR1, in VACV infection. This study was initiated during our initial characterization of VACV E3 protein. E3 protein has two domains (29) : the amino-terminal Z-NA binding domain and the carboxyl-terminal dsRNA-binding domain. Our

data from the characterization study showed that VACVΔ83N, VACV which does not possess the amino-terminal Z-NA binding domain of E3, inhibited eIF2α phosphorylation at 6 hours post infection (an initial-late phase of the infection) as expected but failed to inhibit eIF2α phosphorylation at 9 hours post infection (a delayed-late phase) (99). Interestingly, when cells were treated with IFN to mount the antiviral state, VACVΔ83N did not lead to eIF2 phosphorylation. Our data presented here also showed the same phenomenon observed for VACVΔE3L infection. Since eIF2α phosphorylation is a direct consequence of activation of PKR by binding to dsRNA, our first aim of this study was to determine the amount of dsRNA within VACV infected cells when the cells were pre-treated with IFNs. Our data showed that when the cells were treated with IFN, the amount of dsRNA in VACVΔE3L infection decreased while there was only a modest reduction of dsRNA observed in the VACVΔ83N infection. This data suggested us that there are other IFN-induced proteins that reduce the level of dsRNA during VACV infection. During our literature research, we noticed that ADAR1 is an IFN-induced protein that can deaminate dsRNA (138). Further investigation showed that ADAR1 shares close homology to VACV E3 protein. Thus, in the second aim of the study, we asked if the IFN-induced ADAR1 can complement the loss of E3L in VACVΔE3L. Our data showed that expression of IFN-induced ADAR1 resulted in a significant reduction of dsRNA in VACVΔE3L infection. However, this reduction required the deaminase activity of ADAR1, suggesting that IFN-induced ADAR1 reduces the amount of dsRNA by deaminating it. This data suggest that ADAR1 might be responsible for the reduction of eIF2α phosphorylation in IFN treated cells during VACVΔE3L infection. By expressing ADAR1 in VACVΔE3L, we also found out that ADAR1 can partially rescue

VACVΔE3L replication by restoring viral protein synthesis in infected cells when provided in trans suggesting that ADAR1 plays a pro-viral role during VACV infection.

The role of ADAR1 has been described in several RNA virus systems such as measles virus, influenza virus, and hepatitis D virus (HDV). In Measles virus, a non-segmented negative strand RNA virus, hyper-editing activity of ADAR1 in the matrix (M) mRNA has been shown to contribute to the persistent infection of the central nervous system, leading to the fatal degenerative neurological disease, subacute sclerosing panecephalitis (SSPE) (141). Using ADAR1-knocked-down HeLa cells, it was shown that measles virus infection led to enhanced cytotoxicity and apoptosis correlated with enhanced activation of PKR and IRF3 (201). These data suggests that ADAR1 plays a pro-viral role in measles virus infection. In contrast, ADAR1 has an antiviral role in influenza virus system, a segmented negative-strand RNA virus. It was shown that in the IKK-knocked-down mice, the animal was hyper-susceptible to influenza infection and this was correlated with the lower rate of A-I editing of the viral mRNA in the knocked-down mice compared to the control mice (200). Furthermore, the IFN-induced form of ADAR1 was shown to suppress the influenza virus-induced cytopathic effects (209). These data suggest that ADAR1 plays an antiviral role in influenza infection. ADAR1 also was shown to be both antiviral and proviral at the same time in virus system such as HDV. It was shown that the site-selective editing of viral RNA by the constitutive form of ADAR1 (82) is critical for genome packaging of viral particles, suggesting the proviral of ADAR1 in HDV (34) , a subviral satellite of hepatitis B virus. However, enhanced levels of editing by ADAR1, especially the p150 form upon IFN treatment, can result in an antiviral response in HDV (82). The role of ADAR1 in DNA virus has not been

characterized well. Using mouse polyoma virus (PyV), a member of the *Polyomaviridae*, it was shown that there was extensive A-I editing in early mRNA during late times after infection (95) but the role of ADAR1 in this system has yet been to be identified. Using recombinant virus expressing the IFN-inducible form of ADAR1, we showed here that ADAR1 could result in the reduction of dsRNA in the infected cells; hence rescuing the viral protein synthesis, and subsequently viral replication. Due to difficulties in obtaining the deaminated sequence, the A-I editing activity of ADAR1 in VACV was not shown here. However, our data strongly suggests that the IFN-inducible form of ADAR1 does play a pro-viral role in VACV infection. And this is the first time we show that ADAR1 has a proviral role in DNA virus.

In chapter 1 of this dissertation, we showed that PKR, an IFN-inducible protein, is necessary for the activation of IRF3 in VACVΔE3L infection. On the other hand, in chapter 2 of this dissertation, we showed that the full length form of ADAR1, an IFN-inducible protein, can rescue VACVΔE3L viral protein synthesis and replication. Taken together, we proposed a hypothesized mechanism of how these pathways interact during VACVΔE3L infection (Figure 33). During its life cycle, VACVΔE3L makes dsRNA that can lead to the activation of PKR and RIG-I/MDA5. Activation of PKR can lead to inhibition of protein synthesis via the phosphorylation of eIF2α. Activated PKR can also induce the activation of SAPK/JNK which leads to the phosphorylation of IRF3 in the N-terminal at serine173. This phosphorylation is required for the phosphorylation of IRF3 at the C-terminal by IKKε which is activated by RIG-I/MDA5. This will lead to the production of type I IFN in the infected cells. Thus, PKR is an antiviral gene in VACV infection. On the other hand, IFN can lead to the induction of the full length ADAR1.

ADAR1 can decrease the amount of dsRNA made by VACVΔE3L. This will lead to the inhibition of eIF2α phosphorylation which results in the rescue of viral protein synthesis and viral replication. Thus, ADAR1 is a pro-viral gene in VACV infection.

Our main goal in our lab is to develop VACV as a safer vaccine for smallpox and also a safer and more immunogenic for heterologous vaccine vector for other diseases such as HIV. The first two studies gave us some insights about how the host cellular antiviral mechanism works during VACV infection and how VACV evades these pathways. These two studies also provided increased understanding in virus-host interaction that we utilized during the vaccine development. VACVΔE3L can be greatly immunogenic due to its ability to signal the type I IFN virus-induced cascade. However, it does not replicate well at all in human cell lines due to its protein synthesis being shut down early on during the infection; thus, limiting the amount of antigen presenting to the immune system. Therefore, VACVΔE3L would be a safe vaccine vector but would not be an efficient vaccine vector. Even though, VACVΔE3L::ADAR1 can partially rescue VACVΔE3L, it cannot be considered as a vaccine candidate because ADAR1 possesses the deaminase activity that alters the coding of cellular mRNA, resulting in alteration of cellular protein translation. Thus, in collaboration with the Pox-T cells-Discovery-Consortium group, we developed alternative methods to genetically modify VACV to become a safer and immunogenic vaccine vector. Two strains of VACV have been shown to be a safer vaccine vector: NYVAC and MVA. Even though they have a safe profile due to their being highly attenuated (59), both NYVAC and MVA cannot replicate in human cells, thus preventing production of viral progeny and viral antigens, making these two vectors not immunogenic by themselves. Thus, in the last study, we showed that we

116

have developed a replication competent NYVAC vaccine vector that expresses the HIV-1 antigens as a safe and immunogenic VACV vaccine vector for HIV-1. Our results showed that by reinserting the host ranges genes: K1L and C7L, NYVAC expressing the HIV-1 antigens could highly express HIV-1 antigens and could replicate in human cell lines while still maintaining its attenuation. We also removed the B19R gene of NYVAC in order for our replication competent NYVAC vaccine vector being able to induce pro-inflammatory signaling cascade. Our data showed that deletion of B19R resulted in activation of IRF3, a key transcription factor for the induction of type 1 IFN. This data suggested that replication competent NYVAC in conjunction with the deletion of B19R (NYVAC-C KC ΔB19R) can be immunogenic due to its ability to activate the inflammatory response. This activation of IRF3 is not PKR-dependent since the activation of PKR is inhibited in the NYVAC-C KC ΔB19R infection, which still possesses an intact E3L gene. Unpublished data from our lab indicated that when cells were treated with IFN, VACV infection could lead to activation of IRF3 without activation of PKR. This lead us to a hypothesis in which the loss of B19R leads to virus-induced IFN production which can prime the cells in an antiviral state, thus allowing activation of IRF3 without activation of PKR in NYVAC-C KC ΔB19R infection. This hypothesis has yet to be proven.

Our collaborator also found that both of the constructs: NYVAC-C KC and NYVAC-C KC ΔB19R led to increase in antigen cross-presentation and various pro-inflammatory responses. Unpublished data from our collaborator's non-human primate study using the replication competent virus in the same vacciniation regime as in the Thai trial suggested that the replication competent virus could induce a broader breath of T-

cell response and an increased number in T-cell responses. Taken together, we think that our replication competent vaccine vector can perform better that the replication deficient vector that was used in the Thai trial due to its being safe, being able to express high amount of antigens, being able to establish pro-inflammatory response, and being able to induce strong T-cell response. With this, our replication competent based vaccine vector has been more developed and characterized and will be participating in a phase I clinical study in the third quarter of 2014.
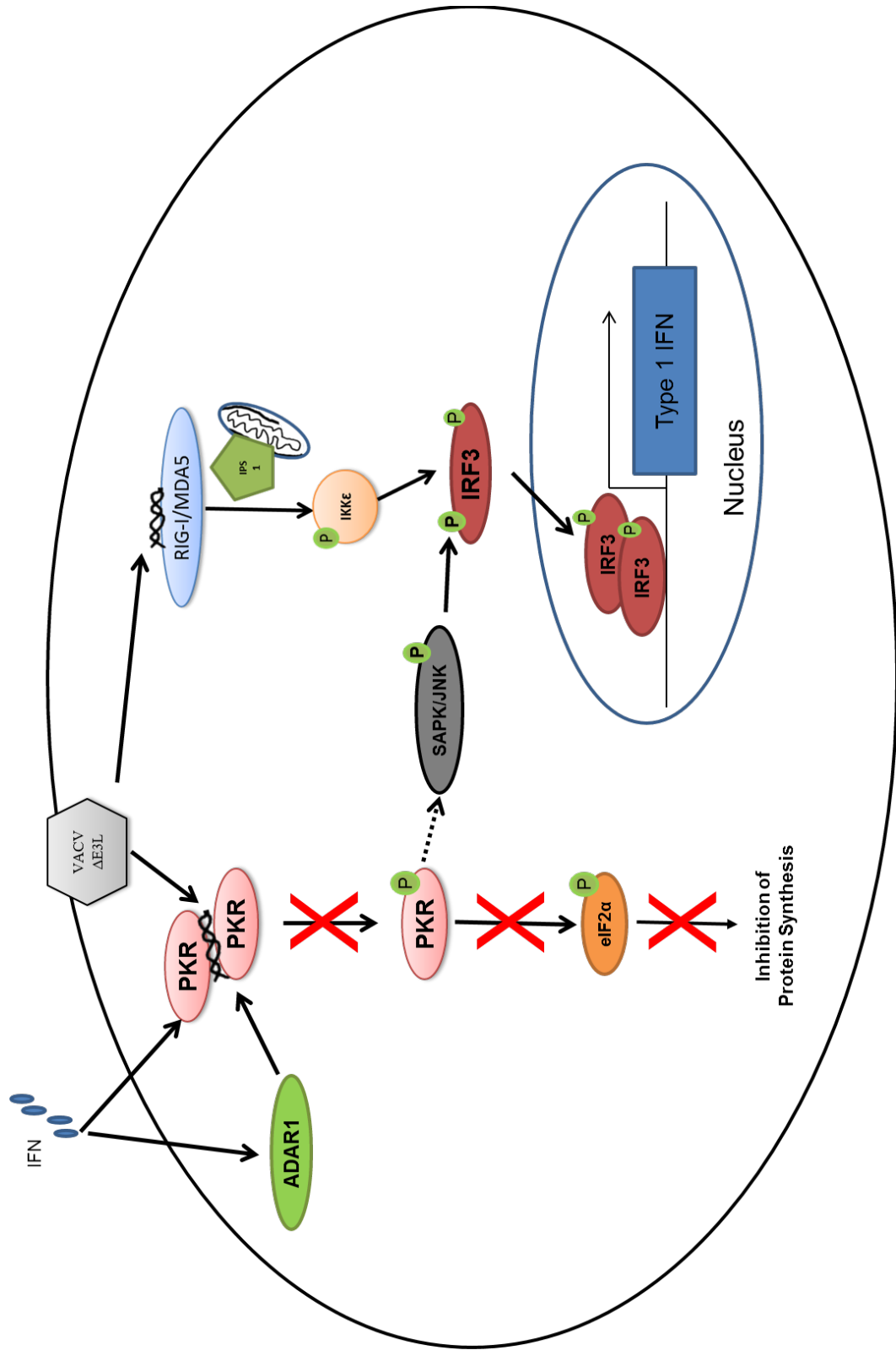
Figure 33 – The role of PKR and ADAR1 during VACVΔE3L infection. During its life cycle, VACVΔE3L makes dsRNA that can lead to the activation of PKR and RIG-I/MDA5. Activation of PKR can lead to inhibition of protein synthesis via the phosphorylation of eIF2α. Activated PKR can also induce the activation of SAPK/JNK which leads to the phosphorylation of IRF3 in the N-terminal at serine173. This phosphorylation is required for the phosphorylation of IRF3 at the C-terminal by IKKε which is activated by RIG-I/MDA5. This will lead to the production of type I IFN in the infected cells. Thus, PKR is an antiviral gene in VACV infection. On the other hand, IFN can lead to the induction of the full length ADAR1. ADAR1 can decrease the amount of dsRNA made by VACVΔE3L. This will lead to the inhibition of eIF2α phosphorylation which results in the rescue of viral protein synthesis and viral replication. Thus, ADAR1 is a pro-viral gene in VACV infection

REFERENCES

1.   **Akira, S.** 2009. Pathogen recognition by innate immunity and its signaling. Proc Jpn Acad Ser B Phys Biol Sci **85:**143-156.

2.   **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783-801.

3.   **Alcami, A., and G. L. Smith.** 1992. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell **71:**153-167.

4.   **Alcami, A., and G. L. Smith.** 2002. The vaccinia virus soluble interferon-gamma receptor is a homodimer. The Journal of general virology **83:**545-549.

5.   **Alcami, A., and G. L. Smith.** 1995. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. J Virol **69:**4633-4639.

6.   **Alcami, A., J. A. Symons, and G. L. Smith.** 2000. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. J Virol **74:**11230-11239.

7.   **Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell.** 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **413:**732-738.

8.   **Alisi, A., A. Spaziani, S. Anticoli, M. Ghidinelli, and C. Balsano.** 2008. PKR is a novel functional direct player that coordinates skeletal muscle differentiation via p38MAPK/AKT pathways. Cell Signal **20:**534-542.

9.   **Alsharifi, M., A. Mullbacher, and M. Regner.** 2008. Interferon type I responses in primary and secondary infections. Immunol Cell Biol **86:**239-245.

10.   **Arsenio, J., Y. Deschambault, and J. Cao.** 2008. Antagonizing activity of vaccinia virus E3L against human interferons in Huh7 cells. Virology **377:**124-132.

11.   **Ashwell, J. D.** 2006. The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol **6:**532-540.

12.   **Baroudy, B. M., S. Venkatesan, and B. Moss.** 1982. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell **28:**315-324.

13.   **Bass, B. L.** 2002. RNA editing by adenosine deaminases that act on RNA. Annual review of biochemistry **71:**817-846.

14. **Bass, B. L., K. Nishikura, W. Keller, P. H. Seeburg, R. B. Emeson, M. A. O'Connell, C. E. Samuel, and A. Herbert.** 1997. A standardized nomenclature for adenosine deaminases that act on RNA. Rna **3:**947-949.

15. **Bass, B. L., and H. Weintraub.** 1987. A developmentally regulated activity that unwinds RNA duplexes. Cell **48:**607-613.

16. **Bayliss, C. D., and R. C. Condit.** 1993. Temperature-sensitive mutants in the vaccinia virus A18R gene increase double-stranded RNA synthesis as a result of aberrant viral transcription. Virology **194:**254-262.

17. **Beattie, E., K. L. Denzler, J. Tartaglia, M. E. Perkus, E. Paoletti, and B. L. Jacobs.** 1995. Reversal of the interferon-sensitive phenotype of a vaccinia virus lacking E3L by expression of the reovirus S4 gene. J Virol **69:**499-505.

18. **Beattie, E., E. B. Kauffman, H. Martinez, M. E. Perkus, B. L. Jacobs, E. Paoletti, and J. Tartaglia.** 1996. Host-range restriction of vaccinia virus E3L-specific deletion mutants. Virus genes **12:**89-94.

19. **Beattie, E., E. Paoletti, and J. Tartaglia.** 1995. Distinct patterns of IFN sensitivity observed in cells infected with vaccinia K3L- and E3L- mutant viruses. Virology **210:**254-263.

20. **Beattie, E., J. Tartaglia, and E. Paoletti.** 1991. Vaccinia virus-encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. Virology **183:**419-422.

21. **Bell, J. K., G. E. Mullen, C. A. Leifer, A. Mazzoni, D. R. Davies, and D. M. Segal.** 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol **24:**528-533.

22. **Bennett, B. L., D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S. S. Bhagwat, A. M. Manning, and D. W. Anderson.** 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A **98:**13681-13686.

23. **Bluyssen, A. R., J. E. Durbin, and D. E. Levy.** 1996. ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. Cytokine Growth Factor Rev **7:**11-17.

24. **Bonjardim, C. A., P. C. Ferreira, and E. G. Kroon.** 2009. Interferons: signaling, antiviral and viral evasion. Immunol Lett **122:**1-11.

25. **Borden, E. C., G. C. Sen, G. Uze, R. H. Silverman, R. M. Ransohoff, G. R. Foster, and G. R. Stark.** 2007. Interferons at age 50: past, current and future impact on biomedicine. Nature reviews. Drug discovery **6:**975-990.

26.   **Bowie, A., E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. O'Neill.** 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc Natl Acad Sci U S A **97:**10162-10167.

27.   **Bowie, A. G., and L. Unterholzner.** 2008. Viral evasion and subversion of pattern-recognition receptor signalling. Nat Rev Immunol **8:**911-922.

28.   **Brandt, T., M. C. Heck, S. Vijaysri, G. M. Jentarra, J. M. Cameron, and B. L. Jacobs.** 2005. The N-terminal domain of the vaccinia virus E3L-protein is required for neurovirulence, but not induction of a protective immune response. Virology **333:**263-270.

29.   **Brandt, T. A., and B. L. Jacobs.** 2001. Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model. J Virol **75:**850-856.

30.   **Brave, A., A. Boberg, L. Gudmundsdotter, E. Rollman, K. Hallermalm, K. Ljungberg, P. Blomberg, R. Stout, S. Paulie, E. Sandstrom, G. Biberfeld, P. Earl, B. Moss, J. H. Cox, and B. Wahren.** 2007. A new multi-clade DNA prime/recombinant MVA boost vaccine induces broad and high levels of HIV-1-specific CD8(+) T-cell and humoral responses in mice. Molecular therapy : the journal of the American Society of Gene Therapy **15:**1724-1733.

31.   **Brochier, B., M. F. Aubert, P. P. Pastoret, E. Masson, J. Schon, M. Lombard, G. Chappuis, B. Languet, and P. Desmettre.** 1996. Field use of a vaccinia-rabies recombinant vaccine for the control of sylvatic rabies in Europe and North America. Revue scientifique et technique **15:**947-970.

32.   **Carroll, K., O. Elroy-Stein, B. Moss, and R. Jagus.** 1993. Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. J Biol Chem **268:**12837-12842.

33.   **Carroll, S. S., J. L. Cole, T. Viscount, J. Geib, J. Gehman, and L. C. Kuo.** 1997. Activation of RNase L by 2',5'-oligoadenylates. Kinetic characterization. J Biol Chem **272:**19193-19198.

34.   **Casey, J. L.** 2006. RNA editing in hepatitis delta virus. Current topics in microbiology and immunology **307:**67-89.

35.   **Cattaneo, R., A. Schmid, D. Eschle, K. Baczko, V. ter Meulen, and M. A. Billeter.** 1988. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. Cell **55:**255-265.

36.   **Chambers, P., B. K. Rima, and W. P. Duprex.** 2009. Molecular differences between two Jeryl Lynn mumps virus vaccine component strains, JL5 and JL2. The Journal of general virology **90:**2973-2981.

37. **Chang, H. W., and B. L. Jacobs.** 1993. Identification of a conserved motif that is necessary for binding of the vaccinia virus E3L gene products to double-stranded RNA. Virology **194:**537-547.

38. **Chang, H. W., L. H. Uribe, and B. L. Jacobs.** 1995. Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. J Virol **69:**6605-6608.

39. **Chang, H. W., J. C. Watson, and B. L. Jacobs.** 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc Natl Acad Sci U S A **89:**4825-4829.

40. **Chen, C. X., D. S. Cho, Q. Wang, F. Lai, K. C. Carter, and K. Nishikura.** 2000. A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains. Rna **6:**755-767.

41. **Clemens, M. J.** 1997. PKR--a protein kinase regulated by double-stranded RNA. Int J Biochem Cell Biol **29:**945-949.

42. **Clerzius, G., J. F. Gelinas, A. Daher, M. Bonnet, E. F. Meurs, and A. Gatignol.** 2009. ADAR1 interacts with PKR during human immunodeficiency virus infection of lymphocytes and contributes to viral replication. J Virol **83:**10119-10128.

43. **Cohen, J.** 1993. AIDS research. Keystone's blunt message: 'it's the virus, stupid'. Science **260:**292-293.

44. **Colamonici, O. R., P. Domanski, S. M. Sweitzer, A. Larner, and R. M. Buller.** 1995. Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. J Biol Chem **270:**15974-15978.

45. **Colby, C., and P. H. Duesberg.** 1969. Double-stranded RNA in vaccinia virus infected cells. Nature **222:**940-944.

46. **Cole, J. L., S. S. Carroll, E. S. Blue, T. Viscount, and L. C. Kuo.** 1997. Activation of RNase L by 2',5'-oligoadenylates. Biophysical characterization. J Biol Chem **272:**19187-19192.

47. **Corbett, M., W. M. Bogers, J. L. Heeney, S. Gerber, C. Genin, A. Didierlaurent, H. Oostermeijer, R. Dubbes, G. Braskamp, S. Lerondel, C. E. Gomez, M. Esteban, R. Wagner, I. Kondova, P. Mooij, S. Balla-Jhagjhoorsingh, N. Beenhakker, G. Koopman, S. van der Burg, J. P. Kraehenbuhl, and A. Le Pape.** 2008. Aerosol immunization with NYVAC and MVA vectored vaccines is safe, simple, and immunogenic. Proc Natl Acad Sci U S A **105:**2046-2051.

48. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-1421.

49. **Davies, M. V., H. W. Chang, B. L. Jacobs, and R. J. Kaufman.** 1993. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. J Virol **67:**1688-1692.

50. **de Veer, M. J., M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, and B. R. Williams.** 2001. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol **69:**912-920.

51. **Desterro, J. M., L. P. Keegan, M. Lafarga, M. T. Berciano, M. O'Connell, and M. Carmo-Fonseca.** 2003. Dynamic association of RNA-editing enzymes with the nucleolus. Journal of cell science **116:**1805-1818.

52. **Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529-1531.

53. **Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa.** 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature **424:**324-328.

54. **Doria, M., F. Neri, A. Gallo, M. G. Farace, and A. Michienzi.** 2009. Editing of HIV-1 RNA by the double-stranded RNA deaminase ADAR1 stimulates viral infection. Nucleic acids research **37:**5848-5858.

55. **Duesberg, P. H., and C. Colby.** 1969. On the biosynthesis and structure of double-stranded RNA in vaccinia virus-infected cells. Proc Natl Acad Sci U S A **64:**396-403.

56. **Eckmann, C. R., A. Neunteufl, L. Pfaffstetter, and M. F. Jantsch.** 2001. The human but not the Xenopus RNA-editing enzyme ADAR1 has an atypical nuclear localization signal and displays the characteristics of a shuttling protein. Molecular biology of the cell **12:**1911-1924.

57. **English, J. M., and M. H. Cobb.** 2002. Pharmacological inhibitors of MAPK pathways. Trends in pharmacological sciences **23:**40-45.

58. **Essbauer, S., M. Bremont, and W. Ahne.** 2001. Comparison of the eIF-2alpha homologous proteins of seven ranaviruses (Iridoviridae). Virus genes **23:**347-359.

59. **Esteban, M.** 2009. Attenuated poxvirus vectors MVA and NYVAC as promising vaccine candidates against HIV/AIDS. Human vaccines **5:**867-871.

60. **Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis.** 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol **4:**491-496.

61. **Floyd-Smith, G., E. Slattery, and P. Lengyel.** 1981. Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. Science **212:**1030-1032.

62. **Fritz, J., A. Strehblow, A. Taschner, S. Schopoff, P. Pasierbek, and M. F. Jantsch.** 2009. RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. Mol Cell Biol **29:**1487-1497.

63. **Gale, M., Jr., and G. C. Sen.** 2009. Viral evasion of the interferon system. J Interferon Cytokine Res **29:**475-476.

64. **Garon, C. F., E. Barbosa, and B. Moss.** 1978. Visualization of an inverted terminal repetition in vaccinia virus DNA. Proc Natl Acad Sci U S A **75:**4863-4867.

65. **George, C. X., Z. Gan, Y. Liu, and C. E. Samuel.** 2011. Adenosine deaminases acting on RNA, RNA editing, and interferon action. J Interferon Cytokine Res **31:**99-117.

66. **George, C. X., M. V. Wagner, and C. E. Samuel.** 2005. Expression of interferon-inducible RNA adenosine deaminase ADAR1 during pathogen infection and mouse embryo development involves tissue-selective promoter utilization and alternative splicing. J Biol Chem **280:**15020-15028.

67. **Gilfoy, F. D., and P. W. Mason.** 2007. West Nile virus-induced interferon production is mediated by the double-stranded RNA-dependent protein kinase PKR. J Virol **81:**11148-11158.

68. **Gillard, S., D. Spehner, R. Drillien, and A. Kirn.** 1986. Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc Natl Acad Sci U S A **83:**5573-5577.

69. **Goh, K. C., M. J. deVeer, and B. R. Williams.** 2000. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. EMBO J **19:**4292-4297.

70. **Gott, J. M., and R. B. Emeson.** 2000. Functions and mechanisms of RNA editing. Annual review of genetics **34:**499-531.

71.   **Greenberg, R. N., and J. S. Kennedy.** 2008. ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. Expert opinion on investigational drugs **17:**555-564.

72.   **Harari, A., P. A. Bart, W. Stohr, G. Tapia, M. Garcia, E. Medjitna-Rais, S. Burnet, C. Cellerai, O. Erlwein, T. Barber, C. Moog, P. Liljestrom, R. Wagner, H. Wolf, J. P. Kraehenbuhl, M. Esteban, J. Heeney, M. J. Frachette, J. Tartaglia, S. McCormack, A. Babiker, J. Weber, and G. Pantaleo.** 2008. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. J Exp Med **205:**63-77.

73.   **Harte, M. T., I. R. Haga, G. Maloney, P. Gray, P. C. Reading, N. W. Bartlett, G. L. Smith, A. Bowie, and L. A. O'Neill.** 2003. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. J Exp Med **197:**343-351.

74.   **Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer.** 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science **303:**1526-1529.

75.   **Hemmi, H., O. Takeuchi, S. Sato, M. Yamamoto, T. Kaisho, H. Sanjo, T. Kawai, K. Hoshino, K. Takeda, and S. Akira.** 2004. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med **199:**1641-1650.

76.   **Herbert, A., J. Alfken, Y. G. Kim, I. S. Mian, K. Nishikura, and A. Rich.** 1997. A Z-DNA binding domain present in the human editing enzyme, double-stranded RNA adenosine deaminase. Proc Natl Acad Sci U S A **94:**8421-8426.

77.   **Hershey, J. W.** 1991. Translational control in mammalian cells. Annual review of biochemistry **60:**717-755.

78.   **Honda, K., A. Takaoka, and T. Taniguchi.** 2006. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity **25:**349-360.

79.   **Iordanov, M. S., J. M. Paranjape, A. Zhou, J. Wong, B. R. Williams, E. F. Meurs, R. H. Silverman, and B. E. Magun.** 2000. Activation of p38 mitogen-activated protein kinase and c-Jun NH(2)-terminal kinase by double-stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. Mol Cell Biol **20:**617-627.

80.   **Isaacs, A., and J. Lindenmann.** 1957. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci **147:**258-267.

81.   **Jacobs, B. L., and J. O. Langland.** 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology **219:**339-349.

82.   **Jayan, G. C., and J. L. Casey.** 2002. Increased RNA editing and inhibition of hepatitis delta virus replication by high-level expression of ADAR1 and ADAR2. J Virol **76:**3819-3827.

83.   **Kagan, J. C., and R. Medzhitov.** 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell **125:**943-955.

84.   **Kalverda, A. P., G. S. Thompson, A. Vogel, M. Schroder, A. G. Bowie, A. R. Khan, and S. W. Homans.** 2009. Poxvirus K7 protein adopts a Bcl-2 fold: biochemical mapping of its interactions with human DEAD box RNA helicase DDX3. Journal of molecular biology **385:**843-853.

85.   **Kang, D. C., R. V. Gopalkrishnan, Q. Wu, E. Jankowsky, A. M. Pyle, and P. B. Fisher.** 2002. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci U S A **99:**637-642.

86.   **Kanneganti, T. D., M. Lamkanfi, and G. Nunez.** 2007. Intracellular NOD-like receptors in host defense and disease. Immunity **27:**549-559.

87.   **Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira.** 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity **23:**19-28.

88.   **Katsoulidis, E., Y. Li, H. Mears, and L. C. Platanias.** 2005. The p38 mitogen-activated protein kinase pathway in interferon signal transduction. J Interferon Cytokine Res **25:**749-756.

89.   **Kawai, T., and S. Akira.** 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. International immunology **21:**317-337.

90.   **Kawai, T., and S. Akira.** 2008. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci **1143:**1-20.

91.   **Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira.** 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol **6:**981-988.

92.   **Kibler, K. V., C. E. Gomez, B. Perdiguero, S. Wong, T. Huynh, S. Holechek, W. Arndt, V. Jimenez, R. Gonzalez-Sanz, K. Denzler, E. K. Haddad, R. Wagner, R. P. Sekaly, J. Tartaglia, G. Pantaleo, B. L. Jacobs, and M. Esteban.** 2011. Improved NYVAC-based vaccine vectors. PloS one **6:**e25674.

93. **Kibler, K. V., T. Shors, K. B. Perkins, C. C. Zeman, M. P. Banaszak, J. Biesterfeldt, J. O. Langland, and B. L. Jacobs.** 1997. Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. J Virol **71:**1992-2003.

94. **Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna.** 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. Blood **103:**1433-1437.

95. **Kumar, M., and G. G. Carmichael.** 1997. Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. Proc Natl Acad Sci U S A **94:**3542-3547.

96. **Lane, J. M., F. L. Ruben, J. M. Neff, and J. D. Millar.** 1969. Complications of smallpox vaccination, 1968. The New England journal of medicine **281:**1201-1208.

97. **Lane, J. M., F. L. Ruben, J. M. Neff, and J. D. Millar.** 1970. Complications of smallpox vaccination, 1968: results of ten statewide surveys. The Journal of infectious diseases **122:**303-309.

98. **Langland, J. O., J. M. Cameron, M. C. Heck, J. K. Jancovich, and B. L. Jacobs.** 2006. Inhibition of PKR by RNA and DNA viruses. Virus research **119:**100-110.

99. **Langland, J. O., and B. L. Jacobs.** 2004. Inhibition of PKR by vaccinia virus: role of the N- and C-terminal domains of E3L. Virology **324:**419-429.

100. **Langland, J. O., J. C. Kash, V. Carter, M. J. Thomas, M. G. Katze, and B. L. Jacobs.** 2006. Suppression of proinflammatory signal transduction and gene expression by the dual nucleic acid binding domains of the vaccinia virus E3L proteins. J Virol **80:**10083-10095.

101. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol **5:**190-198.

102. **Le Bon, A., and D. F. Tough.** 2002. Links between innate and adaptive immunity via type I interferon. Curr Opin Immunol **14:**432-436.

103. **Lee, S. B., and M. Esteban.** 1994. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology **199:**491-496.

104. **Lemaire, P. A., E. Anderson, J. Lary, and J. L. Cole.** 2008. Mechanism of PKR Activation by dsRNA. Journal of molecular biology **381:**351-360.

105. **Leung, S., S. A. Qureshi, I. M. Kerr, J. E. Darnell, Jr., and G. R. Stark.** 1995. Role of STAT2 in the alpha interferon signaling pathway. Mol Cell Biol **15:**1312-1317.

106. **Levy, D. E., and A. Garcia-Sastre.** 2001. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. Cytokine Growth Factor Rev **12:**143-156.

107. **Linnstaedt, S. D., W. K. Kasprzak, B. A. Shapiro, and J. L. Casey.** 2006. The role of a metastable RNA secondary structure in hepatitis delta virus genotype III RNA editing. Rna **12:**1521-1533.

108. **Liptakova, H., E. Kontsekova, A. Alcami, G. L. Smith, and P. Kontsek.** 1997. Analysis of an interaction between the soluble vaccinia virus-coded type I interferon (IFN)-receptor and human IFN-alpha1 and IFN-alpha2. Virology **232:**86-90.

109. **Liu, Y., R. B. Emeson, and C. E. Samuel.** 1999. Serotonin-2C receptor pre-mRNA editing in rat brain and in vitro by splice site variants of the interferon-inducible double-stranded RNA-specific adenosine deaminase ADAR1. J Biol Chem **274:**18351-18358.

110. **Liu, Y., E. G. Shepherd, and L. D. Nelin.** 2007. MAPK phosphatases--regulating the immune response. Nat Rev Immunol **7:**202-212.

111. **Liu, Y., K. C. Wolff, B. L. Jacobs, and C. E. Samuel.** 2001. Vaccinia virus E3L interferon resistance protein inhibits the interferon-induced adenosine deaminase A-to-I editing activity. Virology **289:**378-387.

112. **Lu, J., E. B. O'Hara, B. A. Trieselmann, P. R. Romano, and T. E. Dever.** 1999. The interferon-induced double-stranded RNA-activated protein kinase PKR will phosphorylate serine, threonine, or tyrosine at residue 51 in eukaryotic initiation factor 2alpha. J Biol Chem **274:**32198-32203.

113. **Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki.** 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med **198:**513-520.

114. **Maas, S., A. Rich, and K. Nishikura.** 2003. A-to-I RNA editing: recent news and residual mysteries. J Biol Chem **278:**1391-1394.

115. **Malathi, K., B. Dong, M. Gale, Jr., and R. H. Silverman.** 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature **448:**816-819.

116. **Mann, B. A., J. H. Huang, P. Li, H. C. Chang, R. B. Slee, A. O'Sullivan, M. Anita, N. Yeh, M. J. Klemsz, R. R. Brutkiewicz, J. S. Blum, and M. H.**

Kaplan. 2008. Vaccinia virus blocks Stat1-dependent and Stat1-independent gene expression induced by type I and type II interferons. J Interferon Cytokine Res **28:**367-380.

117. **Martinez, I., J. Dopazo, and J. A. Melero.** 1997. Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants. The Journal of general virology **78 (Pt 10):**2419-2429.

118. **Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya.** 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol **171:**3154-3162.

119. **McCormack, S. J., and C. E. Samuel.** 1995. Mechanism of interferon action: RNA-binding activity of full-length and R-domain forms of the RNA-dependent protein kinase PKR--determination of KD values for VAI and TAR RNAs. Virology **206:**511-519.

120. **McCurdy, L. H., B. D. Larkin, J. E. Martin, and B. S. Graham.** 2004. Modified vaccinia Ankara: potential as an alternative smallpox vaccine. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **38:**1749-1753.

121. **McKenna, S. A., D. A. Lindhout, I. Kim, C. W. Liu, V. M. Gelev, G. Wagner, and J. D. Puglisi.** 2007. Molecular framework for the activation of RNA-dependent protein kinase. J Biol Chem **282:**11474-11486.

122. **Melcher, T., S. Maas, A. Herb, R. Sprengel, M. Higuchi, and P. H. Seeburg.** 1996. RED2, a brain-specific member of the RNA-specific adenosine deaminase family. J Biol Chem **271:**31795-31798.

123. **Melcher, T., S. Maas, A. Herb, R. Sprengel, P. H. Seeburg, and M. Higuchi.** 1996. A mammalian RNA editing enzyme. Nature **379:**460-464.

124. **Mellin, H., J. M. Neff, H. Garber, and J. M. Lane.** 1970. Complications of smallpox vaccination, Maryland 1968. The Johns Hopkins medical journal **126:**160-168.

125. **Meng, X., C. Jiang, J. Arsenio, K. Dick, J. Cao, and Y. Xiang.** 2009. Vaccinia virus K1L and C7L inhibit antiviral activities induced by type I interferons. J Virol **83:**10627-10636.

126. **Meng, X., and Y. Xiang.** 2006. Vaccinia virus K1L protein supports viral replication in human and rabbit cells through a cell-type-specific set of its ankyrin repeat residues that are distinct from its binding site for ACAP2. Virology **353:**220-233.

127. **Meurs, E., K. Chong, J. Galabru, N. S. Thomas, I. M. Kerr, B. R. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62:**379-390.

128. **Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437:**1167-1172.

129. **Mooij, P., S. S. Balla-Jhagjhoorsingh, N. Beenhakker, P. van Haaften, I. Baak, I. G. Nieuwenhuis, S. Heidari, H. Wolf, M. J. Frachette, K. Bieler, N. Sheppard, A. Harari, P. A. Bart, P. Liljestrom, R. Wagner, G. Pantaleo, and J. L. Heeney.** 2009. Comparison of human and rhesus macaque T-cell responses elicited by boosting with NYVAC encoding human immunodeficiency virus type 1 clade C immunogens. J Virol **83:**5881-5889.

130. **Moss, B.** 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A **93:**11341-11348.

131. **Moss, B.** 2007. Poxviridade: The viruses and their replication, 5 ed, vol. 2.

132. **Moss, B., and J. L. Shisler.** 2001. Immunology 101 at poxvirus U: immune evasion genes. Seminars in immunology **13:**59-66.

133. **Mossman, K., P. Nation, J. Macen, M. Garbutt, A. Lucas, and G. McFadden.** 1996. Myxoma virus M-T7, a secreted homolog of the interferon-gamma receptor, is a critical virulence factor for the development of myxomatosis in European rabbits. Virology **215:**17-30.

134. **Mossman, K., C. Upton, R. M. Buller, and G. McFadden.** 1995. Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. Virology **208:**762-769.

135. **Muralinath, M.** 2003. The role of the amino terminus of E3L in vaccinia virus pathogenesis. Ph. D. Arizona State University.

136. **Murphy, D. G., K. Dimock, and C. Y. Kang.** 1991. Numerous transitions in human parainfluenza virus 3 RNA recovered from persistently infected cells. Virology **181:**760-763.

137. **Najera, J. L., C. E. Gomez, E. Domingo-Gil, M. M. Gherardi, and M. Esteban.** 2006. Cellular and biochemical differences between two attenuated poxvirus vaccine candidates (MVA and NYVAC) and role of the C7L gene. J Virol **80:**6033-6047.

138. **Nishikura, K.** 2010. Functions and regulation of RNA editing by ADAR deaminases. Annual review of biochemistry **79:**321-349.

139. **Nishiya, T., E. Kajita, S. Miwa, and A. L. Defranco.** 2005. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem **280:**37107-37117.

140. **Novick, D., B. Cohen, and M. Rubinstein.** 1994. The human interferon alpha/beta receptor: characterization and molecular cloning. Cell **77:**391-400.

141. **Oldstone, M. B.** 2009. Modeling subacute sclerosing panencephalitis in a transgenic mouse system: uncoding pathogenesis of disease and illuminating components of immune control. Current topics in microbiology and immunology **330:**31-54.

142. **Pain, V. M.** 1996. Initiation of protein synthesis in eukaryotic cells. Eur J Biochem **236:**747-771.

143. **Panne, D., T. Maniatis, and S. C. Harrison.** 2007. An atomic model of the interferon-beta enhanceosome. Cell **129:**1111-1123.

144. **Panne, D., S. M. McWhirter, T. Maniatis, and S. C. Harrison.** 2007. Interferon regulatory factor 3 is regulated by a dual phosphorylation-dependent switch. J Biol Chem **282:**22816-22822.

145. **Paoletti, E.** 1996. Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci U S A **93:**11349-11353.

146. **Pastoret, P. P., and B. Brochier.** 1996. The development and use of a vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies; a link between Jenner and Pasteur. Epidemiology and infection **116:**235-240.

147. **Pastoret, P. P., B. Brochier, B. Languet, C. Duret, G. Chappuis, and P. Desmettre.** 1996. Stability of recombinant vaccinia-rabies vaccine in veterinary use. Developments in biological standardization **87:**245-249.

148. **Patterson, J. B., and C. E. Samuel.** 1995. Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. Mol Cell Biol **15:**5376-5388.

149. **Patterson, J. B., D. C. Thomis, S. L. Hans, and C. E. Samuel.** 1995. Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons. Virology **210:**508-511.

150. **Paul, T. A., J. W. Casey, R. J. Avery, and C. A. Sutton.** 2007. Expression of feline immunodeficiency virus Vif is associated with reduced viral mutation rates without restoration of replication of vif mutant viruses. Virology **361:**112-122.

151. **Pearson, G., F. Robinson, T. Beers Gibson, B. E. Xu, M. Karandikar, K. Berman, and M. H. Cobb.** 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine reviews **22:**153-183.

152. **Perkus, M. E., S. J. Goebel, S. W. Davis, G. P. Johnson, K. Limbach, E. K. Norton, and E. Paoletti.** 1990. Vaccinia virus host range genes. Virology **179:**276-286.

153. **Petrilli, V., C. Dostert, D. A. Muruve, and J. Tschopp.** 2007. The inflammasome: a danger sensing complex triggering innate immunity. Curr Opin Immunol **19:**615-622.

154. **Phuphuakrat, A., R. Kraiwong, C. Boonarkart, D. Lauhakirti, T. H. Lee, and P. Auewarakul.** 2008. Double-stranded RNA adenosine deaminases enhance expression of human immunodeficiency virus type 1 proteins. J Virol **82:**10864-10872.

155. **Potter, J. A., R. E. Randall, and G. L. Taylor.** 2008. Crystal structure of human IPS-1/MAVS/VISA/Cardif caspase activation recruitment domain. BMC Struct Biol **8:**11.

156. **Poulsen, H., J. Nilsson, C. K. Damgaard, J. Egebjerg, and J. Kjems.** 2001. CRM1 mediates the export of ADAR1 through a nuclear export signal within the Z-DNA binding domain. Mol Cell Biol **21:**7862-7871.

157. **Qin, B. Y., C. Liu, S. S. Lam, H. Srinath, R. Delston, J. J. Correia, R. Derynck, and K. Lin.** 2003. Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. Nat Struct Biol **10:**913-921.

158. **Quakkelaar, E. D., A. Redeker, E. K. Haddad, A. Harari, S. M. McCaughey, T. Duhen, A. Filali-Mouhim, J. P. Goulet, N. M. Loof, F. Ossendorp, B. Perdiguero, P. Heinen, C. E. Gomez, K. V. Kibler, D. M. Koelle, R. P. Sekaly, F. Sallusto, A. Lanzavecchia, G. Pantaleo, M. Esteban, J. Tartaglia, B. L. Jacobs, and C. J. Melief.** 2011. Improved innate and adaptive immunostimulation by genetically modified HIV-1 protein expressing NYVAC vectors. PloS one **6:**e16819.

159. **Ramanathan, V. D., M. Kumar, J. Mahalingam, P. Sathyamoorthy, P. R. Narayanan, S. Solomon, D. Panicali, S. Chakrabarty, J. Cox, E. Sayeed, J. Ackland, C. Verlinde, D. Vooijs, K. Loughran, B. Barin, A. Lombardo, J. Gilmour, G. Stevens, M. S. Smith, T. Tarragona-Fiol, P. Hayes, S. Kochhar, J. L. Excler, and P. Fast.** 2009. A Phase 1 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 subtype C-modified vaccinia Ankara virus vaccine candidate in Indian volunteers. AIDS research and human retroviruses **25:**1107-1116.

160. **Ratner, L. H., J. M. Lane, and C. N. Vicens.** 1970. Complications of smallpox vaccination: surveillance during an island-wide program in Puerto Rico, 1967-1968. American journal of epidemiology **91:**278-285.

161. **Rebagliati, M. R., and D. A. Melton.** 1987. Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. Cell **48:**599-605.

162. **Rebouillat, D., and A. G. Hovanessian.** 1999. The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. J Interferon Cytokine Res **19:**295-308.

163. **Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J. G. McNeil, D. P. Francis, D. Stablein, D. L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. L. Robb, N. L. Michael, P. Kunasol, J. H. Kim, and M.-T. Investigators.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. The New England journal of medicine **361:**2209-2220.

164. **Riedel, S.** 2005. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent) **18:**21-25.

165. **Rivas, C., J. Gil, Z. Melkova, M. Esteban, and M. Diaz-Guerra.** 1998. Vaccinia virus E3L protein is an inhibitor of the interferon (i.f.n.)-induced 2-5A synthetase enzyme. Virology **243:**406-414.

166. **Romano, P. R., F. Zhang, S. L. Tan, M. T. Garcia-Barrio, M. G. Katze, T. E. Dever, and A. G. Hinnebusch.** 1998. Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: role of complex formation and the E3 N-terminal domain. Mol Cell Biol **18:**7304-7316.

167. **Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald.** 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. J Immunol **175:**5260-5268.

168. **Sadler, A. J., and B. R. Williams.** 2008. Interferon-inducible antiviral effectors. Nat Rev Immunol **8:**559-568.

169. **Saha, S. K., and G. Cheng.** 2006. TRAF3: a new regulator of type I interferons. Cell Cycle **5:**804-807.

170. **Samuel, C. E.** 2011. Adenosine deaminases acting on RNA (ADARs) are both antiviral and proviral. Virology **411:**180-193.

171. **Samuel, C. E.** 2001. Antiviral actions of interferons. Clin Microbiol Rev **14:**778-809, table of contents.

172.   **Sandstrom, E., C. Nilsson, B. Hejdeman, A. Brave, G. Bratt, M. Robb, J. Cox, T. Vancott, M. Marovich, R. Stout, S. Aboud, M. Bakari, K. Pallangyo, K. Ljungberg, B. Moss, P. Earl, N. Michael, D. Birx, F. Mhalu, B. Wahren, G. Biberfeld, and H. I. V. I. S. Team.** 2008. Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. The Journal of infectious diseases **198:**1482-1490.

173.   **Sansam, C. L., K. S. Wells, and R. B. Emeson.** 2003. Modulation of RNA editing by functional nucleolar sequestration of ADAR2. Proc Natl Acad Sci U S A **100:**14018-14023.

174.   **Schwartz, T., M. A. Rould, K. Lowenhaupt, A. Herbert, and A. Rich.** 1999. Crystal structure of the Zalpha domain of the human editing enzyme ADAR1 bound to left-handed Z-DNA. Science **284:**1841-1845.

175.   **Seeburg, P. H., M. Higuchi, and R. Sprengel.** 1998. RNA editing of brain glutamate receptor channels: mechanism and physiology. Brain research. Brain research reviews **26:**217-229.

176.   **Sen, G. C.** 2001. Viruses and interferons. Annu Rev Microbiol **55:**255-281.

177.   **Senkevich, T. G., J. J. Bugert, J. R. Sisler, E. V. Koonin, G. Darai, and B. Moss.** 1996. Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. Science **273:**813-816.

178.   **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell **122:**669-682.

179.   **Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott.** 2003. Triggering the interferon antiviral response through an IKK-related pathway. Science **300:**1148-1151.

180.   **Sharp, T. V., F. Moonan, A. Romashko, B. Joshi, G. N. Barber, and R. Jagus.** 1998. The vaccinia virus E3L gene product interacts with both the regulatory and the substrate binding regions of PKR: implications for PKR autoregulation. Virology **250:**302-315.

181.   **Shors, S. T., E. Beattie, E. Paoletti, J. Tartaglia, and B. L. Jacobs.** 1998. Role of the vaccinia virus E3L and K3L gene products in rescue of VSV and EMCV from the effects of IFN-alpha. J Interferon Cytokine Res **18:**721-729.

182.   **Shors, T., and B. L. Jacobs.** 1997. Complementation of deletion of the vaccinia virus E3L gene by the Escherichia coli RNase III gene. Virology **227:**77-87.

183. **Shors, T., K. V. Kibler, K. B. Perkins, R. Seidler-Wulff, M. P. Banaszak, and B. L. Jacobs.** 1997. Complementation of vaccinia virus deleted of the E3L gene by mutants of E3L. Virology **239:**269-276.

184. **Silverman, R. H.** 2007. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol **81:**12720-12729.

185. **Slattery, E., N. Ghosh, H. Samanta, and P. Lengyel.** 1979. Interferon, double-stranded RNA, and RNA degradation: activation of an endonuclease by (2'-5')An. Proc Natl Acad Sci U S A **76:**4778-4782.

186. **Smith, E. J., I. Marie, A. Prakash, A. Garcia-Sastre, and D. E. Levy.** 2001. IRF3 and IRF7 phosphorylation in virus-infected cells does not require double-stranded RNA-dependent protein kinase R or Ikappa B kinase but is blocked by Vaccinia virus E3L protein. J Biol Chem **276:**8951-8957.

187. **Smith, G. L., J. A. Symons, A. Khanna, A. Vanderplasschen, and A. Alcami.** 1997. Vaccinia virus immune evasion. Immunological reviews **159:**137-154.

188. **Sroller, V., V. Ludvikova, L. Maresova, P. Hainz, and S. Nemeckova.** 2001. Effect of IFN-gamma receptor gene deletion on vaccinia virus virulence. Archives of virology **146:**239-249.

189. **Stack, J., I. R. Haga, M. Schroder, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie.** 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J Exp Med **201:**1007-1018.

190. **Stark, G. R.** 2007. How cells respond to interferons revisited: from early history to current complexity. Cytokine Growth Factor Rev **18:**419-423.

191. **Strehblow, A., M. Hallegger, and M. F. Jantsch.** 2002. Nucleocytoplasmic distribution of human RNA-editing enzyme ADAR1 is modulated by double-stranded RNA-binding domains, a leucine-rich export signal, and a putative dimerization domain. Molecular biology of the cell **13:**3822-3835.

192. **Suspene, R., M. Renard, M. Henry, D. Guetard, D. Puyraimond-Zemmour, A. Billecocq, M. Bouloy, F. Tangy, J. P. Vartanian, and S. Wain-Hobson.** 2008. Inversing the natural hydrogen bonding rule to selectively amplify GC-rich ADAR-edited RNAs. Nucleic acids research **36:**e72.

193. **Symons, J. A., A. Alcami, and G. L. Smith.** 1995. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. Cell **81:**551-560.

194. **Symons, J. A., D. C. Tscharke, N. Price, and G. L. Smith.** 2002. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. The Journal of general virology **83:**1953-1964.

195. **Takada, Y., H. Ichikawa, A. Pataer, S. Swisher, and B. B. Aggarwal.** 2007. Genetic deletion of PKR abrogates TNF-induced activation of IkappaBalpha kinase, JNK, Akt and cell proliferation but potentiates p44/p42 MAPK and p38 MAPK activation. Oncogene **26:**1201-1212.

196. **Takahasi, K., H. Kumeta, N. Tsuduki, R. Narita, T. Shigemoto, R. Hirai, M. Yoneyama, M. Horiuchi, K. Ogura, T. Fujita, and F. Inagaki.** 2009. Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. J Biol Chem **284:**17465-17474.

197. **Tartaglia, J., M. E. Perkus, J. Taylor, E. K. Norton, J. C. Audonnet, W. I. Cox, S. W. Davis, J. van der Hoeven, B. Meignier, M. Riviere, and et al.** 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology **188:**217-232.

198. **Taylor, D. R., M. Puig, M. E. Darnell, K. Mihalik, and S. M. Feinstone.** 2005. New antiviral pathway that mediates hepatitis C virus replicon interferon sensitivity through ADAR1. J Virol **79:**6291-6298.

199. **Taylor, G., E. J. Stott, G. Wertz, and A. Ball.** 1991. Comparison of the virulence of wild-type thymidine kinase (tk)-deficient and tk+ phenotypes of vaccinia virus recombinants after intranasal inoculation of mice. The Journal of general virology **72 (Pt 1):**125-130.

200. **Tenoever, B. R., S. L. Ng, M. A. Chua, S. M. McWhirter, A. Garcia-Sastre, and T. Maniatis.** 2007. Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. Science **315:**1274-1278.

201. **Toth, A. M., Z. Li, R. Cattaneo, and C. E. Samuel.** 2009. RNA-specific adenosine deaminase ADAR1 suppresses measles virus-induced apoptosis and activation of protein kinase PKR. J Biol Chem **284:**29350-29356.

202. **Toth, A. M., P. Zhang, S. Das, C. X. George, and C. E. Samuel.** 2006. Interferon action and the double-stranded RNA-dependent enzymes ADAR1 adenosine deaminase and PKR protein kinase. Progress in nucleic acid research and molecular biology **81:**369-434.

203. **Ueda, Y., S. Morikawa, and Y. Matsuura.** 1990. Identification and nucleotide sequence of the gene encoding a surface antigen induced by vaccinia virus. Virology **177:**588-594.

204. **Upton, C., S. Slack, A. L. Hunter, A. Ehlers, and R. L. Roper.** 2003. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol **77:**7590-7600.

205. **Uze, G., G. Lutfalla, and I. Gresser.** 1990. Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. Cell **60:**225-234.

206. **van Boxel-Dezaire, A. H., M. R. Rani, and G. R. Stark.** 2006. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity **25:**361-372.

207. **Verardi, P. H., L. A. Jones, F. H. Aziz, S. Ahmad, and T. D. Yilma.** 2001. Vaccinia virus vectors with an inactivated gamma interferon receptor homolog gene (B8R) are attenuated In vivo without a concomitant reduction in immunogenicity. J Virol **75:**11-18.

208. **Wang, T., T. Town, L. Alexopoulou, J. F. Anderson, E. Fikrig, and R. A. Flavell.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med **10:**1366-1373.

209. **Ward, S. V., C. X. George, M. J. Welch, L. Y. Liou, B. Hahm, H. Lewicki, J. C. de la Torre, C. E. Samuel, and M. B. Oldstone.** 2011. RNA editing enzyme adenosine deaminase is a restriction factor for controlling measles virus replication that also is required for embryogenesis. Proc Natl Acad Sci U S A **108:**331-336.

210. **Watson, J. C., H. W. Chang, and B. L. Jacobs.** 1991. Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNA-dependent protein kinase. Virology **185:**206-216.

211. **Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan.** 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol **80:**5059-5064.

212. **Wehrle, P. F.** 1980. A reality in our time--certification of the global eradication of smallpox. The Journal of infectious diseases **142:**636-638.

213. **Weiss, R. A.** 1993. How does HIV cause AIDS? Science **260:**1273-1279.

214. **White, S. D., K. Conwell, J. O. Langland, and B. L. Jacobs.** 2011. Use of a negative selectable marker for rapid selection of recombinant vaccinia virus. BioTechniques **50:**303-309.

215.	**White, S. D., and B. L. Jacobs.** 2012. The amino terminus of the vaccinia virus E3 protein is necessary to inhibit the interferon response. J Virol **86:**5895-5904.

216.	**Williams, B. R., I. M. Kerr, C. S. Gilbert, C. N. White, and L. A. Ball.** 1978. Synthesis and breakdown of pppA2'p5'A2'p5'A and transient inhibiton of protein synthesis in extracts from interferon-treated and control cells. Eur J Biochem **92:**455-462.

217.	**Winter-Vann, A. M., and G. L. Johnson.** 2007. Integrated activation of MAP3Ks balances cell fate in response to stress. J Cell Biochem **102:**848-858.

218.	**Wreschner, D. H., J. W. McCauley, J. J. Skehel, and I. M. Kerr.** 1981. Interferon action--sequence specificity of the ppp(A2'p)nA-dependent ribonuclease. Nature **289:**414-417.

219.	**Xiang, Y., R. C. Condit, S. Vijaysri, B. Jacobs, B. R. Williams, and R. H. Silverman.** 2002. Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. J Virol **76:**5251-5259.

220.	**Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira.** 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science **301:**640-643.

221.	**Yoneyama, M., and T. Fujita.** 2008. Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity **29:**178-181.

222.	**Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita.** 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol **175:**2851-2858.

223.	**Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol **5:**730-737.

224.	**Yu, Y. X., M. Bearzotti, P. Vende, W. Ahne, and M. Bremont.** 1999. Partial mapping and sequencing of a fish iridovirus genome reveals genes homologous to the frog virus 3 p31, p40 and human eIF2alpha. Virus research **63:**53-63.

225.	**Zahn, R. C., I. Schelp, O. Utermohlen, and D. von Laer.** 2007. A-to-G hypermutation in the genome of lymphocytic choriomeningitis virus. J Virol **81:**457-464.

226. **Zhang, B., M. Li, L. Chen, K. Yang, Y. Shan, L. Zhu, S. Sun, L. Li, and C. Wang.** 2009. The TAK1-JNK cascade is required for IRF3 function in the innate immune response. Cell Res **19:**412-428.

227. **Zhang, P., J. O. Langland, B. L. Jacobs, and C. E. Samuel.** 2009. Protein kinase PKR-dependent activation of mitogen-activated protein kinases occurs through mitochondrial adapter IPS-1 and is antagonized by vaccinia virus E3L. J Virol **83:**5718-5725.

228. **Zhang, P., and C. E. Samuel.** 2008. Induction of protein kinase PKR-dependent activation of interferon regulatory factor 3 by vaccinia virus occurs through adapter IPS-1 signaling. J Biol Chem **283:**34580-34587.

APPENDIX A

FOOTNOTE
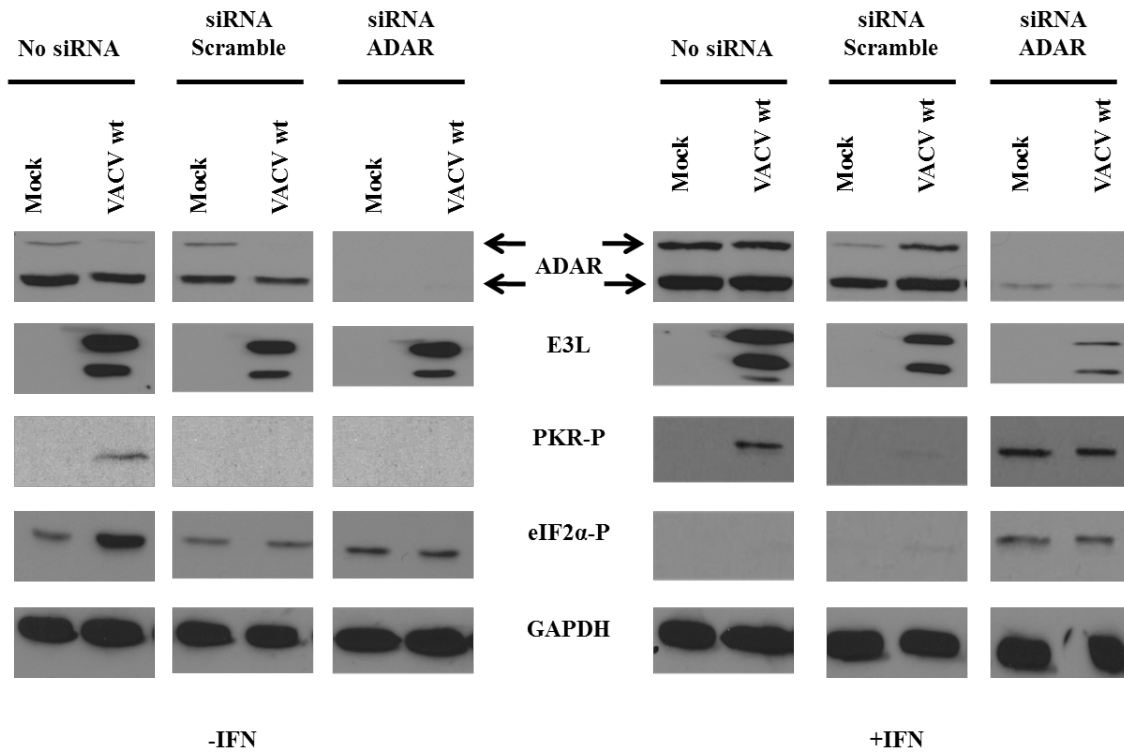
APPENDIX B

SUPPLEMENTARY FIGURE

Figure S1 – Knock down of ADAR1 leads to inhibit of E3 synthesis in IFN treated cells. Hela cells were mock transfected or transfected with either scramble siRNA or siRNA against ADAR. After 30 hours post transfection, cells were mock treated or treated with 1000U/ml of IFN. Cells were mock infected or infected with VACV wt after 18 hours of treatment. . Cell lysates were prepared at 9 hours post infection. Cell lysates were resolved on a 10% poly-acrylamide gel followed by Western blotting using specified antibodies