Role of the Vaccinia Virus E3 protein and its poxvirus orthologues in suppressing innate immune responses activated by RNA-based pathogen-associated molecular patterns

by

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Dedication

This thesis is dedicated to my parents, Rod and Lori Myskiw, my brother, Jarett Myskiw, and my grandmother, Grace Towns, whose support and encouragement from the beginning has allowed me to achieve my goals in life.

The charm of fishing is that it is the pursuit of what is elusive but attainable, a perpetual series of occasions for hope. - John Buchan

Those that know me well know that fishing and science are two big passions in my life. For me, the above quote applies to science as much as it does to fishing.

Abstract

Poxviruses are a diverse family of double-stranded DNA viruses. A characteristic feature of poxviruses is that they express a vast array of immuno-modulatory proteins. Vaccinia virus is the prototypic member of the *Orthopoxvirus* genus, which also includes variola virus, the causative agent of smallpox. The vaccinia E3 protein is required for virus replication *in vivo* and in numerous cell culture systems. Although E3 function has received considerable study, many aspects of E3 biology remain to be addressed. While E3 can inhibit cytokine expression, the pathways targeted by E3 to block cytokine expression have not been identified. Furthermore, the factor(s) which stimulate innate immune responses during vaccinia infection are not known. In this study, E3 was found to target PKR and RIG-I-like receptor mediated signal transduction to differentially block expression of IFN-β, TNF-α and IL-6 in HeLa cells. RNA species generated in vaccinia infected cells were identified as pathogen-associated molecular patterns capable of inducing cytokine expression and activating apoptosis. Furthermore, PKR, RIG-I and MDA5 play non-overlapping and essential roles in mediating the innate immune response to these RNA species.

Orthologues of E3 are encoded by all poxviruses which infect vertebrate animals except the *Avipoxviruses* and molluscum contagiosum virus. However, orthologues of vaccinia E3 remain essentially uncharacterized. A comparative analysis of the ability of E3 orthologues encoded by sheeppox, yaba monkey tumour, swinepox and myxoma virus to complement deletion of E3 was performed. E3 orthologues of myxoma virus and swinepox virus suppress PKR activation and interferon induced antiviral activity and restore the host range function of E3 in culture. In contrast, the E3 orthologues of sheeppox virus and yaba monkey tumour virus are unable to inhibit PKR activation.

While the sheeppox orthologue cannot restore the host range function of E3, the yaba monkey tumour virus orthologue partially restores E3 deficient vaccinia replication. However, none of these E3 orthologues restore pathogenicity to E3 deficient vaccinia *in vivo*. In summary, these results highlight the role of the vaccinia virus E3 protein and its poxvirus orthologues in suppressing innate immune responses activated by RNA-based pathogen-associated molecular patterns.

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List of abbreviations

ADAR1	adenosine deaminase 1
AdV	adenovirus
AIM2	absent in melanoma 2
araC	cytosine arabinoside
bp	base-pair
CARD	caspase recruitment domain
CBP	CREB binding protein
CEV	cell associated virion
CSF	colony stimulating factor
Ct	cycle threshold
DNase	Deoxyribonuclease
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EBER	Epstein-Barr virus encoded RNA
EEV	extracellular enveloped virion
EGFP	enhanced green fluorescent protein
eIF2α	eukaryotic initiation factor 2 alpha
EMCV	encephalomyocarditis virus
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCV	hepatitis C virus
HDAC	histone deacetylase
hpi	hours post-infection
hpt	hours post-transfection
HSV1	herpes simplex virus type 1
IEV	intracellular enveloped virion
IFI16	interferon inducible protein 16
IFN	interferon
IFNAR	interferon alpha/beta receptor

IκB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
IL	interleukin
IMV	intracellular mature virion
INHBA	inhibin beta A
IPS-1	interferon promoter stimulator 1
IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISRE	interferon stimulated response element
Jak	Janus associated kinase
kbp	kilo base-pair
LGP2	laboratory of genetics and physiology 2
LTA	lymphotoxin A
MAPK	mitogen activated protein kinase
MDA5	melanoma differentiation-associated gene-5
ml	millilitre
MK2	mitogen activated protein kinase-activated protein kinase 2
MYXV	myxoma virus
NDV	Newcastle disease virus
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NLR	nucleotide oligomerization domain-like receptor
ORF	open reading frame
p38	p38 mitogen activated protein kinase
PACT	PKR-activating protein
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
pC	polycytidylic acid
PCR	polymerase chain reaction
PDGF	platelet derived growth factor

PFU	plaque forming units
pI	polyinosinic acid
pIC	polyinosinic-polycytidylic acid
PKR	dsRNA activated protein kinase
PRR	pattern recognition receptor
RIG-I	retinoic acid inducible gene-I
RLR	RIG-I-like receptor
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
Ser	serine
SeV	Sendai virus
SPPV	sheeppox virus
SPV	swinepox virus
ssRNA	single-stranded RNA
Stat	signal transducer and activator of transcription
TBK	TRAF family member-associated NF-κB activator binding kinase
TGF	transforming growth factor
Thr	threonine
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
µg	microgram
UTR	untranslated region
VACV	Vaccinia virus
VAI	AdV associated RNA
VCP	Vaccinia complement control protein
VEGF	vascular endothelial growth factor
VSV	Vesicular stomatitis virus
WNV	West Nile virus
YMTV	Yaba monkey tumour virus

Chapter I

Introduction

1 - The Poxviridae

The *Poxviridae* comprise a large family of double-stranded DNA (dsDNA) viruses that replicate exclusively in the cytoplasm of infected cells[156]. Therefore, poxviruses must encode most of the genes required for viral DNA replication and gene transcription and are relatively independent from their host cells in comparison to other DNA viruses. The *Poxviridae* are divided into two subfamilies, the *Chordopoxvirinae* and *Entomopoxvirinae*, which infect vertebrate and insect hosts, respectively. The *Chordopoxvirinae* are further divided into eight genera. Amongst these, the *Orthopoxviruses* are the most intensively studied genus. Orthopoxviruses include numerous human pathogens, the most notorious of which is variola virus, the causative agent of smallpox. Throughout history, smallpox infections are estimated to have killed several hundred million people[101], a staggering number by today's public health standards. Monkeypox virus, another orthopoxvirus, is an emerging zoonosis also capable of causing lethal disease in humans. Since the cessation of smallpox vaccination, the incidence of monkeypox virus infections in Africa has been increasing[193]. Although most cases of monkeypox infection are confined to Africa, the first monkeypox virus outbreak in North America occurred in 2003, with a total of fifty-three suspected human infections occuring[32]. Poxviruses can also cause serious disease in a variety of animals. For example, sheeppox virus can cause fatal disease in livestock and is a significant economic burden in some developing countries[8]. Given the serious public health threat associated

with poxvirus infection, and the increasing incidence of poxviral zoonoses like monkeypox, a greater understanding of the mechanisms by which poxviruses cause disease and evade immune detection is required. Such knowledge will aid in the development of improved vaccines and antivirals for the treatment of poxvirus infection. Insight into the interplay between poxviruses and the immune system will also be important for the ongoing development of poxvirus-based recombinant vaccine vectors for infectious diseases and cancer.

2 - Biology of the Poxviridae

The linear dsDNA genomes of poxviruses range from 130-300 kilobase pairs (kbp)[156]. The genome of vaccinia virus (VACV), the prototypic member of the *Orthopoxvirus* genera, is approximately 192 kbp, encoding for over 200 potential proteins[92]. All poxviruses examined to date share similarities in genome structure and organization. The highly conserved genes of poxviruses encoding proteins required for essential functions such as transcription, translation and viral morphogenesis tend to be located in the central region of the genome[233]. In contrast, the terminal regions of the genome generally encode the less conserved genes, many of which play a species-specific role in regulating intracellular signalling and evasion of host immune responses. These genes undergo a higher rate of diversifying selection than genes in the core region, reflecting the role of terminally located genes in mediating virus-host interactions[61]. Another feature of the terminal regions of poxvirus genomes is the presence of inverted terminal repeats which are adenine and thymine rich and facilitate viral DNA replication[156]. Given that poxviruses replicate in the cytoplasm, poxviral mRNAs do not have access to the host splicing machinery and do not contain introns.

Morphologically, poxviruses are some of the largest known viruses and feature barrel-shaped, enveloped virions (Fig. 1). The dimensions of the VACV virion are approximately 310 x 240 x 140 nm[48], and are visible by light microscopy. The membrane of the virion contains an array of viral proteins and host derived lipids with a unique composition of each present in the inner and outer layers of the membrane. Within the virion are found the viral core, as well as one to two lateral bodies oriented on opposing sides of the core. The core wall, similar to the virion membrane, is also composed of two layers of different width and houses the DNA genome. In total, 73 distinct viral proteins have thus far been identified in the virion and 47 of these proteins are found specifically in the virus core[48]. Besides playing a role in virion structure, many of these proteins are required for early events proceeding virus entry including regulation of early gene transcription and immune evasion[156].

2.1 - Poxvirus entry

The poxvirus infectious cycle begins with attachment of the virion to the host cell surface and entry of the virion into the cytoplasm of the cell (Fig. 2). Much of the understanding of poxvirus replication has come from studies using VACV, and although poxviruses are a very diverse group of viruses, the core mechanisms of virus replication are highly conserved[24]. VACV enters cells predominantly through a low-pH dependent endosomal pathway[229], although fusion with the plasma membrane also occurs[31]. More specifically, it has been reported that phosphatidylserine exposed on the outer surface of the virion stimulates macropinocytosis of the virus in a process that resembles the uptake of apoptotic bodies[143]. However, a cellular receptor responsible for binding to phosphatidylserine or any other molecule exposed on the surface of VACV virions to

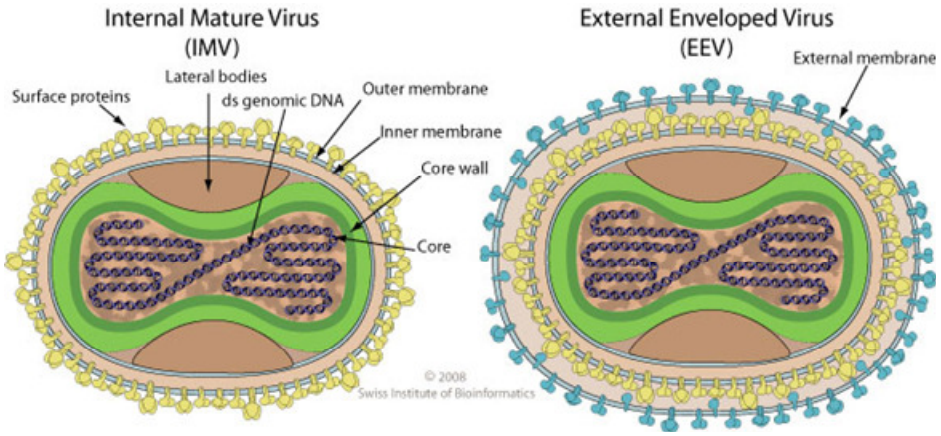

Figure 1: The virion structure of intracellular mature and extracellular enveloped vaccinia virus. Reproduced with permission from ViralZone, Swiss Institute of Bioinformatics.

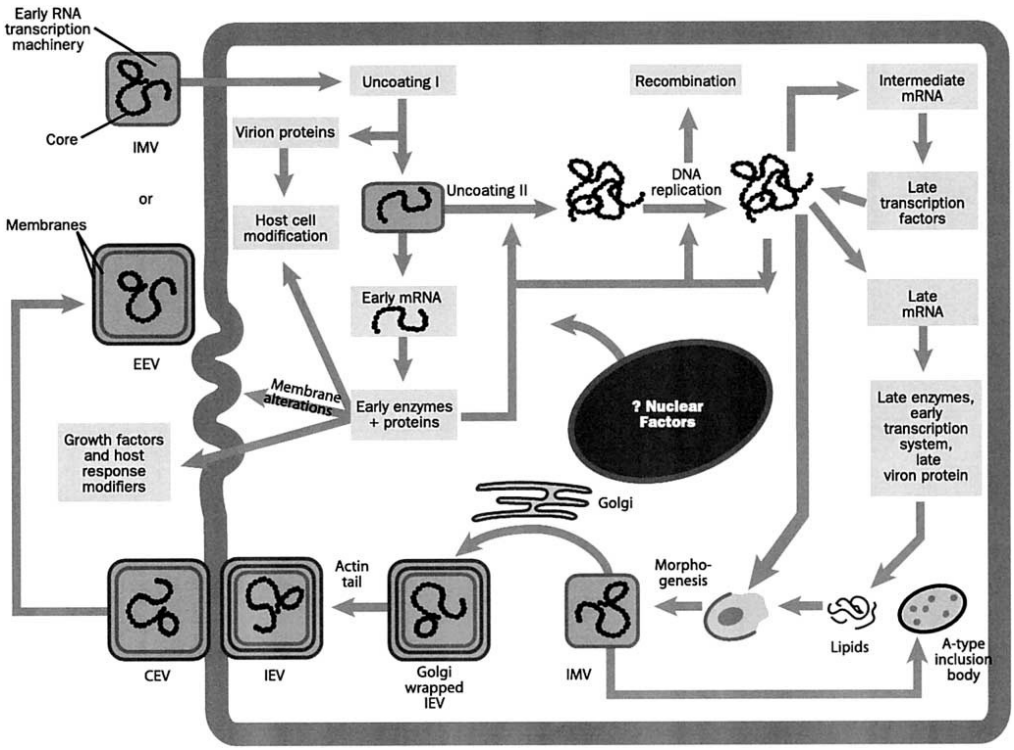

Figure 2: Entry, replication and morphogenesis of poxviruses. Bray, M., Buller, M. Looking back at smallpox. Clinical infectious diseases. 2004. 38:882–9. Reproduced with permission from Oxford University Press.

facilitate virus entry has yet to be identified. Numerous viral proteins mediate the entry and membrane fusion process[195] and assemble into a large complex localized within the virion membrane[214].

2.2 - Poxvirus gene expression and genome replication

Following entry, the virion core is transported along actin microtubules to sites of virus replication termed viral factories[156]. These factories become surrounded by extensions of the endoplasmic reticulum during virus replication, but are otherwise void of cellular organelles[228]. VACV gene expression proceeds through a cascade-like mechanism consisting of three steps; early, intermediate and late gene expression[24]. All the proteins required to initiate early VACV mRNA synthesis are housed within the core such that early viral transcription begins shortly after virus entry and before uncoating of the core[95]. Early mRNAs are extruded through the virion core into the cytoplasm of the cell and then translated[161]. Early mRNAs encode transcription factors required for intermediate gene expression, as well as modulators of host signal transduction which create a permissive intracellular environment for virus replication. This class of VACV genes accounts for approximately 50% of the total number of expressed transcripts[169]. Following early gene expression, the viral core uncoats, the viral genome is released into the cytoplasm and genome replication begins.

VACV DNA replication begins at about 2 hours post-infection (hpi)[230] with the formation of a nick near the end of the genome[186]. This generates a free 3′ end which serves as a primer for the viral DNA polymerase which, in complex with several other viral proteins, initiates DNA synthesis. Through a strand displacement mechanism the entire viral genome is replicated, with the formation of concatemeric genomes[156,159].

After resolution of the concatemers, complete, full-length viral genomes are produced. Transcription of intermediate and late class genes begins only after DNA replication has begun. This is believed to result from the inaccessibility of intermediate/late transcription factors to the viral DNA before DNA replication, however; DNA replication results in opening of the DNA structure to facilitate transcription[100]. Intermediate gene products include transcription factors required for late gene expression. Late gene products include proteins required for virus assembly as well as early gene transcription which will be packaged into progeny virions. Following synthesis, VACV mRNAs are capped at the 5′ end with a 7-methylguanosine cap by the VACV encoded D1[152] and D12[167] capping complex and are also polyadenylated[94] by virally encoded enzymes[157]. Given the step-wise production of transcription factors, each stage of poxvirus transcription is critically dependant on the preceding stage for the production of the necessary enzymes.

2.3 - Generation of double-stranded RNA during poxvirus transcription

Double-stranded RNA (dsRNA) produced during virus replication is a potent stimulator of innate immunity[21]. These dsRNAs are sensed as "danger" signals by host cells because, in general, dsRNA is largely absent in the cytoplasm of mammalian cells, although numerous dsRNAs, such as microRNAs, exist in the nucleus. In the case of poxviruses, dsRNA is generated primarily during convergent transcription of intermediate and late class genes (Fig. 3), with considerably less dsRNA generated during early gene transcription[19,46,131]. Transcription of early genes is efficiently terminated at UUUUUNU sequences approximately 20-50 base-pairs (bp) from the 3' end of the mRNA[250]. However, this termination sequence is not recognized in the mRNA sequence of intermediate and late genes, where it is sometimes found in the middle of these

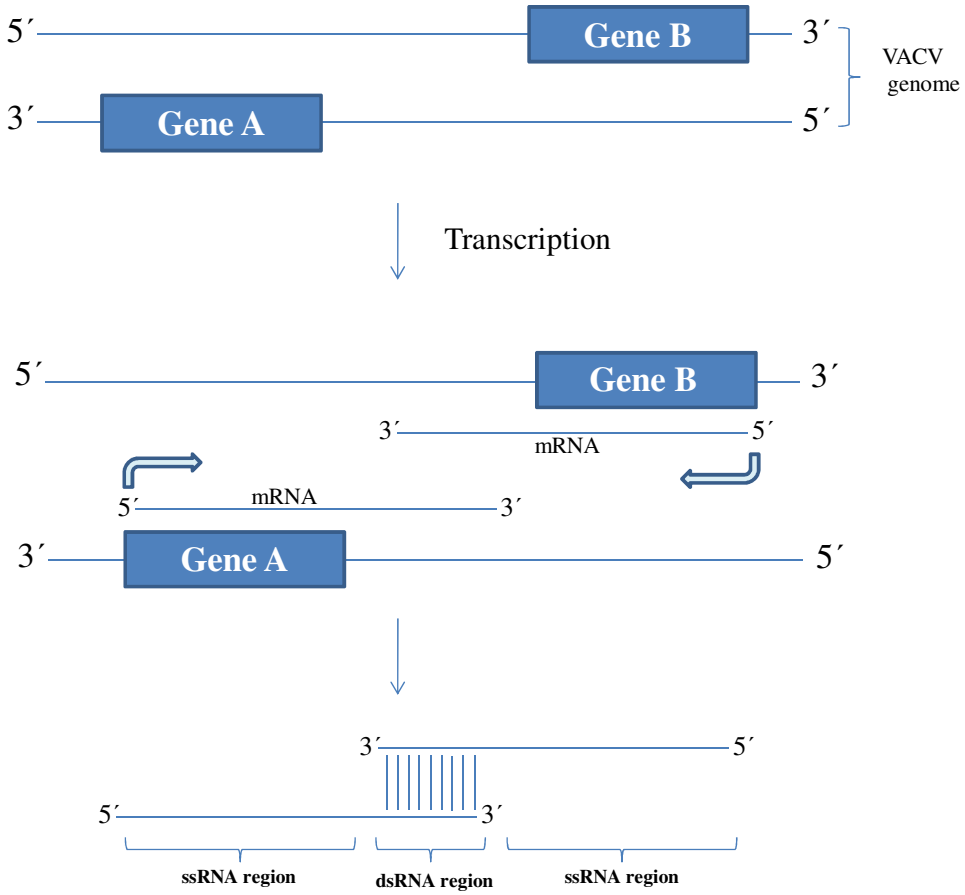

Figure 3: Model for the generation of dsRNA during VACV transcription. Gene A and B represent hypothetical intermediate and/or late VACV genes. Convergent transcription of each gene generates mRNA with complementary sequences at the 3′ end. These mRNAs can hybridize to produce an RNA molecule containing ssRNA and dsRNA regions.

transcripts. For this reason transcription of intermediate/late class genes tends to run into adjacent regions of the genome, generating mRNAs with heterogeneous 3' sequences. Therefore, when two intermediate/late genes in close proximity are transcribed in opposing directions from opposite strands of the genome, there is a potential to form two RNA transcripts harbouring complementary 3' regions which can anneal to form dsRNA. It is also possible that VACV mRNAs, through sequence self-complementarity, could form dsRNA secondary structures. Transcription of early genes also produces some dsRNA, although significantly less than intermediate/late gene expression because of the recognition of transcription stop signals in these genes. Although dsRNA species produced from intermediate and late gene transcription have been proposed to stimulate the innate immune system during poxvirus infection, direct evidence to support this claim is lacking.

2.4 - Poxvirus morphogenesis

Similar to poxvirus entry, the mechanisms and function of viral proteins in assembly of poxvirus particles is far from understood[195]. Generation of the mature virion proceeds through numerous steps which begin in the viral replication factory and occur simultaneously with intermediate and late gene expression. Early investigations into the assembly of VACV virions revealed that the first structures to form are crescent-shaped membranes[50]. As these structures grow in size, they begin to surround a region of dense material containing viral proteins, eventually forming fully enclosed spheres. The DNA genome is transported into the assembling membrane before it has completely closed[151]. Following proteolytic processing of proteins within the enclosed virus core, the virus takes on its characteristic barrel shape and is termed an intracellular mature virion

(IMV)[156]. IMVs contain a single membrane layer; however, intracellular enveloped virions (IEVs) are formed when the IMV obtains a second membrane derived from the Golgi apparatus[209]. IEVs which are released from the cell but remain in contact with the extracellular surface of the cell are termed cell-associated virions (CEV). While many CEVs remain attached to the cell surface, when a CEV is released from the cell surface it is called an extracellular enveloped virion (EEV). While all four forms of the VACV virion are infectious, EEVs are believed to mediate dissemination of virus within the host[195]. However, lysis of infected cells can also release IMVs which can contribute to cell-to-cell spread of the virus. Released virions then bind to and enter neighbouring cells to begin the replication cycle anew. It is likely that the precise mechanism(s) used by poxviruses to enter cells depends not only on the specific form of virion encountered by the cell (IMV versus EEV) but also on the virus strain and cell type[144,204].

3 - VACV as a model system to study poxvirus subversion of host antiviral defence

Despite the diverse nature of the members of the *Poxviridae*, most poxviruses employ conserved mechanisms for virus entry and replication[156]. VACV is the prototypic member of the orthopoxvirus genera and serves as the model virus for the study of orthopoxvirus biology. VACV was used as a live vaccine for the eradication of smallpox during the World Health Organization's smallpox eradication program in the 1970s[78]. A unique facet of VACV biology is that the virus represents one of, if not the most, successful vaccine agent ever utilized and yet the evolutionary origins of the virus and a natural host reservoir species remain to be identified[195]. The efficacy of VACV vaccination against smallpox stems from the fact that VACV shares greater than 90% genome sequence identity with variola virus[233], and many of the key antigenic proteins

are highly conserved. Thus, VACV often serves as a surrogate virus for the study of variola and other pathogenic poxviruses such as monkeypox virus which require high containment facilities.

The accidental or intentional release of smallpox and the increasing incidence of human monkeypox virus infections represent potentially serious public health threats. Currently, a large percentage of the global population is not vaccinated against smallpox. Furthermore, there are no licensed antivirals to treat human poxvirus infection[178]. Therefore, a key goal in poxvirology is to understand the mechanisms by which poxviruses antagonize host innate and adaptive immune responses since this knowledge is expected to aid in the design of more effective strategies to block poxvirus infection. Furthermore, there is a considerable effort underway to develop recombinant poxvirus based vaccine vectors and understanding poxvirus interactions with the immune system will be critical for optimizing vaccine efficacy[183]. Thus, analysis of VACV immune evasion strategies remains particularly relevant. Before delving into the specific mechanisms used by VACV to suppress innate immunity, the defence system employed by mammalian cells to recognize and combat virus infection will first be considered.

4 - Innate immune signalling in antiviral defence

The human innate immune system serves not only as the first-line of defence against invading pathogens, but also mediates both the direction and amplitude of adaptive immunity and is therefore critical for the overall response to infection[148,87]. The basis for activation of innate immunity involves the detection of conserved molecular structures associated with infection termed pathogen-associated molecular patterns (PAMPs) by host cell receptors termed pattern recognition receptors (PRRs)[21]. PAMPs

are present on the surface of invading pathogens or are generated during their replication. Examples of PAMPs include single-stranded RNA (ssRNA) and dsRNA species produced during viral infections as well as lipopolysaccharide and other cell wall components of bacteria. PAMPs become bound by PRRs located at the host cell surface, within the endosomal compartment or within the cytoplasm of infected cells. Three distinct groups of PRRs have been identified. These include the Toll-like receptors (TLRs), the retinoic acid inducible-gene I (RIG-I) -like receptors (RLRs) and the nucleotide oligomerization domain (NOD)-like receptors (NLRs)[148]. The TLRs and the RLRs primarily mediate the expression of Type I and Type III IFN[171], while NLRs regulate pro-inflammatory IL-1β expression via activation of the inflammasome. Several NLRs, including NOD2 and NOD5, can also regulate the induction of Type I IFN in response to infection[4,149,174]. Although cells typically contain a multitude of various cellular ssRNA and dsRNA species, they are either unable to stimulate PRRs due to structural modifications, or they do not have access to these receptors. Therefore, PRRs are generally not activated in uninfected cells and their interaction with PAMPs serves as a danger signal indicating infection. Following the interaction between a PAMP and its cognate receptor, intracellular signalling cascades are stimulated to activate innate defence systems. In this manner, the cell has evolved a mechanism to sense invasion by foreign pathogens and is able to mount an appropriate response to clear the infection. Conversely, viruses, which have co-evolved with their hosts as obligate intracellular parasites, must circumvent these responses in order to successfully replicate and have developed elaborate strategies to do so.

4.1 – Nucleic-acid pattern recognition receptors

Nucleic-acids are the major viral PAMPs[164]. The TLRs and RLRs are the two families of PRRs which detect nucleic-acid based PAMPs. The TLRs TLR3, TLR7, TLR8 and TLR9 are localized primarily to the inner face of the endosomal membrane, where they recognize distinct viral and bacterial DNA and RNA structures. While TLR3 binds to dsRNA, TLR7 and TLR8 detect ssRNA. In contrast, TLR9 recognizes unmethylated DNA harbouring CpG motifs, a characteristic feature of some viral and bacterial genomes. While the endosomal compartment is surveyed by the TLRs, the cytoplasm harbours the RLRs which consist of three members; RIG-I[249], melanoma differentiation-associated gene-5 (MDA5)[5] and laboratory of genetics and physiology 2 (LGP2)[249]. RIG-I and MDA5 bind viral nucleic acids in the cytoplasm of infected cells and activate signalling cascades aimed at inhibiting virus replication. In contrast, LGP2 plays a regulatory role in RIG-I and MDA5 dependent signalling.

4.2- RIG-I-like receptor signalling

Since the discovery of RIG-I and MDA5 as sensors of virus infection, considerable progress has been made towards understanding the mechanisms regulating their activity and selectivity towards unique RNA ligands. The molecular structure of the two proteins is similar, and consists of two tandem caspase-recruitment domains (CARDs) located at the N-terminus and a central DExD/H box RNA helicase and ATPase domain[164]. While both proteins function in a similar manner, the regulation of RIG-I activation has been studied in more detail than MDA5. The C-terminal domain of RIG-I is referred to as the repressor domain, since in resting cells this domain is proposed to mediate intramolecular interactions with the CARD domain which blocks RIG-I activity[201]. The C-terminal region of MDA5, however, lacks such a repressor function.

During virus infection, dsRNA may be present in the cytoplasm as a consequence of the dsRNA nature of the virus genome itself or as a by-product of virus replication. It is also clear that the genome of ssRNA viruses can form dsRNA secondary structures which can be recognized by RLRs[208]. Upon dsRNA binding to the C-terminal region of RIG-I[49], the physical interaction between the repressor domain and the CARD domain is relieved (Fig. 4). The protein then multimerizes, leading to its interaction with interferon-β promoter stimulator 1[99] (IPS-1, also known as MAVS[216], VISA[244] and Cardif[147]). IPS-1 is the central adaptor protein mediating both RIG-I and MDA5-dependant signal transduction and is localized to the outer surface of the mitochondrial membrane. Interaction of RIG-I and IPS-1 coordinates the formation of a signalling complex triggering downstream activation of the transcription factors nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 (IRF3)[96,98], as well as IRF7[180].

In resting cells, both NF-κB, and IRF3/7 are retained in an inactive state in the cytoplasm. However, different mechanisms are used to trigger activation of these transcription factors. The NF-κB family of transcription factors are sequestered in the cytoplasm through association with inhibitor of NF-κB (IκB) proteins[77]. IκB proteins are phosphorylated by the IκB kinase complex (IKK), a trimeric complex consisting of IKKα, IKKβ and NEMO (NF-κB essential modulator, also known as IKKγ). This phosphorylation event marks IκB for ubiquitination and proteasomal degradation, freeing NF-κB to translocate to the nucleus where it transactivates target genes. Similarly, IRF3 and IRF7 are maintained in an inactive state in the cytoplasm of unstimulated cells. Phosphorylation of IRF3 and IRF7 by two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKKε, results in IRF activation and nuclear translocation[217]. These phosphorylation events occur at serine residues in the C-terminus of IRF3[153,215] and

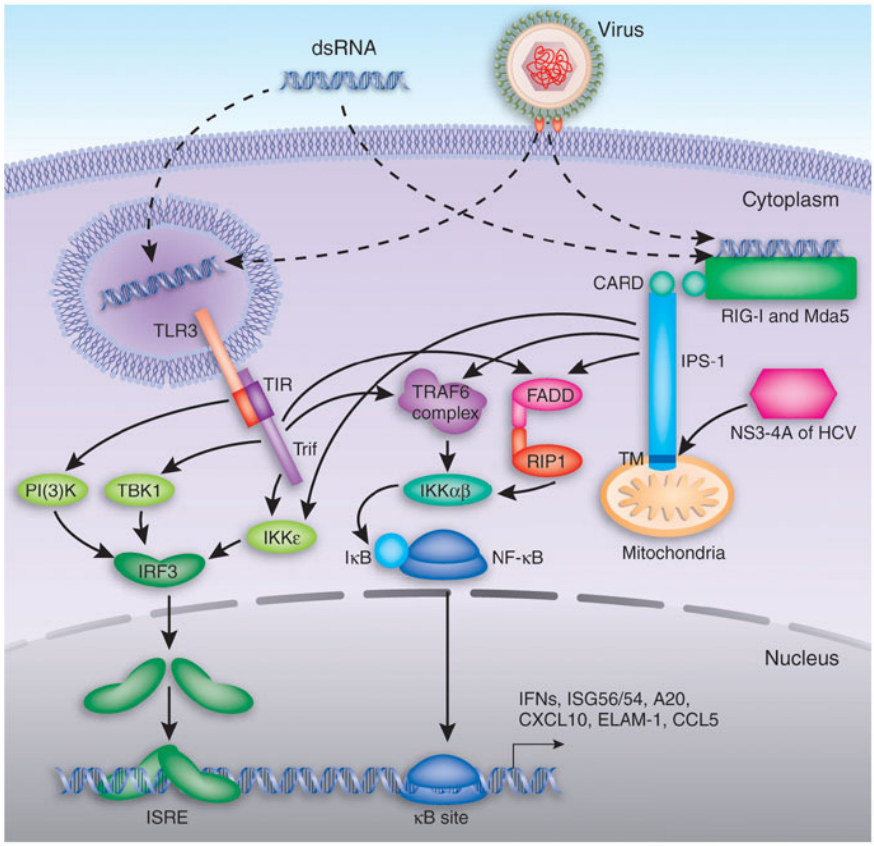

Figure 4: Recognition of virus infection by the RNA helicases RIG-I and MDA5. Sen, G., Sarkar, S. Hitching RIG to action. Nature Immunology. 2005. 6, 1074 - 1076. Reproduced with permission from Nature Publishing Group.

IRF7[180] and are believed to promote IRF activation either through dimerization of the protein, or by relieving auto-inhibitory interactions[80]. Following nuclear localization, IRF3 interacts with CREB binding protein (CBP)/p300, forming a complex which can then stimulate expression of target genes. The regulatory subunit of the IKK complex, NEMO, is essential for RIG-I mediated activation of both NF-κB and IRF pathways upstream of TBK1[256]. Therefore, NEMO represents the point at which signalling through the RLR pathway branches towards both NF-κB and the IRFs. Thus, the culminating event of RLR signalling is the activation of NF-κB and IRF3/7, leading to transcription of genes encoding various antiviral cytokines and chemokines. Given the potent pro-inflammatory and potentially harmful cellular effects of many cytokines and chemokines, RLR signalling is tightly regulated.

4.3- Regulation of RLR signalling

Numerous positive and negative regulators of RIG-I and MDA5 signalling have been discovered. Indeed, the third member of the RLR family, LGP2, modulates RLR signalling. Similar to RIG-I and MDA5, LGP2 contains a DExD/H box RNA helicase and ATPase domain, but lacks the CARD domains and is therefore unable to transduce downstream activating signals to IPS-1[150]. Experiments in cell culture initially demonstrated that LGP2 inhibits virus-induced IRF3[248] and NF-κB[199] activation and suggested that LGP2 may function through sequestering dsRNA ligands away from RIG-I and/or MDA5. An interaction between IPS-1 and LGP2 has also been proposed to explain the inhibitory activity of LGP2 in RLR signaling[106]. Overall, these findings suggested that LGP2 was a negative regulator of RLR signalling. However, two recent *in vivo* studies with LGP2 deficient mice demonstrated a positive role for this RNA helicase

in the antiviral response against EMCV infection[205,235]. These reports describe contradictory findings in regards to the role of LGP2 in the response to synthetic RNA agonists and RNA viruses other than EMCV. A positive role for LGP2 in the *in vivo* cytokine response to *Listeria monocytogenes* and VACV has also been described[187]. Most of the cell culture experiments described for LGP2 involve over-expression of the protein which likely does not reflect the physiological conditions under which LGP2 functions. *In vivo* experiments with knockout mice are likely more biologically relevant to LGP2 biology, and suggest that overall, LGP2 is a positive regulator of the RLR pathway. It has been suggested that LGP2 may function through modulating RNA structure or localization to facilitate RLR signalling[205]. However, further experiments are necessary to clarify the physiological role of LGP2 in RLR signalling.

Ubiquitination has emerged as a key post-translational modification regulating RLR signalling. The A20 protein is a negative regulator of RIG-I signalling and blocks RIG-I dependent activation of IRF3 and NF-κB[126]. This inhibitory activity requires a zinc finger motif present in the ubiquitin ligase domain of A20. It was not demonstrated whether or not A20 can ubiquitinate RIG-I, but it was shown that A20 does not affect RIG-I protein levels, suggesting that A20 may target an adaptor of RLR signalling. Direct ubiquitination of RIG-I has been demonstrated in other studies. For example, negative regulation of RLR signalling occurs through ring finger protein 125 (RNF125) mediated ubiquitination and proteasomal degradation of both RIG-I and MDA5 [6]. Conversely, RIG-I can also be activated through ubiquitination. The second CARD of RIG-I undergoes tripartite motif containing 25 protein (TRIM25) dependent, lysine-63 linked ubiquitination, an event required for RIG-I signalling activity[67]. Another ubiquitin ligase, Riplet, also functions in the activation of RIG-I signalling and is essential for the

in vivo antiviral response to RNA virus infection[172]. MDA5 however, is not targeted by this mechanism, but its activity is instead enhanced following SUMOylation by protein inhibitor of activated stat 2 beta (PIAS2β)[65]. Interestingly, the CARD domain of RIG-I can bind directly to unanchored polyubiquitin chains and such binding activates RIG-I, suggesting that dsRNA and ubiquitin binding may cooperatively promote RIG-I signalling[253]. Thus, ubiquitin regulates the RIG-I pathway in both a positive and negative manner. The numerous proteins regulating the activity of RIG-I and MDA5 underscores the need to tightly control activation of innate immunity to balance the antiviral response with unnecessary inflammation and cell death mediated by immune activation.

4.4 - Recognition of virus infection and specific RNA ligands by RIG-I and MDA5

To date, the role of RIG-I and MDA5 in innate immune responses has largely focused on recognition of RNA virus infection. RIG-I and MDA5 recognize different RNA structures and therefore differentially respond to RNA virus infection. Initially, it was shown that RIG-I recognizes *in vitro* transcribed RNA, while MDA5 recognizes polyinosinic: polycytidylic acid (pIC)[98]. Furthermore, Newcastle disease virus (NDV)-, vesicular stomatitis virus (VSV)-, Sendai virus (SeV)- and influenza virus-induced IFN-β expression was found to require RIG-I, while MDA5 mediated the induction of IFN-β in response to encephalomyocarditis virus (EMCV). Subsequent to this, a wide range of both negative and positive-sense RNA viruses were found to be specifically recognized by RIG-I, whereas MDA5 only appears to recognize positive-sense RNA viruses[129]. Both RIG-I and MDA5 contribute to IFN-β expression following infection of cells with reovirus[128], Japanese encephalitis virus[98], West Nile virus (WNV)[64], and Dengue virus[128], suggesting that these viruses produce RNA agonists for RIG-I and MDA5. Studies

utilizing knockout mice have also demonstrated the essential role of RIG-I and MDA5 in the protective immune response to RNA viruses *in vivo*[96,98]. Loss of IPS-1, the central adaptor of RLR signalling, also increases the susceptibility of mice to RNA virus infection[111].

The initial finding that RIG-I and MDA5 specifically respond to distinct viruses suggested that these PRRs recognize unique and generally non-overlapping RNA ligands. The identification of the molecular features of RNA which contribute to this specificity has been and continues to be an intense area of research[164]. Similar to the structure-function studies investigating regulation of RLR signalling, studies in this field have mainly focused on RIG-I. Initially, the presence of a triphosphate moiety at the 5' end of ssRNA and dsRNAs generated by *in vitro* transcription was reported to be a structural signature recognized by RIG-I, but not MDA5[82]. Similarly, the influenza virus ssRNA genome is recognized by RIG-I in a 5'-triphosphate-dependent manner[181]. These results suggested that 5'-triphosphate ssRNAs, in addition to dsRNAs, are a ligand for RIG-I. However, more detailed experiments demonstrated that the *in vitro* transcription reactions used in these types of studies generate unintended by-product RNA species which can form dsRNA hairpin secondary structures[207], providing evidence that RIG-I ligands are actually blunt-ended dsRNAs bearing a 5'-triphosphate group[208].

The overall length of RNA also affects the specificity of recognition by RIG-I and MDA5. Originally, pIC was classified as an MDA5 ligand, however, shortening the length of pIC (normally 4-8 kbp) through RNase digestion to about 300 bp generates pIC which is a ligand for RIG-I and not MDA5[97]. Shorter *in vitro* transcribed dsRNAs also preferentially activate RIG-I, while dsRNAs of 3-4 kbp activate MDA5. An elegant experiment in which the small, medium and large segments of reovirus genomic RNA

were individually isolated and transfected into cells also demonstrated that RIG-I is preferentially activated by shorter dsRNAs and MDA5 by longer dsRNAs. Although the 5'-triphosphate group is required for shorter dsRNA (<100 bp) to activate RIG-I, longer dsRNAs do not require a 5'-triphosphate to activate RIG-I[16]. On such longer dsRNA, RIG-I is found bound along the entire length of the RNA molecule, and the presence of a greater number of RIG-I binding sites on longer dsRNA may obviate the requirement for a 5'-triphosphate. Therefore, RIG-I ligands are currently best defined as short dsRNAs bearing 5'-triphosphates and longer dsRNAs (<1000 bp) which do not require a 5'-triphosphate to activate RIG-I. Although much less work has been done on MDA5, MDA5 ligands include longer dsRNAs (>1000bp) which do not require a 5'-triphosphate group for their stimulatory activity.

Thus, the presence of a 5'-triphosphate moiety, RNA length and overall dsRNA content contribute to the selectivity of RIG-I and MDA5 towards unique RNA ligands. The addition of a 7-methylguanosine cap to 5'-triphosphate dsRNA abolishes its ability to stimulate RIG-I activation[82]. The cytoplasm of mammalian cells is largely devoid of dsRNA molecules and the 5' triphosphate group present on cellular mRNA is hidden by capping. Thus, the detection of cytoplasmic dsRNA species (with or without 5'-triphosphates) by RIG-I and MDA5 provides the cell with a means of discriminating between self and non-self RNA as an indicator of virus infection. This recognition process results in the activation of antiviral defence mechanisms to block virus replication and/or to destroy virus infected cells. Viruses, in turn, have evolved mechanisms to negatively regulate the RLR pathway to avoid the antiviral outcome of RIG-I and MDA5 signalling.

4.5 - Suppression of RIG-I and MDA5 dependant signalling by viruses

Given the pivotal role of RIG-I and MDA5 in the innate response to virus infection, it is not surprising that many viruses have evolved means to inhibit RLR signalling. One of the first viral proteins demonstrated to specifically target the RLR pathway was the NS3/4A protease of hepatitis C virus (HCV). NS3/4A cleaves IPS-1, inactivating signalling downstream of RIG-I and MDA5 and thereby blocking interferon beta (IFN-β) production in HCV infected cells[147]. The importance of IPS-1 as an adaptor for both RIG-I and MDA5 dependant signalling is highlighted by the fact that many other viruses, including hepatitis B virus[112,240] and influenza virus[75], also encode suppressors of IPS-1 activity. The NS1 protein of influenza virus interacts with TRIM25 to inhibit RIG-I-dependent IFN production[66]. In contrast, MDA5, but not RIG-I, is inhibited through interactions with the V proteins of paramyxoviruses[5,40]. Many viruses also encode dsRNA binding proteins which are believed to non-specifically suppress RLR function through sequestering dsRNA ligands. An example of such a dsRNA binding protein demonstrated to inhibit RLR signalling is the VP35 protein of Ebola virus[29]. The crystal structure of the VP35 IFN inhibitory domain in complex with dsRNA elegantly demonstrates that VP35 not only binds dsRNA, but also interacts with dsRNA in such a way as to form a cap structure over the RNA termini, thereby blocking access of the 5'-triphosphate group to RIG-I[122]. In addition to these factors which directly target RIG-I, MDA5 and IPS-1, numerous viral proteins target downstream signalling components of the RLR pathway, including the IKK complex, NF-κB and IRFs to ultimately block IFN production[236]. That most, if not all, viruses have evolved mechanisms to suppress the expression of IFNs underscores the importance of this group of cytokines in the antiviral response.

4.6 - The role of interferons in antiviral defence

The IFNs represent one of the most important families of genes whose expression is induced following RLR pathway activation[21]. IFNs are small, secreted cytokines that mediate a vast range of biological functions including antiviral and antitumor activities. IFNs are classified into three different groups; Type I, Type II and Type III IFNs[227]. RLR signalling primarily activates the expression of Type I IFNs, which in humans include IFN-β and twelve functional subtypes of IFN-α, as well as Type III IFNs[171]. Collectively these IFNs play a pivotal role in the innate immune response to virus infection.

Triggering of RLR or TLR signalling following virus infection results in the expression and secretion of IFN-β, which binds to the Type I IFN receptor on the surface of neighbouring cells (Fig. 5). The Type I IFN receptor is a heterodimer composed of the IFN-α/β receptor 1[168,234] (IFNAR1) and IFN-α/β receptor 2 (IFNAR2) proteins. IFNAR1 and IFNAR2 are associated with the Janus-activated kinases (Jaks) Tyk2 and Jak1, respectively[185]. Binding of IFN-β stimulates autophosphorylation of Tyk2 and Jak1, which then phosphorylate members of the signal-transducer and activator of transcription (Stat) family of proteins. Although various Stat containing complexes can form, a phosphorylated heterodimer of Stat1 and Stat2 is among the most well characterized. The Stat1-Stat2 heterodimer interacts with IRF9 to form IFN-stimulated gene factor 3 (ISGF3)[206]. This complex translocates to the nucleus and binds to IFN-stimulated response elements[191] (ISRE) present in the promoter region of IFN stimulated genes (ISGs). This process activates the expression of hundreds of ISGs, many of which have direct antiviral activity. By this means, IFN-induced ISG expression generates an antiviral state within the cell which can block virus replication and/or destroy virus

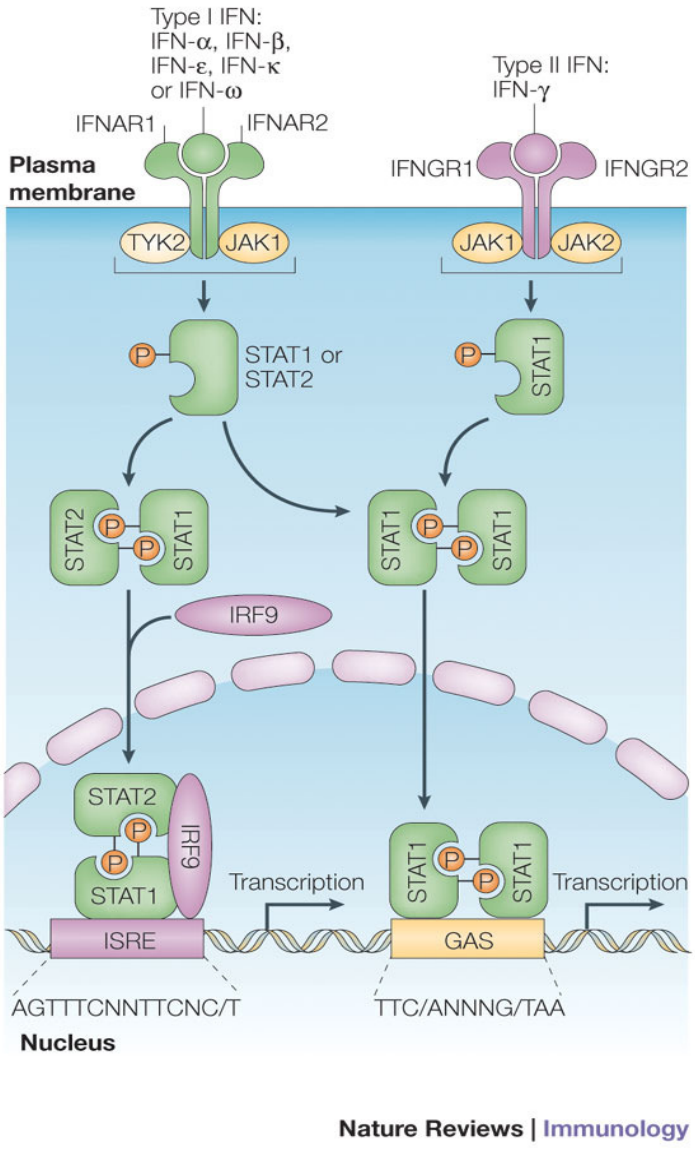

Figure 5: Interferon receptor signalling. Platanius, L. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature Reviews Immunology. 2005. ***5***, 375-386. Reproduced with permission from Nature Publishing Group.

infected cells. Although functional studies of most ISGs have yet to be performed, PKR represents an ISG for which a considerable amount of research has been undertaken.

5 - The double-stranded RNA activated kinase in innate immunity

In addition to RIG-I and MDA5, double-stranded RNA activated kinase (PKR) represents another cytosolic dsRNA sensor capable of activating antiviral responses. Although PKR expression can be induced by IFN and it is therefore classified as an ISG, its basal expression level in many cell types is high enough to facilitate a functional antiviral response. Among the multitude of antiviral functions ascribed to PKR, the control of host cell translation, transcription and apoptosis have been well characterized[69,184] (Fig. 6). PKR has been implicated in controlling the replication of a wide range of DNA and RNA viruses including VACV[254], EMVC[145], HCV[37,93], and WNV[203] among others.

PKR is composed of two tandem, N-terminal dsRNA binding motifs and a C-terminal serine (Ser)/threonine (Thr) kinase domain[166]. There remains some debate about the precise mechanism by which PKR becomes activated following dsRNA binding[47]. However, in unstimulated cells, PKR exists primarily as a monomer[121] in an extended conformation[139]. DsRNA binding serves to promote dimerization of PKR, which is critical for activation of the kinase activity of the enzyme[121]. PKR dimers then phosphorylate other PKR dimers or monomers leading to full activation of the enzyme. The Thr-446 and Thr-451 residues of PKR are critical phosphorylation sites[197]. Thus, the available evidence best supports a mechanism whereby dsRNA binding to PKR promotes a conformational change in PKR which favours dimerization of the protein. This process

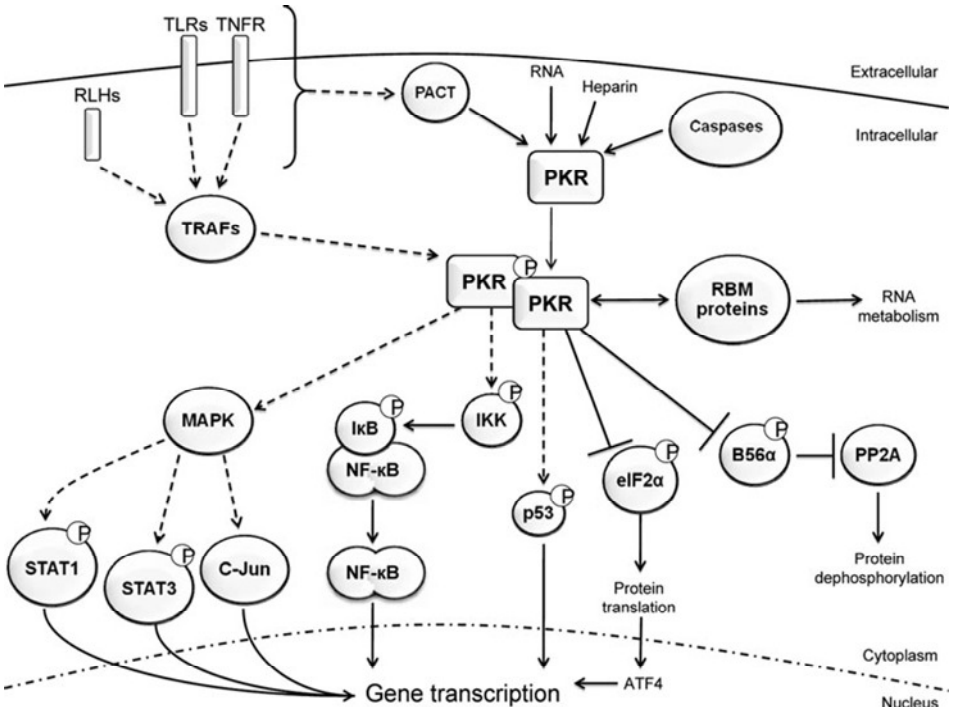

Figure 6: Modulation of intracellular signalling by PKR. Pindel, A., Sadler, A. The role of Protein Kinase R in the interferon response. Journal of interferon and cytokine research. 2011. 31:59-70. Reproduced with permission from Mary Ann Liebert, Inc.

activates the kinase activity of PKR, and subsequent autophosphorylation of PKR results in full activation of the enzyme.

One of the most well characterized substrates of PKR is the alpha subunit of eukaryotic initiation factor 2 (eIF2α). EIF2 is a protein translation initiation factor responsible for binding the initiator methionine-tRNA (Met-tRNA) and guanosine triphosphate (GTP) and complexing with the 40S ribosomal subunit and the mRNA to be translated[44]. Translational initiation at the AUG start codon requires hydrolysis of eIF2-bound GTP to guanosine diphosphate (GDP). Subsequent rounds of initiation require the GDP to be exchanged for GTP, a reaction catalyzed by eIF2B. Phosphorylation of the Ser-51 residue of eIF2α stabilizes its interaction with eIF2B[200]. As a result, eIF2 becomes locked in an inactive, GDP-bound state and global translation within the cell becomes impaired. The crystal structure of the PKR kinase domain: eIF2α interaction has been solved[51]. It consists of a dimer of PKR kinase domains, with each individual kinase domain interacting with one molecule of eIF2α, wherein the Ser-51 residue of eIF2α is exposed to the PKR catalytic site. Based on this structure, it has been proposed that PKR monomers in a PKR dimer do not auto-phosphorylate each other since the kinase domains face in opposing directions. Rather, auto-phosphorylation occurs via the action of one PKR dimer on another dimer or PKR monomer. The consequence of PKR-mediated eIF2α phosphorylation is the global inhibition of protein translation, thereby blocking virus replication.

5.1 - PKR regulation of cell signalling pathways

Cytokines are pivotal regulators of both innate and adaptive immune responses. Besides phosphorylating eIF2α, PKR also regulates numerous signalling pathways

mediating the expression of cytokines and chemokines. In some cases, these processes require PKR kinase activity, whereas in others it appears PKR plays a purely structural role[69]. In human and mouse cells, PKR regulates cytokine expression following virus infection or treatment of cells with pIC [33,74,146,222]. Although the precise mechanism by which PKR contributes to induction of cytokine expression is unknown in many cases, PKR can activate several transcription factors. For example, PKR has been linked to activation of NF-κB, which is a central mediator of cytokine and chemokine expression. Stimulation of NF-κB by PKR involves PKR-mediated activation of the IKK complex[18,86]. PKR also activates p38, a member of the mitogen-activated protein kinase (MAPK) family, which can regulate IFN signalling and the cytokine response to adenovirus (AdV) infection[123,146,188]. PKR-mediated p38 activation occurs through an interaction between PKR and MAPK kinase 6 (MKK6)[222]. This interaction results in MKK6 phosphorylation and downstream activation of p38. In addition to its role in the transcriptional control of cytokine gene expression, it also appears that PKR can post-transcriptionally regulate the stability of select mRNAs. For example, it was recently reported that PKR controls the stability of IFN-β mRNA following EMCV infection[210]. In the absence of PKR, IFN- β protein levels are reduced *in vitro* and *in vivo* in response to EMCV and therefore, PKR plays a protective role in host defence against EMCV infection by sustaining IFN- β production. A few recent reports have also implicated PKR in RLR signalling. For example, both PKR and MDA5 are required for IFN-β expression in response to infection with Sindbis virus harbouring a point mutation in the nsP2 protein[27]. However, it remains unclear as to how PKR contributes to RLR signalling. In addition to regulating cytokine responses, PKR also mediates apoptosis as part of antiviral defence.

Apoptosis is a common mechanism employed to destroy virus-infected cells and thereby prevent virus spread[68]. Apoptosis is mediated by a family of cysteine proteases termed caspases which sequentially cleave target proteins which are often also caspases. These cleavage events result in the condensation of the chromatin structure, fragmentation of genomic DNA and death of the cell. Apoptotic responses can be activated by both extrinsic and intrinsic mechanisms. Extrinsic mechanisms are initiated when death receptors (such as the TNF-α receptor) dimerize at the cell surface, resulting in the proteolytic activation of caspase 8[226]. Caspase 8 then cleaves the executioner caspases-3 and -7. The intrinsic apoptotic pathway is initiated following activation of B-cell lymphoma 2 (BCL-2) homology 3 (BH3)-only proteins which induce permeabilization of the mitochondrial outer membrane. This results in the release of pro-apoptotic proteins, such as cytochrome *c*, which then facilitate the formation of the apoptosome and activation of caspase-3 and -7. Collectively, caspase -3 and caspase -7 activation can stimulate nuclease activity to degrade genomic DNA. Furthermore, permeabilization of the mitochondrial membrane impairs mitochondrial functions. As a result of these actions, the cell dies and in the context of infection, the virus is unable to replicate.

PKR is a central regulator of several pro-apoptotic pathways[72]. For example, both dsRNA and tumour necrosis factor-alpha (TNF-α)-induced activation of apoptosis have been linked to PKR[56,247]. The mechanism by which PKR contributes to pro-apoptotic signalling is only partially understood. Phosphorylation of the Ser-51 residue of eIF2α by PKR has been reported to play a role in dsRNA and TNF-α-induced cell death[71,225]. Therefore, at least in some systems, the catalytic activity of PKR is required to stimulate apoptosis. Furthermore, PKR-dependent apoptosis may also be mediated by activation of

NF-κB, which is known to mediate pro- and anti-apoptotic signalling in response to different stimuli. PKR also stimulates apoptosis in response to virus infection. For example, the apoptotic response to EMCV[246], influenza virus[9], and VACV[120,254] is dependent on PKR. Thus host cell translation, transcription and apoptosis can be modulated by PKR following dsRNA-induced activation.

5.2- Ligand specificity of PKR

PKR binds specifically to dsRNA[15], and not ssRNA or dsDNA, and many details regarding the specificity of this interaction are now understood[166]. PKR can bind to dsRNAs as short as 16 bp, however, these short dsRNAs cannot activate PKR. Instead, PKR activation requires a minimum of approximately 34 bp of dsRNA, although more efficient activation and binding occurs with dsRNAs ranging from 55 bp to 2 kbp[136]. These findings support a model whereby PKR activation requires dsRNA of sufficient length such that a single dsRNA molecule can bridge the first and second dsRNA binding motifs of each monomer in a PKR dimer[166]. Thus, short dsRNAs at high concentration inhibit PKR because they only bind to the first PKR dsRNA binding motif and cannot bridge both. Furthermore, these short dsRNAs are too small to accommodate a PKR dimer. While dsRNA length is an important factor for recognition by PKR, this process occurs in a sequence-independent manner[136,166].

SsRNAs which can form dsRNA hairpin secondary structures can also activate PKR[257]. This activation is dependent on the presence of ssRNA tails of the hairpin RNA and also on the length of such tails. Similar to the ligand specificity of RIG-I, activation of PKR by these short RNAs also requires a 5'-triphosphate moiety, whereas activation by longer dsRNAs does not[165]. Besides activation by viral dsRNAs and synthetic dsRNA

molecules, PKR can also be activated by cellular mRNAs as part of translational feedback mechanisms. For example, the IFN-γ mRNA activates PKR in a manner dependent on a double-stranded pseudoknot region located in the 5' untranslated region (UTR) of the IFN-γ mRNA[13]. The pseudoknot region is approximately 33 bp long and therefore of sufficient length to activate PKR[45]. This mechanism is proposed to modulate IFN-γ mRNA levels in the context of viral infection. Lastly, PKR can also be activated by cellular proteins including the PKR-activating protein (PACT)[179]. PACT can activate PKR in absence of dsRNA through protein-protein interactions between the dsRNA binding domains of each protein.

5.3- Inhibition of PKR by viruses

Viruses have evolved many unique mechanisms to block PKR activity, including the expression of RNA and protein inhibitors[114]. Viral inhibitors of PKR can be broadly categorized based on their proposed mechanism of inhibition (Table 1). Viruses exploit the length-dependent activation (or inhibition) of PKR. The AdV virus associated (VA) RNA, VAI[105] and the Epstein-Barr virus encoded RNAs EBER1 and EBER2[43] are ssRNAs which form stem-loop secondary structures and inhibit PKR activity. The inhibitory properties of the VAI RNA are believed to result from its unique tertiary structure which only accommodates binding of PKR monomers, thereby inhibiting dimer formation, the active conformation of PKR[114,118,140].

Viral protein inhibitors of PKR are proposed to function through sequestering dsRNA or via direct inhibitory interactions with PKR. However, for many of these proteins, the details regarding the specific means by which they inhibit PKR remains to be clarified. For example, many dsRNA binding proteins can inhibit PKR, including the

Table 1: Inhibition of PKR by viral proteins and RNAs

Regulation of PKR by viral products

Mechanism	Virus	Gene product
I. dsRNA binding proteins	Vaccinia vims	E3L
	Reovirus	σ3
	Influenza virus	NS1
	Rotavirus group C	NSP3
	Rotavirus group A	NSP5?
	Herpes simplex virus	Us11
	Epstein-Barr virus	SM
II. RNA inhibitors	Epstein-Barr virus	EBER RNA
	Adenovirus	VAI RNA, VAII RNA?
	Hepatitis C virus	IRES
III. PKR interaction	Hepatitis C virus	NS5A, E2
	Influenza virus	p58
	Vaccinia virus	E3L
	Baculovirus	PK2
	Herpes simplex virus-1	Us11
	Epstein-Barr virus	SM
	Human herpes virus-8	vIRF-2
IV. Competitive inhibitor	Vaccinia virus	K3L
	Ambystoma tigrinum virus	ReIF2H
	HIV	Tat
V. PKR degradation	Polio virus	Protease
VI. eIF2α phosphatase	Herpes virus	γ34.5
	Papilloma virus	E6 (GADD34/PP1α)
	SV-40	Large-T antigen

Langland, J., Cameron, J., Heck, M., Jancovich, J., Jacobs, B. Inhibition of PKR by RNA and DNA viruses. Virus Research. 2006. 199:100-110. Reproduced with permission from Elsevier.

reovirus sigma 3 protein[84], Ebola virus VP35[211] and influenza virus NS1[130]. These proteins are believed to inhibit PKR activation in part by sequestering dsRNA. However, it is difficult to specifically analyze the role of dsRNA binding since dsRNA binding domains often mediate protein-protein interactions in addition to simply binding dsRNA. For this reason, whether proteins harbouring dsRNA binding domains inhibit PKR primarily through simply sequestering dsRNA, by protein-protein interactions, or a combination of the two functions remains to be determined.

Molecular mimicry is another mechanism employed by viruses to inhibit PKR. VACV expresses the K3 protein which is structurally similar to eIF2α[12,30] and acts as a pseudo-substrate of PKR, thereby sequestering PKR during infection and preventing the antiviral outcome of the PKR- eIF2α interaction. However, the role of K3 is unclear given that it does not seem to be able to inhibit PKR function in the absence of VACV E3, one of the most well characterized inhibitors of PKR (discussed in detail in the following sections). This mimicry mechanism is also employed by *Ambystoma tigrinum* virus, a dsDNA virus of the Ranavirus family. The 57R ORF expressed by this virus is homologous to eIF2α and prevents virus-induced eIF2α phosphorylation[89].

6 - Pattern recognition receptor activation by DNA viruses

The cellular antiviral response to RNA virus infection has received comparably more attention than the response to DNA virus infection. Nonetheless, mammalian cells also mount effective innate immune responses to DNA viruses through the recognition of PAMPs[189]. Many TLRs respond to DNA virus infection. For example, at the extracellular surface, TLR2, which can recognize glycoproteins and lipoproteins, plays an important role in the antiviral response to cytomegalovirus infection through the

recognition of viral envelope proteins during virus entry[17]. Intracellular PRRs have been implicated in sensing DNA viruses, including TLR3, TLR7, TLR8 and TLR9. In addition to the TLRs, specific DNA-sensing PRRs have also recently been identified, including absent in melanoma 2 (AIM2)[81]. However, the physiological relevance of these PRRs in antiviral responses has yet to be confirmed with more rigorous experimentation *in vivo*[189]. There have been very few reports describing a role for either RIG-I or MDA5 in sensing DNA virus infection, but it has been shown that infection of macrophages with herpes simplex virus-type 1 (HSV1) induces IFN-β expression dependent on MDA5, but not RIG-I[142]. Moreover, one of the more interesting mechanisms employed to recognize DNA virus infection involves RNA polymerase III and the RLR pathway. Synthetic DNAs, as well as the EBV RNAs EBER-1 and EBER-2, are transcribed by RNA polymerase III into 5'-triphosphate bearing RNA ligands activating RIG-I and Type I IFN expression[1]. The RNA polymerase III/RIG-I pathway also appears to mediate IFN expression following infection of cells with other DNA viruses, including AdV and HSV1[41]. Thus, RNA polymerase III is proposed to facilitate the recognition of cytosolic DNA by reverse transcribing DNA genomes or DNA fragments into RNA ligands activating RIG-I. Although the innate immune response to VACV has been studied, few details have been reported on the nature of VACV PAMPs and the identity of their cognate PRRs.

7 - Inhibition of innate immune responses by VACV

A characteristic feature of VACV, and other poxviruses infecting vertebrate animals, is that they encode a plethora of proteins that suppress cellular innate and adaptive immunity[158]. IFNs and pro-inflammatory cytokines such as TNF-α play a

pivotal role not only in the initial generation of an antiviral state during innate immunity, but also in directing adaptive immunity. VACV expresses secreted homologues of the IFN-α/β (B18) and IFN-γ (B8) receptors which function to bind secreted IFNs, thereby preventing receptor binding and downstream antiviral signalling[158]. VACV also expresses a secreted receptor for IL-1β and some strains of VACV also express A53, a secreted receptor for TNF-α[3]. The evolution of such a diverse array of decoy receptors by poxviruses underscores the key role of antiviral cytokines and chemokines in the defence against poxvirus infection.

Many VACV proteins have been found to redundantly target the same antiviral signalling pathway. For example, in addition to inhibiting binding of the IFNs to their cellular receptors via the expression of decoy receptors, VACV also expresses a phosphatase, H1, which dephosphorylates Stat1[163], a key protein mediating the effects downstream of IFN receptor binding. Another signalling pathway targeted by several VACV proteins is the NF-κB pathway. The A46 and A52 proteins of VACV function to suppress TLR signalling[20], which if activated, stimulates NF-κB activity and the expression of genes with antiviral function. NF-κB signalling is targeted by a different mechanism by VACV K1, which blocks degradation of IκB proteins, preventing the nuclear translocation of NF-κB and its ability to stimulate gene transcription[219]. VACV B14 also targets NF-κB by interacting with IKKβ[39]. This interaction prevents phosphorylation of IKKβ and IκB, thereby blocking NF-κB activity.

The complement system is another innate immune mechanism used by host cells to neutralize invading viruses or destroy infected cells[63]. The complement system can be activated by the binding of the C1 complex either directly to virus virions, or to antibody-antigen complexes. This process activates C1, resulting in the cleavage of another

complement protein, C4, into C4b and C4a. C4b can then assemble as part of a C3 convertase complex which can form the membrane-attack complex to destroy both virions and infected cells. Similar to the secreted viral homologues of the IFN and TNF-α receptors, VACV expresses a secreted protein termed VACV complement control protein (VCP) which suppresses the complement system by binding to C4b and destabilizing the C3 convertase[109,141]. The activity of VCP is sufficient to prevent neutralization of VACV IMVs by the complement system[85]. Mutants lacking VCP are attenuated *in vivo*, demonstrating the important role of this protein during VACV infection in animals.

7.1 - The E3 protein of VACV

The E3L gene of VACV encodes a 25 kilodalton, dsRNA binding protein expressed during the early phase of virus replication[242]. The E3 protein is composed of a carboxy-terminal dsRNA binding domain and an amino-terminal Z-DNA binding domain. The earliest functional studies of E3 demonstrated that it was an inhibitor of PKR activity[36]. Since this initial characterization, the functions of E3 have expanded and revealed that this protein plays a pivotal role in regulating host antiviral defence to facilitate a productive infection. While wild-type VACV has a broad cellular tropism, VACV mutants lacking E3L (vvΔE3L) have a restricted replication phenotype. For example, in contrast to the wild-type virus, vvΔE3L cannot replicate in human HeLa cells, Vero cells or murine L929 cells[11,35]. In contrast, E3 is not required for virus replication in BHK21 or RK13 cells. The replication of E3 deficient virus aborts at the stage of intermediate protein translation[131]. While the dsRNA binding domain is required for replication in cell culture, the Z-DNA binding domain is dispensible[35].

Another defining characteristic of VACV is that it is highly resistant to the antiviral effects of IFNs[173]. However, vvΔE3L is sensitive to the antiviral effects of both type I and II IFNs[7]. In cell culture, treatment of vvΔE3L infected cells with IFN blocks viral protein synthesis[10] at the stage of intermediate protein translation[7]. Ectopic expression of E3 is able to rescue the replication of IFN sensitive viruses such as VSV[220]. The IFN resistance function of E3 maps to the dsRNA binding domain. The precise mechanism by which E3 promotes virus replication in the presence of IFNs remains to be determined; however, IFNs generally function through inducing expression of ISGs which can have direct antiviral activity. The antiviral effect of IFN on vvΔE3L replication in Huh7 cells is partially mediated by PKR[7]. E3 has also been reported to block the activity of several other ISGs, including 2'-5' oligoadenylate synthetase[194] and RNA-specific adenine deaminase 1 (ADAR1)[127]. Overall, the precise contribution of various ISGs to blocking vvΔE3L replication likely depends on the cell line and context of infection (*in vitro* versus *in vivo*).

Several viral and bacterial dsRNA binding proteins have been reported to complement deletion of E3L from the VACV genome. For example, expression of the ribonuclease III (RNase III) gene of *Escherichia coli* in vvΔE3L restores the ability of the virus to replicate in HeLa cells[221]. Moreover, the IFN-resistant phenotype of VACV can be restored in vvΔE3L by expression of the reovirus S4 gene, and this requires the dsRNA binding function of the encoded protein[10]. While the E3L gene is relatively well conserved amongst orthopoxviruses, there is comparatively less sequence conservation among more distantly related members of the *Poxviridae*. To date, only the Orf virus E3L orthologue has been studied and was shown to restore IFN resistance to E3L deficient VACV in cell culture[238].

7.2 - E3 is a potent inhibitor of PKR activity

The primary function of E3 identified to date is the inhibition of PKR function[36,242]. Deletion of the terminal 25 amino acids from the dsRNA binding domain of E3, as well as select point mutations within this domain abrogate dsRNA binding and PKR inhibition[34,79]. These results demonstrate that the dsRNA binding domain is essential for the inhibition of PKR function. Thus, in the absence of E3, PKR becomes phosphorylated during VACV infection and virus replication aborts at the stage of intermediate protein translation[131]. Phosphorylation of eIF2α by PKR has been proposed as the mechanism by which PKR suppresses replication of vvΔE3L. However, recent evidence suggests that PKR-dependant formation of cytoplasmic antiviral granules, an event downstream of eIF2α phosphorylation, is responsible for the inhibition of vvΔE3L[223]. The replication of vvΔE3L can be partially rescued in cells lacking PKR[254] or proteins required for antiviral granule formation[223], as well as by expression of a mutant of eIF2α which cannot be phosphorylated at Ser-51. Thus, the overall contribution of downstream targets of PKR to the inhibition of VACV replication in cell culture remains to be clarified.

The precise mechanism by which E3 inhibits PKR activity has still not been well defined. Given that E3 binds dsRNA, it has been proposed that E3 simply sequesters dsRNA ligands from binding to and activating PKR. However, this model appears too simple and is not supported by some findings. For example, while expression of RNase III in place of E3L can restore the replication of vvΔE3L, mutants of RNase III which still bind dsRNA but lack RNase catalytic activity poorly rescue the virus[221]. Thus, dsRNA may be necessary, but not sufficient for E3 mediated inhibition of PKR. It is likely that

protein-protein interactions between E3 and PKR are required for complete inhibition. Indeed, dsRNA binding domains are known mediators of protein-protein interactions. E3 interacts with both the dsRNA binding domain and the kinase domain of PKR[198,218]. The region of the kinase domain that interacts with E3 was putatively identified as that region of PKR that interacts with eIF2α. These experiments were performed *in vitro* in the absence of dsRNA, demonstrating that dsRNA is not required as a "bridging" molecule to bring E3 and PKR together. Experiments in yeast also demonstrate that E3 interacts with PKR, and that this interaction is abolished by mutations in E3 which prevent dsRNA binding[198]. Despite these findings, it is not clear whether the primary mechanism by which E3 inhibits PKR occurs through sequestering dsRNA, by protein-protein interactions, a combination of both or through some unknown mechanism. Given that the dsRNA binding domain of E3 is required for dsRNA binding and regulation of protein-protein interactions, it is difficult to separate the ability of E3 to bind dsRNA from its ability to interact with PKR and other proteins, making determination of its precise mechanism of action difficult. To date, no studies have directly demonstrated that E3 binds to viral dsRNA within infected cells.

7.3 - Inhibition of cell signalling and cytokine expression by E3

Appropriation of cellular signalling pathways is a common strategy employed by viruses to create a cellular environment permissive for virus replication. E3 targets host signalling pathways which lead to the expression of antiviral cytokines and chemokines. Phosphorylation of IRF3 and IRF7 is critical in the signalling downstream of RLR activation and leads to the expression of IFNs. The first evidence that E3 could suppress this pathway was the finding that NDV-induced IRF3 and IRF7 phosphorylation could be

blocked by E3[224]. Furthermore, this pathway is independent of PKR, demonstrating that E3 is capable of targeting multiple host signalling cascades. Infection of cells with vvΔE3L, but not wild-type VACV, also activates IRF3 and leads to the expression of IFN-β[243]. Other cytokines, including TNF-α and IL-6 are also induced by vvΔE3L infection[53]. The ability of E3 to inhibit TNF-α expression requires the dsRNA binding domain[116]. Although the expression of antiviral and pro-inflammatory cytokines in vvΔE3L infected cells has been correlated with activation of PKR, p38, IRF3 and NF-κB pathways, there is no direct evidence to support this link. Direct evidence has been reported to demonstrate that E3 suppresses cytokine expression through the RLR pathway. For example, in murine keratinocytes the induction of IFN-β, along with other cytokines such as IL-6 during vvΔE3L infection occurs through IPS-1, the adaptor of RLR signalling[54]. However, whether this signalling occurs through RIG-I, MDA5, or a combination of the two has not been addressed. Therefore, although the inhibition of cytokine production by E3 has been reported, for the most part it remains unclear which host signalling pathways are targeted by E3 in this response. Importantly, it remains to be determined which of the two major cytosolic PRRs, RIG-I or MDA5, mediates vvΔE3L-induced IFN-β expression.

While wild-type VACV is pathogenic in mice, numerous studies have demonstrated that vvΔE3L is highly attenuated[22,23,192,243]. The same factors which inhibit vvΔE3L replication *in vitro* also appear to block replication of the virus in mice, although only a few studies have been performed to investigate this phenomenon. Initially, it was demonstrated that intranasal infection of PKR and ribonuclease L (RNaseL)-double knockout mice with vvΔE3L does not result in severe disease[243]. However, in this study, the authors endpoint measurement for disease severity in mice was mortality, with no

monitoring of weight-loss provided. Therefore, this model system would only be able to detect very obvious phenotypes resulting from loss of PKR and RNaseL. In a more recent study, an intratracheal model of VACV infection was described[192]. In this model, when using doses of 10^8 plaque forming units (PFU) per mouse (a dose considerably higher than previous studies), vvΔE3L infection of PKR/RNaseL double knockout mice, but not wild-type mice, resulted in severe disease with infected animals losing approximately 30% body weight. Under these conditions, it was also stated that the vvΔE3L virus disseminated to the liver, spleen and brain, similar to wild-type virus, which has not previously been observed. Thus, although the number of studies detailing the role of E3 *in vivo* is limited, it appears that inhibition of PKR and RNaseL activity in live animals is one mechanism by which E3 promotes viral replication. Future studies on the importance of RLR signalling (including RIG-I and MDA5 knockout mice) and antiviral cytokines (Type I IFN receptor and TNF-α receptor knockout mice) in limiting vvΔE3L replication *in vivo* will be useful to delineate the function of E3 in live animal systems.

7.4 - Role of the Z-DNA binding domain of E3

Although the amino-terminal domain of E3 is dispensable in cell culture for virus replication, dsRNA binding and the inhibition of PKR activity[34,35] it is required for pathogenicity *in vivo*[23], where it contributes to neurovirulence of the virus[22]. The amino terminus of E3 harbours a Z-DNA binding domain. Z-DNA is a left-handed conformation of DNA that has a propensity to form in DNA regions featuring purine and purimidine repeats[239]. The presence of Z-DNA has been correlated with active regions of transcription in the genome. Furthermore, the presence of Z-DNA is implicated in

several disease pathologies, including cancer. The Z-DNA binding domain of E3 has been demonstrated to bind to Z-form DNA structures, and the ability of E3 to bind Z-DNA has been correlated with viral pathogenicity in mice[103]. Apart from these findings, the mechanism by which the Z-DNA binding domain promotes VACV replication *in vivo* is unknown. It has been reported that in cell culture, the Z-DNA binding domain suppresses PKR activation at late times post-infection[115]. Furthermore, PKR activation assays in yeast also demonstrate that the E3 Z-DNA binding domain functions in the inhibition of PKR activity[198]. However, numerous studies have shown that deletion of the entire Z-DNA binding domain of E3 has no effect on virus replication in cell culture[35], and therefore if the inhibition of PKR by the E3 N-terminus is truly of biological significance, it may only manifest *in vivo*. This would not be surprising, given that the context of virus infection is drastically different between live animals and cell culture. Others findings suggest that the Z-DNA binding domain of E3 may be involved in blocking apoptosis and IL-6 gene expression[113]. However, these experiments were carried out by ectopic expression of E3 in the absence of virus infection. Therefore, it is unknown whether the N-terminus of E3 functions in a similar manner in the context of VACV infection. Thus, given that deletion of the Z-DNA binding domain of E3 only affects virus replication *in vivo*, detailed experiments in mouse models will be required for a more complete understanding of the contribution of this domain to E3 function.

General Rationale

The signalling pathways targeted by E3 to inhibit cytokine expression have not been identified. The identification of the pathways and proteins involved will help to explain the molecular mechanisms by which E3 suppresses cytokine production. It is also unknown what stimulates the expression of cytokines during vvΔE3L infection. Although it has been speculated that RNA species generated in VACV infected cells are PAMPs responsible for activating innate immune responses, little direct experimental evidence to support this hypothesis has been reported. Moreover, currently no cellular PRRs have been shown to be activated specifically by VACV PAMPs.

Given the important role of E3 in VACV host-range, it might be expected that the E3 orthologues of other poxviruses also have essential roles in determining host-range. However, very little is known about the function of E3 orthologues, especially those encoded by poxviruses outside the Orthopoxvirus genus. Thus, the study of poxvirus orthologues of E3 may lead to a greater understanding of how poxvirus proteins inhibit innate immune responses related to virus host-range.

Thus, the **central hypothesis** of this study is that RNA species generated in VACV infected cells are PAMPs activating cytokine expression and apoptosis. Furthermore, E3, and its poxvirus orthologues, inhibit cytokine expression during VACV infection by suppressing host PRRs activated by these RNA species. The **general objectives** of this project are to **1)** Identify the host signalling pathways regulating vvΔE3L-induced cytokine expression, **2)** Characterize the immuno-stimulatory properties of RNA species generated in VACV infected cells and **3)** Determine the ability of poxvirus orthologues of E3 to complement important functions of E3.

In summary, this study seeks to determine the molecular mechanisms by which VACV E3 and its poxvirus orthologues inhibit cytokine expression and other innate immune responses. The potential for RNA species generated in VACV infected cells to serve as PAMPs activating cytokine expression and apoptosis will also be examined. Furthermore, the cellular receptors for VACV RNAs will be investigated. Overall, the results from this study should contribute to an understanding of how VACV evades immune recognition. Such information will be important for the design of novel strategies to block poxvirus infection and will also aid in the design of poxvirus-based vaccine vectors and oncolytic agents.

Chapter II

Materials and Methods

Cell culture and viruses - HeLa (human cervical cancer epithelial cells), A549 (human lung adenocarcinoma epithelial cells), Huh7 (human hepatoma cells), BHK21 (baby hamster kidney cells), RK13 (rabbit kidney cells), PK15 (porcine kidney cells) , OA3.Ts (ovine testes cells) and CV-1 (African green monkey kidney cells) cells were maintained in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% fetal-calf serum (FCS) and 1% penicillin/streptomycin, at 37^{o}C, 5% CO_2. Culturing of OA3.Ts cells and all work involving these cells was carried out at the National Centre for Foreign Animal Disease. Over the course of passaging, cells were maintained in the conditions described above. Cells were split when they reached approximately 90-100% confluency by removing the culture media, washing the cells in phosphate buffered saline (PBS), and dissociating the cells at 37^{o}C with TrypLE Express (Gibco). In general, approximately 15-20% of the cells were used to seed the subsequent passage.

The Copenhagen strain (Cop) of VACV and myxoma virus (Lausanne strain) were generous gifts from Dr. Grant McFadden, University of Florida. The Western Reserve strain (WR) of VACV and yaba monkey tumour virus were obtained from ATCC. Swinepox virus (Kaza strain) was a generous gift from Dr. Richard Moyer, University Of Florida. Nigeria sheeppox virus was from the Canadian Centre for Foreign Animal Disease collection.

Generation of recombinant viruses - All recombinant viruses were generated by the poxvirus homologous recombination technique[73,155]. For generation of recombinant

viruses based on the Copenhagen strain of VACV, the left and right flanking regions of the E3L gene were PCR amplified using VACV (Copenhagen strain) genomic DNA as the template. The left flanking region (E3L_FL) was synthesized with E3L_FL forward and reverse primers, while the right flanking region (E3L_FR) was synthesized with E3L_FR forward and reverse primers. The left and right flanking sequences were ligated by a second round of PCR with primers bridging the two PCR products and inserted into the cloning vector pBS-KS+ (Stratagene) between the XhoI and SacI sites, resulting in plasmid pΔE3L. Sequence fidelity was confirmed by DNA sequencing. The xanthine-guanine phosphoribosyltransferase (gpt) gene, driven by an early and late poxvirus promoter, p7.5, was subcloned into pΔE3L at the SmaI site within the flanking regions. A cassette containing enhanced green-fluorescent protein (EGFP) gene, driven by a synthetic early and late poxvirus promoter, was cloned between the NheI and NdeI sites of the flanking regions, yielding the VACV (Copenhagen) E3L deletion vector, pΔE3L/EGFP. A schematic of the pΔE3L/EGFP is provided (Fig. 7). The complete vector was transformed into JM109 cells and grown overnight. Plasmid DNA was isolated using the Qiagen Miniprep kit. The concentration of plasmid DNA was determined by measuring solution absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

5 micrograms (μg) of pΔE3L/EGFP was transfected into BHK21 cells infected with wild-type VACV (Copenhagen strain) using Effectene Transfection Reagent (Qiagen), according to the manufacturer's instructions. Recombinant poxvirus was grown in selective media containing mycophenolic acid (MPA)[62] (25 μg/mL), xanthine (300 μg/mL) and hypoxanthine (12.5 μg/mL). MPA blocks synthesis of xanthine monophosphate by inhibiting inosine monophosphate dehydrogenase, resulting in the

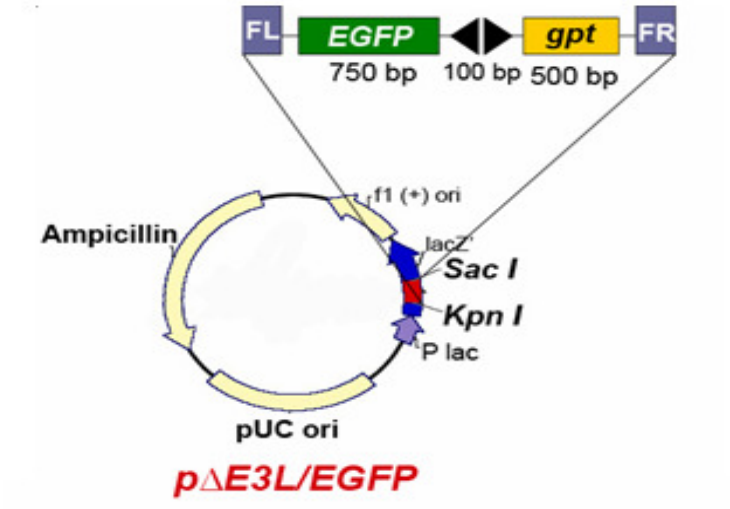

Figure 7: The pΔE3L/EGFP vector used to generate recombinant viruses. FL and FR refer to the flanking left (FL) and flanking right (FR) genomic regions of the E3L gene. The (◄) and (►) symbols denote synthetic poxvirus early/late promoters.

depletion of purine nucleotides in MPA-treated cells. In this manner, MPA treatment inhibits VACV replication. However, this inhibition can be relieved by expression of the gpt gene, which allows cells to use xanthine to generate purine nucleotides[160]. Therefore, the recombinant viruses are constructed to express gpt, while the parental wild-type virus does not. During selection of recombinant virus, only virus (ie recombinant) expressing gpt will be able to replicate and in this way the wild-type virus can be diluted out upon serial passaging in selective media. Recombinant virus was amplified by three rounds of growth in selective media. After each round of selection, the virus was released by three cycles of freeze and thaw and supernatant from this was used to infect fresh cells. Recombinant virus was further purified by four rounds of plaque purification until there was no indication of the presence of wild-type virus. The resulting virus, which is based on the Copenhagen strain of VACV and lacks the E3L gene was termed vvCopΔE3L.

To construct the revertant control virus, the E3L gene was re-inserted into the E3L locus. The E3L gene, including its authentic promoter region, was PCR amplified from VACV (Copenhagen strain) genomic DNA with E3L forward and reverse primer pairs. The E3L gene was cloned into pΔE3L/EGFP plasmid, resulting in plasmid pΔE3L-Rev. 5 μg of pΔE3L-Rev was transfected into BHK21 cells infected with vvCopΔE3L. Recombinant poxvirus was passaged in HeLa cells and purified by plaque purification. The resulting virus, which is based on the Copenhagen strain of VACV and expresses the E3L gene was termed vvCopRev. Recombinant VACV expressing E3 deleted of either the N-terminal domain (amino acids 1-80) (vvCopE3LΔN) or the last 25 amino acids of the C-terminal domain (vvCopE3LΔC25) were constructed in a similar manner to vvCopΔE3L.

The purity of the recombinant viruses was determined by PCR amplification of the E3L gene from extracted genomic DNA. Genomic DNA was isolated from BHK21 cells infected with each recombinant virus. Virus was released by three cycles of freeze and thaw. Supernatant was then mixed in a 1:1 ratio with phenol:chloroform:isoamyl alcohol. The sample was then centrifuged at approximately 17000 x g for 2 minutes and the top layer was transferred to a fresh tube. Two more phenol:chloroform:isoamyl alcohol extractions were then performed. After the final extraction, the top layer of supernatant was mixed 1:1 with chloroform. After a 2 minute centrifugation at 14,000 rpm, the top layer of the sample was transferred to a fresh tube and mixed 10:1 with sodium acetate. Two volumes of 100% ethanol were then added to the sample, which was placed at -20^{o}C overnight. The following day, the precipitated DNA was washed several times with 70% ethanol, air dried, and then resuspended in distilled water. The purity of vvCopΔE3L and vvCopRev was confirmed by PCR with forward and reverse primers flanking the E3L open reading frame (E3L locus). Expression of the E2L and E4L open reading frames, adjacent to the E3L gene, was also verified by PCR to ensure they were not disrupted during the recombination process.

Generation of the E3L-deficient virus (vvWRΔE3L) and revertant control virus in which the E3L gene was re-inserted into the E3L locus (vvWR-Rev) based on the Western Reserve strain was performed using protocols similar to generation of the viruses based on the Copenhagen strain. Recombinant viruses expressing sheeppox virus SPPV34L (vvWR-SPPV34L), yaba monkey tumour virus YMTV34L (vvWR-YMTV34L), swinepox virus SPV032L (vvWR-SPV032L) and myxoma virus M029L (vvWR-M029L) in place of E3L were generated in a similar manner to vvWRΔE3L. Gene sequences for the SPPV34L and YMTV34L genes were obtained from Genscript.

The SPV032L gene sequence was amplified from swinepox virus genomic DNA and the M029L gene sequence was amplified from myxoma virus genomic DNA. The purity of the recombinant viruses based on the Western Reserve strain of VACV was determined as described for recombinants based on the Copenhagen strain. A summary of all viruses used in this study is shown in Table 2. The sequences of primers used in the generation of recombinant viruses is shown in Table 3.

Chemical Reagents – Polyinosinic: polycytidylic acid (pIC), polycytidylic acid (pC) and polyinosinic acid (pI) were purchased from GE Healthcare. Cytosine arabinoside (araC) was purchased from Sigma.

Antibodies – All antibodies used in this study are listed in Table 4.

Small-interfering RNA (siRNA) knockdown: ~1.5 x 10^5 HeLa or A549 cells were seeded in 6-well plates. The next day, cells were transfected with 100 nM of siRNA (Dharmacon) using HiPerFect (Qiagen), according to the manufacturer's protocol. For the double-knockdown experiments, cells were transfected with each siRNA at 50 nM, for a total of 100 nM siRNA. Control siRNAs included a scrambled siRNA (Ambion) and a mutant of the MDA5 siRNA with a 2 bp mismatch. All siRNA target sequences are listed in Table 5.

Isolation and transfection of viral RNA - Confluent HeLa cells and BHK21 cells were infected with vvCopRev or vvCopΔE3L at an MOI of 5 and RNA was collected at 2 hpi (early RNA) and 8 hpi (late RNA). Cells were also pre-treated with 50 µg/mL of

Table 2: Summary of vaccinia virus recombinants generated

Vaccinia Virus Recombinants			
Virus Name	*Vaccinia Strain*	*E3L gene status*	*Transgene*
vvCopRev	Copenhagen	**+**	-
vvCopΔE3L	Copenhagen	**-**	-
vvCopE3LΔN	Copenhagen	**+** (amino acids 81-190)	-
vvCopE3LΔC25	Copenhagen	**+** (amino acids 1-165)	-
vvWR-Rev	Western Reserve	**+**	-
vvWRΔE3L	Western Reserve	**-**	-
vvWR-SPPV34L	Western Reserve	**-**	SPPV E3 orthologue
vvWR-YMTV34L	Western Reserve	**-**	YMTV E3 orthologue
vvWR-SPV032L	Western Reserve	**-**	SPV E3 orthologue
vvWR-M029L	Western Reserve	**-**	MYXV E3 orthologue

*All viruses express EGFP.

Table 3: PCR primer sequences

PCR Primers	Sequence (5´- 3´)
A1	For: ATGGCTAAGCGAGTAAGCCTTCCAGAT Rev: TTACAATAAACTCCGTAGAGAAATATC
A17	For: ATGAGTTATTTAAGATATTACAATATGCTT Rev: TCGTCAGTATTTAAACTGTTAAATGTTGGT
D12	For: ACCTCAGCGCACGCAATAAACTGTTCA Rev: AGTCATACTAGAATAAAGCAGCGAGT
E3L_FL (Cop)	For: ATTACTCGAGTGATGTTCATATATTGGTTCATACAT Rev: TTGTCCCGGGTTGAAGCTTACTACATATGAGAATGCTAGCTAG A TTCTGATTCTAGTTATCAATAACA
E3L_FR (Cop)	For: ATCTAGCTAGCATTCTCATATGTAGTAAGCTTCAACCCGGGAC AACCAGCAATAAAACTGAACCTACT Rev: TGGCGAGCTCTCAAGAATATAGGTAATACAAAATCTAA
E3L (Cop)	For: TGGATCCATTTCGCAATCTTAATGT Rev: ATAACCCGGGTCAGAATCTAATGATGACGTAACCAA
E3L locus (Cop)	For: TGATAAAGTAGGT TCAGTTTTATTGCTGGTTGT Rev: TGTTATTGATAACTAGAA TCAGAATCTA
E3L (WR)	For: TGGATCCATTTCGCAATCTTAATGT Rev: ATAACCCGGGTCAGAATCTAATGATGACGTAACCAA
E3L locus (WR)	For: ACGACGAACCACCAGAGGATGATGAA Rev: AGAGAATATACTAGTCGCGTTAATAGTA
E3L_FL (WR)	For: TTGGTACCTCTCTTATGAATCGTATATCATCAT Rev: AGATCTACTACATATGAGTCGACGAATGCTAGCTATT CGATAAGGCAGATGGAAAATCTA
E3L_FR (WR)	For: GCTAGCATTCGTCGACTCATATGTAGTAGATCTAATCTCTGCG TTAGAACGCTCGTCGA Rev: TAGAGCTCAATAAACCGTCTATTGCCACAAATT
E2L	For: TGGATTCTGTCCAATGATGATGAAACG Rev: TCTTCCCTCTATCATGTTCACTACTGG
E4L	For: ATACATTAGTAGTTACTCATCCAA Rev: ATCATCCTCTGGTG GTTCGTCGTT
GAPDH	For: AAGGTGAAGGTCGGAGTCAA Rev: TTACTCCTTGGAGGCCATGT
IFN-β	For: TGTCTCCTCCAAATTGCTCTC Rev: TCCTTGGCCTTCAGGTAATG
IL-6	For: ATGAACTCCTTCTCCACAAGCGCCT Rev: ATTTGTGGTTGGGTCAGG
INHBA	For: ATGCCCTTGCTTTGGCTGAGAGGAT Rev: TGCTGGAAGAGGCGGATGGT
M029L	For: TTAAGAATTCATGTACCCA TACGATGTTCCAGATTACGCTATG GATCCCATTAACACGCTATGGCACA Rev: TAATCCCGGGTTAAAACTTTATAACGACG TGTTTTAGT
SPPV34L	For: TCACGAATTCATGTACCCATACGATGTTCCAGATTACG CTATG TATTCTTGTG ATGAAGTAGATTCTT Rev: TCATCCCGGGTT AAAATTTGATAATAGATGTATTGATT
SPV032L	For: ATTAGGATCCATGTACCCATACGATGT TCCAGATTACGCTATG TGTTCTGATATCTCAAATGAAGA Rev: TGCACCCGGGTTAGAATTTTATAATAGTTTTATTTAATATGA
TNF-α	For: ATGAGCACTGAAAGCATGATCCGGG Rev: TGGTAGGAGACGGCGATGC
YMTV34L	For: TTAAGGATCCATGTACCCATACGATGTTCCAGATTACGCTATG GACTCTCCCGGTTGTGAGAACGA Rev: AT TACCCGGGTTAAAATTTTACAATTGACGATTTTAATATCTC TTCCA

Table 4: Antibodies used in this study

Antibody	Company
Actin	Sigma
Caspase-7, cleaved	Cell Signalling Technologies
E3	GenScript
eIF2α	Invitrogen
eIF2α, phospho-Ser51	Invitrogen
HA	Sigma
IPS-1	Bethyl Laboratories
IRF3	Epitomics
MDA5	Alexis Biochemicals
MK2	Cell Signalling Technologies
MK2, phospho-Thr334	Cell Signalling Technologies
NF-κB p50	Epitomics
NF-κB p65	Epitomics
PARP, cleaved	Cell Signalling Technologies
p38	Cell Signalling Technologies
p38, phospho-Thr180/Tyr182	Cell Signalling Technologies
PKR	Cell Signalling Technologies
PKR, phospho-Thr446	Cell Signalling Technologies
PKR, phospho-Thr451	Epitomics
RIG-I	Dr. Rongtuan Lin, McGill University

Table 5: Small-interfering RNA target sequences

Small-interfering RNA	Target Sequence (5´- 3´)
IRF3	AGACAUUCUGGAUGAGUUA
IPS-1	CAUCCAAAGUGCCUACUAG
MDA5	CAAUGAGGCCCUACAAAUU
MDA5 mutant control	CAA***CC***AGGCCCUACAAAUU
NF-κB p50	GAUGGGAUCUGCACUGUAA
NF-κB p65	GGAUUGAGGAGAAACGUAA
p38	GGAAUUCAAUGAUGUGUAU
PKR	GCGAGAAACUAGACAAAGU
RIG-I	CCACAACACUAGUAAACAA

araC, infected with vvCopRev or vvCopΔE3L and RNA was collected at 8 hpi (late RNA + araC). RNA was also isolated from uninfected HeLa cells (HeLa RNA). Total RNA was extracted using TRIzol (Invitrogen). Residual genomic DNA contamination was removed using the TURBO DNA-free kit (Ambion) by incubating the isolated RNA samples with DNase for 1 hour at 37°C. Total RNA concentration was determined by measuring absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Cells were either mock transfected or transfected with the TRIzol extracted total RNA using Attractene (Qiagen), according to the manufacturer's protocol. Cells for RNA transfection experiments were transfected when they were approximately 70-80% confluent.

Western blotting - Cells were collected in 1 mL of PBS and pelleted by centrifugation at 6000 rpm for 2 minutes. Cell pellets were lysed in 200 µl of protein loading buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, 0.1% bromophenol blue, 10% glycerol and 100 mM beta-mercapto-ethanol). Protein samples were separated on Criterion XT pre-cast gels (Bio-Rad) and transferred to Hybond-C nitrocellulose membranes (Amersham Bioscience) using a Tyler apparatus (Tyler Research). Transfer buffer consisted of 48 mM Tris, 39 mM glycine, 1.3 mM sodium dodecyl sulphate and 20% methanol. Membranes were blocked for three hours at room temperature in 5% skim milk in Tris buffered saline with Tween (containing 0.2% Tween 20, TBST). Membranes were then incubated with the indicated antibodies overnight at 4°C. The next day, membranes were washed three times in TBST, with at least twenty minutes per wash. Next, membranes were incubated with secondary antibodies conjugated to horseradish peroxidise for 1 hour at room temperature. Membranes were

again washed three times in TBST, with at least twenty minutes per wash. Blots were developed with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) and exposed using Kodak X-OMAT Blue film.

Reverse-transcription PCR (RT-PCR) - RNA was extracted using the RNeasy Mini Kit (Qiagen). RT-PCR was performed on 1.0-1.5 µg of RNA using the Advantage RT-For-PCR Kit (Clontech) or the Quantitect Reverse-transcription kit (Qiagen). PCR was performed using GoTaq Green (Promega) using a standard PCR cycle of an initial incubation for 2 minutes at 95°C, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and an extension time of 1 minute per kbp. A final incubation at 72°C for 5 minutes was used to complete all PCR reactions. An Eppendorf Mastercycler epGradientS thermocycler was used for all PCR reactions. Primer sequences used for RT-PCR are listed in Table 3.

Real time PCR - 0.5 µg of RNA was reverse transcribed using the Quantitect Reverse transcription kit. Real time PCR was performed using Taqman Gene Expression Master Mix and primer/probe sets (Applied Biosystems) on a StepOnePlus or a 7500 FAST 96-well real time PCR machine (Applied Biosystems). Reactions were performed in MicroAmp Fast Optical 96-well plates (Applied Biosystems). The real time PCR cycle consisted of an initial step of 50°C for 2 minutes, followed by 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Actin expression was used as an internal normalization control to account for total RNA loading. Sequences for real time PCR primers and probes are listed in Table 6.

Table 6: Real time PCR primer/probe sequences

Real time PCR primer/probes	Sequence (5´- 3´)
Actin	For: CACACTGT GCCCATCTACGA Rev: GCCAGCCAGGTCCAGA C Probe: CCCATGCCATCCTGC
IFN-β	For: TGGCTGGAATGAGACTATTGTTGAG Rev: CAGGACTGTCTTCAGATGGTTTATCT Probe: CCTCCTGGCTAATGTC
IL-6	For: AGATGGATGCTTCCAATCTGGATTC Rev: TCAAACTCCAAAAGACCAGTGATGA Probe: ACCAGGCAAGTCTCCTCA
TNF-α	For: GCC CCTCCACCCATGTG Rev: GGTTGACCTTGGTCT GGTAGGA Probe: ACCCACACCATCAGCC

Real Time PCR Arrays - The RT^2 Profiler PCR Array System/Common Human Cytokines (SuperArray) was used to quantify cytokine mRNA expression. For cytokine profiling during virus infection, confluent HeLa cells in 6-well plates were infected with vvCopRev or vvCopΔE3L at an MOI of 5 and collected 12 hpi. For cytokine profiling during RNA transfection, HeLa cells were transfected with 3 μg of the indicated RNA sample and cells were collected at 6 hours post-transfection (hpt). In each experiment, RNA was extracted using the RNeasy kit (Qiagen). Residual genomic DNA contamination was removed using the TURBO DNA-free kit (Ambion) by incubating the RNA samples with DNase for 1 hour at 37°C. 0.75 μg of RNA from each sample was reverse transcribed into cDNA using the RT^2 First Strand Kit (SuperArray). Real time PCR was performed using the RT^2 SYBR Green qPCR Master Mix (SuperArray) on an ABI 7500 FAST 96-well real time PCR machine (Applied Biosystems). The real time PCR reaction cycle consisted of an initial incubation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. SYBR green fluorescence was collected during the annealing step of 60°C for 1 minute. Results were analyzed using the PCR Array Data Analysis Web Portal (SuperArray). GAPDH and actin were chosen for data normalization and total RNA loading controls for standardization between samples.

In vitro transcription – The coding sequence of EGFP was cloned into the pDP19 transcription vector (Ambion). SsRNA copies of the EGFP gene (ssEGFP) were transcribed from both template DNA strands using the MEGAscript T3/T7 kit (Ambion). Reactions were treated with DNase to remove template DNA using the MEGAscript T3/T7 kit. SsRNAs were purified following transcription using the RNeasy kit (Qiagen).

Equal amounts of T3 and T7-transcribed RNA were annealed together at 37°C for 1 hour to produce a double-stranded EGFP RNA (dsEGFP) of approximately 800 bp.

RNase A and RNase III digests – RNA samples (where applicable) were incubated for 1 hour at 37°C with RNase III reaction buffer (50 mM NaCl, 10 mM Tris, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol) (Ambion) in the presence or absence of 15 units of RNase III (Ambion). 3 µg of ssEGFP or dsEGFP RNA or 3 µg of VACV late RNA were incubated at 37°C for 1 hour with RNase A digestion buffer (10 mM Tris-HCl, 10 mM EDTA, 400 mM NaCl, pH7.9), in the absence (mock digested) or presence (RNase A digested) of 100 ng of recombinant RNase A (Ambion). Electrophoresis of RNA before and after digestion was carried out on an RNA gel using the NorthernMax system (Ambion). Briefly, 1 gram of RNase-free agarose was dissolved in 90 mL of RNase-free water. Then 10 mL of Denaturing Buffer (Ambion) containing formaldehyde was added and the gel was poured. RNA samples were mixed with three volumes of Formaldehyde Load Dye (Ambion) and ethidium bromide was added to each RNA sample to a final concentration of 10 µg/mL. RNA samples were then incubated at 65°C for 15 minutes and run on the RNA gel using MOPS Gel Running buffer (Ambion).

Fluorescence activated cell sorting- Semi-confluent HeLa cells in 6-well plates were transfected with 10 µg of late RNA using Attractene. After 12 hours, cells were collected by scraping and centrifuged at 5000 rpm for 5 minutes. Cell pellets were washed with 1 mL PBS. Cells were then lysed in hypotonic propidium iodide buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 50 µg/mL propidium iodide and 100 µg/mL of RNase A. Following a 30 minute incubation in the dark at room temperature,

cells were analyzed on a Becton Dickinson FACScalibur flow cytometer. Propidium iodide fluorescence was collected on the FL3 channel in logarithmic data collection mode. Cellular debris, evident as particles of minimal size and fluorescence, was gated out in a dot plot of forward scatter (FSC) versus FL3 fluorescence. Nuclei to the left of the G1 peak in the DNA fluorescence histogram were considered apoptotic.

Myxoma, swinepox, yaba monkey tumour and sheeppox virus infections – Fully confluent cells were pre-treated with or without 50 µg/mL of araC for 1 hour at 37°C. The reference cell lines OA3.Ts, CV-1, PK15 and RK13 were then infected with sheeppox virus, yaba monkey tumour virus, swinepox virus or myxoma virus, respectively. Infections were performed in the presence or absence of araC and cells were collected at 1, 3 and 8 hpi. RNA was extracted from cells and RT-PCR was used to detect expression of each viral gene. Primer sequences are listed in Table 3. Sheeppox virus infections were performed at the National Centre for Foreign Animal disease in a biosafety level III laboratory.

Virus yield assays – Fully confluent confluent cells in 6-well plates were infected with the indicated viruses. After a 1 hour incubation at 37°C, the virus inoculum was removed and the cells were washed three times with PBS. Fresh growth media was then added and cells were collected at the indicated time points. The virus was released from the sample by three cycles of freeze and thaw. Serial dilutions of each sample were then added to confluent BHK21 cells in 12-well plates. All samples were analyzed in triplicate. EGFP positive plaques were counted at 24 hpi by fluorescent microscopy.

Interferon Sensitivity Assays - Huh7 cell monolayers in 12-well plates were treated overnight at 37°C with or without 1000 units/mL of human IFN-β (PBL Biomedical Laboratories). Fully confluent cell monolayers were infected with the indicated viruses at an MOI of 1 and incubated at 37°C for 1 hour. Media containing the virus was then aspirated and replaced with fresh media with or without 1000 units/mL IFN-β. Cells were collected at 5 and 48 hpi and virus yields were determined by plaque assays in BHK21 cells.

DsRNA binding assays - To prepare beads for pull-down assays, cyanogen bromide-activated Sepharose 4B beads (GE Healthcare) were conjugated with a 10 mg/mL solution of pIC (Sigma) in coupling buffer (0.1M $NaHC0_3$, pH 8.3, 0.5M NaCl). Unconjugated active sites were blocked by incubating the beads with 0.1M Tris-HCl, pH 8.0, for 2 hours at room temperature. Beads were subsequently washed in alternating cycles of low pH buffer (0.1M sodium acetate, pH 4.0, 0.5M NaCl) and high pH buffer (0.1M Tris-HCl, pH 8.0, 0.5M NaCl). Beads were stored at 4^oC in 0.1M Tris-HCl, pH 7.0. For pull-down assays, confluent BHK21 cells were infected with the indicated viruses at an MOI of 1. Cells were collected at 16 hpi and washed with PBS. Cells were then resuspended in non-denaturing lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA) in the presence of protease inhibitors (Roche) and incubated on ice for 30 minutes. Samples were then centrifuged at 5000 x g for 10 minutes at 4^oC and the supernatant was collected. An aliquot of this supernatant was collected and mixed 1:1 with protein loading buffer as a pre-pull-down sample. The rest of the supernatant was then incubated with end-over-end mixing for 2

hours at room temperature with pIC coated sepharose beads. Beads were then washed three times with lysis buffer, and incubated for 10 minutes at 95^{o}C in protein loading buffer to elute associated proteins. Samples were then analyzed by Western blotting.

In vivo pathogenicity assay - All animal experiment protocols were reviewed and approved by the National Microbiology Laboratory Animal Care Committee of the Canadian Science Centre for Human and Animal Health. 4-6 week old, female Balb/c mice were obtained from Charles River and housed in the National Microbiology Laboratory Animal Facilities. Mice were acclimatized for at least one week before being used. Animals (4 per group) were housed in separate cages for each experimental group. For infections, mice were anesthetised with isoflurane. Virus inoculum was diluted in PBS to a total volume of 50 µl and each mouse was intranasally inoculated with 10^6 plaque forming units of the indicated virus using a stepper repetitive pipette (Tridak). Animals were monitored daily for clinical signs and weight loss. Animals were euthanized at an end-point defined as weight loss >20% in combination with clinical signs including piloerection, inactivity in the cage and a hunched posture.

Chapter III

Results

Section I - *Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of PKR, NF-κB and IRF3 pathways (Journal of Virology, 83: 6757-68)*

Rationale, hypothesis and specific objectives

Cytokines play a key role in regulating innate immunity to virus infection and also mediate the magnitude and direction of adaptive immune responses. The cytokines IFN-β, TNF-α and IL-6 play an important role in controlling VACV replication *in vivo*[107,124,196]. The VACV dsRNA binding protein, E3, has been demonstrated to inhibit the expression of these cytokines[53,116]. However, few details regarding the molecular mechanisms of this inhibition have been described. Identifying the host signalling pathways targeted by E3 to inhibit cytokine expression will lead to a better understanding of how this viral protein functions to antagonize the host immune system.

The hypothesis that drives this work is that E3 inhibits cytokine expression in VACV-infected cells by suppressing dsRNA-activated host signalling pathways, including those mediated by PKR, which culminate in cytokine expression.

The specific objectives of this work are to; **(A)** Identify cytokines whose expression is inhibited by E3 during VACV infection, **(B)** Determine the role of the Z-DNA binding and dsRNA binding domains of E3 in the inhibition of cytokine expression and **(C)** Identify the host proteins and/or signalling pathways which E3 targets to block cytokine expression.

1.1 - E3 suppresses expression of a diverse array of cytokines. The effect of E3 on cytokine expression was investigated using real time PCR arrays. HeLa cells were mock infected or infected with a VACV E3L deletion mutant virus (vvCopΔE3L), or a revertant virus based on vvCopΔE3L with the E3L gene re-inserted back into the viral genome (vvCopRev). The relative expression of 84 cytokines, including IFNs, interleukins (IL) and members of the TNF, transforming-growth factor (TGF) and platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) super-families were determined using real time PCR arrays. Cytokines detected with a cycle threshold (Ct) value of $\geq$35 were scored as not significantly expressed and excluded from the analysis. Infection of cells with both viruses induced the detectable expression of the majority of cytokines (Table 7). Many cytokines were regulated to similar degrees by both viruses. For example, IFN-α8 was up-regulated 8.7 (vvCopRev) and 7.4 (vvCopΔE3L) fold compared to mock infected cells. The expression of several cytokines was unaffected (fold change < 2) by infection with either virus, including IL-1β, and colony-stimulating factor (CSF) 1 and CSF2. While cellular stress due to the cytopathic effect of infection could explain the increased cytokine expression to some degree, infection of cells with vvCopΔE3L, as compared to vvCopRev, resulted in significant up-regulation of several pro-inflammatory and antiviral cytokines. IFN-β, TNF-α and inhibin beta-A (INHBA) were all up-regulated approximately 10-fold in vvCopΔE3L infected cells as compared to cells infected with vvCopRev. IL-6, IL-8, IL-20 and lymphotoxin A (LTA) were also up-regulated by 4.2, 4.1, 8.3 and 4.5 fold, respectively, in vvCopΔE3L infected cells. Some cytokines, including IL-12A, were more strongly induced by infection with vvCopRev as compared to vvCopΔE3L.

Table 7: E3 blocks expression of a diverse array of cytokines during infection

Cytokines	vvCopRev	vvCopΔE3L
Interleukin family		
IL20	16.9692	141.16
IL8	5.38	21.89
IL6	5.15	21.69
IL13	26.18	20.21
IL11	6.36	17.75
IL21	12.66	14.07
IL12B	8.21	13.69
IL12A	67.24	10.66
IL24	3.28	9.98
IL1A	2.92	8.28
IL22	13.08	7.35
IL1F7	24.11	6.39
IL15	4.85	6.24
IL19	1.25	5.82
IL17C	6.35	5.69
IL18	3.15	5.09
TXLNA	4.78	3.22
IL7	1.96	2.67
IL1B	2.00	-1.04
Interferon family		
IFNB1	8.11	84.41
IFNA5	5.88	10.07
IFNA8	8.71	7.42
IFNA2	13.71	7.35
IFNA4	15.32	7.35
IFNK	12.36	7.26
IFNA1	4.29	2.82
Platelet-derived/Vascular-endothelial growth factor family		
FIGF	8.59	3.37
PDGFA	1.50	-1.17
Tumour-necrosis factor family		
TNF-α	12.95	123.78
LTA	8.21	37.21
TNFSF14	10.23	14.08
LTB	8.21	11.38
TNFSF13	8.21	9.43
TNFSF11	8.99	6.60
TNFSF13B	7.72	6.47
TNFRSF11B	-1.13	3.85
TNFSF12	-1.15	2.12
CD70	2.04	1.54
TNFSF10	-2.56	-4.21
Transforming growth factor family		
INHBA	3.37	36.43
GDF5	8.21	26.28
BMP5	13.6	15.93
GDF9	7.91	12.12
GDF10	1.80	11.12
BMP2	7.29	10.16
TGFB2	3.50	7.46
MSTN	21.11	7.35
BMP6	6.02	5.17
BMP4	3.34	4.57
INHA	4.76	4.27
NODAL	2.81	2.51
BMP1	3.74	2.09
TGFB3	5.22	1.98
GDF11	1.66	1.51
TGFA	3.19	1.39
BMP8B	1.50	-1.10
TGFB1	1.59	-1.33
Others		
CSF2	1.96	-1.03
CSF1	-1.20	-1.78

HeLa cells were infected at an MOI of 5 with the indicated virus. Cells were collected at 12 hpi and cytokine expression was analyzed using real time PCR arrays. Shown is the fold change in gene expression in comparison to mock infected cells. Results are representative of three independent experiments.

It was next tested whether the observed cytokine induction requires expression of intermediate and/or late gene products, or whether early gene products alone are sufficient to up-regulate cytokine expression. Intermediate and late genes, but not early genes, require viral DNA replication before they can be expressed[24], and viral DNA replication (and therefore intermediate/late gene expression) can be blocked by treating cells with araC. To this end, HeLa cells were infected with vvCopRev or vvCopΔE3L in the presence or absence of araC and collected at several time-points post-infection. The expression of IFN-β, TNF-α, IL-6 and INHBA was determined by RT-PCR. A slight up-regulation of IL-6, TNF-α and INHBA was observed by 1 hpi in cells infected with either virus (Fig. 8A). At late times post-infection (12 hpi), expression of TNF-α, IFN-β and INHBA was significantly higher in cells infected with vvCopΔE3L in comparison to vvCopRev infected cells. IL-6 expression was induced by vvCopΔE3L at 6 hpi and sustained until 12 hpi. Pre-treatment of cells with araC abolished the induction of IFN-β in cells infected with vvCopΔE3L. In contrast, induction of IL-6 was unaffected by araC treatment. Induction of INHBA was greatly reduced by araC, whereas TNF-α induction was only slightly reduced by araC. To ensure that araC treatment had blocked viral DNA replication, expression of the viral late gene A17 (which requires viral DNA replication to be transcribed) was analyzed. A17 expression was completely blocked in araC treated cells. The replication of vvCopΔE3L is inhibited at the stage of intermediate protein synthesis [131,254], and thus A17 is not expressed by vvCopΔE3L. The expression of GAPDH was measured as a loading control. The level of GAPDH remained constant except at late times (6 and 12 hpi), when both viruses induced a shut-off in host gene expression, as previously described[154]. Treatment of cells with araC was able to restore GAPDH transcription to levels comparable to mock infected cells.

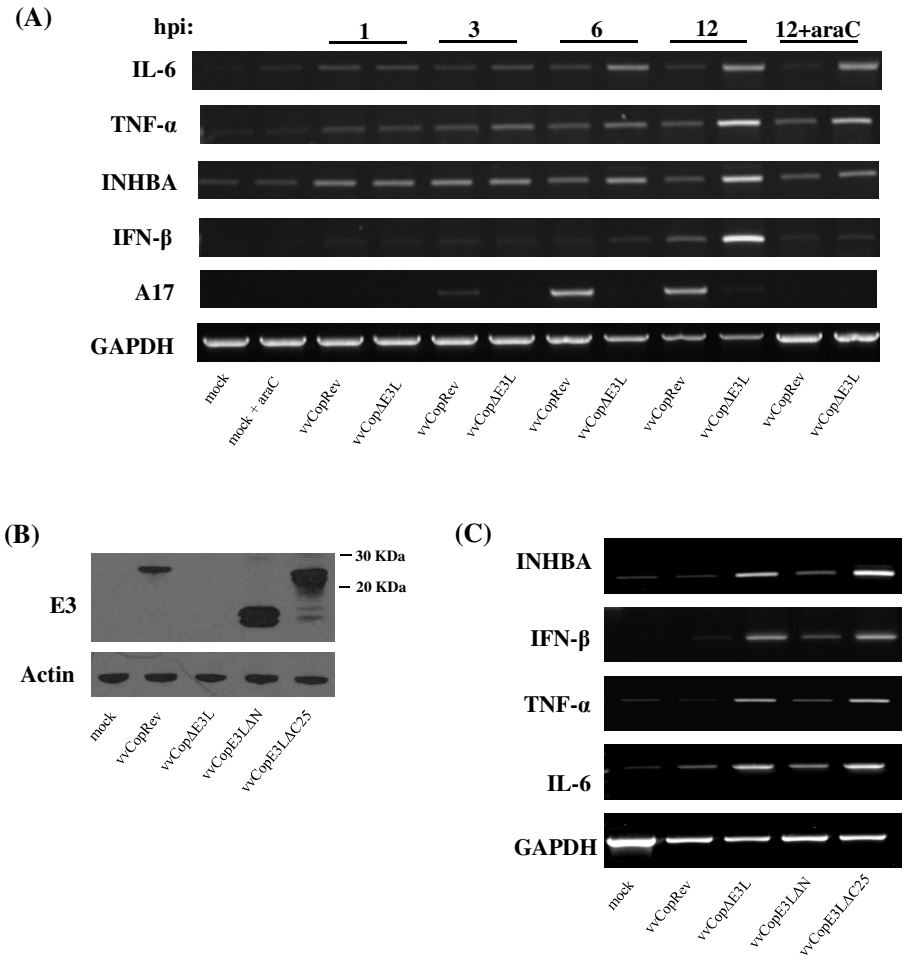

Figure 8: Induction of cytokines by virus infection. (A) Confluent HeLa cells in 6-well plates were either mock infected or infected with vvCopRev or vvCopΔE3L at an MOI of 5 and collected at the times indicated. Select wells were also treated with 50 µg/mL of araC 1 hour prior to infection. Cytokine expression was analyzed by RT-PCR. (B) Confluent BHK21 cells were infected with vvCopRev, vvCopΔE3L, vvCopE3LΔN or vvCopE3LΔC25 at a MOI of 5 and collected at 6 hpi. The expression of E3 and actin was determined by Western blotting. (C) Confluent HeLa cells were mock infected or infected with vvCopRev, vvCopΔE3L, vvCopE3LΔN or vvCopE3LΔC25 at an MOI of 5. Cells were collected at 12 hpi and RNA was extracted for RT-PCR. Results are representative of three independent experiments.

To determine the role of the individual N-terminal Z-DNA binding and C-terminal dsRNA binding domains of E3 in the inhibition of cytokine expression, HeLa cells were infected with vvCopRev, vvCopΔE3L or VACV recombinants expressing E3 truncated of the last 25 amino acids of the C-terminal domain (vvCopE3LΔC25) or the entire N-terminal domain (vvCopE3LΔN). Previously it has been shown that mutation of several residues in the terminal 25 amino acids of E3 abolishes its ability to bind dsRNA[79]. To verify the expression of the E3 truncations, BHK21 cells were infected with each of the recombinant viruses. The E3 truncations were detected at the correct molecular weight (Fig. 8B). Next, HeLa cells were infected with the recombinant viruses, collected at 12 hpi and cytokine expression was analyzed by RT-PCR. Cells infected with vvCopΔE3L and vvCopE3LΔC25 expressed significantly higher levels of IFN-β, TNF-α, IL-6 and INHBA than cells infected with vvCopRev or vvCopE3LΔN (Fig. 8C). Expression of IFN-β, TNF-α and INHBA was also moderately higher in cells infected with vvCopE3LΔN in comparison to vvCopRev infected cells.

1.2- PKR, p38 and IPS- 1 contribute to vvCopΔE3L-induced cytokine expression.

E3 has been demonstrated to inhibit PKR and p38 activation[116]. However, the mechanism by which E3 inhibits p38 phosphorylation is not known. Moreover, both PKR and p38 are known to regulate the cytokine response to virus infection[33,74,188], but it remains to be determined whether these proteins play a role in VACV-induced cytokine expression. Therefore, to study the signalling pathways which may regulate cytokine expression in VACV infected cells, we analyzed activation of PKR and p38, as measured by phosphorylation, following infection of HeLa cells with vvCopRev or vvCopΔE3L. The levels of total and phosphorylated p38 and PKR were determined by Western

blotting and the level of actin was determined as a loading control. A slight and transient increase in phosphorylated p38 was detected in cells infected with either virus at 1 hpi which returned to basal levels by 3 hpi (Fig. 9A). A sustained increase in PKR and p38 phosphorylation was detected in vvCopΔE3L infected cells as early as 3 hpi. At 8 hpi, considerably higher levels of PKR and p38 phosphorylation were detected in vvCopΔE3L infected cells in comparison to vvCopRev infected cells. The total level of p38 and β-actin remained constant throughout the time-course. The total PKR band appeared as a double-band and was less intense in vvCopΔE3L infected cells after 3 hpi, which correlated with increased phosphorylation of PKR. Infection of cells with vvCopRev resulted in minimal PKR phosphorylation at late times post-infection. The phosphorylation of p38 and PKR during vvCopΔE3L infection was blocked by treatment of cells with araC.

The direct role of PKR and p38 in VACV-induced cytokine expression was examined next. Furthermore IPS-1, the adaptor of the RLR pathway, was also analyzed since it was recently reported to mediate VACV-induced IFN-β and IL-6 expression in mouse cells[54]. HeLa cells were transfected with PKR, p38 or IPS-1 specific siRNAs, and then infected with either vvCopRev or vvCopΔE3L. The siRNA knockdown efficiency was determined by Western blotting (Fig. 9B). Expression of cytokines in infected cells treated with each of the siRNAs was analyzed by RT-PCR (Fig. 9C). The level of GAPDH was determined as a loading control. In cells infected with vvCopΔE3L, a significant decrease in TNF-α, INHBA, and to a lesser degree, IL-6 and IFN-β induction was observed in cells treated with PKR siRNAs. VvCopΔE3L-induced expression of INHBA, but not the other cytokines tested, was reduced in p38 siRNA treated cells. In contrast, transfection with IPS-1 siRNAs abolished IFN-β expression in vvCopΔE3L

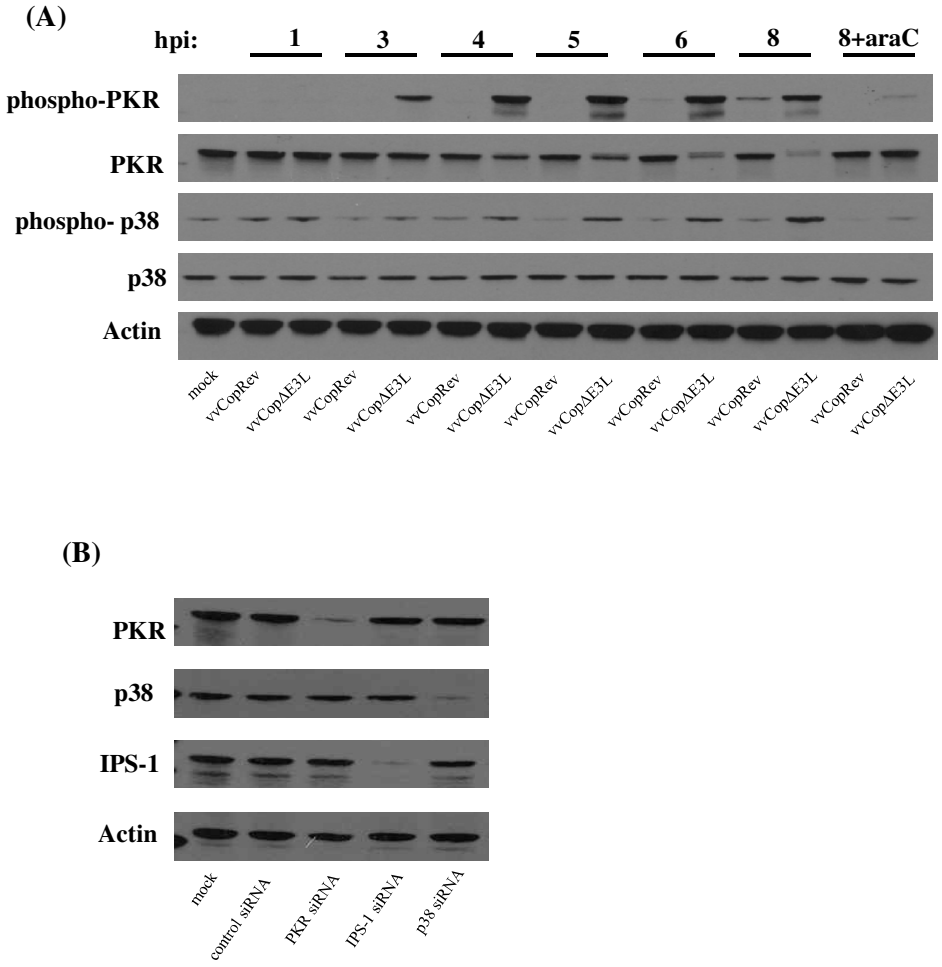

Figure 9: PKR, p38 and IPS-1 mediate the cytokine response to vvCopΔE3L. (A) Confluent HeLa cells in 6-well plates were either mock infected or infected with vvCopRev or vvCopΔE3L at an MOI of 5. Cells were collected at the times indicated. Select wells were also treated with 50 µg/mL of araC 1 hour prior to infection. Western blotting was performed to detect total and phosphorylated PKR and p38. (B) $1.5x10^5$ HeLa cells were seeded in 6-well plates. Cells were then transfected with 100 nM control, PKR, p38 or IPS-1 specific siRNAs for 72 hours. Western blotting was performed to determine the efficiency of the siRNA knockdown. Results are representative of three independent experiments.

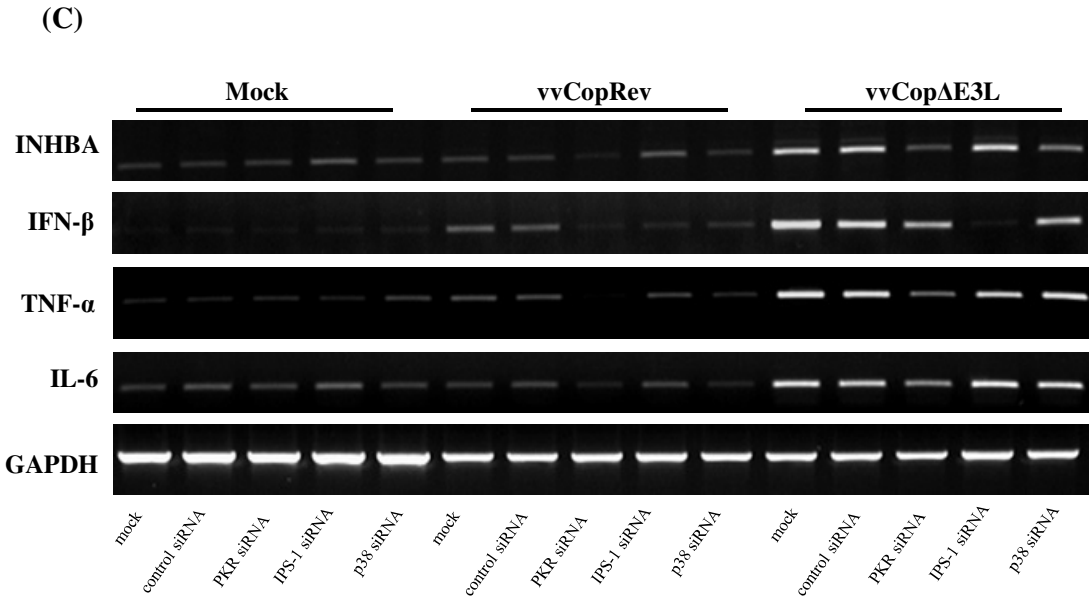

Figure 9: PKR, p38 and IPS-1 mediate the cytokine response to vvCopΔE3L. (C) Cells were transfected as in Fig. 9B and then either mock infected or infected with vvCopRev or vvCopΔE3L at an MOI of 5. Cells were collected at 12 hpi and RNA was extracted for RT-PCR. Results are representative of three independent experiments.

infected cells but did not affect the induction of TNF-α, INHBA or IL-6. It should be noted from Figure 9C that none of the siRNAs used activated the IFN response as measured by IFN-β expression.

1.3 - PKR is required for p38 activation during vvCopΔE3L infection. PKR has been identified as an upstream mediator of p38 activation and cytokine expression[222]. Both PKR and p38 were observed to play a role in INHBA expression (Fig. 9C). Given that E3 is a known PKR inhibitor, it was hypothesized that E3 inhibits p38 activation through a PKR-dependant mechanism. To address this possibility, PKR expression was suppressed in HeLa cells using PKR specific siRNAs and cells were then infected with either vvCopRev or vvCopΔE3L. PKR expression was significantly abrogated in cells transfected with PKR specific siRNAs and the phosphorylation of PKR was not detected (Fig. 10A). VvCopΔE3L-induced phosphorylation of p38 was blocked in PKR siRNA treated cells (Fig. 10B). Furthermore, in cells treated with PKR siRNAs, the phosphorylation of MAPK-activated protein kinase 2 (MK2), a substrate of activated p38, was inhibited.

1.4 - NF-κB p65, NF-κB p50 and IRF3 contribute to vvCopΔE3L-induced cytokine expression. IPS-1 mediates IFN-β induction through activation of NF-κB and IRF3[99]. As shown in Figure 9C, IPS-1 mediates vvCopΔE3L-induced IFN-β expression. Therefore, the roles of NF-κB and IRF3 in the cytokine response to VACV were investigated. HeLa cells were transfected with siRNAs targeting IRF3 or the NF-κB p65 or p50 subunits and then infected with vvCopRev or vvCopΔE3L. The siRNA knockdown efficiency was determined by Western blotting (Fig. 11A). The expression of INHBA, IFN-β, TNF-α, and IL-6 was determined by RT-PCR (Fig. 11B). The

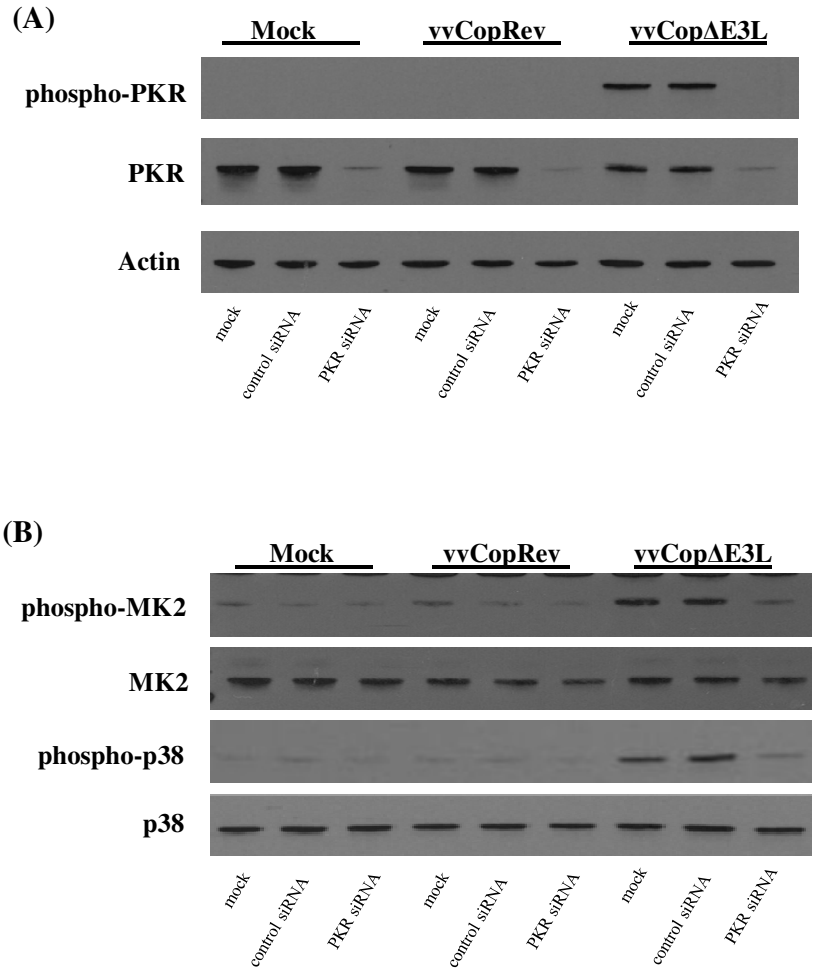

Figure 10: E3 inhibits p38 activation through a PKR-dependant mechanism. (A, B) $1.5x10^5$ HeLa cells were seeded in 6-well plates. Cells were then transfected with 100 nM PKR specific siRNAs or control siRNAs for 72 hours. Cells were then either mock infected or infected with vvCopRev or vvCopΔE3L at an MOI of 5 and collected at 6 hpi for Western blotting. Results are representative of three independent experiments.

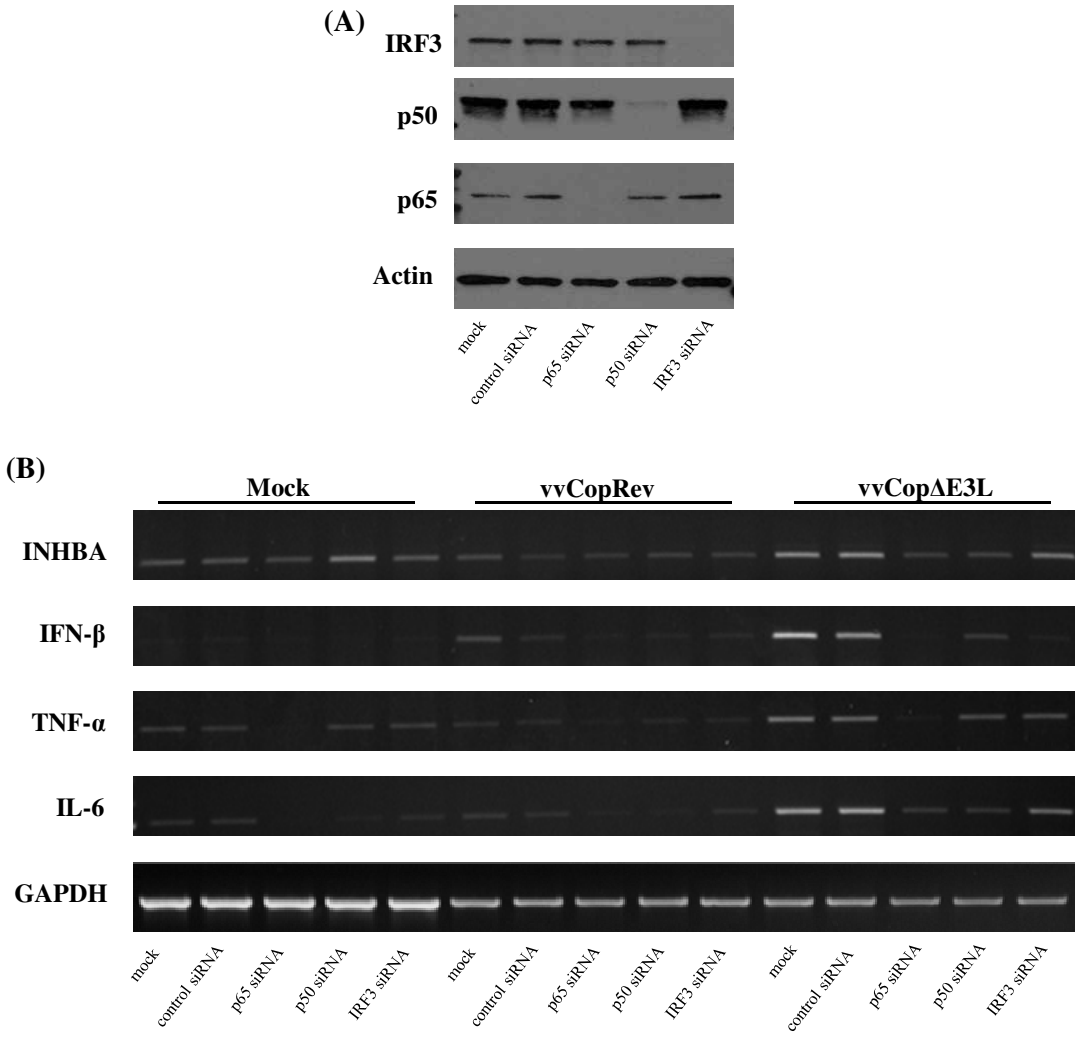

Figure 11: E3 suppresses cytokine expression through NF-κB and IRF3 mediated pathways. (A) $1.5x10^5$ HeLa cells were seeded in 6-well plates. Cells were then transfected with 100 nM control, NF-κB p65, NF-κB p50 or IRF3 specific siRNAs for 72 hours. Western blotting was performed to determine the efficiency of the siRNA knockdown. (B) Cells were transfected as above and then either mock infected or infected with vvCopRev or vvCopΔE3L at an MOI of 5. Cells were collected at 12 hpi and RNA was extracted for RT-PCR. Results are representative of three independent experiments.

expression of INHBA, IFN-β, TNF-α, and IL-6 was significantly reduced in vvCopΔE3L-infected cells treated with p65 siRNAs, while the expression of INHBA, IFN-β and IL-6, but not TNF-α, was reduced in cells treated with NF-κB p50 siRNAs. In contrast, the expression of IFN-β, but not INHBA or TNF-α, was significantly reduced in vvCopΔE3L-infected cells treated with IRF3 siRNAs. IL-6 expression was slightly reduced in vvCopΔE3L-infected cells treated with IRF3 siRNAs.

1.5 - PKR is required for NF-κB nuclear translocation during vvCopΔE3L infection.

Previous work has demonstrated that E3 can inhibit NF-κB nuclear translocation, but the role of PKR in this process was not determined[116]. Given that both PKR and NF-κB contribute to vvCopΔE3L-induced TNF-α and INHBA expression, the role of PKR in regulating NF-κB nuclear translocation, a hallmark of NF-κB activation, was investigated. HeLa cells were transfected with control or PKR specific siRNAs and infected with vvCopRev or vvCopΔE3L. Cytoplasmic and nuclear extracts were prepared at 8 hpi for Western blotting. As a control to determine fractionation efficiency, the level of histone deacetylase 2 (HDAC2), an enzyme localized primarily to the nucleus, was determined in both cytoplasmic and nuclear fractions. Considerably more HDAC2 was detected in the nuclear fraction in comparison to the cytoplasmic fraction (Fig. 12). Significant nuclear translocation of p65 was observed in vvCopΔE3L infected cells. However, p65 nuclear translocation was blocked in vvCopΔE3L infected cells treated with PKR siRNAs. In comparison to vvCopRev infected cells, more NF-κB p50 was present in the nucleus of mock and vvCopΔE3L infected cells. A reduced level of p50 was observed in mock and vvCopΔE3L infected cells treated with PKR siRNAs. In contrast, suppression of PKR expression did not affect the level of IRF3 in the nucleus.

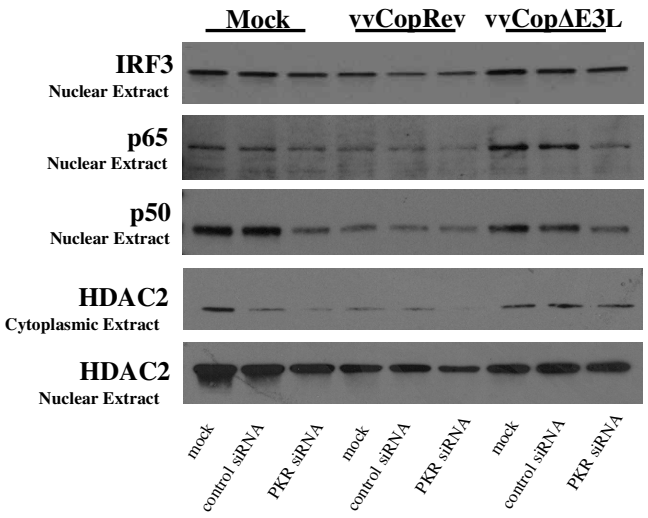

Figure 12: E3 antagonizes PKR-dependant NF-κB nuclear translocation. $1.5x10^5$ HeLa cells were seeded in 6-well plates. Cells were then transfected with 100 nM control or PKR siRNA for 48 hours. Cells were then mock infected or infected with vvCopRev or vvCopΔE3L at a MOI of 5 and nuclear extracts were prepared at 8 hpi for Western blotting. Results are representative of three independent experiments.

Section I discussion

Modulation of cellular signalling cascades is a common mechanism by which viruses suppress the host immune response[148]. Cytokines play a pivotal role in host defence against virus infection, in part through inducing expression of antiviral genes and recruiting immune cells to sites of infection. Although E3 has been reported to suppress cytokine expression, the signalling pathways regulating VACV-induced cytokine expression remain largely unknown. In this study, the molecular mechanisms by which the VACV E3 protein inhibits cytokine expression were investigated. E3 was found to suppress cytokine induction by antagonizing several distinct but interlinked signalling cascades mediated by PKR, p38 and IPS-1, as well as the transcription factors NF-κB and IRF3. Although E3 has been previously shown to antagonize both p38 and NF-κB activation[116], as well as cytokine expression[53], no direct evidence has been provided to link these processes to its inhibition of PKR. In this study, E3 was found to regulate cytokine expression in infected cells by modulating PKR-dependent activation of p38 and NF-κB. Furthermore, E3 was found to inhibit IPS-1-dependent expression of IFN-β, implicating E3 in modulation of RLR signalling. These results help to delineate the signalling pathways targeted by E3 to block expression of distinct cytokines.

Using real time PCR arrays, a subset of cytokines whose expression is inhibited by E3 were identified, and include members of the IFN, IL, TNF and TGF cytokine families (Table 7). The requirement of the Z-DNA binding and dsRNA binding domains of E3 for this inhibitory function was investigated by the use of viruses expressing E3 truncations. The Z-DNA binding domain of E3 appears to play some role in the inhibition of cytokine expression since cells infected with vvCopE3LΔN expressed

slightly higher levels of IFN-β, TNF-α and INHBA than cells infected with vvCopRev (Fig. 8C). The Z-DNA binding domain of E3 has been demonstrated to play a role in PKR inhibition at late times post-infection[116]. Thus, the moderate induction of cytokines by the vvCopE3LΔN virus may result from incomplete inhibition of PKR or other host antiviral factors. However, deletion of the terminal 25 amino acids of the dsRNA binding domain of E3 completely abolished the inhibition of cytokine expression. Therefore, the dsRNA binding domain is the primary domain of E3 required to suppress expression of IFN-β, TNF-α, IL-6 and INHBA and likely other cytokines during VACV infection. Since mRNA levels often do not correlate with protein levels, an attempt was made to quantify IFN-β protein by ELISA. However, in HeLa cells, very little IFN-β protein was detected following virus infection (data not shown). This could be due to the cell death and overall cytopathic effect induced by VACV infection, in particular vvCopΔE3L, which may destroy the cells before cytokine protein can be produced and secreted. Therefore, cytokine expression could not be quantified at the protein level.

Poxvirus genomes are transcribed via a cascade-like mechanism, with the transcription of early genes occurring prior to viral DNA replication, while intermediate and late genes are transcribed only after viral DNA replication has commenced[24]. Several studies have demonstrated that VACV intermediate and late gene transcripts are the major source of viral dsRNA generated in VACV infected cells[19,46,131]. VACV-induced cytokine expression was differentially sensitive to araC treatment, a potent inhibitor of DNA replication, and therefore intermediate and late gene transcription (Fig. 8A). AraC treatment abolished vvCopΔE3L-induced IFN-β expression, suggesting that a PAMP associated with the intermediate/late phase of virus infection ("late" PAMP) is essential for the induction of IFN-β. In contrast, IL-6 induction is not sensitive to araC treatment,

while TNF-α and INHBA expression are partially sensitive. Thus, a PAMP(s) associated with the early phase of replication ("early" PAMP) is capable of inducing IL-6, TNF-α and INHBA expression. Since araC blocks intermediate/late gene transcription, a process known to form dsRNA, it is likely that the observed induction of IFN-β is due to dsRNA species produced during virus infection. The role of other PAMPs in activation of IL-6, INHBA and TNF-α expression cannot be ruled out, as induction of these cytokines is not completely blocked by araC treatment during vvCopΔE3L infection. Thus, it is possible that other components of VACV, such as the dsDNA genome, also function as PAMPs to activate cytokine expression.

Treatment of cells with araC blocked vvCopΔE3L-induced PKR phosphorylation (Fig. 9A). Although this would be expected to block PKR kinase activity, it is unlikely that araC affects any purely structural roles of PKR. PKR is required for full expression of IL-6 (Fig. 9C), yet IL-6 expression is not affected by araC treatment (Fig. 8A). Therefore, PKR may play a structural role in regulating IL-6 expression, independent of its catalytic activity. It has been reported that catalytically inactive PKR mutants can still activate NF-κB under some conditions[42,86]. This may explain why IL-6 induction, which requires PKR for full expression, is unaffected by araC treatment, even though araC blocks PKR phosphorylation. Taken together, these results suggest that E3 is capable of suppressing expression of cytokines induced by both "early" and "late" PAMPs.

The signalling pathways which regulate cytokine expression following infection of cells with vvCopΔE3L were investigated. PKR was found to be required for vvCopΔE3L-induced TNF-α, INHBA, and to lesser degree, IL-6 and IFN-β gene transcription (Fig. 9C). Experiments based on siRNA-mediated knockdown of PKR provide direct evidence that PKR is required for p38 activation in response to

vvCopΔE3L infection (Fig. 10B). Efficient induction of INHBA requires both PKR and p38, and thus PKR-mediated p38 activation likely plays a key role in the regulation of this cytokine. Although E3 has been reported to inhibit p38 activation[116], these results demonstrate that E3 blocks INHBA induction by antagonizing PKR-dependant p38 activation.

In addition to PKR and p38, INHBA expression also requires NF-κB. E3 was found to suppress PKR-dependant NF-κB nuclear translocation (Fig. 12). P38 is also known to regulate NF-κB phosphorylation and transcriptional activity[91,252]. Infection of cells with vvCopΔE3L, but not vvCopRev, resulted in PKR-dependant activation of p38 (Fig. 10B). Thus, the induction of INHBA in vvCopΔE3L infected cells may potentially be explained by the activation of p38 signalling and its regulation of NF-κB function. It was also observed that MK2, a substrate of activated p38, was phosphorylated to its active state in a PKR-dependant manner in cells infected with vvCopΔE3L. Activation of the p38-MK2 pathway can enhance the stability of cytokine mRNAs harbouring adenine/uridine (A/U) rich elements within the 3' UTR[108,117]. Therefore, it is also possible that the stability of INHBA mRNA, which does contain A/U rich sequences in the 3' UTR, is enhanced by p38-MK2 activity and this may explain the increase in INHBA mRNA levels in vvCopΔE3L infected cells. Collectively, these results are the first to provide direct evidence of the mechanism by which E3 suppresses p38 activation and NF-κB nuclear translocation in response to VACV infection and how these events modulate cytokine expression.

Efficient induction of TNF-α expression requires PKR and NF-κB, but not p38 (Fig. 9C, 11B). Therefore, the pathway(s) regulating TNF-α expression is distinct from that for INHBA. PKR has been demonstrated to activate the IKK complex and NF-κB

dependant transcription[251]. The N-terminus of PKR is responsible for the interaction with, and activation of, IKKβ and NF-κB[18]. Thus, PKR-dependant NF-κB activation in the absence of E3 likely contributes to TNF-α expression during vvCopΔE3L infection. That TNF-α induction only requires p65, while INHBA requires both p65 and p50 NF-κB subunits demonstrates that several unique NF-κB complexes are formed in response to vvCopΔE3L infection and these different complexes regulate distinct cytokines. This finding supports the concept that several distinct, but interlinked pathways regulate the cytokine response to VACV. A surprising number of VACV proteins in addition to E3 are capable of inhibiting NF-κB[39,58,70,76,219]. Even though the vvCopΔE3L virus expresses these NF-κB inhibitors, NF-κB nuclear translocation was still observed following infection, in agreement with previous reports[116]. Therefore, these proteins are unable to fully inhibit the activation of NF-κB in cells infected with vvCopΔE3L. It is possible that PKR activation mediated by vvCopΔE3L signals to NF-κB through a mechanism not targeted by the alternate VACV NF-κB inhibitors.

The pathway mediating IL-6 and IFN-β expression differs from the TNF-α and INHBA pathways in several ways. First, induction of IL-6 and IFN-β gene transcription is less dependent on PKR than is expression of TNF-α and INHBA (Fig. 9C). Second, activation of the IFN-β pathway is highly sensitive to inhibition of viral DNA replication, whereas activation of the IL-6 pathway is unaffected by inhibition of viral DNA replication (Fig. 8A). Under the conditions used in these studies, very little IRF3 appears to translocate to the nucleus in vvCopΔE3L infected cells, even though IFN-β expression is highly up-regulated. It was recently reported that PKR mediates IRF3 nuclear translocation in response to vvCopΔE3L[255]. The explanation for this conflict remains unclear and could be due to experimental conditions. However, the results described

herein are supported by the finding that loss of PKR in mouse cells does not affect the induction of IFN-β by vvCopΔE3L[243]. Moreover, E3 inhibits NDV-induced IRF3 phosphorylation in a PKR-independent manner[224]. Similar to a recent report in murine cells[54], E3 was also found to inhibit IPS-1-dependent IFN-β expression (Fig. 9C). In mouse keratinocytes, E3 also inhibits IL-6 expression through an IPS-1-dependant mechanism[54]. These findings are in contrast to the results reported here wherein IPS-1 was dispensable for IL-6 expression. This discrepancy may be due to differences in species and tissue specific immune responses, and/or the method of gene silencing.

A large complex of proteins termed the IFN-β enhanceosome is required for full transcriptional activation of the IFN-β gene[175]. This complex consists of IRF3, IRF7, NF-κB p65, NF-κB p50, CBP, ATF-2 and c-Jun[241]. The promoter region of the IFN-β gene contains four positive regulatory domains (PRD), each of which is bound by distinct transcription factor components of the enhanceosome. The PRDs coordinate the sequential recruitment of transcription factors into the enhanceosome structure following virus infection. For example, the NF-κB p50 and p65 bind to PRDII, while IRFs (including IRF3) bind to PRDI and PRDIII. Downstream of IPS-1, activation of both IRF3 and NF-κB participate in IFN-β expression following RLR activation[99]. In the context of vvCopΔE3L infection, the combined function of NF-κB p65 and IRF3 is essential for activation of IFN-β gene transcription, as siRNA-mediated silencing of either gene alone completely abolished IFN-β expression (Fig. 11B). The effect of suppressing p50 expression on IFN-β gene transcription was not as drastic as compared to p65 silencing. This could be due to the finding that p50, unlike p65, is constitutively bound to the IFN-β promoter, which may stabilize p50 protein levels in the cell[241]. Overall, these results demonstrate that E3 suppresses the signalling mediated by multiple components of

the IFN-β enhanceosome in order to block IFN-β expression. That IFN-β was the only cytokine tested which requires IRF3 expression again demonstrates that unique pathways mediate VACV-induced cytokine expression.

In summary, the VACV E3 protein can suppress a diverse array of cytokines (including members from IL, IFN, TNF and TGF families) whose induction involves both "early" and "late" PAMPs associated with VACV infection. Furthermore, the distinct, but interlinked signalling pathways regulating the induction of these cytokines during virus infection were characterized. PKR, p38 and NF-κB were found to play important roles in regulating VACV-induced cytokine expression. Moreover, PKR was required for the activation of p38 signal transduction and NF-κB nuclear translocation. These results highlight the role of E3 in suppressing these cellular signalling pathways to limit cytokine expression associated with both "early" and "late" PAMPs. A largely PKR-independent response mediated by IPS-1, NF-κB and IRF3 was found to regulate VACV-induced IFN-β expression associated with a "late" PAMP. As VACV E3 and its orthologues in other orthopoxviruses are highly conserved, these findings should contribute to the understanding of the strategies used by orthopoxviruses to suppress the host immune response. In addition, the results described in this study may aid in the development of safer, more effective VACV-based recombinant vaccines.

<u>Section II</u> - ***RNA species generated in vaccinia virus infected cells activate cell type-specific MDA5 or RIG-I-dependant interferon gene transcription and PKR-dependant apoptosis (Virology, 413: 183-93)***

Rationale, hypothesis and specific objectives

RNA species produced during virus replication are PAMPs triggering cellular innate immune responses including induction of type I IFN expression and apoptosis[148]. PRRs for these RNAs include RIG-I, MDA5 and PKR. While the interaction between RNA-based PAMPs of RNA viruses and their cellular PRRs are well characterized, comparatively less is known about the role of RNA species generated by DNA viruses as PAMPs. While RNAs generated in poxvirus-infected cells have been hypothesized to function as PAMPs, there is no direct evidence to support this claim. Understanding the role of poxvirus RNAs in stimulating innate immune responses will help to define how VACV and other poxviruses interact with the immune system. This knowledge will be important for the design of novel strategies to inhibit poxvirus replication. Moreover, the development of poxvirus-based recombinant vaccine vectors and oncolytic agents remain active and promising fields of research. Optimizing the efficacy of such vaccines and oncolytic agents will require a greater understanding of the interplay between poxviruses and the immune system[183].

<u>The hypothesis that drives this work is that RNA species generated in VACV infected cells are PAMPs triggering PRR-mediated innate immune responses.</u>

The specific objectives of this work are to; **(A)** Determine whether RNA species generated in VACV infected cells function as PAMPs which can activate innate immune responses, including induction of cytokine expression and the activation of apoptosis, **(B)**

Analyze the role of host PRRs RIG-I, MDA5 and PKR in triggering cytokine expression and apoptosis in response to VACV RNA species and **(C)** Analyze the role of host PRRs RIG-I, MDA5 and PKR in triggering cytokine expression and apoptosis in response to E3L deficient VACV.

2.1 - RNA species generated in VACV infected cells activate expression of IFN-β and a select group of pro-inflammatory cytokines. It is generally believed that transcription of poxvirus intermediate and late genes can produce viral dsRNA species [19,46,131] which activate host PRRs to stimulate innate immune responses. However, the direct role of RNAs generated in the course of VACV infection in the activation of innate immune responses has not been confirmed. It was hypothesized that if VACV RNAs are PAMPs, then isolation of total RNAs from VACV infected cells and introduction of these RNAs back into cells should result in activation of innate immune responses. To investigate this, HeLa cells were infected with vvCopRev or vvCopΔE3L at an MOI of 5 and RNA was collected at 2 hpi (early RNA) and 8 hpi (late RNA). A set of cells was also pre-treated prior to infection with araC to inhibit viral DNA replication and intermediate/late gene transcription and RNA was isolated at 8 hpi (late RNA + araC). This sample therefore contains mainly poxvirus early RNA species in addition to cellular RNAs. RNA was also isolated from mock infected HeLa cells (HeLa RNA) and therefore only contains cellular RNA. All RNA samples were digested with DNase to remove DNA contamination.

The ability of these RNA preparations to stimulate cytokine expression was analyzed using real time PCR arrays following transfection of HeLa cells with 3 µg of each RNA sample. The relative expression of 84 cytokines representing members of the

IFN, IL, TNF, TGF and PDGF/VEGF super-families were determined. Cytokines detected at a Ct value of ≥35 were scored as not significantly expressed and excluded from the analysis. Expression of the majority of the cytokines tested was not significantly induced by early or late RNA transfection (Table 8). However, robust transcriptional activation of several cytokine genes was observed in cells transfected with RNA isolated from late-stage VACV infections (late RNA). IFN-β expression was up-regulated 18.7-fold in cells transfected with late RNA as compared to uninfected total HeLa cell RNA. Similarly, TNF-α expression was up-regulated 11.4-fold in late RNA transfected cells. In contrast, late RNA isolated from araC treated cells (late RNA + araC) stimulated IFN-β and TNF-α expression comparably to HeLa RNA and early RNA. Additionally, several members of the IL family were transcriptionally activated by late RNA including IL-20, IL-8, IL-13 and IL1A. In general, transfection of cellular RNA alone, early RNA and late RNA isolated from araC treated cells poorly activated cytokine gene transcription. However, expression of several cytokines such as IFN-β, were also induced to some degree by early RNA transfection.

2.2 - Characterization of immuno-stimulatory RNA generated in VACV infected cells. RNA-induced expression of IFN-β, TNF-α and INHBA was confirmed by RT-PCR (Fig. 13A). Cells were also transfected with 1 μg of pI or pIC, as ssRNA and dsRNA controls, respectively. Transfection of HeLa cells with pIC, but not pI, resulted in increased IFN-β, TNF-α and INHBA expression. In agreement with the real time PCR array data, transfection of HeLa cells with RNA from mock infected HeLa cells only slightly up-regulated cytokine expression. Early RNA isolated from either vvCopRev or vvCopΔE3L infected cells induced cytokine expression comparable to cellular RNA alone. In contrast, late RNA transfection resulted in a significant induction of IFN-β and

Table 8: RNA species generated during VACV infection stimulate cytokine expression

Cytokines	*HeLa RNA*	*Early RNA*	*Late RNA*	*Late RNA + araC*
Interferon family				
IFN-β1	5.79	10.37	108.04	5.79
IFN-γ	1.73	4.44	2.21	2.91
IFN-κ	2.02	2.41	2.72	2.14
IFN-α8	2.13	1.62	2.66	-1.06
IFN-α2	1.73	1.76	2.21	6.29
IFN-α1	1.76	2.20	1.91	1.50
Interleukin family				
IL19	9.07	19.64	19.43	5.47
IL20	1.65	5.92	14.37	3.69
IL1F7	2.0	6.44	11.78	5.28
IL6	3.58	6.15	11.55	2.27
IL8	2.70	4.05	10.9	1.90
IL1A	2.32	3.99	10.46	1.67
IL22	1.73	9.55	4.36	2.91
IL13	1.54	1.05	8.46	1.75
IL1B	1.83	4.11	4.18	1.34
IL12A	1.46	1.67	2.86	3.29
IL5	1.73	1.76	3.21	2.91
IL1F9	1.73	2.39	2.21	2.91
IL17C	1.82	1.76	2.21	2.91
IL11	1.52	1.96	2.66	1.64
IL21	-1.16	2.16	1.09	2.26
IL7	1.46	2.24	2.09	1.18
IL24	1.63	2.16	2.10	1.70
IL15	1.46	1.85	2.12	1.64
IL10	1.29	1.56	1.83	1.42
IL18	1.26	1.42	1.66	1.33
TXLNA	1.26	1.61	1.41	1.24
IL12B	-1.15	1.07	1.40	1.08
IL17B	-1.34	-1.32	-1.05	1.24
Tumour-necrosis factor family				
TNF-α	3.15	6.19	35.98	1.58
LTB	1.73	1.76	3.68	2.91
TNFSF11	2.09	1.23	1.37	3.27
LTA	-1.05	1.92	1.20	3.07
TNFSF10	2.74	1.99	2.45	1.35
TNFSF12	-1.18	1.76	1.90	2.43
TNFSF13	1.26	1.81	1.36	1.52
TNFSF13B	1.35	1.10	-1.21	1.40
CD70	1.05	1.74	1.40	1.31
TNFRSF11	1.04	1.16	1.20	-1.07
TNFSF14	1.00	-1.10	-1.17	-1.18

Cytokines	*HeLa RNA*	*Early RNA*	*Late RNA*	*Late RNA + araC*
Transforming growth factor family				
BMP4	-6.56	1.67	2.18	1.45
INHBA	1.63	2.68	4.06	1.64
INHA	1.87	4.62	2.21	2.91
GDF9	3.53	3.35	2.11	1.69
MSTN	1.73	1.76	2.21	3.21
GDF5	1.73	1.76	2.21	3.16
NODAL	1.08	1.10	1.38	3.05
BMP3	1.72	1.70	2.14	2.82
BMP2	1.41	2.58	1.85	1.14
GDF2	2.51	1.19	1.66	1.98
TGFA	1.26	1.54	1.40	2.18
TGFB2	1.42	1.76	2.12	1.60
BMP5	-1.31	-1.30	1.04	1.84
GDF11	1.27	1.64	1.1	1.11
TGFB1	1.25	1.57	1.33	1.21
TGFB3	1.16	1.45	1.53	1.28
BMP8B	-1.04	-1.05	1.46	1.34
BMP1	-1.09	1.21	1.00	-1.06
BMP6	-1.13	-1.55	-1.07	1.02
Platelet-derived/vascular-endothelial growth factor family				
FIGF	1.30	1.33	2.02	2.36
PDGFA	1.32	1.54	1.51	1.33
Others				
FAM3B	1.73	2.2	13.1	2.91
CSF2	1.60	2.59	8.01	1.15
CSF1	1.21	1.65	1.73	1.31

HeLa cells were mock transfected or transfected with 3 µg of the indicated RNA sample isolated from vvCopRev infected HeLa cells. Cells were collected at 6 hpt and the expression of cytokines was determined by real time PCR arrays. Shown is the fold change in gene expression in comparison to mock transfected cells. Results are representative of three independent experiments.

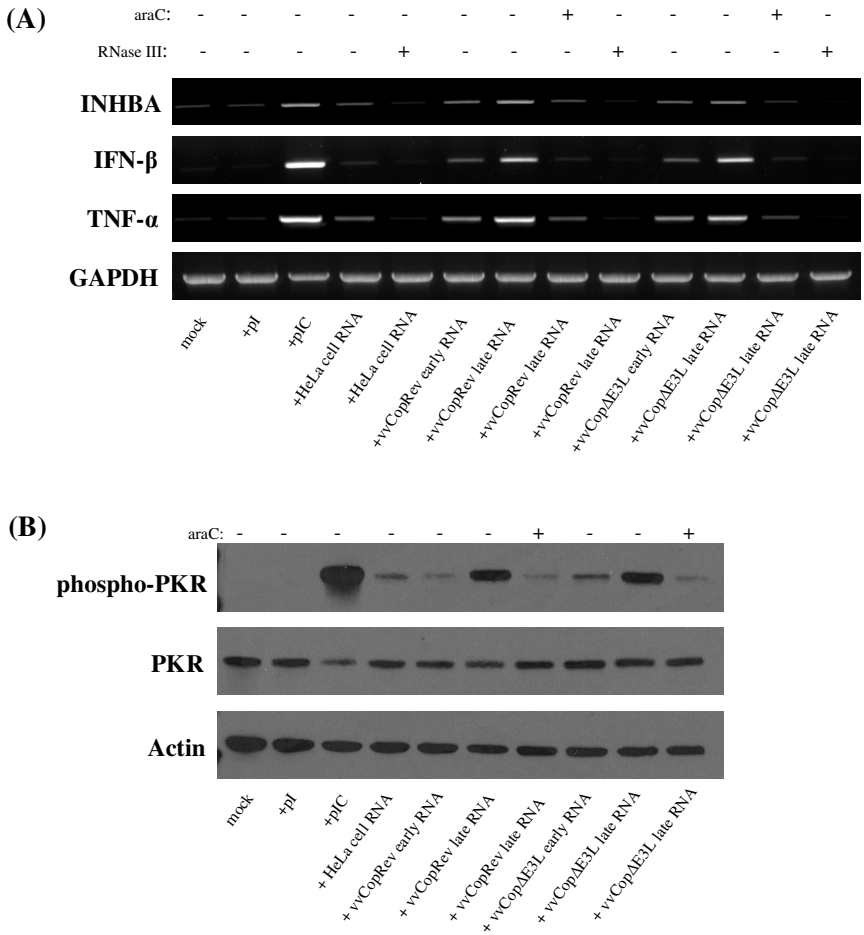

Figure 13: Induction of cytokine expression and PKR phosphorylation by RNA species generated during VACV infection. (A) Early, late and late RNA+ araC RNA samples were isolated from vvCopRev and vvCopΔE3L infected HeLa cells. HeLa cells were transfected with 5 µg of the indicated RNA samples, or 1 µg of pI or pIC. A set of RNA samples was also digested with RNase III prior to transfection. Cells were collected at 6 hpt and cytokine expression was examined by RT-PCR. (B) HeLa cells were transfected with the RNA samples described in (A) and collected at 6 hpt. The levels of actin, PKR and phosphorylated PKR were examined by Western blotting.

TNF-α expression. Late RNA isolated from either vvCopRev or vvCopΔE3L infected cells stimulated cytokine expression to a comparable degree. In comparison to IFN-β and TNF-α, INHBA expression was induced to a lesser degree by both pIC and late RNA, in agreement with the real time PCR array data. Again, transfection of late RNA isolated from araC treated cells induced cytokine expression to levels comparable to that observed for transfection of cellular RNA or early RNA. To investigate the structural requirements of the RNA for the stimulation of cytokine expression, a set of RNA samples was digested with RNase III which degrades dsRNA. Digestion of RNA samples with RNase III abolished the observed induction of cytokines. An attempt was also made to determine whether IL-6 expression could be induced by transfection of cells with late RNA. However, the results were inconclusive as IL-6 was induced more strongly by late RNA, but the induction of IL-6 could not be completely blocked by RNase III or RNase A digestion (data not shown). The activation of PKR was also examined following transfection of the above RNA samples into HeLa cells (Fig. 13B). Minimal PKR activation was observed in cells transfected with cellular RNA, early RNA, or RNA isolated from late stage infections in araC treated cells. In contrast, robust PKR activation was observed in cells transfected with late RNA isolated from either vvCopRev or vvCopΔE3L infected cells.

Digestion of VACV late RNA with RNase III abolished the induction of IFN-β by the various RNA preparations. The requirement for ssRNAs was examined next by digesting late RNA with RNase A under conditions which specifically degrade ssRNA but not dsRNA. As controls, *in vitro* transcribed ssRNA and dsRNA of approximately 800 bp corresponding to the coding sequence for EGFP were also digested. Using these conditions, ssEGFP RNA, but not dsEGFP RNA, was selectively degraded by RNase A

(Fig. 14A). The two prominent RNA bands present in the mock digested late RNA sample represent the 28S and 18S ribosomal ssRNAs. The 28S and 18S RNAs appeared to be completely degraded by RNase A digestion. Therefore, it is likely that most, if not all ssRNA in the late RNA sample was digested. Digestion of late RNA with RNase A did not abolish the stimulatory potential of the RNA sample on IFN-β induction (Fig. 14B). Virus-specific RNA bands were not detected in the late RNA sample on the RNA gel. This is likely because viral RNA species produced in VACV infected cells are very diverse in size and structure[19]. Therefore, viral RNAs would be expected to be present throughout the lane of the gel and not observed as any discreet band.

To further characterize these RNA preparations, the presence of VACV early, intermediate and late gene transcripts were analyzed in each sample. RT-PCR was used to detect VACV D12 (early), A1 (intermediate) and A17 (late) transcripts (Fig. 14C). As expected, D12 was detected in all viral RNA samples. A1 transcripts were detected in each viral RNA sample, with higher levels detected in late RNA as compared to early RNA and late RNA collected from araC treated cells. Although expression of the intermediate gene A1 should be blocked in araC treated VACV-infected cells, it is possible that a run-on transcript under the control of an early promoter proceeds through the A1 locus, resulting in the band detected in the late RNA + araC sample. Several such run-on transcripts are expressed from the genomic region adjacent to the A1 gene[245]. It is also possible, given the sensitivity of PCR, that even minute amounts of A1 expressed in the presence of araC would be detectable. A17 transcripts were detected in late RNA samples, but were not detected in early RNA preparations or late RNA isolated from araC treated cells.

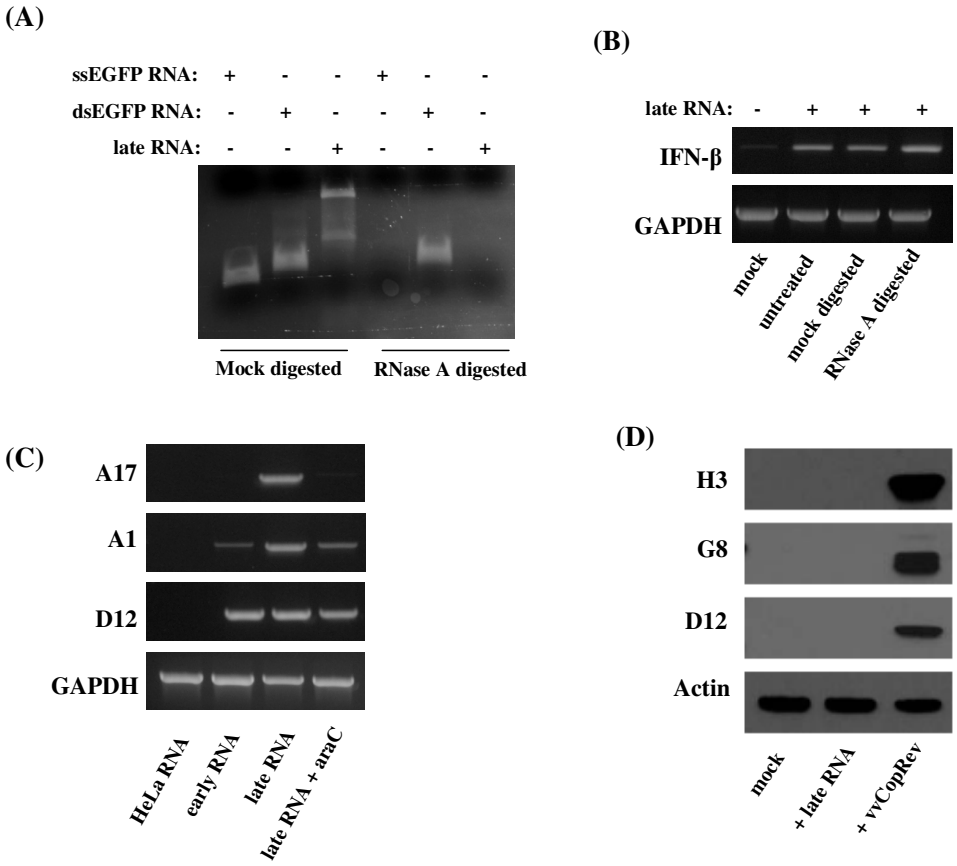

Figure 14: Characterization of immuno-stimulatory RNA isolated from VACV infected HeLa cells. (A) 3 µg of ssEGFP RNA, dsEGFP RNA and late RNA (from vvCopRev-infected cells) were incubated at 37^{o}C for 1 hour in RNase A digestion buffer in the absence (mock digested) or presence of 100 ng of recombinant RNase A (RNase A digested). Electrophoresis of the digested samples was carried out on an RNA gel. (B) Late RNA (3 µg) that was mock digested or digested with RNase A, along with untreated late RNA, was transfected into HeLa cells. IFN-β expression was measured by RT-PCR at 6 hpt. (C) Expression of VACV early (D12), intermediate (A1) and late (A17) transcripts was measured by RT-PCR in each RNA preparation. (D) HeLa cells were left untreated, or were transfected with 3 µg of late RNA or infected with vvCopRev. The expression of H3, G8, D12 and actin was analyzed by Western blotting at 8 hpi/hpt.

It is possible that proteins are being translated from transfected viral mRNAs and are responsible for the observed activation of cytokine expression. To address this, cells were transfected with late RNA or infected with vvCopRev. The expression of VACV D12 (early), G8 (intermediate) and H3 (late) proteins was analyzed by Western blotting. Expression of these proteins was readily detectable in cells infected with vvCopRev (Fig. 14D). However, these proteins were not detected in cells transfected with late RNA, even with a long exposure of the Western blot.

Next, it was tested whether the cell-type in which the infections were done, and the RNA was collected from, could affect the ability of the isolated RNA to induce IFN-β expression and activate PKR. Total RNA was isolated from vvCopRev infected hamster BHK21 in an identical manner to the extracts described from human HeLa cells. RNAs isolated from HeLa and BHK21 cells were transfected back into HeLa cells and the induction of IFN-β was determined by RT-PCR (Fig. 15A). The expression of GAPDH was measured as a loading control. Late RNA isolated from either infected HeLa and BHK21 cells activated IFN-β gene transcription to a similar degree. The activation of PKR, as measured by phosphorylation of Thr-446, was determined by Western blotting in HeLa cells transfected with the RNA preparations isolated from HeLa and BHK21 cells. Activated PKR was only detected in cells transfected with late RNA, regardless of the cell-type from which the RNAs were isolated (Fig. 15B). Again, no difference in the ability of RNA isolated from infected BHK21 or HeLa cells to stimulate PKR activity was observed. Furthermore, increased phosphorylation of eIF2α was detected in late RNA transfected cells, correlated with the activation of PKR.

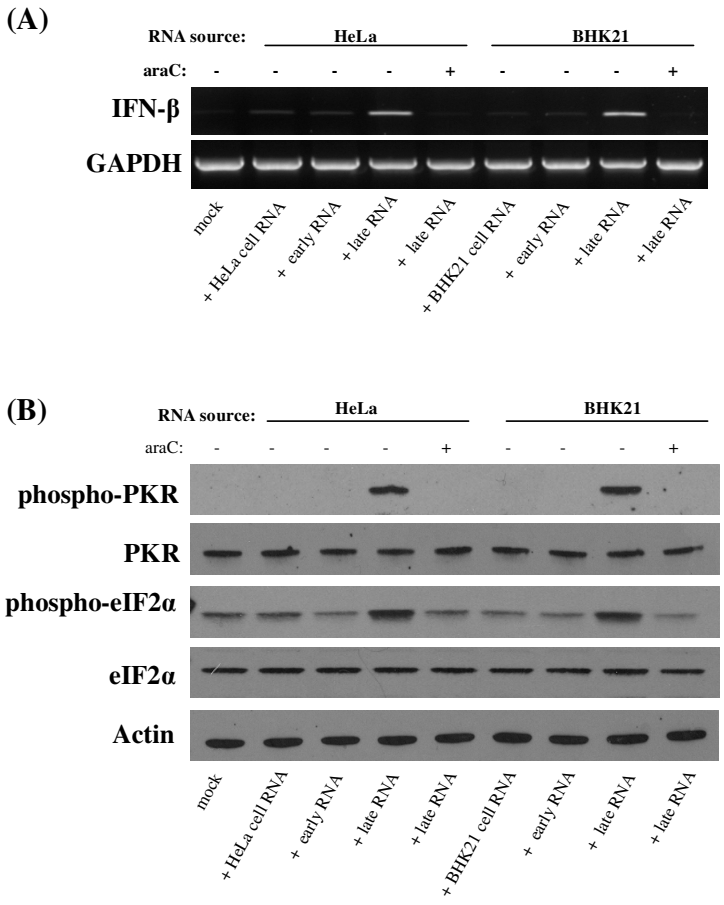

Figure 15: Total RNA isolated from VACV infected HeLa and BHK21 cells stimulate comparable IFN-β expression and PKR activation. (A) Total RNA samples were isolated from vvCopRev infected HeLa and BHK21 cells. HeLa cells were transfected with 3 μg of the indicated RNA preparations isolated from either infected HeLa or BHK21 cells. IFN-β expression was measured by RT-PCR at 6 hpt. (B) Duplicate samples as described in (A) were also collected at 6 hpt for Western blotting.

2.3 - RNA species generated in VACV infected cells can activate cell type-specific MDA5 or RIG-I-dependant IFN-β expression. Next, the host PRRs which mediate late RNA-induced IFN-β gene transcription were investigated. Following the finding that VACV RNAs activate PKR and cytokine expression, it was reported that RNA isolated from VACV infected cells induces IFN-β expression dependant on MDA5 in murine cells[182]. However, the role of RIG-I was not investigated. Thus, it remains to be determined if other PRRs can detect these RNA species. To this end, the role of both RIG-I and MDA5 in the induction of IFN-β by RNA isolated from VACV infected human HeLa cells was tested. It was first confirmed whether MDA5 and RIG-I-dependant signalling are functional in HeLa cells. MDA5 and PKR have been shown to regulate EMCV-induced IFN-β expression[55,98]. Therefore, HeLa cells were infected with EMCV for 16 hours and total RNA was isolated by TRIzol extraction, followed by DNase digestion. HeLa cells were treated with MDA5, RIG-I or PKR specific siRNAs, or a combination of both MDA5 and RIG-I siRNAs to assess the effects of a double knockdown. As a negative control, cells were also treated with a mutant MDA5 siRNA (control siRNA) containing a 2 bp mismatch compared to the targeting MDA5 siRNA. The knockdown efficiency was determined 48 hpt by Western blotting (Fig. 16A). SiRNA-treated cells were then transfected with the RNA isolated from EMCV infected cells. At 6 hpt, RNA was collected for real time PCR. IFN-β induction in response to EMCV RNA was significantly reduced in MDA5 and PKR siRNA treated cells, but not in RIG-I siRNA treated cells (Fig. 16B).

The activity of RIG-I-dependant signalling was examined next. *In vitro* transcribed dsRNA has been shown to be a ligand activating RIG-I, but not MDA5[98]. HeLa cells treated with siRNA specific to MDA5, RIG-I or PKR or a combination of

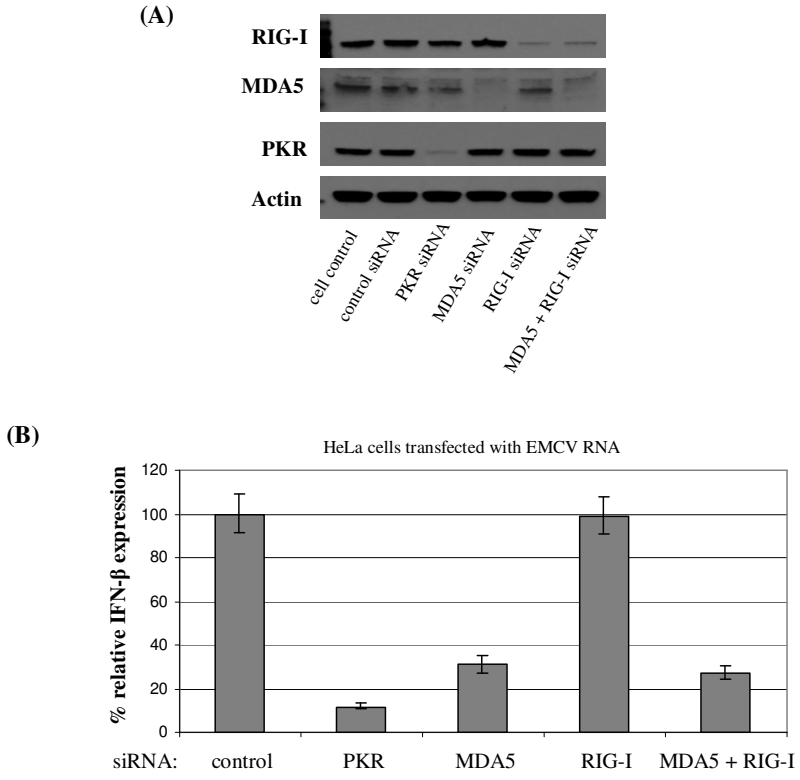

Figure 16: RIG-I and MDA5-dependant signalling are functional in HeLa cells. (A) $1.5x10^5$ HeLa cells were seeded in 6-well plates. Cells were then treated with 100 nM control, PKR, MDA5 or RIG-I siRNAs or a combination of MDA5 and RIG-I siRNAs for 48 hours. Western blotting was performed to determine the efficiency of the siRNA knockdown. (B) HeLa cells were transfected with siRNA as in (A) and then transfected with 100 ng of RNA from EMCV infected HeLa cells. IFN-β expression was measured by real time PCR 6 hpt. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

MDA5 and RIG-I siRNAs were transfected with *in vitro* transcribed dsEGFP RNA. IFN-β induction was significantly reduced in RIG-I siRNA treated cells, but not MDA5 or PKR siRNA treated cells (Fig. 16C). Thus, both MDA5 and RIG-I-dependant induction of IFN-β are functional in HeLa cells. Furthermore, the observed requirements for MDA5 and RIG-I in the response to EMCV RNA and *in vitro* transcribed RNA are in agreement with previous reports[98].

Next, the PRRs for RNA species generated in VACV infected cells were studied. HeLa cells were transfected with siRNAs targeting MDA5, RIG-I or a combination of MDA5 and RIG-I siRNAs for 48 hours. Cells were then transfected with VACV late RNA and collected 6 hpt to analyze IFN-β expression. In comparison to control siRNA treated cells, a significant reduction of IFN-β expression was observed in cells treated with MDA5 siRNAs, but not in RIG-I siRNA treated cells (Fig. 17). IFN-β expression was further reduced in cells with a combination of MDA5 and RIG-I knockdown compared to cells with a single MDA5 knockdown.

To determine whether MDA5 also functions as the primary sensor of VACV RNAs in a different human cell line, A549 cells were treated with MDA5 or RIG-I siRNAs, or a combination of both siRNAs. Cells were then transfected with late RNA. The knockdown efficiency was determined 48 hpt by Western blotting (Fig. 18A). Surprisingly, in contrast to HeLa cells, late RNA-induced IFN-β expression was significantly reduced in RIG-I siRNA treated A549 cells, even though RIG-I expression was only moderately reduced by siRNA treatment (Fig. 18B). Treatment of A549 cells with MDA5 siRNAs had no effect on IFN-β induction. A549 cells were also transfected with RNA isolated from EMCV infected cells. Again, in contrast to the HeLa cells, IFN-

(C)

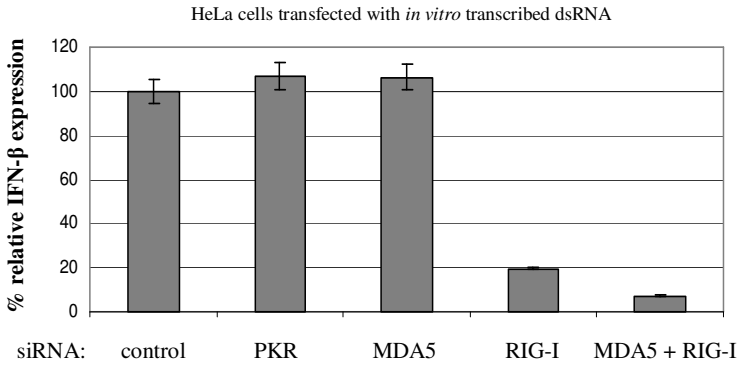

Figure 16: RIG-I and MDA5-dependant signalling are functional in HeLa cells. (C) HeLa cells were transfected with siRNA as in Fig. 16A and then transfected with 3 μg of *in vitro* transcribed dsEGFP RNA. IFN-β expression was measured by real time PCR 6 hpt. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

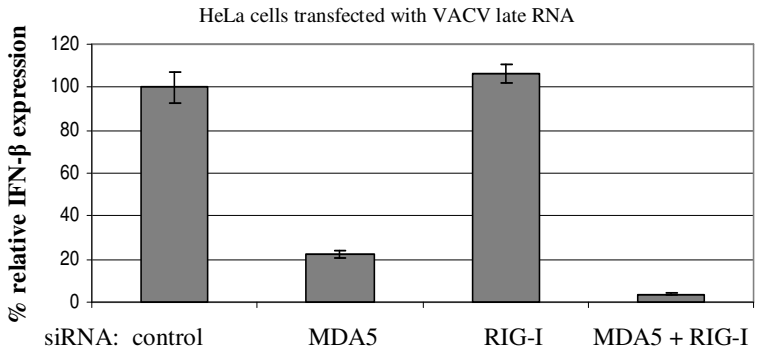

Figure 17: RNA species generated in VACV infected cells stimulate MDA5-dependent IFN-β expression in HeLa cells. HeLa cells were treated with siRNAs as described in Fig. 16A. Cells were then transfected with 3 μg of late RNA isolated from vvCopRev infected cells. IFN-β expression was quantified at 6 hpt by real time PCR. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

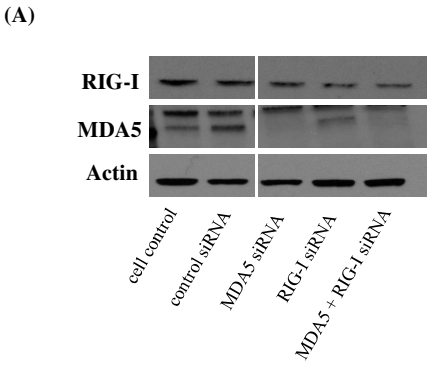

Figure 18: RNA species generated in VACV infected cells stimulate RIG-I-dependent IFN-β expression in A549 cells. (A) A549 cells were transfected with the indicated siRNAs for 48 hours. Western blotting was used to detect expression of RIG-I, MDA5 and actin. Results are representative of three independent experiments.

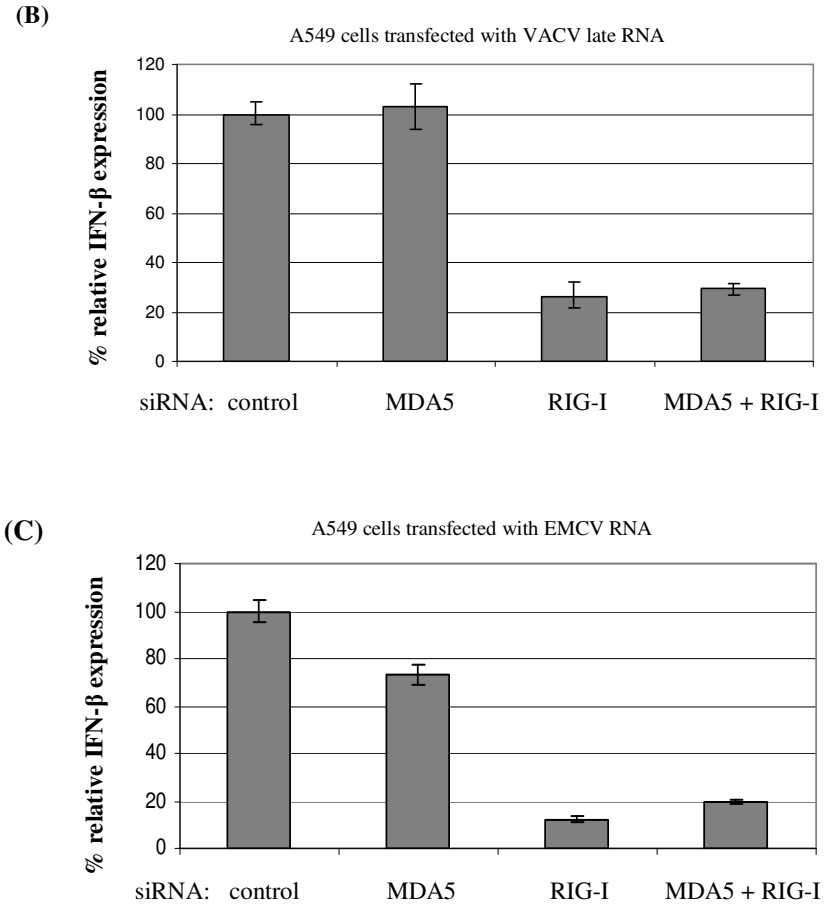

Figure 18: RNA species generated in VACV infected cells stimulate RIG-I-dependent IFN-β expression in A549 cells. (B) A549 cells were transfected with siRNAs as in Fig. 18A and then transfected with 3 μg of late RNA. The expression of IFN-β was quantified using real time PCR. (C) A549 cells were transfected with siRNAs as in Fig. 18A and then transfected with 100 ng of RNA from EMCV infected HeLa cells. The expression of IFN-β was quantified using real time PCR. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

β induction was significantly reduced in RIG-I siRNA treated cells and only moderately reduced in MDA5 siRNA treated cells (Fig. 18C).

2.4 - RNA species generated in VACV infected cells can activate MDA5 or RIG-I-dependant IFN-β expression in HeLa cells of different passage history. Given the contrasting roles of MDA5 and RIG-I in HeLa and A549 cells, additional variables which may affect the use of PRRs in HeLa cells were tested. Although the HeLa cells used for the above experiments have been passaged extensively in our laboratory, no phenotypic changes in cell morphology or papilloma virus E1, E6 and E7 expression have been observed. Nonetheless, fresh, low passage HeLa cells were ordered from ATCC. These low passage HeLa cells were treated with siRNAs and then transfected with late RNA. In the low passage HeLa cells, similar to A549 cells, suppression of RIG-I expression significantly reduced the induction of IFN-β, while suppression of MDA5 expression slightly reduced IFN-β expression (Fig. 19A). To our knowledge, the only difference between these cells and the HeLa cells used in the initial transfection experiments is passage history. Whether repeated passage of HeLa cells (fresh from ATCC) would result in phenotypic changes in the use of either RIG-I or MDA5 to sense VACV RNAs was tested. After approximately 20 passages of the cells, it was observed that late RNA-induced IFN-β expression was reduced in MDA5 siRNA treated cells, but not RIG-I siRNA treated cells (Fig. 19B) in a manner similar to the initial transfection experiments (Fig. 17 vs. Fig 19B).

It is likely that free dsRNA species, such as those isolated in VACV late RNA, contribute to IFN-β expression during vvCopΔE3L infection, wherein E3 is not present to sequester these RNAs, or inhibit their interaction with host PRRs. In Results Section I,

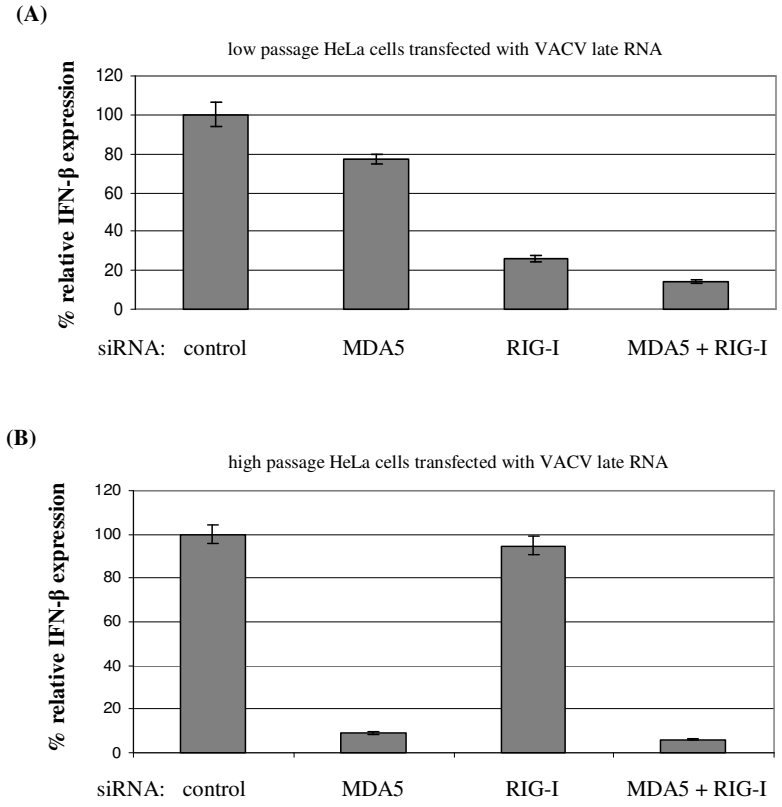

Figure 19: RNA species generated in VACV infected cells activate cell type-specific RIG-I or MDA5-dependant IFN-β expression in HeLa cells of different passage history. (A) Fresh, low passage HeLa cells obtained from ATCC were treated with the indicated siRNAs for 48 hours and then transfected with 3 μg of late RNA. The expression of IFN-β was quantified using real time PCR. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation. (B) The same HeLa cells used in (A) were passaged approximately 20 times in culture and then treated with the indicated siRNAs for 48 hours. Cells were then transfected with 3 μg of late RNA and expression of IFN-β was quantified using real time PCR. Error bars represent the mean, plus and minus one standard deviation.

the induction of IFN-β following infection of HeLa cells with vvCopΔE3L was found to be dependent on IPS-1, IRF3 and NF-κB p65/p50 expression. Given that IPS-1 is the major adaptor protein of RLR signalling, and the finding that MDA5 and RIG-I can contribute to VACV late RNA-induced IFN-β expression, the role of MDA5 and RIG-I during vvCopΔE3L infection was investigated. First, low passage HeLa cells were transfected with MDA5 or RIG-I siRNAs and then infected with vvCopΔE3L. The induction of IFN-β expression was measured by real time PCR. In comparison to control siRNA treated cells, IFN-β induction stimulated by vvCopΔE3L infection was slightly reduced in MDA5 siRNA treated cells and significantly reduced in RIG-I siRNA treated cells (Fig. 20A). Next, more extensively passaged HeLa cells, which displayed a MDA5-dependant phenotype for IFN-β induction following RNA transfection, were tested. When these HeLa cells were treated with siRNAs and infected with vvCopΔE3L, IFN-β induction was moderately reduced in MDA5 siRNA treated cells and more significantly reduced in RIG-I siRNA treated cells (Fig. 20B).

2.5 - Downstream components of the RLR pathway mediate IFN-β expression in response to RNAs generated in VACV-infected cells. The role of downstream components of the MDA5 and RIG-I pathways were also analyzed utilizing a siRNA knockdown approach. High passage HeLa cells were transfected with siRNAs targeting IPS-1, the adaptor of the MDA5 and RIG-I pathways, IRF3, NF-κB p65 or NF-κB p50. The knockdown efficiency was analyzed by Western blotting at 48 hpt (Fig. 21A). Cells treated with siRNA were then transfected with late RNA and collected 6 hpt to analyze IFN-β expression. The activation of IFN-β gene transcription, in comparison to control siRNA treated cells, was significantly reduced in cells treated with IPS-1, IRF3, and p65

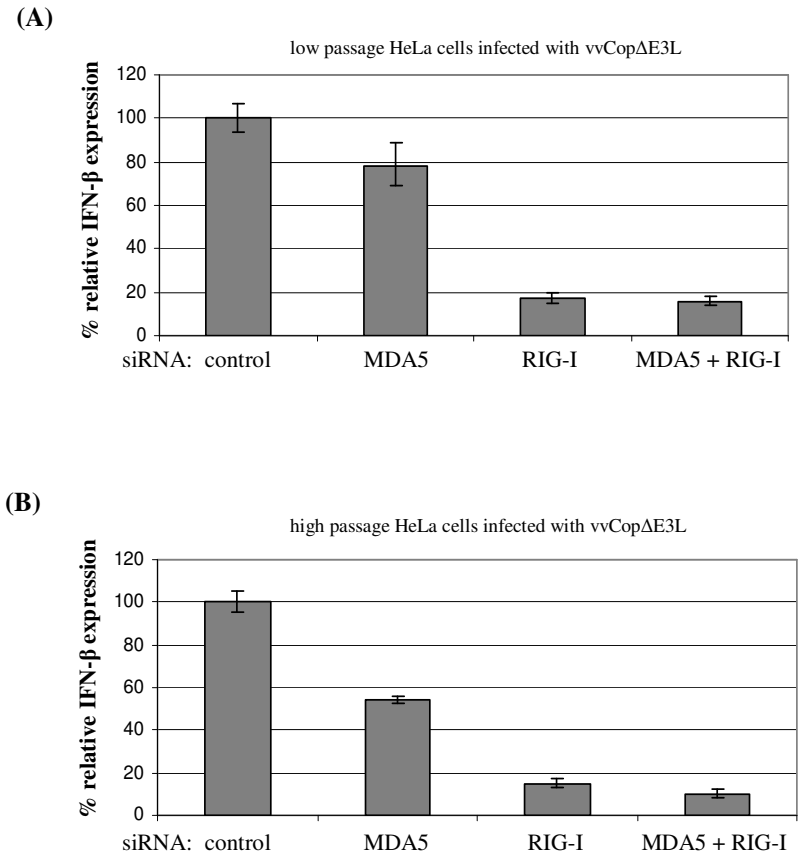

Figure 20: Both RIG-I and MDA5 mediate IFN-β expression during vvCopΔE3L infection. Low passage (A) or high passage (B) HeLa cells were treated with the indicated siRNAs for 48 hours. Cells were then infected with vvCopΔE3L at an MOI of 5 and the expression of IFN-β was quantified using real time PCR at 10 hpi. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

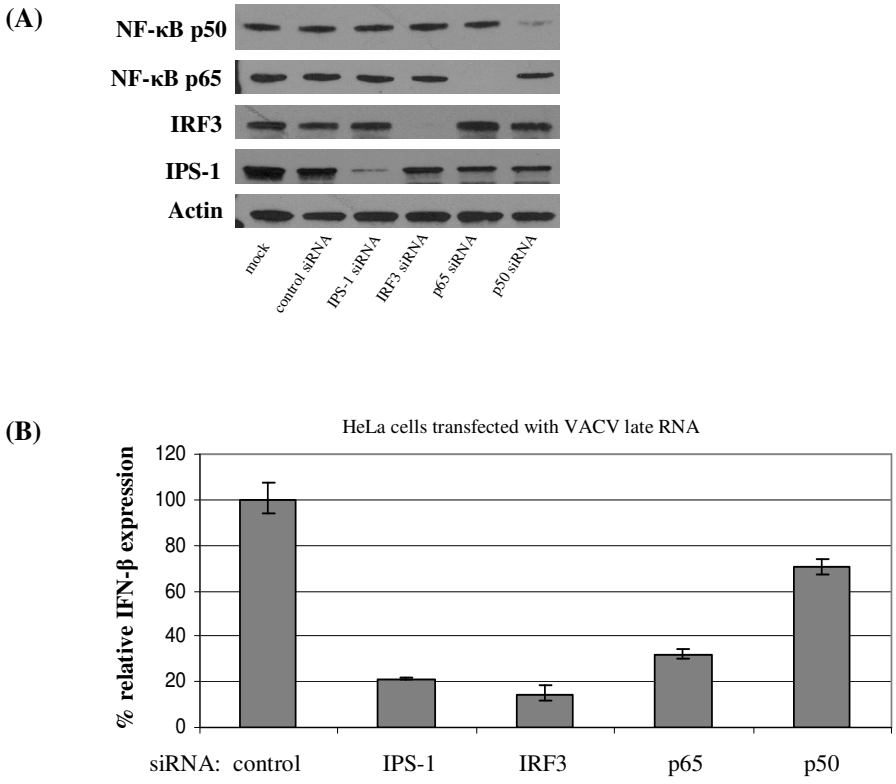

Figure 21: RNA species generated in VACV infected cells stimulate IPS-1, IRF3 and NF-κB p65-dependent IFN-β expression. (A) HeLa cells were treated with 100 nM control, IPS-1, IRF3, NF-κB p50 or NF-κB p65 specific siRNAs for 48 hours. Western blotting was performed to determine the efficiency of the siRNA knockdown. (B) Cells were treated as in (A) and transfected with 3 μg of late RNA. IFN-β expression was quantified by real time PCR at 6 hpt. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

siRNAs and moderately reduced in p50 siRNA treated cells (Fig. 21B). These results are very similar to the results obtained from vvCopΔE3L infection of HeLa cells (Fig. 9C, 11B).

2.6 - RNA species generated in VACV infected cells activate PKR-dependant apoptosis. Infection of cells with vvCopΔE3L, but not wild-type VACV, induces apoptosis[120] in a PKR-dependant manner[254]. Although this has been speculated to be a result of free dsRNA present during infection with vvCopΔE3L, no studies have directly confirmed the role of RNAs generated in VACV infected cells in inducing apoptosis. To address this possibility, RNA isolated from VACV infected HeLa cells was tested for its ability to activate apoptosis as measured by cleavage of caspase-7 and poly (ADP-ribose) polymerase (PARP). HeLa cells (high passage history) were transfected with the previously described RNA preparations. While all the RNA transfections resulted in a small increase in the cleavage of caspase-7 and PARP, significantly higher levels of cleaved caspase-7 and cleaved PARP were detected in cells transfected with VACV late RNA (Fig. 22A). The cleaved caspase-7 antibody does not detect full-length caspase-7 and therefore the full length form of the protein does not appear in the Western blot. While the PARP antibody does detect both the full length and cleaved forms, the band indicating the full-length protein was excluded. Detectable full-length PARP was reduced in cells where the cleaved form was detected (data not shown). RNA isolated from cells treated with araC and collected late during infection activated caspase-7 and PARP cleavage to a similar level as seen in HeLa RNA and early RNA-transfected cells.

Induction of apoptosis by these RNA preparations was then quantified using propidium iodide staining and flow cytometry to measure genomic DNA fragmentation.

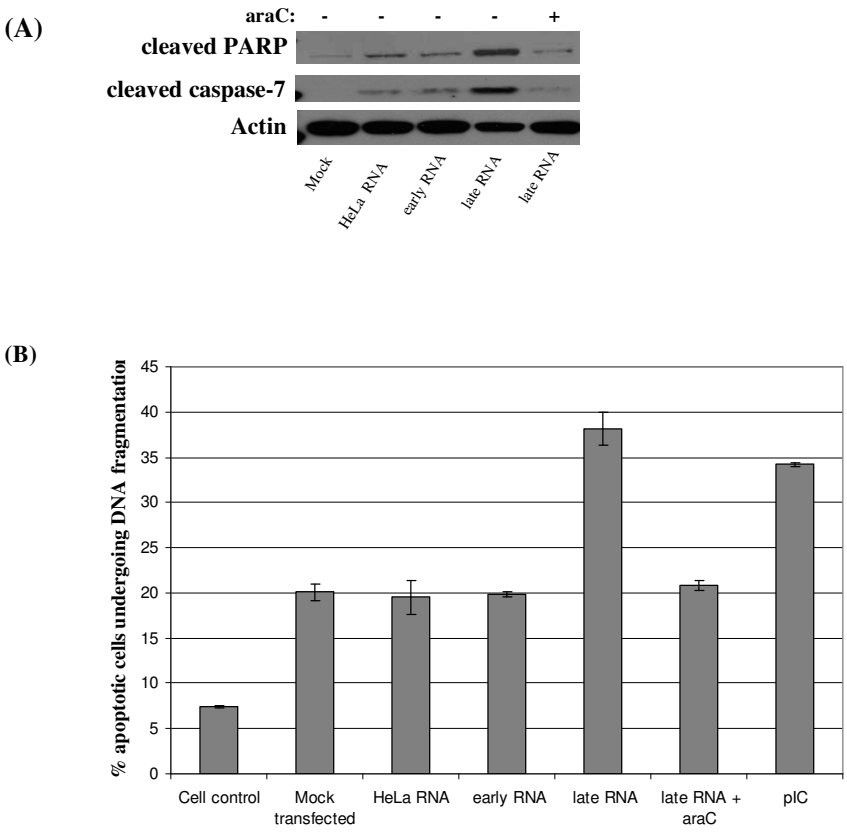

Figure 22: RNA species generated during VACV replication activate PKR-dependant apoptosis. (A) HeLa cells were transfected with 10 μg of the indicated RNA preparation and collected 12 hpt. The cleavage of caspase-7 and PARP was determined by Western blotting. (B) Cells were treated as in (A), and an additional well was transfected with 10 μg of pIC. At 12 hpt, cells were collected, washed in PBS and then lysed in propidium iodide buffer. The fragmentation of cellular genomic DNA was analyzed by flow cytometry. Shown is the percentage of apoptotic cells undergoing DNA fragmentation. Results are representative of three independent experiments. Error bars represent the mean, plus and minus one standard deviation.

Treatment of cells with transfection reagent alone, cellular RNA or RNA collected at early times post-infection increased the percentage of cells undergoing apoptosis by approximately 13% in comparison to untreated controls (Fig. 22B). Late RNA isolated from araC treated cells stimulated apoptosis similar to that observed in HeLa RNA and early RNA-transfected cells. The percentage of apoptotic cells increased by 31% in late RNA-transfected cells as compared to untreated cells. Thus, the transfection of late RNA increased the number of apoptotic cells by 18% in comparison to transfection of all other RNA samples. As a positive control, DNA fragmentation induced by transfection of cells with pIC was also assessed. Treatment of cells with pIC increased DNA fragmentation approximately 27% as compared to untreated controls.

The role, if any, of the cytosolic PRRs, PKR, MDA5 and RIG-I in this apoptotic response was then studied. HeLa cells were transfected with PKR, MDA5 or RIG-I-specific siRNAs for 48 hours. Cells were then transfected with late RNA and the cleavage of caspase-7 and PARP was measured by Western blotting. Treatment of cells with MDA5 or RIG-I siRNAs had no effect on the cleavage of caspase-7 or PARP (Fig. 22C). In contrast, cleavage of both caspase-7 and PARP induced by late RNA was greatly reduced in cells treated with PKR siRNAs. Similar results were obtained when measuring DNA fragmentation induced by late RNA. Treatment of cells with PKR siRNA reduced late RNA induced DNA fragmentation by 13% in comparison to control siRNA treated cells (Fig. 22D). This corresponds to approximately a 35% decrease in the level of apoptosis induced specifically by late RNA treatment. In contrast, treatment of cells with MDA5 or RIG-I siRNA did not reduce the level of DNA fragmentation.

PKR has been reported to mediate apoptosis induced by a VACV mutant lacking the E3L gene[254]. Another report found no effect on E3L mutant virus-induced apoptosis

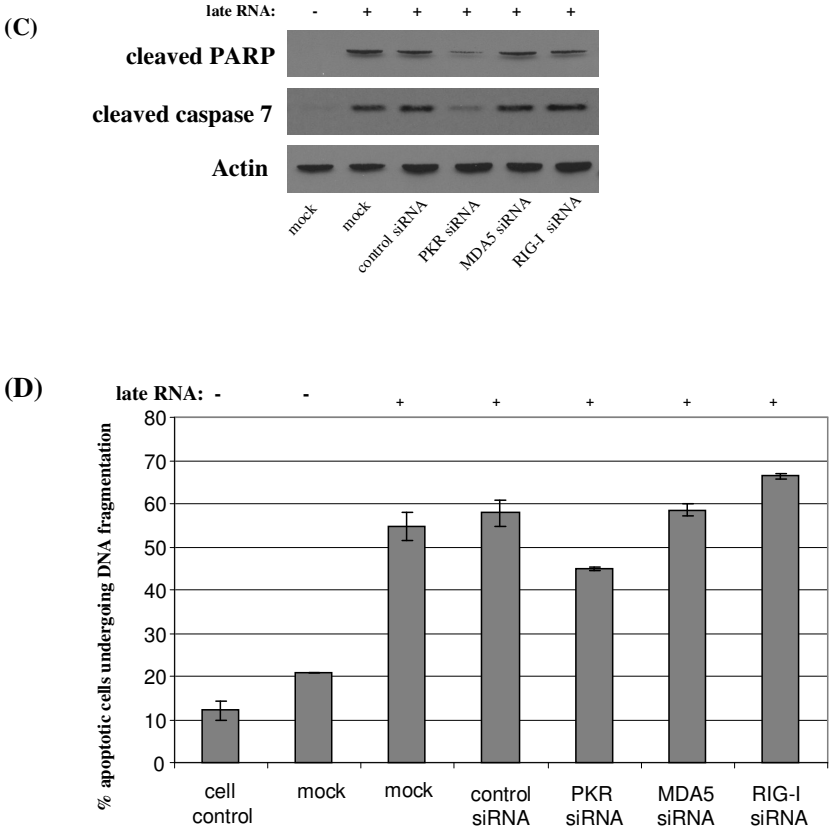

Figure 22: RNA species generated during VACV replication activate PKR-dependant apoptosis. (C) HeLa cells were transfected with control, PKR, MDA5 or RIG-I siRNAs. After 48 hours, cells were transfected with 10 μg of late RNA and collected at 12 hpt. The cleavage of caspase-7 and PARP was analyzed by Western blotting. Results are representative of three independent experiments. (D) Cells were treated as in (C) and washed in PBS and then lysed in propidium iodide buffer. The fragmentation of cellular genomic DNA was analyzed by flow cytometry. Shown is the percentage of apoptotic cells undergoing DNA fragmentation. Results are representative of two independent experiments. Error bars represent the mean, plus and minus one standard deviation.

in cells treated with MDA5 and RIG-I siRNAs[255]. However, in this study the efficiency of the siRNA knockdown of RIG-I and MDA5 was not determined, and thus whether the MDA5 and RIG-I protein levels were reduced sufficiently to observe a phenotype was not addressed. Therefore, the role of MDA5 and RIG-I, which are not required for late RNA induced apoptosis, was examined during vvCopΔE3L-induced apoptosis. Cells were treated with siRNAs targeting PKR, MDA5 or RIG-I and then infected with vvCopΔE3L. Similar to previous reports, cleavage of caspase-7 and PARP was reduced in vvCopΔE3L-infected cells treated with PKR-specific siRNAs (Fig. 22E). In contrast, treatment of cells with MDA5 or RIG-I specific siRNAs had no effect on caspase-7 or PARP cleavage.

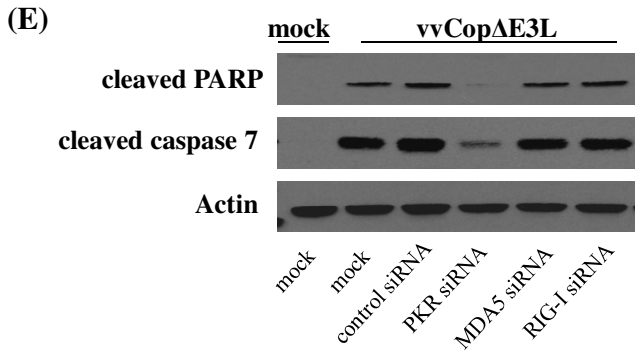

Figure 22: RNA species generated during VACV replication activate PKR-dependant apoptosis. (E) HeLa cells were transfected with siRNA as in Figure 22C. Cells were then mock infected or infected with vvCopΔE3L at an MOI of 5 and collected at 8 hpi. The cleavage of caspase-7 and PARP was determined by Western blotting. Results are representative of three independent experiments.

Section II discussion

Compared with RNA viruses, considerably less is known about the sensing of RNAs generated during replication of dsDNA viruses, such as poxviruses. Deletion of the E3L gene from VACV results in a virus (vvΔE3L) which activates cytokine expression and apoptosis[53,116,120,162]. DsRNA species associated with VACV intermediate and late gene transcription have been suggested to be one of the major PAMPs responsible for inducing cellular innate immune responses. The vvΔE3L phenotype (activation of PKR, cytokine expression and apoptosis) is believed to be due to activation of innate immune responses by viral dsRNAs. During wild-type VACV infection, these responses would be antagonized by the E3 protein. However, to date, no direct evidence has been published to confirm the role of RNA species associated with VACV replication in induction of such innate immune responses. The finding that E3 can inhibit IFN-β expression induced by ssRNA and dsDNA, in addition to dsRNA[137], suggests that several VACV PAMPs may contribute to vvΔE3L-induced cytokine expression. In Results Section II, the specific role of RNA species generated during VACV infection on the activation of PKR and the induction of cytokine expression and apoptosis was investigated. Moreover, the signalling pathways mediating the cytokine and apoptotic responses were studied.

Total RNA was isolated from VACV infected cells using TRIzol. If viral proteins such as E3 sequester viral RNA during infection, these RNA species should be released during extraction and present in the final RNA sample. Upon transfection of these RNA species into HeLa cells, the VACV RNAs should be free to be engaged by host PRRs to activate innate immune responses, as viral immuno-modulatory proteins such as E3 are absent. Moreover, in this system the effect of VACV RNAs are studied in isolation as

other viral PAMPs are absent. Therefore, this approach provides a direct means to study the potential of RNA species generated during VACV infection to serve as PAMPs. It should be noted that TRIzol extraction of RNA will dissociate RNA-protein interactions. If an RNA-protein interaction is required for a complex to be immuno-stimulatory, this effect will be lost following RNA extraction. The possibility that TRIzol extraction may alter the structure and/or dsRNA content of the isolated RNA also cannot be excluded. In addition, the combinatorial effect of other viral PAMPs and/or factors is excluded from this system. Therefore, while this system offers the advantage that VACV RNAs can be studied in isolation, it should be noted that this context differs from live virus infection.

Real time PCR array profiling revealed that RNA species generated during the intermediate/late stage of VACV replication are potent activators of IFN-β and TNF-α gene expression, as well as several members of the IL family (Table 8). However, some cytokines, including IFN-β and IL-19, were also induced to some degree by early RNA. Transcription of early VACV genes is believed to produce some dsRNA, although considerably less than transcription of intermediate and late class genes[19,46]. In our system, it is possible that VACV early dsRNA functions as a PAMP to stimulate expression of some cytokines, but the quantity of early dsRNA produced may be insufficient to stimulate significant IFN-β expression. Therefore, the slight induction of cytokines by early RNA may be explained by small amounts of VACV dsRNA present in the early RNA preparations. Furthermore, it was found that the early RNA preparation also contains some intermediate viral transcripts (Fig. 14C) which may also contribute to cytokine expression. Late RNA isolated from araC treated cells induced cytokine expression comparable to early RNAs samples. The enhanced cytokine response in cells transfected with late RNA (from untreated cells) suggests that intermediate and/or late

VACV mRNA species, and not early gene transcripts, are the major stimulatory RNAs generated by VACV. Since mRNA levels often do not correlate with protein levels, an attempt was made to quantify IFN-β protein by ELISA. However, in HeLa cells, very little IFN-β protein was detected following VACV RNA transfection (data not shown). This could be due to the activation of apoptosis by VACV late RNA, which may destroy the cells before IFN-β protein can be produced and secreted. Therefore, IFN-β expression could not be quantified at the protein level.

A robust cytokine response can be induced in HeLa cells transfected with late RNA collected from either vvCopRev or vvCopΔE3L infected cells, but not from uninfected HeLa cells (Fig. 13A). Furthermore, transfection of vvCopRev or vvCopΔE3L late RNA samples into cells results in robust PKR activation (Fig. 13B). It might be expected that vvCopRev infected cells would contain more dsRNA than vvCopΔE3L infected cells given that vvCopRev produces both intermediate and late viral transcripts associated with the ability to form dsRNA, while vvCopΔE3L only produces intermediate transcripts as a consequence of its incomplete replication cycle. However, cytokine expression and PKR activation were equally induced by late RNA from vvCopRev and vvCopΔE3L infected cells. It is possible that the vvCopΔE3L virus produces dsRNA quantities above a threshold of dsRNA required for maximal induction of these responses and therefore RNA from cells infected with either virus induces cytokine expression and PKR activation to a comparable degree. It is also possible that intermediate gene transcripts are the predominant immuno-stimulatory RNA species generated in VACV infected cells. Interestingly, total RNAs isolated from uninfected HeLa cells also induced a low level of cytokine expression and PKR phosphorylation. DsRNA species such as double-stranded primary microRNAs (which naturally exist in

the nucleus) are isolated in our total RNA extracts. Moreover, these RNA extracts likely contain a considerable amount of uncapped host mRNA with exposed 5'-triphosphate groups originating from the nucleus which could potentially function as PAMPs activating PKR and RIG-I. Therefore, transfection of uninfected HeLa cell RNA back into HeLa cells is likely to introduce ssRNA and dsRNA into the cytoplasm with the potential to activate PKR and cytokine expression. This may account for the minimal induction of cytokines and PKR activation observed even in cells transfected with cellular HeLa RNA.

Several lines of evidence demonstrate that late RNA-induced cytokine expression is due to specific detection of dsRNAs in the samples and not to other potential VACV PAMPs such as ssRNA, viral proteins or DNA. The VACV early (D12), intermediate (G8) and late (H3) proteins were not detected in late RNA-transfected cells (Fig. 14D), suggesting that viral proteins are not being translated from transfected RNAs. Thus, the observed induction of cytokines following transfection is unlikely to be due to viral proteins. Digestion of late RNA with RNase III, a nuclease specific for dsRNA, prior to transfection abolished the ability of the RNA to induce cytokine expression (Fig. 13A). Furthermore, digestion of late RNA with RNase A to specifically degrade ssRNA had no effect on late RNA-induced IFN-β expression (Fig. 14A, B). In addition, transfection of HeLa cells with ssRNA molecules such as pI does not induce cytokine expression or PKR phosphorylation (Fig. 13A, B). Collectively, these results provide evidence to support the role of dsRNA present in the late RNA, and not ssRNA, viral proteins or DNA, as the activator of PKR and cytokine expression. Alternatively, a virally-induced cellular dsRNA could also function as an activator of these responses. Overall, these results are the first direct evidence to demonstrate that RNA species produced within VACV

infected cells function as PAMPs capable of initiating an innate immune response including PKR activation and cytokine induction.

Following the initial finding that VACV RNA are PAMPs, as described in Results Section II, it was reported that MDA5 regulates IFN-β induction in cells transfected with RNA isolated from VACV infected cells[182]. However, the role of RIG-I in this system was not addressed. Utilizing a siRNA knockdown approach, it was observed that MDA5 and RIG-I, as well as IPS-1, IRF3 and NF-κB participate in activating IFN-β expression following VACV late RNA transfection (Fig. 17, 18B, 19A, B, 21A, B). One striking observation of this data is that the use of PRRs for VACV RNAs can be cell-type and even cell state-specific. In A549 cells, RIG-I was found to be the sensor for VACV RNAs. Although RIG-I protein levels were only moderately reduced in A549 cells by siRNA treatment, several observations suggest that the effect of the RIG-I siRNA is not due to non-specific effects. First, in high passage HeLa cells, the RIG-I siRNA has no effect on IFN-β expression induced by VACV late RNA or EMCV RNA. Second, several different RIG-I siRNAs tested also only moderately reduce RIG-I protein levels in A549 cells but significantly reduce IFN-β expression. In low passage HeLa cells, late RNA-induced IFN-β expression was RIG-I-dependent. However, maintaining these same cells in culture over time resulted in a phenotypic change such that late RNA-induced IFN-β expression became MDA5-dependent. That the mechanism(s) for recognition of RNA-based PAMPs can be cell-type specific is also supported by the finding that EMCV RNA-induced IFN-β expression requires MDA5 in high passage HeLa cells (Fig. 16B) and RIG-I in A549 cells (Fig. 18C). While an understanding for the basis of this observation is currently lacking, it is possible that an unknown adaptor protein required

for RIG-I or MDA5-dependant IFN-β gene transcription is differentially expressed and/or regulated in the two cell lines and potentially under different cellular conditions such as passage history. Also, unique RNase expression profiles may exist in different cell lines which modify incoming RNAs and affect their ability to specifically stimulate MDA5 or RIG-I. For example, RNase L, an antiviral enzyme activated during vvΔE3L infection[194], can produce RIG-I and MDA5 ligands by cleavage of cellular self-RNAs[134]. RNase L-mediated cleavage of HCV RNA also produces RNA ligands which activate RIG-I, but not MDA5[135]. It is possible that RNase L also cleaves VACV RNAs, which could affect the capacity of these RNAs to activate RIG-I or MDA5. Thus, differential expression of RNase L, or other RNA modifying factors, in different cell lines or cellular states could potentially affect PRR specificity.

Since these results demonstrate that RNA species generated during VACV infection function as PAMPs, the requirement for RIG-I and MDA5 during vvCopΔE3L infection was also investigated. Both RIG-I and MDA5 mediate IFN-β gene transcription following vvCopΔE3L infection (Fig. 20A, B). During vvCopΔE3L infection, RIG-I knockdown consistently had a greater effect on IFN-β expression than did MDA5 knockdown, regardless of the passage history of the cells. However, with HeLa cells maintained in culture over time, RIG-I does not appear to facilitate late RNA-activated IFN-β expression (Fig. 17, 19B). The reason for the difference between the results from these two approaches is likely the complex nature of virus infection, wherein numerous viral PAMPs, such as dsRNA, dsDNA and viral proteins may contribute to overall cytokine expression. In viral RNA transfection experiments, only RNA species are present, while other immuno-stimulatory molecules are absent. The finding that both MDA5 and RIG-I can regulate viral RNA and vvCopΔE3L-mediated IFN-β expression

supports the concept that E3 functions to suppress viral dsRNA-dependent RLR activation and expression of IFN-β during actual VACV infection.

In addition to suppressing cytokine expression during virus infection, E3 can also block VACV-induced apoptosis[120] through the inhibition of PKR function[254]. In order to investigate the role of RNAs generated during VACV infection in activating apoptosis, a series of transfection experiments were performed. Results from these experiments demonstrate that RNA species generated late in VACV-infected cells are capable of stimulating apoptosis (Fig. 22A, B). Transfection of cells with late RNA isolated from araC treated cells induces significantly less apoptosis than late RNA isolated from untreated cells. This demonstrates that intermediate and/or late viral RNAs are required for the activation of apoptosis. PKR, but not MDA5 or RIG-I, regulates the apoptotic response to transfection of cells with RNAs generated during VACV infection (Fig. 22C, D). These results strongly suggest that the apoptotic response to vvCopΔE3L infection, which is also dependent on PKR (Fig. 22E), is due to VACV RNA-induced PKR activation. Previously, both MDA5 and RIG-I have been shown to mediate apoptotic responses to RNA agonists[14]. Furthermore, during virus infection, the RLR can activate apoptosis. For example, apoptosis in SeV infected cells requires RIG-I-mediated activation of IRF3[38]. Therefore, the mechanism(s) regulating apoptosis in VACV infected cells is distinct from the response to SeV and likely other viruses. Cumulatively, these results directly demonstrate that RNA-based PAMPs of VACV trigger the induction of apoptosis through PKR, independent of MDA5 and RIG-I.

Understanding the molecular structure of RNA-based PAMPs remains an important aim of the field of innate immunity[164]. The precise structural nature of VACV RNA-based PAMPs is currently unknown. Convergent transcription of complementary

strands of the VACV genome produces RNA transcripts which can anneal to form RNA molecules consisting of both dsRNA and ssRNA regions[19]. Based on this mechanism, it would be expected that these RNA species would be composed of a central based-paired region (dsRNA) with two 5' ssRNA tails (Fig. 3). Indeed, dsRNA generated during VACV infection has been shown to be highly variable in length, and can have a complex, branched structure[19]. Thus, the RNA species produced in VACV-infected cells are diverse in length, the percentage of dsRNA content and overall structure. It is also possible that VACV mRNAs can self-anneal to form regions of dsRNA secondary structure through sequence self-complementarity. Given that PKR, MDA5 and RIG-I recognize a multitude of different RNA structures, the result that all three PRRs can recognize VACV RNAs is not unexpected. However, it is novel that VACV RNAs may be sensed by either RIG-I or MDA5 in a cell-type/state specific manner. Also, that VACV RNA-induced IFN-β expression is primarily regulated by the RLRs, while apoptosis is regulated by PKR highlights that several unique mechanisms are employed to initiate antiviral responses following the detection of VACV RNA-based PAMPs.

It is likely that a combination of RNA length, dsRNA content and structure, and the presence of terminal phosphate groups collectively affect the activation of PKR, MDA5 and RIG-I by VACV RNAs. Whether the majority of VACV intermediate/late RNA species have the capacity to stimulate IFN-β expression and apoptosis or if only a small number of these RNAs with unique structures are immuno-stimulatory is not known. The finding that RNA isolated from vvCopΔE3L infected cells activates PKR and IFN-β expression comparably to RNA from vvCopRev infected cells suggests that VACV intermediate gene transcripts are sufficient to induce these responses. Thus, while VACV late transcripts may also function as PAMPs, in the context of vvCopΔE3L

infection, intermediate gene transcripts likely constitute the primary PAMPs activating cytokine expression and apoptosis. A recent study demonstrated that the stimulatory potential of VACV RNAs resides in high molecular weight complexes composed of ssRNA and dsRNA[182]. However, whether the ssRNA or the dsRNA component of the complex was required for the stimulation of IFN-β expression was not determined. To analyze the immuno-stimulatory potential of ssRNAs in VACV late RNA-induced IFN-β expression, RNase A was used to selectively digest ssRNA. Results from these experiments, as well as the RNase III digest data, provide evidence that ssRNAs in VACV-infected cells do not contribute to the induction of IFN-β (Fig. 13A, 14A, B). Thus, dsRNAs (or ssRNAs with the propensity to form dsRNA secondary structures) generated following DNA replication during VACV infection are the primary PAMPs activating IFN-β expression.

In summary, RNA species generated during the intermediate and/or late stage of VACV replication serve as PAMPs activating IFN-β expression and apoptosis. Moreover, both MDA5 and RIG-I are key PRRs of the IFN-β response to these RNA species. The use of PRRs for VACV RNAs appears to be cell-type and cell state-specific in regards to HeLa and A549 cells. Both MDA5 and RIG-I also participate in vvCopΔE3L-induced IFN-β expression. Furthermore, PKR, but not the RLRs, plays a major role in the activation of apoptosis induced by VACV RNAs and vvCopΔE3L infection. Collectively, although it has long been hypothesized that dsRNA generated in VACV-infected cells activates innate immune responses, these results provide the first direct evidence to support this hypothesis. Moreover, these findings better define the molecular mechanisms by which cellular PRRs engage RNA-based PAMPs produced within VACV-infected cells and are the first to implicate RIG-I in sensing VACV

infection. Overall, these observations will contribute to the understanding of PAMP-PRR interactions and to how VACV evades immune recognition.

Section III - ***Comparative analysis of poxvirus orthologues of the vaccinia virus E3 protein: Modulation of PKR activity, cytokine responses and virus pathogenicity (Journal of Virology, in press)***

Rationale, hypothesis and specific objectives

Poxviruses are important human and animal pathogens that have evolved elaborate strategies for antagonizing host innate and adaptive immunity. The E3 protein of VACV, the prototypic member of the orthopoxviruses, functions as an inhibitor of innate immune signalling and is essential for VACV replication *in vivo*[23] and in many human cell culture systems[11,35]. While sequence similarity is high for orthologues of E3 within the *Orthopoxviridae*, E3 orthologues encoded by other poxvirus genera display considerably more sequence divergence. Currently, the function of orthologues of E3 expressed by poxviruses of other genera with different host specificity remains largely unknown. Given the essential role of E3 in VACV biology and the fact that all vertebrate poxviruses encode orthologues of E3, with the exception of the Avipoxviruses and molluscum contagiosum virus, it is likely that orthologues of E3 also play important roles in regulating poxvirus biology. Therefore, studying the function of orthologues of E3 will be important for understanding the mechanisms by which poxvirus proteins antagonize host antiviral defence.

The hypothesis that drives this work is that orthologues of E3L encoded by poxviruses outside the orthopoxvirus genus will vary in their ability to complement important functions of VACV E3, including restoration of virus host-range, modulation of PKR activity and suppression of cytokine responses.

The specific objectives of this work are to; **(A)** Generate recombinant VACVs expressing Nigeria goat and sheeppox virus (SPPV) (genus *Capripoxvirus*) SPPV34L[231], yaba monkey tumour virus (YMTV) (genus *Yatapoxvirus*) YMTV34L[25], swinepox virus (SPV) (genus *Suipoxvirus*) SPV032L[2] and myxoma virus (MYXV) (genus *Leporipoxvirus*) M029L[28] E3L orthologues in place of E3L. These recombinant viruses will then be used as tools to **(B)** Determine which of these E3 orthologues can complement the host-range function of VACV E3 through the inhibition of PKR function, **(C)** Determine the capacity of these E3 orthologues to modulate cytokine responses in infected cells and **(D)** Determine if these E3 orthologues can restore pathogenicity to E3 deficient VACV *in vivo*.

3.1 - Amino acid alignment of poxvirus orthologues of VACV E3. An amino acid alignment of the E3, SPPV34, YMTV34, SPV032 and M029 proteins was generated (Fig. 23). Overall, only a minority of amino acid residues are conserved across all the sequences examined. Furthermore, in comparing E3 with any single E3 orthologue, overall conservation of amino acid sequence identity is low. By comparison, E3 and the variola virus (another orthopoxvirus) E3 orthologue share approximately 92% amino acid sequence identity, with 100% identity shared in the last 59 amino acids of the dsRNA binding domain (data not shown). The C-terminal dsRNA binding domains display comparatively more amino acid conservation than do the N-terminal Z-DNA binding domains. It should also be noted that the myxoma virus M029 protein has two large deletions in the N-terminus of the protein in comparison to VACV E3. Previously, amino acid residues E124, F135, F148, G164 K167, R168, K171 and A174 have been shown to contribute to E3 dsRNA binding[34,79] and are highlighted in red. E124, F148, G164 and

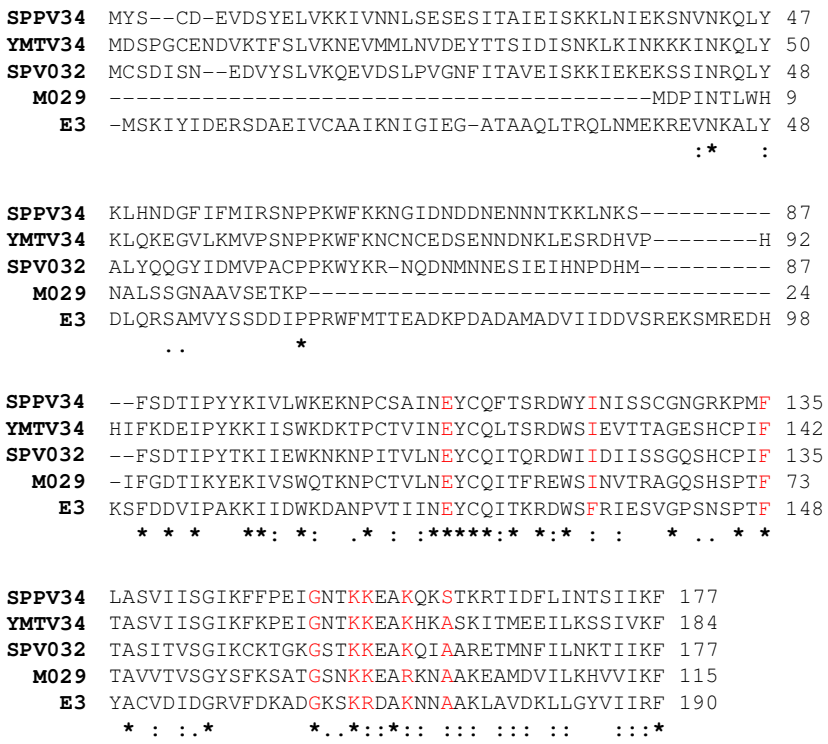

Figure 23: Poxvirus orthologues of VACV E3 display significant amino acid divergence. Protein sequence alignments were generated using the ClustalW2 program. The "**.**" symbol denotes semi-conserved substitutions, the "**:**" symbol denotes conserved substitutions and the "*" symbol denotes complete conservation of the residue across all sequences. The amino acids highlighted in red indicate residues previously identified as contributing to E3 dsRNA binding.

K167 are conserved in each of the E3 orthologues in comparison to E3. F135I and R168K conserved substitutions are present in the sequence of each orthologue in comparison to E3. The K171 residue is also conserved in all the orthologues except M029, where a K→R substitution has occurred while A174 is conserved in each orthologue except SPPV34 where an A→S substitution has occurred. Overall, there is considerable divergence in the amino acid sequence of E3 orthologues encoded by poxviruses outside the orthopoxvirus genus.

3.2 - The E3L orthologues SPPV34L, YMTV34L, SPV032L and M029L are early genes. Next**,** it was determined whether SPPV, YMTV, SPV and MYXV express their respective E3L orthologue and whether these genes belong to the early class of poxvirus genes, as E3L does. To this end, reference cell lines OA3.Ts, CV-1, PK15 and RK13 were infected with SPPV, YMTV, SPV and MYXV, respectively. Infections were performed in the presence or absence of araC to inhibit viral DNA replication, and thus intermediate and late poxvirus gene expression. Therefore, if expression of a poxvirus gene is unaffected by araC treatment, it is generally classified as an early gene. Each of the viruses expressed the respective E3L orthologue within infected cells (Fig. 24). M029L expression was detected as early as 1 hpi, while expression of SPV032L, YMTV34L and SPPV34L was not detected until 3 hpi, with increased transcript levels present at 8 hpi. The expression of these E3L orthologue genes was not significantly inhibited by araC treatment, indicating that SPPV34L, YMTV34L, SPV032L and M029L are early genes.

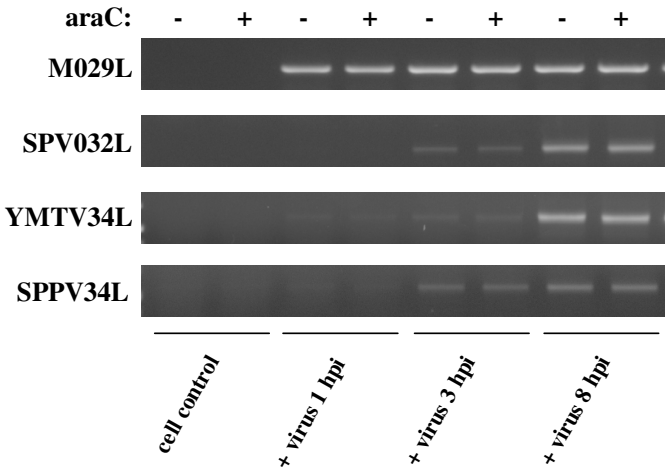

Figure 24: Poxvirus orthologues of VACV E3L are early genes. OA3.Ts, CV-1, PK15 and RK13 cells were infected with SPPV, YMTV, SPV and MYXV, respectively. Infections were performed in the presence or absence of araC and cells were collected at 1, 3 and 8 hpi. The expression of SPPV34L, YMTV34L, SPV032L and M029L was detected by RT-PCR in cells infected with the respective virus.

3.3 - SPV032 and M029, but not SPPV34 or YMTV34, restore the host-range function of E3 in CV-1 and HeLa cells. To investigate the potential of poxvirus orthologues of the VACV E3 protein to complement the deletion of E3, recombinant VACVs expressing the sheeppox virus SPPV34L (vvWR-SPPV34L), yaba monkey tumour virus YMTV34L (vvWR-YMTV34L), swinepox virus SPV032L (vvWR-SPV032L) and myxoma virus M029L (vvWR-M029L) genes in place of the E3L locus were constructed. As one aim of this study is to test the pathogenicity of the viruses in mice, all recombinant viruses were generated using the Western Reserve strain of VACV since this strain is pathogenic in mice while the Copenhagen strain is not. A hemagglutinin (HA) tag was added to the amino-terminus of each orthologue gene to facilitate detection of the proteins, a modification which has been shown to have no effect on the ability of E3 to bind dsRNA or inhibit PKR[198]. A VACV mutant lacking the E3L gene based on the Western Reserve strain (vvWRΔE3L) was also generated, as was a control revertant virus based on vvWRΔE3L in which the E3L gene was re-inserted into the original E3L locus (vvWR-Rev). BHK21 cells were infected with the recombinant viruses to detect expression of the gene products. As expected, E3 was only detected in vvWR-Rev infected cells (Fig. 25A). Expression of SPPV34, YMTV34, SPV032 and M029 was detected in cells infected with the respective recombinant virus.

The capacity of each virus to replicate in BHK21 cells, which are permissive for vvWRΔE3L, and CV-1 and HeLa cells, which are not[11], was investigated. BHK21 cells were infected with each of the recombinant viruses and collected at 5 and 48 hpi to determine the virus yield. All the recombinant viruses produced comparable viral yields in BHK21 cells (Fig. 25B). Next, CV-1 and HeLa cells were infected with each of the recombinant viruses. Cells were collected at 5 and 48 hpi and the virus yield was

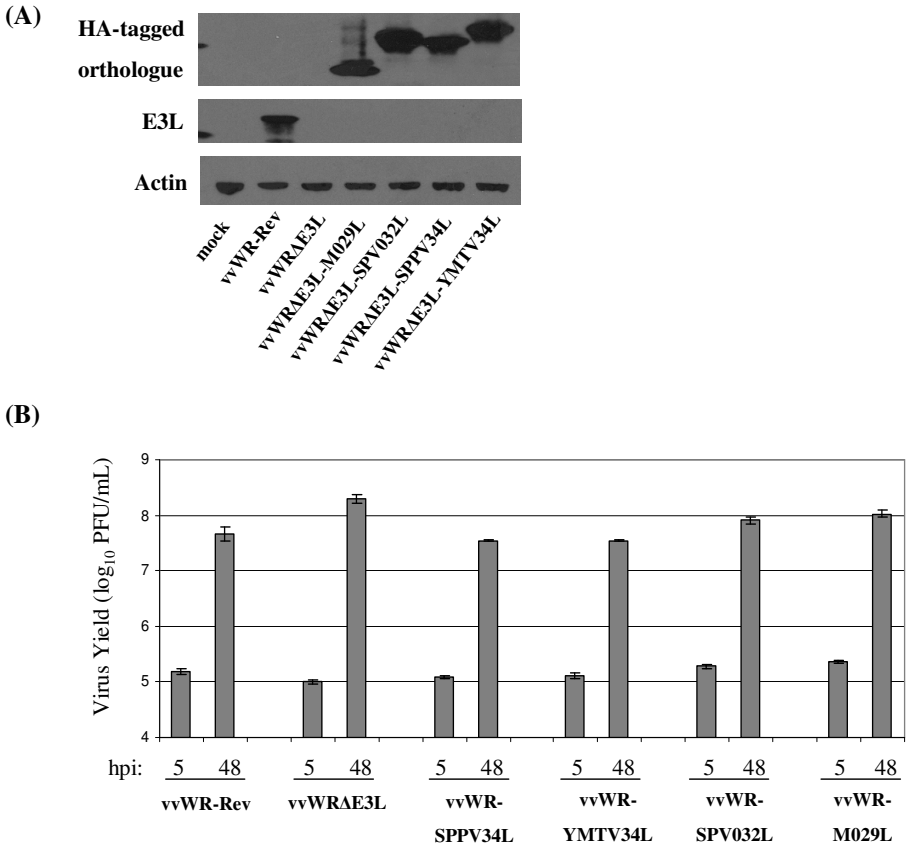

Figure 25: SPV032 and M029 restore vvWRΔE3L replication in CV-1 and HeLa cells. (A) BHK21 cells were infected with the indicated viruses and collected at 24 hpi. Western blotting was used to detect expression of actin, E3, and HA-tagged SPPV34, YMTV34, SPV032 and M029. (B) BHK21 cells were infected with the indicated viruses at an MOI of 0.5 and collected at 5 and 48 hpi. Virus yields were determined by plaque assays in BHK21 cells. Error bars represent the standard error of the mean. Results are representative of three independent experiments.

determined by plaque assays. Similar to vvWRΔE3L, vvWR-SPPV34L was unable to replicate in either CV-1 (Fig. 25C) or HeLa cells (Fig. 25D). An intermediate virus yield was obtained from CV-1 and HeLa cells infected with vvWR-YMTV34L. In contrast, vvWR-M029L replicated to titres comparable to vvWR-Rev in both CV-1 and HeLa cells. Virus yields from vvWR-SPV032L infected CV-1 cells were moderately lower than vvWR-Rev yields, but yields were comparable between the two viruses in HeLa cells.

Each of the recombinant viruses expresses EGFP under the control of a poxvirus early/late promoter. At late times post-infection, EGFP expression is driven primarily by VACV late promoter activity. Therefore, detection of EGFP expression serves as a measure of VACV late transcription. EGFP expression was detected in infected HeLa cells by fluorescent microscopy at 24 hpi (Fig. 25E). Robust expression of EGFP was detected in vvWR-Rev, vvWR-SPV032L and vvWR-M029L infected cells and a lower level of EGFP was detected in vvWR-YMTV34L infected cells. EGFP was not easily detectable in vvWRΔE3L and vvWR-SPPV34L infected cells. Since the hallmark of the restricted replication phenotype of E3L-deficient VACV in HeLa cells is a block in intermediate and late protein translation[131], the expression of the VACV D12 (early) and H3 (late) proteins was analyzed in infected HeLa cells. D12 protein expression was comparable in all infected cells (Fig. 25F). In contrast, the H3 protein was almost undetectable in vvWRΔE3L and vvWR-SPPV34L infected cells, while a faint band was detected in vvWR-YMTV34L infected cells. Robust expression of H3 was observed in vvWR-Rev, vvWR-SPV032L and vvWR-M029L infected cells. Thus, late gene transcription and late protein translation are consistent with the virus yield data obtained from HeLa cells.

(C)

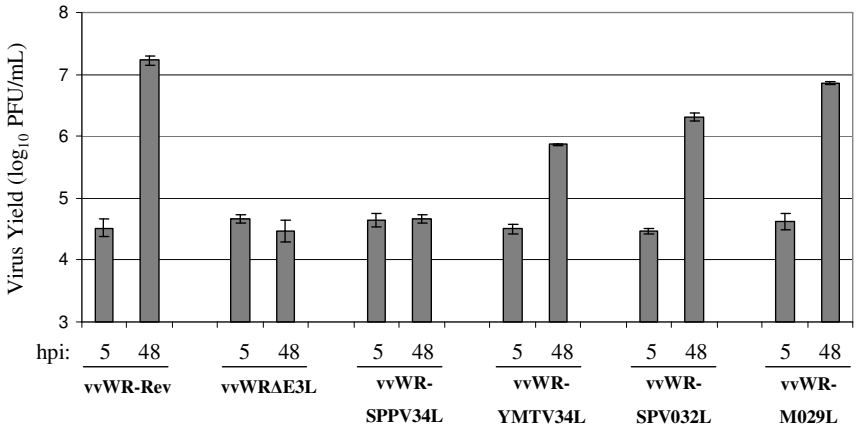

Figure 25: SPV032 and M029 restore vvWRΔE3L replication in CV-1 and HeLa cells. (C) CV-1 cells were infected with the indicated viruses at an MOI of 0.5 and collected at 5 and 48 hpi. Virus yields were determined by plaque assays in BHK21 cells. Error bars represent the standard error of the mean. Results are representative of three independent experiments.

(D)

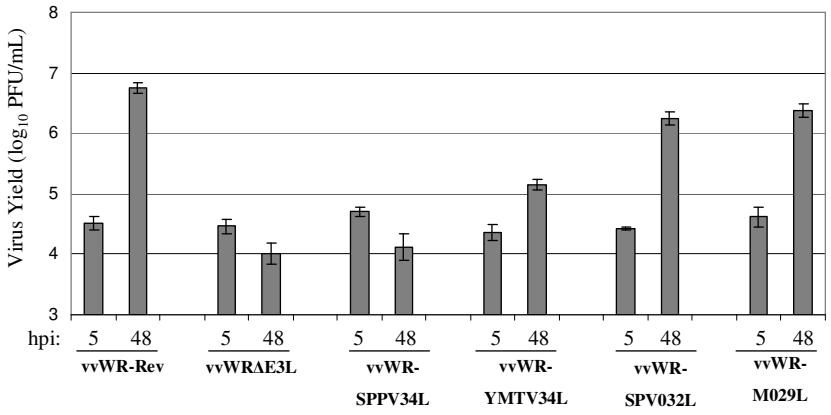

Figure 25: SPV032 and M029 restore vvWRΔE3L replication in CV-1 and HeLa cells. (D) HeLa cells were infected with the indicated viruses at an MOI of 0.5 and collected at 5 and 48 hpi. Virus yields were determined by plaque assays in BHK21 cells. Error bars represent the standard error of the mean. Results are representative of three independent experiments.

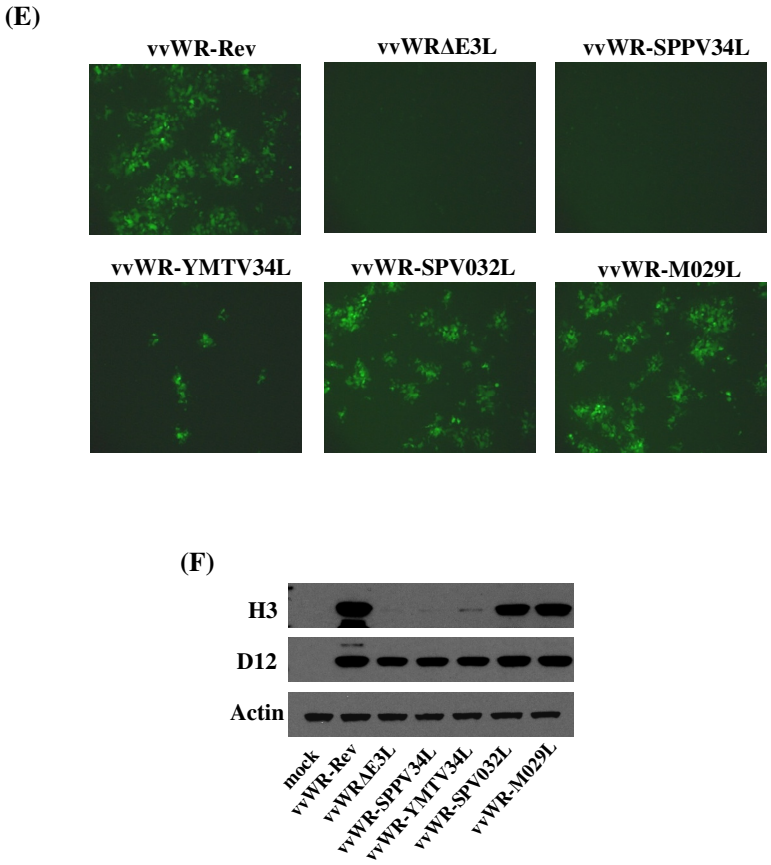

Figure 25: SPV032 and M029 restore vvWRΔE3L replication in CV-1 and HeLa cells. (E) HeLa cells were infected with the indicated viruses at an MOI of 0.01 and EGFP expression was analyzed by fluorescent microscopy at 24 hpi. Microscope settings were kept consistent for all images taken. (F) HeLa cells were infected with each of the indicated viruses at an MOI of 5 and collected at 6 hpi. Western blotting was used to analyze expression of actin, VACV D12 and H3. Results are representative of three independent experiments.

3.4 - SPV032 and M029, but not SPPV34 or YMTV34, inhibit PKR antiviral activity. PKR is a major antiviral protein limiting the replication of E3L-deficient VACV in HeLa cells[254]. Therefore, the activation of PKR, as measured by phosphorylation of Thr-446 and Thr-451, and its downstream substrate, eIF2α, was examined by Western blotting. Robust phosphorylation of PKR (Thr-446 and Thr-451) and eIF2α was detected in vvWRΔE3L, but not vvWR-Rev, infected HeLa cells (Fig. 26A). Significant PKR and eIF2α phosphorylation were also observed in vvWR-SPPV34L and vvWR-YMTV34L infected cells. In contrast, phosphorylation of PKR and eIF2α was significantly inhibited in vvWR-SPV032L infected cells and completely blocked in vvWR-M029L infected cells. The phosphorylation of PKR in infected CV-1 cells was also determined. A high level of phosphorylation of the Thr-446 residue was observed in CV-1 cells infected with vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L (Fig. 26B). PKR phosphorylation was significantly reduced in vvWR-SPV032L infected CV-1 cells, and completely suppressed in vvWR-M029L infected CV-1 cells. The total PKR antibody did not cross-react with PKR expressed in CV-1 cells and therefore the total PKR levels could not be determined. In addition to mediating eIF2α phosphorylation, PKR also regulates apoptosis induced by E3L-deficient VACV[254]. The activation of apoptosis was examined in HeLa cells infected with each of the recombinant viruses by Western blotting to detect cleavage of caspase-7 and PARP as apoptotic markers. Cleavage of both caspase-7 and PARP was detected in vvWRΔE3L and vvWR-SPPV34L infected cells and to a lesser degree in vvWR-YMTV34L infected cells (Fig. 26C). In contrast, cleavage of these apoptotic markers was not detected in vvWR-Rev and vvWR-M029L infected cells, and minimal cleavage of caspase-7 was detected in vvWR-SPV032L infected cells.

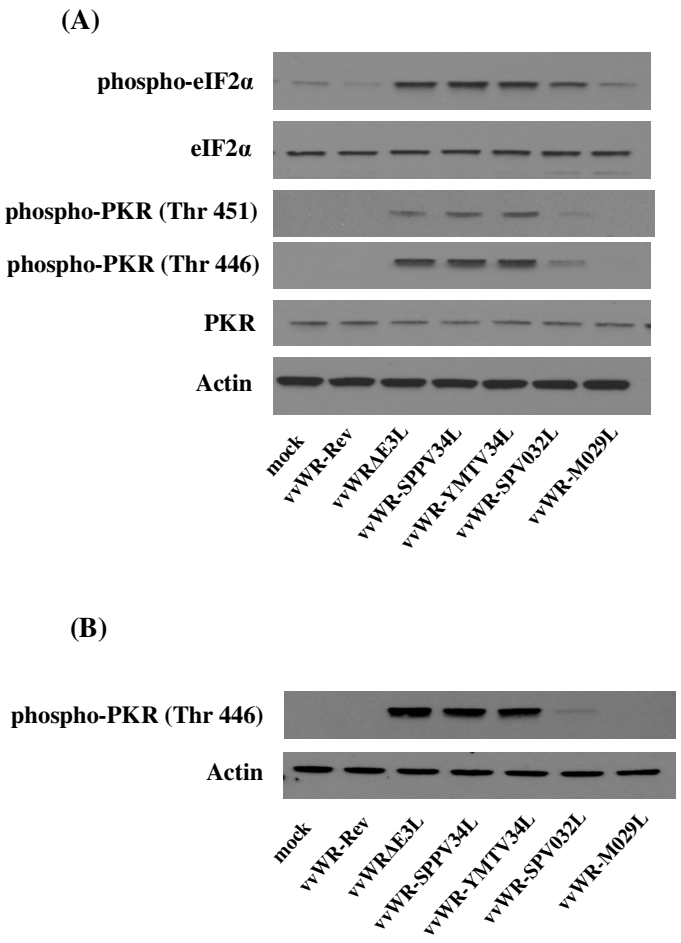

Figure 26: SPV032 and M029 suppress PKR activation. (A) HeLa cells were infected with the indicated viruses at an MOI of 5 and collected at 6 hpi. The phosphorylation of PKR and eIF2α was assessed by Western blotting. Results are representative of four independent experiments. (B) CV-1 cells were infected with the indicated viruses at an MOI of 5 and collected at 6 hpi. The phosphorylation of PKR was assessed by Western blotting.

(C)

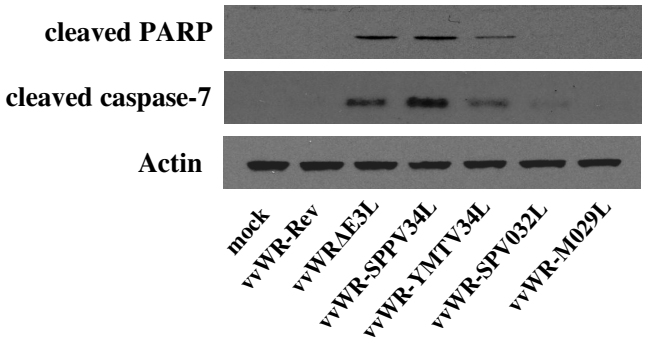

Figure 26: SPV032 and M029 suppress PKR activation. (C) HeLa cells were infected with the indicated viruses at an MOI of 5 and collected at 9 hpi. Cleavage of caspase-7 and PARP was assessed by Western blotting. Results are representative of four independent experiments.

Given that PKR suppresses vvWRΔE3L replication, the capacity of PKR to inhibit the replication of the E3L orthologue expressing viruses was investigated. To this end, HeLa cells were treated with control or PKR-specific siRNAs and the efficiency of the siRNA knockdown was determined by Western blotting (Fig. 27A). SiRNA transfected cells were then infected with each of the recombinant viruses and virus yields were determined at 24 hpi. In comparison to control siRNA treated cells, treatment of cells with PKR siRNAs significantly increased virus yields in vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L infected cells (Fig. 27B). Virus yields were also moderately increased in vvWR-SPV032L infected cells treated with PKR siRNAs. Suppression of PKR expression only slightly increased virus yields in vvWR-Rev and vvWR-M029L infected cells in comparison to control siRNA treated cells. Expression of virally expressed EGFP at 24 hpi in control and PKR siRNA treated cells (Fig. 27C) correlated well with the virus yield data.

3.5 - SPPV34, YMTV34, SPV032 and M029 bind dsRNA. Binding to dsRNA is believed to be a critical function of E3 for suppression of PKR activation. The dsRNA binding capacity of the E3 orthologues was tested using sepharose beads conjugated to pIC, a synthetic dsRNA. BHK21 cells were infected overnight with each of the recombinant viruses and lysates from these cells were used in pull-down assays. As controls, the presence of actin and VACV D12, which are not known to bind dsRNA were examined. Although actin and D12 were detected in pre-pull-down samples, neither was detected following pull-down with pIC coated beads (Fig. 28). All E3 orthologues were pulled-down using the pIC coated beads. However, significantly less SPPV34 was found associated with the pIC beads in comparison to the other E3 orthologues. E3 was

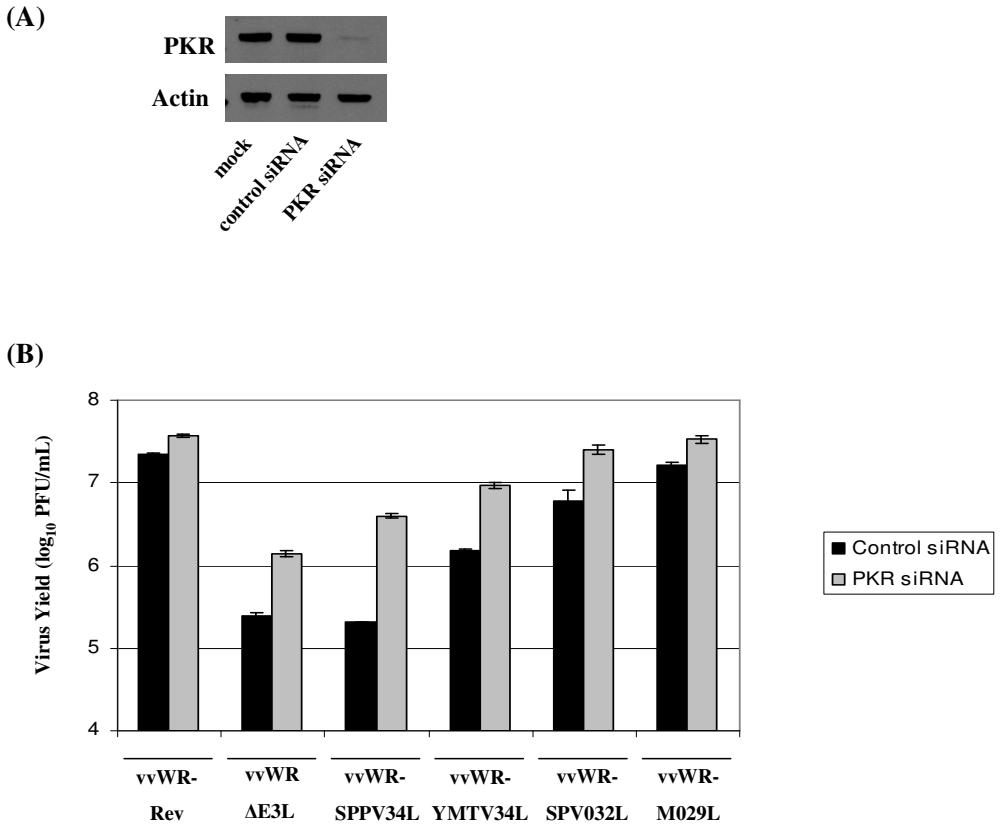

Figure 27: Suppression of PKR expression enhances replication of vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L. (A) HeLa cells were mock transfected, or transfected with control or PKR siRNA at 100 nM for 48 hours. Western blotting was used to determine the expression of PKR and actin. (B) HeLa cells were treated as in (A) and then infected at an MOI of 1 with each of the indicated viruses. Cells were collected at 5 and 24 hpi and virus yields were determined by plaque assays in BHK21 cells.

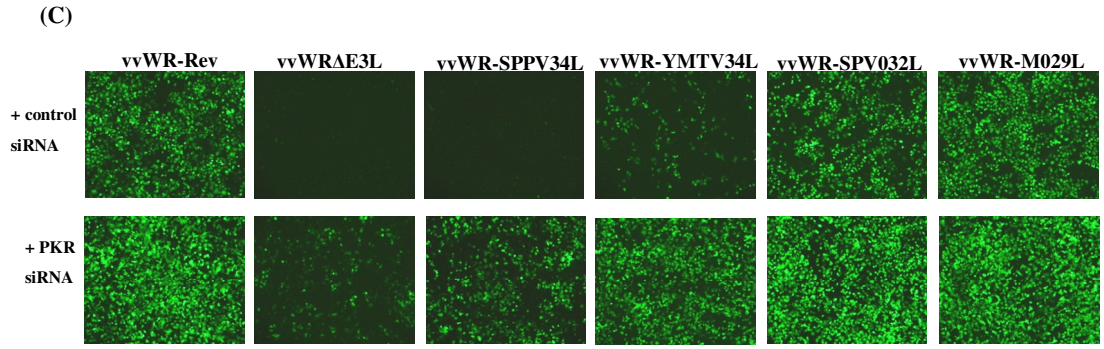

Figure 27: Suppression of PKR expression enhances replication of vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L. (C) EGFP expression was analyzed by fluorescent microscopy at 24 hpi in the siRNA treated, virus infected HeLa cells from Figure 27B. Microscope settings were kept consistent for all images taken. Results are representative of three independent experiments.

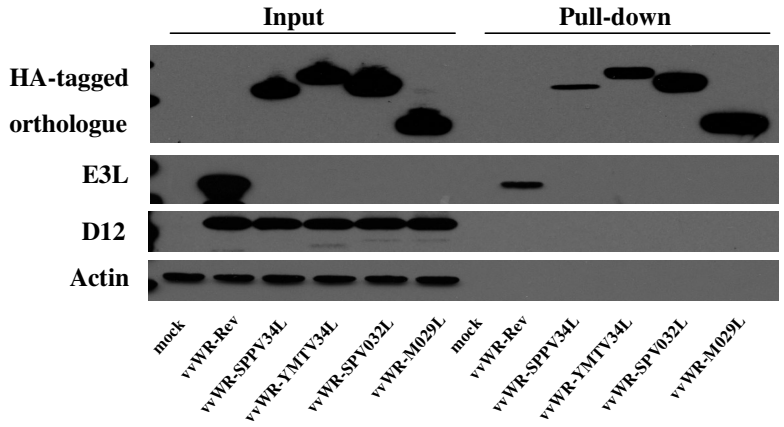

Figure 28: Double-stranded RNA binding capacity of orthologues of the E3 protein. BHK21 cells were infected with the indicated viruses at an MOI of 1 for 16 hours. Cells were then collected and lysed in non-denaturing lysis buffer. Lysates were incubated with pIC conjugated beads and proteins associated with the beads were isolated using pull-down assays. The presence of actin, D12, E3, and HA-tagged SPPV34, YMTV34, SPV032 and M029 were analyzed by Western blotting. Results are representative of four independent experiments.

also pulled-down using the pIC coated beads. It was observed that less E3 associated with the pIC beads in comparison to YMTV34, SPV032 and M029.

3.6 - Differential suppression of cytokine expression by E3 orthologues. In Results Section I and II, it was demonstrated that E3 suppresses cytokine induction through inhibition of PKR, IRF3, NF-κB, IPS-1, RIG-I and MDA5-dependant pathways. Thus, the ability of the poxvirus orthologues to complement the function of E3 in regards to suppressing cytokine induction was investigated. HeLa cells were infected with the recombinant viruses and collected during the late phase of infection. The expression of IFN-β, TNF-α and IL-6 was quantified by real time PCR (Fig. 29A, B, C). Consistent with previous reports[53,54,116,243], infection of cells with vvWRΔE3L resulted in increased IFN-β, TNF-α and IL-6 gene transcription. In comparison to vvWR-Rev infected cells, expression of IFN-β was higher in cells infected with each of the E3 orthologue expressing viruses with the exception of vvWR-M029L. In comparison to vvWRΔE3L infected cells, IFN-β expression was moderately reduced in vvWR-SPPV34L infected cells and more significantly reduced in vvWR-YMTV34L and vvWR-SPV032L infected cells. TNF-α gene transcription was moderately reduced in vvWR-SPPV34L and vvWR-YMTV34L infected cells in comparison to cells infected with vvWRΔE3L and more significantly reduced in vvWR-SPV032L and vvWR-M029L infected cells. Infection of cells with all the recombinant viruses except vvWR-M029L also induced significantly greater expression of IL-6 in comparison to vvWR-Rev. IL-6 expression was slightly reduced in vvWR-SPPV34L and vvWR-SPV032L infected cells in comparison to vvWRΔE3L infected cells. Expression of IL-6 was comparable in vvWRΔE3L and vvWR-YMTV34L infected cells.

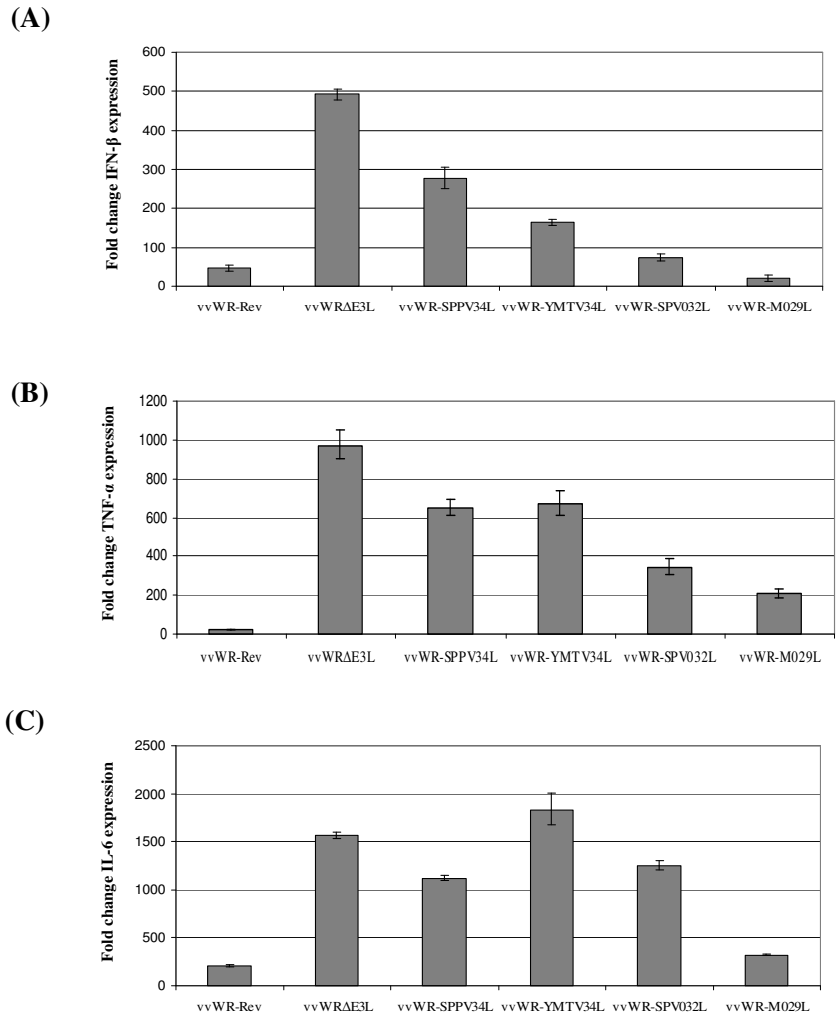

Figure 29: Inhibition of cytokine expression by orthologues of the E3 protein. HeLa cells were infected with the indicated viruses at an MOI of 5 and collected at 9 hpi. The expression of IFN-β (A), TNF-α (B) and IL-6 (C) was quantified by real time PCR. Shown is the gene expression fold change in infected cells in comparison to mock infected cells. Actin expression was used as an internal normalization control for total RNA loading. Error bars represent the mean, plus and minus one standard deviation. Results are representative of four independent experiments.

3.7 - YMTV34, SPV032 and M029, but not SPPV34, inhibit IFN-β induced antiviral activity. Wild-type VACV is highly resistant to IFN treatment, while deletion of the E3L gene results in an IFN sensitive phenotype[7,10]. It was next examined if SPPV34, YMTV34, SPV032 or M029 could compensate for the function of E3 in inhibiting the antiviral effects induced by IFN-β. To this end, Huh7 cells were treated with or without IFN-β overnight and then infected with each of the recombinant viruses. Virus yields were determined at 5 and 48 hpi by plaque assays. IFN-β treatment completely blocked the replication of vvWRΔE3L, but did not affect vvWR-Rev replication (Fig. 30A). Replication of vvWR-SPPV34L was also completely abolished in IFN-β treated cells. In contrast, IFN-β treatment had no effect on vvWR-SPV032L and vvWR-M029L replication, while replication of vvWR-YMTV34L was slightly inhibited in the presence of IFN-β. The phosphorylation of PKR and eIF2α was examined in IFN-β treated Huh7 cells infected with each of the recombinant viruses. IFN-β treatment up-regulated the expression of PKR in all samples (Fig. 30B). The phosphorylation of PKR was enhanced in IFN-β treated cells infected with vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L. In vvWR-Rev, vvWR-SPV032L and vvWR-M029L infected Huh7 cells, PKR is not phosphorylated in the absence of IFN-β. However, following IFN-β treatment, a low level of PKR phosphorylation was detected during infection with these viruses. In the absence of IFN-β treatment, phosphorylation of eIF2α generally correlated with the activation of PKR. However, IFN-β induced enhancement of PKR expression and activation did not increase the level of phosphorylated eIF2α. Phosphorylation of eIF2α was also slightly lower in IFN-β treated cells infected with vvWR-SPPV34L in comparison to untreated vvWR-SPPV34L infected cells. While this result was consistently observed, the basis for this observation is not currently understood.

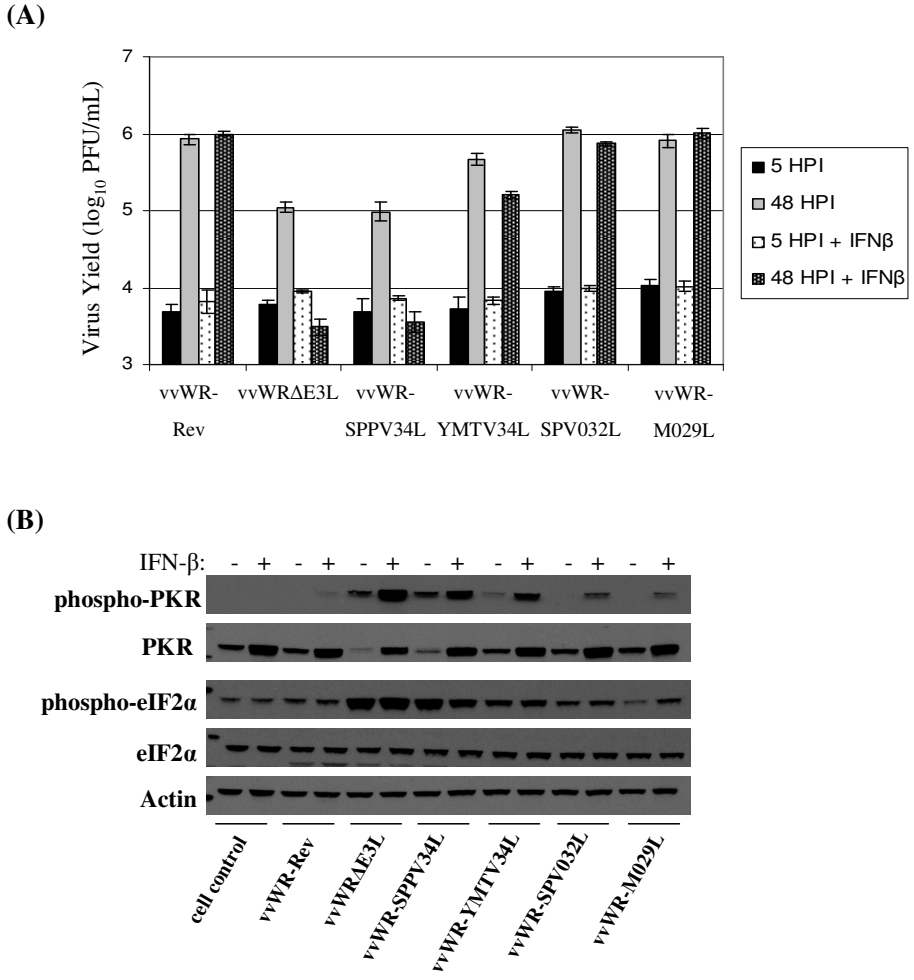

Figure 30: Inhibition of the antiviral effects of IFN-β by orthologues of the E3 protein. (A) Huh7 cells were treated overnight with 1000 units/mL of recombinant human IFN-β. Cells were then infected with the indicated viruses in the presence or absence of 1000 units/mL of IFN-β and collected at 5 and 48 hpi. Virus yields were determined by plaque assays in BHK21 cells. (B) Huh7 cells were treated overnight with 1000 units/mL of recombinant human IFN-β. Cells were then infected with the indicated viruses at an MOI of 5 and collected at 6 hpi. The phosphorylation of PKR and eIF2α was analyzed by Western blotting. Results are representative of three independent experiments. Error bars represent the standard error of the mean.

3.8 - SPPV34, YMTV34, SPV032 and M029 do not restore pathogenicity to E3-deficient VACV in mice. The pathogenesis of the recombinant viruses was tested in a Balb/c mouse model of VACV infection via intranasal inoculation. First, the pathogenicity of wild-type VACV was compared to that of the control virus, vvWR-Rev. No significant differences were found in either weight-loss or survival following infection of mice with 10^6 PFUs/mouse of either wild-type VACV or vvWR-Rev (Fig. 31A, B). Next, mice were infected (4 mice per experimental group) with 10^6 PFUs/mouse of the indicated E3 orthologue expressing recombinant virus. Animals were monitored daily for weight-loss and clinical signs. Mice infected with vvWR-Rev began to lose weight at dpi 1 and reached greater that 20% weight loss by dpi 4 (Fig. 32). All animals in the vvWR-Rev group were euthanized at dpi 4 on the basis of weight-loss and severe clinical signs including piloerection, inactivity and hunched posture. In contrast, the vvWRΔE3L virus was not pathogenic and mice infected with this virus displayed only minor clinical signs. These results for vvWR-Rev and vvWRΔE3L are similar to previous results with wild-type and E3L deficient VACV[23,192,243]. Similar to vvWRΔE3L, all of the recombinant viruses expressing orthologues of E3L did not cause serious disease in this model. Mice infected with vvWR-SPPV34L, vvWR-YMTV34L, vvWR-SPV032L or vvWR-M029L reached a maximum weight loss at dpi 2-3, with vvWR-YMTV34L and vvWR-SPV032L infected mice experiencing the greatest weight loss (approximately 13%). Clinical signs in mice infected with the E3 orthologue expressing recombinant viruses were mild and transient.

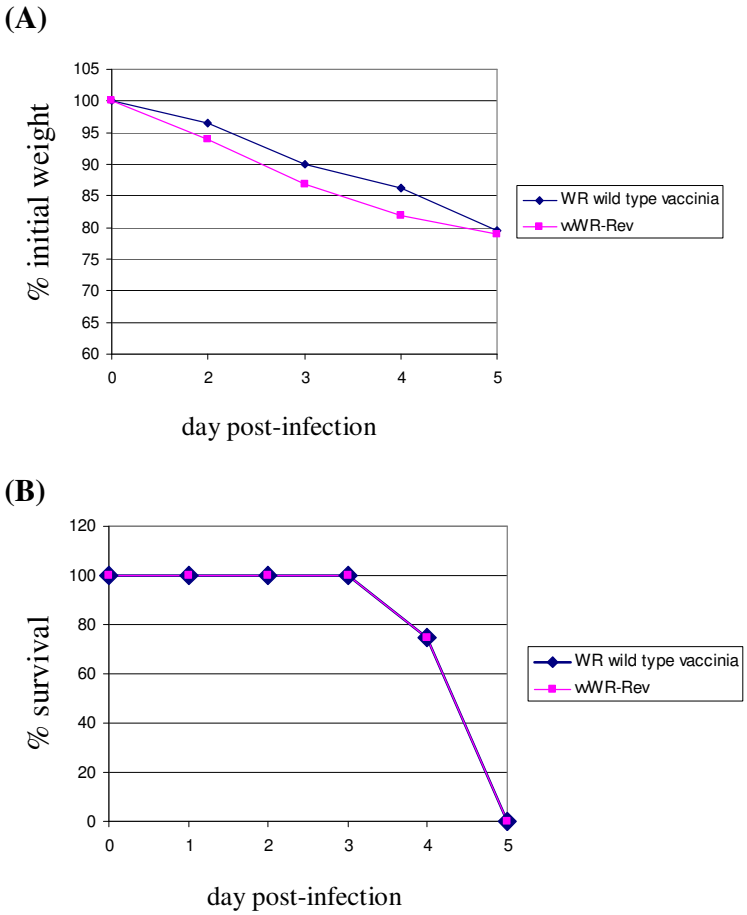

Figure 31: Comparison of the pathogenicity of wild-type VACV and vvWR-Rev in mice. (A, B). Balb/c mice were infected with 10^6 PFUs per mouse of the indicated virus via intranasal inoculation. Animals were weighed daily and monitored for clinical signs. Mice were euthanized when they reached > 20% weight loss in combination with clinical signs including inactivity, hunched posture and piloerection.

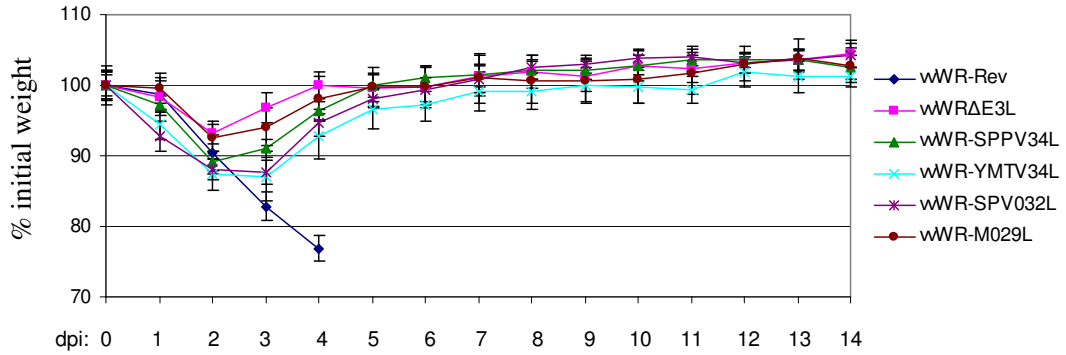

Figure 32: SPPV34, YMTV34, SPV032 and M029 cannot complement the deletion of E3 *in vivo.* Balb/c mice were infected with 10^6 PFUs per mouse of the indicated viruses via intranasal inoculation. Animals were weighed daily and monitored for clinical signs. Mice were euthanized when they reached > 20% weight loss in combination with clinical signs including inactivity, hunched posture and piloerection.

Section III discussion

All vertebrate poxviruses encode orthologues of E3, with the exception of the *Avipoxviruses* and molluscum contagiosum virus. Currently, little is known about the function of orthologues of E3 encoded by other poxviruses. Orthologues of E3 encoded by poxviruses outside the orthopoxvirus genus were chosen for study, as sequence similarity is high for orthologues of E3 within the *Orthopoxviridae*. The E3, SPPV34, YMTV34, SPV032 and M029 proteins display considerable amino acid sequence divergence, particularly in the Z-DNA binding domain (Fig. 23). Since the N-terminus of E3 is required for *in vivo* pathogenesis[23], it is possible that the divergence of the N-terminal domains plays a role in the species specificity of the viruses naturally expressing these E3 orthologues. The majority of residues highly conserved between E3 and its orthologues are located in the dsRNA binding domain and amino acids previously identified to be important for E3 dsRNA binding[34,79] are relatively well conserved among SPPV34, YMTV34, SPV032 and M029.

SPV032 and M029 restored replication of vvWRΔE3L in CV-1 (Fig. 25C) and HeLa cells (Fig. 25D, E, F). In contrast, YMTV34 only moderately restored vvWRΔE3L replication in CV-1 and HeLa cells while SPPV34 was unable to rescue replication of vvWRΔE3L in either of these cell lines. These results demonstrate that YMTV34, SPV032 and M029 are able to at least partially complement the deletion of E3, while SPPV34 cannot. To identify the mechanisms restricting replication of vvWR-SPPV34L and vvWR-YMTV34L, the activation of PKR, which is known to inhibit the replication of E3-deficient VACV in HeLa cells, was analyzed[254]. In both HeLa and CV-1 cells, M029 could completely suppress PKR activation, while SPV032 also significantly inhibited PKR activation (Fig. 26A, B). In contrast, neither SPPV34 nor YMTV34 were

capable of inhibiting PKR phosphorylation in CV-1 or HeLa cells. Phosphorylation of the Thr-451 residue is required in addition to Thr-446 phosphorylation for the activation of PKR[197]. It was found that E3, M029 and SPV032 could also suppress phosphorylation of Thr-451. These results are the first to demonstrate that E3, as well as M029 and SPV32, can inhibit the phosphorylation of both residues required for PKR activation. In support of these results, suppression of PKR expression significantly enhanced the replication of vvWRΔE3L, vvWR-SPPV34L and vvWR-YMTV34L, demonstrating that SPPV34 and YMTV34 do not inhibit PKR antiviral activity. Thus, the inability of SPPV34 to block PKR function explains the non-permissive infection of HeLa cells by vvWR-SPPV34L.

Although robust PKR activation occurs during vvWR-YMTV34L infection, this virus is still capable of replicating to some degree in CV-1 and HeLa cells, as evidenced by virus yield assays. Further evidence for the ability of vvWR-YMTV34L to replicate was obtained from examining EGFP fluorescence and VACV late protein expression in HeLa cells. The activation of apoptosis is slightly reduced in vvWR-YMTV34L infected cells in comparison to vvWRΔE3L (Fig. 26C) but this effect is unlikely to be related to virus replication in cell culture as it was previously shown that inhibition of apoptosis does not rescue vvΔE3L[254]. Although YMTV34 does not suppress PKR phosphorylation, it is possible that it inhibits PKR through a mechanism such as protein-protein interactions. However, the finding that suppression of PKR expression significantly enhances vvWR-YMTV34L replication (Fig. 27B) argues against YMTV34 having significant PKR inhibitory function in HeLa cells. Therefore, YMTV34 may block an alternate, PKR-independent antiviral mechanism active against vvWRΔE3L. Such a mechanism is likely to exist, given that suppression of PKR expression does not fully

restore E3L deficient VACV replication in HeLa cells[254] (Fig. 27B). Although E3 also inhibits activation of the 2'-5' oligoadenylate synthetase/RNaseL system[194], RNaseL is expressed at very low levels in HeLa cells and suppression of RNaseL expression in HeLa cells has no effect on vvWRΔE3L replication (data not shown). Therefore, this antiviral mechanism does not contribute to the replication defective phenotype of vvWRΔE3L or vvWR-YMTV34L in HeLa cells. The formation of cytoplasmic, antiviral granules in cells infected with vvΔE3L occurs downstream of PKR activation and eIF2α phosphorylation and limits the replication of the virus[223]. Thus, it remains a possibility that although YMTV34 does not block PKR activation, it could function downstream of PKR in the inhibition of antiviral granule formation. Analysis of the function of the YMTV34 protein should prove useful in future studies to identify the mechanism(s) by which YMTV34, and possibly E3, promote viral replication.

Significant variability in the dsRNA binding capacity of the E3 orthologues was observed (Fig. 28). SPPV34 had the lowest dsRNA binding capacity, while SPV032 and M029 displayed the highest dsRNA binding capacity, correlated with their ability to suppress PKR activation. YMTV34 binds dsRNA much more efficiently than SPPV34, but is still unable to inhibit the activation of PKR. Although different antibodies must be used to detect E3 and the E3 orthologues, it seems E3 binds dsRNA with less affinity than YMTV34, SPV032 and M029. Nonetheless, E3 can potently inhibit PKR activation. These results support the concept that dsRNA binding is not the sole factor in PKR inhibition, which may additionally require protein-protein interactions between E3 and PKR[198,218]. The A175 residue in E3 is also required for dsRNA binding and inhibition of PKR (Jingxin Cao, unpublished data). The corresponding alanine residue is conserved in SPV032 and M029. In YMTV34, an A→S substitution has occurred, while in SPPV34

an A→T substitution has occurred. Thus, the presence of alanine at this residue correlates with the ability of the protein to suppress PKR activation and to promote viral replication. It will be interesting to investigate further the functional consequence of mutation of this residue in E3, SPPV34 and YMTV34.

E3 suppresses expression of cytokines during VACV infection[53,116,243] and also antagonizes the antiviral effects of IFN-β[7,10]. All the E3 orthologues were able to inhibit the induction of IFN-β expression, although to varying degrees (Fig. 29A). M029 was the most effective inhibitor of IFN-β expression in this system, and SPPV34, the least effective. However, the partial inhibition of cytokine gene expression was the only function identified for SPVV34 in this study. In Results Section II, it was found that IFN-β induction is regulated by both RIG-I and MDA5 during vvCopΔE3L infection of HeLa cells (Fig. 20). Thus, although the recombinant viruses expressing E3 orthologues are based on a different strain (Western Reserve) of VACV, it appears that each of the E3 orthologues can inhibit RIG-I/MDA5 signalling to some extent. However, whether this is a consequence of sequestering viral dsRNA, or occurs through an alternate mechanism remains unknown. YMTV34, SPV032 and M029, but not SPPV34, were also able to suppress the antiviral effects of IFN-β (Fig. 30A). These results highlight the ability of E3 and its orthologues to inhibit both the induction and effector arms of the IFN response.

In Results Section I, it was found that E3 suppresses expression of TNF-α and IL-6 through a mechanism partially dependent on PKR. SPPV34 and YMTV34 moderately reduced TNF-α expression during virus infection while SPV032 and M029 more significantly inhibited TNF-α expression (Fig. 29B), correlating with the ability of each viral protein to suppress PKR function. M029 was also the only E3 orthologue capable of significantly suppressing IL-6 expression in this system (Fig. 29C). In Results Section I,

IFN-β was found to be induced by late gene products in vvCopΔE3L infected cells, while TNF-α and IL-6 can be induced by early gene products. Since the E3 orthologues inhibit IFN-β expression to a greater extent than TNF-α and IL-6, these viral proteins are more effective at suppressing cytokines induced by late gene products as opposed to early gene products.

Despite results obtained in cell culture, none of the E3 orthologues tested could restore pathogenicity to vvWRΔE3L *in vivo* (Fig. 32). Likewise, expression of the Orf virus E3 orthologue in place of E3L is sufficient to rescue virus replication *in vitro* but not *in vivo*[238]. Currently, the role of host proteins such as PKR in the *in vivo* response to vvWRΔE3L infection remains to be clarified. In one study, intranasal inoculation of PKR/RNaseL double-deficient mice with vvWRΔE3L failed to result in severe disease[243]. However, in an alternate, intratracheal mouse model, inoculation with higher doses of vvWRΔE3L resulted in severe disease in ~80% of PKR/RNaseL double-deficient mice[192]. Thus, although SPV032 and M029 can suppress PKR activation and the antiviral effect of IFN-β *in vitro*, these functions may not be sufficient to promote viral replication following intranasal infection of mice.

SPPV, YMTV, SPV and MYXV naturally replicate in distinct host species and the N-terminal divergence of the E3 orthologues encoded by these viruses may reflect species specific evolution and the need to adapt specifically to unique host environments. It has been shown that the N-terminal domain of E3 is required for pathogenesis and neurovirulence in mice[22,23]. Residues important for Z-DNA binding by ADAR1 have been identified, as have the corresponding residues in the E3 Z-DNA binding domain[103]. Overall, many of these residues, including Tyr-48 and Pro-63, are conserved in the E3 orthologues examined. However, several of these residues, including Ile-62 and Arg-41

of E3 are not conserved in any of the E3 orthologues. The effect of Ile-62 and Arg-41 mutations has not been tested but mutation of Tyr-48 in E3 is correlated with reduced pathogenicity *in vivo*. Therefore, the inability of the recombinant viruses expressing orthologues of E3 to replicate *in vivo* may be a result of the sequence variation of the N-terminal domain.

In summary, this work details the first characterization of the SPPV34, YMTV34, SPV032 and M029 poxvirus proteins. SPPV34 and YMTV34 cannot suppress PKR activity in HeLa cells. However, YMTV34 promotes VACV replication through a PKR-independent mechanism. It cannot be ruled out that SPPV34 and YMTV34 can inhibit PKR in the natural host during SPPV and YMTV infection, respectively. While SPV032 and M029 can complement the deletion of E3 *in vitro* through the inhibition of PKR activity, they cannot complement E3 function *in vivo*. Therefore, further work is required to understand the mechanism by which E3 supports VACV replication *in vivo*. It will also be interesting to study the phenotype of SPPV, YMTV, SPV and MYXV deficient in expression of their respective E3 orthologue. Overall, these results contribute to an understanding of how poxviral proteins subvert host innate immune signalling to support virus replication.

Chapter IV

General discussion

Poxviruses have, and continue to serve as excellent models for studying the mechanisms by which viruses subvert host immune responses[213]. VACV expresses a large and diverse repertoire of proteins which antagonize innate and adaptive immunity. The E3 protein encoded by VACV is one such antagonist of innate immune signalling. E3 was initially identified as a soluble factor present in extracts from VACV-infected cells capable of inhibiting PKR activation[242]. Since this initial discovery, E3 has been demonstrated to perform several other functions during infection, including inhibition of pro-apoptotic signalling and suppression of cytokine expression. The activation of some of these responses during E3L-deficient VACV infection have been found to be PKR-dependant[254], while other responses have been found to be PKR-independent[243]. Thus, it is now clear that E3 targets alternate host antiviral factors in addition to PKR. However, how E3 antagonizes innate immune responses is incompletely understood and represents a gap in understating how VACV suppresses host antiviral defences. Moreover, despite the identification and characterization of a multitude of VACV immuno-modulatory proteins like E3, an understanding of what actually activates the innate immune system during VACV infection has not been thoroughly investigated.

Previously, E3 has been reported to suppress expression of IFN-β, TNF-α and IL-6[53,116,243] but the mechanism(s) responsible for this inhibition has not been studied. Although PKR can regulate p38 and NF-κB, which are known to regulate cytokine expression, inhibition of PKR by E3 has only been correlated to its ability to inhibit cytokine expression. The results presented herein demonstrate that E3 targets both PKR-

dependant and PKR-independent signal transduction to block cytokine gene transcription (Fig. 9C). The pathways regulating TNF-α and IL-6 expression during vvCopΔE3L are at least partially PKR-dependant. The pathway regulating TNF-α expression can be significantly suppressed by E3, SPV032 and M029, while the pathway regulating IL-6 can be blocked by E3 and M029 (Fig. 29B, C). In contrast SPPV34 and YMTV34 only moderately inhibit these pathways. Interestingly, the capacity of the E3 orthologues to bind dsRNA (Fig. 28) does not correlate with the inhibition of IL-6 expression (Fig. 29C). This suggests that E3 and M029 antagonize the pathway regulating IL-6 through a function independent of their ability to bind dsRNA. A largely PKR-independent activation of IFN-β expression occurs during vvCopΔE3L/vvWRΔE3L infection, and full transcriptional activation requires MDA5, RIG-I (Fig. 20A, B), IPS-1, IRF3 and NF-κB p65 and p50 (Fig. 11B). This response can be inhibited by E3, SPV032 and M029. Collectively, these results detail the signalling pathways mediating cytokine expression during vvΔE3L infection and highlight the ability of E3, SPV032 and M029 to inhibit these responses. Moreover, these findings are the first direct evidence to show that VACV infection is sensed by both MDA5 and RIG-I and that E3, SPV032 and M029 can block RLR signalling to inhibit IFN-β expression.

It is possible that inhibition of cytokine production by E3 contributes to VACV replication *in vivo*. Several of the cytokines suppressed by E3 have been demonstrated to play a pivotal role in host defence against VACV infection *in vivo*. For example, IL-6 is pivotal mediator of the immune response and can function as a co-stimulatory molecule for T-cell activation and can modulate the balance between T-helper 1 (Th1) and Th2 immune responses[57]. IL-6 deficient mice display a defective cytotoxic T-cell response against VACV, and support 1000-fold greater viral replication in the lungs[107]. It has been

reported that the VACV-specific T-cell response is diminished in transgenic mice expressing E3[59]. Therefore, it is possible that suppression of IL-6 expression by E3 may dampen the antiviral activities of T-cells against VACV *in vivo*. Furthermore, TNF-α treatment has no effect on VACV replication in cell culture, but potently suppresses VACV replication in mice[124,202]. TNF-α is an important mediator of pro-inflammatory signalling and can activate immune cells such as macrophages, as well as cellular antiviral defence mechanisms including apoptosis[177]. The cytokine activin A, a homodimer of INHBA subunits, can regulate the differentiation of monocytes into dendritic cells and enhance antibody production by B-cells[170,212]. Moreover, natural killer cells are activated to proliferate and secrete effecter molecules during VACV infection in a manner dependant on type I IFN receptor signaling[138]. Although many cytokines do not display their full biological activity in cell culture, the enhanced innate cytokine response which occurs in the absence of E3 may help explain the replication defective phenotype of E3L-deficient vaccinia virus in mice. Moreover, since none of the E3 orthologues tested can fully compensate for the ability of E3 to inhibit TNF-α and/or IL-6 expression (Fig. 29B, C), it is possible that induction of these cytokines during infection may result in activation of mechanisms to clear virus-infected cells. This may explain, at least in part, the inability of the recombinant viruses expressing E3 orthologues to replicate *in vivo*.

Previously, the effect of VACV dsRNA species on the induction of cytokines and apoptosis has been inferred from the phenotypes of vvΔE3L or VACV recombinants expressing E3 mutants unable to bind dsRNA. Few studies have directly assessed the immuno-stimulatory activity of RNA species generated during VACV replication in the absence of other viral factors such as viral DNA or proteins. It was recently shown that

the dsRNA binding domain of E3, as well as single amino acids essential for dsRNA binding, also account for the ability of E3 to inhibit ssRNA and dsDNA-induced signalling[137]. Thus, although the induction of cytokine expression and apoptosis during vvΔE3L infection is speculated to be stimulated by viral RNA species, it is possible that other alternative viral PAMP(s) could be the stimulatory molecule. Therefore, a more direct model was required to assess the specific role of RNA species generated in VACV-infected cells to function as PAMPs. In this project, RNA species generated during VACV infection were directly introduced into the cytoplasm of cells and subsequently cytokine expression and apoptosis were analyzed. Interestingly, these VACV RNA species stimulate IFN-β expression through the same signalling pathways which mediate vvΔE3L-induced IFN-β expression. This provides evidence that during vvΔE3L infection, RNA species are major PAMPs activating IFN-β expression. This finding is significant because it provides the first direct evidence to support the role of VACV dsRNAs in stimulating host immune responses during VACV infection. It also supports the concept that a major function of E3 is to block the activation and/or signalling of host PRRs activated by these RNAs.

In Results Section I, the cytokine response to vvCopΔE3L infection was described (Table 7) and in Results Section II, the cytokine response to transfected RNA was examined (Table 8). Comparing the data between the two experiments offers insight into the potential PAMPs responsible for activating cytokine expression during virus infection. First, both vvCopΔE3L infection and late RNA transfection result in up-regulation of IFN-β, TNF-α and IL-20. This suggests that RNA species generated in VACV-infected cells are capable of inducing expression of these cytokines during vvCopΔE3L infection. However, they do not rule out the stimulatory role of other

PAMPs produced during VACV infection. In contrast, vvCopΔE3L infection up-regulates LTA expression while RNA transfection does not. Thus, induction of LTA during virus infection likely results from the recognition of an alternative viral PAMP such as viral DNA or proteins and not from recognition of viral RNAs. Some indirect evidence exists to suggest that VACV DNA can serve as a PAMP during virus infection. For example, VACV infection results in inflammasome activation dependent on the cytosolic DNA sensor, AIM2[81,190]. Although AIM2 is classified as a DNA sensor, and binds to dsDNA, it has not been shown to bind VACV DNA. IFN inducible protein 16 (IFI16), an AIM2-like protein, is another cytosolic DNA sensor[232]. A synthetic 70 bp dsDNA corresponding to an adenine-thymine rich region present in the terminal region of the VACV genome induces IFI16-dependent IFN-β expression when transfected into cells. However, during VACV infection, it is unlikely that this 70 bp region of the VACV genome would be recognized by IFI16 in a similar manner since it is present in the context of the entire viral genome. Furthermore, a role for IFI16 during actual VACV infection has not been established. Therefore, although VACV infection has been linked to DNA sensing pathways, it remains to be determined whether VACV DNA is recognized by host PRRs as a PAMP.

In Results Section II, VACV RNA species were found to stimulate PKR, MDA5 and RIG-I activity. This situation is similar to reovirus infection, where these three PRRs can be activated, and at least RIG-I and MDA5 are specifically activated by reovirus genomic RNA[84,97]. RIG-I and MDA5 generally bind to distinct, and non-overlapping RNA structures, while PKR can bind to dsRNA in a manner similar to both RIG-I and MDA5. For example, on shorter dsRNA (which does not activate MDA5), both PKR and RIG-I are activated by binding to the 5'-triphosphate group[165,208]. PKR can also be

activated by long dsRNA of several kbps[166], similar to MDA5. Given the diversity of RNA structures identified in VACV-infected cells, which vary in length between only a few hundred bp to several kbps[19], it is not surprising that RNA ligands for PKR, MDA5 and RIG-I are produced. That both VACV and EMCV RNA-based PAMPs can be sensed in a cell-type specific manner has interesting implications for the specificity of RNA induced activation of RIG-I and MDA5. These results suggest that additional factors, which could include other RNA modifying enzymes, may have as significant an impact on the selectively of RIG-I or MDA5 activation as does the intrinsic RNA binding properties of RIG-I and MDA5 themselves.

It was found that VACV RNA-based PAMPs are generated predominantly during the intermediate and late phase of infection, and likely originate from intermediate and/or late gene transcripts. Recently, RNA isolated from VACV-infected cells was reported to trigger MDA5 dependent IFN-β expression[182]. The stimulatory RNA species were present in a high molecular weight complex composed of both ssRNA and dsRNA, although the roles of the different RNA components were not reported. Such a high molecular weight RNA complex was not observed in the late RNA preparations used in this study. However, it would be interesting to size fractionate the late RNA sample to see if the immuno-stimulatory RNAs are present in a specific range of RNA length/molecular weight. In Results Section II, it was found that dsRNA, and not ssRNA, is the RNA-based PAMP generated during VACV replication responsible for inducing IFN-β expression (Fig. 13A, Fig. 14A, B). Therefore, it appears that the 5'-ssRNA tails which are likely present on some VACV dsRNAs (Fig. 3) do not play an important role in stimulating RLR activity. However, these results do not rule out the possibility that ssRNAs which can form dsRNA secondary structures can function as PAMPs in VACV

infected cells. Interestingly, since PKR, MDA5 and RIG-I can be activated by unique RNA ligands, E3 has evolved to counteract PRR activation by diverse RNA agonists.

The precise mechanism(s) by which E3, SPV032 and M029 inhibit PKR, MDA5 and RIG-I remains to be determined. Sequestering of dsRNA and protein-protein interaction are two possible mechanisms. In the case of PKR, a combination of both mechanisms may contribute to PKR inhibition since E3 can bind dsRNA[34,79], and can also form protein-protein interactions with PKR[198,218]. However, separation of these two potential mechanisms is difficult because the dsRNA binding domain is responsible for both functions. Moreover, single amino acid residues required for dsRNA binding are also required for interaction with PKR[79,198]. Based on the results presented in this project, and the work of others[192,254], it is evident that in many systems, inhibition of PKR activity is a key factor in determining VACV host-range. The ability of each E3 orthologue studied to inhibit PKR activation correlated with the restoration of virus replication in cell culture, but not *in vivo*. Thus, other factors in addition to PKR may contribute to *in vivo* host-range and likely affect poxvirus species specificity. The YMTV34 protein can bind dsRNA with similar affinity to SPV032 and M029, and potentially with even higher affinity than E3 (Fig. 28). However, unlike E3, SPV032 and M029, YMTV34 cannot inhibit PKR phosphorylation or downstream signalling in HeLa cells (Fig. 26A, B, C). Overall, this supports the concept that dsRNA binding might be necessary, but not sufficient for PKR inhibition. However, it remains to be determined if SPPV34 and YMTV34 can inhibit PKR activation during infection of the natural hosts for SPPV and YMTV.

In contrast to PKR, very little is known about the function of E3 in antagonizing RLR function. It has been shown that RIG-I can be activated by binding to the 5'-

triphosphate of shorter dsRNAs[208] and along the entire length of longer RNA molecules[16]. If E3 functions by sequestering long dsRNA away from RIG-I, then E3 would have to bind along the entire length of dsRNA in order prevent RIG-I activation. To date, no direct experimental evidence has been reported to show that E3 binds to viral dsRNA in infected cells. Moreover, if short dsRNAs function as PAMPs in VACV-infected cells to activate RIG-I and/or PKR, E3 would also have to bind these RNAs in such a way as to mask the 5'-triphosphate. The role of 5'-triphosphate groups in the immuno-stimulatory activity of VACV RNAs has not been tested. However, VACV mRNAs are capped[60] and therefore it is unlikely that 5'-triphosphate groups are exposed on these RNAs. Treatment of VACV late RNA with phosphatases to remove the 5'-triphosphate and then transfecting this RNA into cells would be one approach to address the contribution of 5'-triphosphate groups in the immuno-stimulatory activity of VACV RNAs. However, if VACV RNAs do contain immuno-stimulatory 5'-triphosphates, removing these motifs would only be expected to abolish the activation of RIG-I by short dsRNA, and not by longer dsRNA and therefore the effect of 5'-triphosphate removal might be subtle. Furthermore, MDA5 activation would not be affected by this treatment since it does not sense RNA in a 5'-triphosphate-dependent manner. The structural requirements of RNA required for MDA5 binding, besides length, have not been determined, but since E3 inhibits IFN-β expression during infection which is partially dependent on MDA5, E3 likely also binds dsRNA in such a way as to prevent MDA5 activation.

The results obtained in this study should contribute to the design of vaccines and oncolytic agents. Poxvirus-based recombinant vectors represent a promising vaccine platform since they are highly immunogenic, easy to manipulate and can express high levels of foreign genes[62,88]. Human immuno-deficiency virus[176], avian influenza[26] and

malaria[125] are examples of human infectious diseases for which the efficacy of poxvirus-based vaccines are currently being investigated. VACV expressing mutants of E3 are also being investigated as a vaccine platform since they are safer than wild-type VACV[90,237]. A better understanding of the interplay between poxviruses and the immune system will be critical for improving these vaccines[183]. E3-deficient VACV vectors might be more immunogenic because they induce more cytokine expression than wild-type VACV via the activation PKR (Fig. 9C), MDA5 and RIG-I (Fig. 20), as detailed in this study. Moreover, poxviruses are being investigated as potential oncolytic agents to destroy cancer cells[104]. For example, MYXV has demonstrated efficacy in glioma[133] and other cancers, and can also selectively eliminate leukemic cells from hematopoietic grafts while sparing normal hematopoietic stem cells[102]. Results from the comparison of E3 orthologues suggest that deletion of M029 from the MYXV genome may result in a virus which induces higher levels of cytokine expression (Fig. 29). Furthermore, because M029 was found to inhibit apoptosis (Fig. 26C), it is possible that M029 deficient MYXV will more effectively induce apoptosis in cancer cells, a desirable outcome for cancer treatment.

Lastly, viral RNAs may also have significant therapeutic applications. There is a great deal of interest in developing potent RNA-based ligands for the selective activation of PKR, MDA5 and RIG-I, as well as the TLRs, as a means to trigger antiviral defence in the context of treatment for viral infections[52,207]. Typically, live-attenuated and whole-killed vaccines are more immunogenic than protein subunit vaccines. This likely stems from the fact that exposing cells to the actual virus, which may replicate to some degree, more effectively stimulates innate immunity which would subsequently affect adaptive immunity. In contrast, protein subunit vaccines do not typically stimulate innate immune

responses as effectively. However, many current vaccine technologies are based upon subunit vaccines because they are generally safer and easier to manipulate. The inclusion of immuno-stimulatory RNAs as adjuvants may boost the effectiveness of subunit vaccines by stimulating innate immune responses. For example, both dsRNA and Type I IFNs have been demonstrated to have potent adjuvant activity by enhancing the antibody response to protein antigens[119]. Since viral RNA-based PAMPs induce expression of Type I IFN and pro-inflammatory cytokines, it is possible they could increase the efficacy of vaccines. Indeed, inclusion of a RIG-I agonist has been shown to improve the antibody response of an influenza vaccine[132]. RNA ligands activating PKR, MDA5 and RIG-I may also prove useful to stimulate anti-tumour responses in cancer. It has already been demonstrated that synthetic MDA5 and RIG-I RNA ligands can induce pro-apoptotic signalling in melanoma[14] and ovarian cancer cells[110].

In order to improve the effectiveness of RNA-based adjuvants which stimulate PRR activity, it will be necessary to better define the *in vivo* RNA agonists of these proteins. The identification and characterization of viral ligands for PKR, MDA5 and RIG-I will provide a valuable starting point for the rational design of such synthetic therapeutic RNA ligands. In this study, it was found that VACV RNAs induce apoptosis through PKR (Fig. 22C, D) and also activate MDA5 and RIG-I-dependent cytokine production (Fig. 17, 18B, 19A, B). Therefore, VACV RNAs have the potential to be used directly as adjuvants or to serve as a model for the design of synthetic RNAs. At present, there has been no reported method to selectively purify immuno-stimulatory VACV RNAs from infected cells, but such a technique would prove useful for determining the structure of poxvirus RNA-based PAMPs and for the development of RNA-based therapeutics.

In conclusion, the findings presented in this study contribute to an understanding of how E3 and its poxvirus orthologues suppress innate immune responses activated by poxvirus RNA-based PAMPs. This information will lead to a greater appreciation of the interplay between poxviruses and the human immune system, as well as the mechanisms regulating poxvirus host-range and species specificity. Such knowledge will be important for the development of novel strategies to inhibit poxvirus infection in both human and animal hosts. Furthermore, the results described herein will be beneficial to the design of poxvirus-based vaccine vectors and oncolytic agents, as well as for the development of therapeutic RNAs for the treatment of infectious diseases and cancer.

Future Directions

The results obtained in this study raise intersting questions on the function of VACV E3 and on the nature of immuno-stimulatory VACV RNA species. For example, little is known about the mechanism by which E3 inhibits the RLR pathway. It is not known if E3 can directly interact with RIG-I and/or MDA5 and whether this could contribute to the inhibition of these RLRs, but immuno-precipitation experiments could be used to determine this. Furthermore, studies similar to those employed to detect RIG-I bound along the entire length of long dsRNA using atomic force microscopy[16] should also be applied to E3 to determine its mode of interaction with dsRNA. Results from these experiments will contribute to a better understanding of how E3 can inhibit RIG-I and MDA5 signal transduction.

How E3 inhibits PKR is also not fully understood. Data presented here, and by others, suggests that dsRNA binding alone cannot explain the ability of E3 to inhibit PKR, which may also depend of protein-protein interactions. One approach to address the specific contribution of E3 interactions with PKR would be to investigate the ability of E3 to inhibit dsRNA-independent activation of PKR. For example, PKR can be activated in the absence of dsRNA by heparin[83], as well as by PACT[179]. Treating cells with heparin, or over-expressing PACT, and then monitoring the activation of PKR in presence or absence of E3 might provide evidence for the specific role of E3-PKR protein-protein interaction in the inhibition of PKR.

Given the importance of E3 for the suppression of antiviral and proinflammatory cytokine expression, and the role these cytokines play in immune defence against virus infection, it will be interesting to determine whether the pathogenicity of E3L-deficient vaccinia virus is restored in mice deficient for the receptors of IFN-β, TNF-α, IL-6 or

INHBA. Furthermore, the importance of RIG-I and MDA5 could also be examined *in vivo* by the use of gene-specific knockout mice. The results from these types of studies could underscore the importance of the ability of E3 to inhibit cytokine expression.

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