dsRNA SIGNALING IN INNATE IMMUNITY AND VIRAL INHIBITION

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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We also certify that written approval has been obtained for any proprietary material contained therein.
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ACKNOWLEDGEMENTS

Parts of this work were presented at the CWRU MSTP winter and summer retreats, the Cleveland Clinic Research Day Retreats, the 2006 ASV meeting in Madison, Wisconsin, the 2008 Innate immune signaling mechanisms Keystone meeting in Keystone, Colorado, 2008 Lepow Day at CWRU and 2008 Research ShowCASE at CWRU.

Veronique Lefebvre at the Cleveland Clinic provided advice for the ISG56 knockout mouse targeting construct and Southern strategy. The ISG56 knockout mouse targeting vector was injected into ES cells at the Embryonic Stem Cell Core in collaboration with Herbert Virgin IV at Washington University in St. Louis. Mamta Puri under Curt Horvath at Northwestern University cloned V protein wildtype and mutant expression vectors. Sun Qiang under John Hiscott at McGill University provided purified IRF3 and IRF3 constructs for the \textit{in vitro} kinase assays. Minghao Sun under Biao He at Pennsylvania State University provided wildtype and mutant PIV5 viruses and guidance in their use. Ulrich Siebenlist at NIAID provided TBK1 and IKKe expression vectors. Xiaoxia Li at Cleveland Clinic provided TRIF expression vector. Serge Fuchs at the University of Pennsylvania provided HA tagged ubiquitin expression vector. Adam Geballe at University of Washington in Seattle provided mCMV and hCMV ORF expression vectors. Jae Jung at University of Southern California provided MHV68 ORF expression vectors and also related reagents for which data have not been presented here: wildtype MHV68 virus, GFP tagged MHV68 virus and ORF27 and 58 deficient MHV68 (originally constructed by Ren Sun at University of California at Los Angeles).
In the Sen lab, Kevin Smith provided invaluable technical support in the projects involved with viral proteins. Kristi Peters cloned the 561-TK construct and was instrumental in the construction of the cell line itself. Sauman Sarkar, Theresa Rowe and Heather Smith constructed and kindly provided the parental cells TLR3-293. Srabani Pal provided technical support in the cloning and Southern for the ISG56 (and ISG69) knockout mouse project and Theresa Rowe and Tina Gaughan provided much expertise in the design of the knockout and Southern strategies. In work not presented here, Avanti Desai contributed by conducting MHV68 plaque assays. Christine White provided mice for making macrophages and Difernando Vanegas and Michi Yamashita provided technical assistance with mice. Finally, Neil Halmagyi provided technical support for cloning in the V protein project.

In addition, other past and present members of the Sen lab provided invaluable assistance. In the Signaling group, Kristi Peters, Saumen Sarkar, Chris Elco, Neil Halmagyi, Theresa Rowe, Heather Smith, Michi Yamashita, Boni Pal, Kevin Smith, Mitali Pandey, Pritha Majumder, Jazz Singh, Tammy Guo and Andy the rotating CCF med student whose last name I can’t remember. Chris in particular was helpful in writing and editing this thesis. In the PACT/PKR group, Christine White, Greg Peters, Ben Dickerman, Avanti Desai, Shoudong Li, Mark Rizzi and Joao Marques. In the P56 group, Fulvia Terenzi, Parama Saikia, Danny Hui, Wen Li and Volker Fensterl. In the ACE group, Saurabh Chattopadhyay, Jeff Chang, Difernando Vanegas, Sean Kessler and Tina Gaughan.
Lastly, I thank my committee members George Stark, Clifford Harding, Phil Pellet, Robert Silverman and Jon Karn for guiding me through my studies, and my advisor Ganes Sen for his mentorship.
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<td>Epstein Barr virus</td>
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<tr>
<td>CARD</td>
<td>caspase activated recruitment domain</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPG</td>
<td>Cytosine Guanine</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IRFs</td>
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<tr>
<td>DAMP</td>
<td>damage associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DExD</td>
<td>H box RNA helicases motif</td>
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<tr>
<td>Dpi</td>
<td>days post infection</td>
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<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
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<tr>
<td>eIF</td>
<td>elongation initiation factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
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<tr>
<td>GAS</td>
<td>gamma-activated site</td>
</tr>
<tr>
<td>GCV</td>
<td>ganciclovir</td>
</tr>
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<td>Hepatitis C virus</td>
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<tr>
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<td>Hendra virus</td>
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<td>HHV8</td>
<td>human herpesvirus 8</td>
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<td>human Parainfluenza virus 2</td>
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<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IFIT1</td>
<td>interferon induced tetracopeptide 1</td>
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<tr>
<td>IKKe</td>
<td>Inhibitor of κB kinase ε</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPS-I</td>
<td>IFNβ promoter stimulator 1</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
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<tr>
<td>ISG</td>
<td>interferon stimulate gene</td>
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<td>ISRE</td>
<td>interferon stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi Sarcoma Herpesvirus</td>
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<tr>
<td>LMB</td>
<td>leptomycin B</td>
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<td>LPS</td>
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<td>LRR</td>
<td>leucine-rich repeat</td>
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<td>mda-5</td>
<td>melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MHV68</td>
<td>murine γ-herpesvirus 68</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MeV</td>
<td>Measles virus</td>
</tr>
<tr>
<td>MuV</td>
<td>Mumps virus</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor κ B</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>pIC</td>
<td>Polyninosinic-polycytidylic acid</td>
</tr>
<tr>
<td>PIV5</td>
<td>Parainfluenza virus 5</td>
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<tr>
<td>PKR</td>
<td>protein kinase RNA regulated</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>RIG-I</td>
<td>retinoic acid inducible gene</td>
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<tr>
<td>SeV</td>
<td>Sendai virus</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>ssRNA</td>
<td>single-stranded RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TIR</td>
<td>toll/IL-1R homology domain</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TPR</td>
<td>tetracopeptide repeat</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll-IL1R domain containing adaptor inducing IFNβ</td>
</tr>
<tr>
<td>VH</td>
<td>V protein from hPIV2</td>
</tr>
<tr>
<td>VM</td>
<td>V protein from MuV</td>
</tr>
<tr>
<td>VP</td>
<td>V protein from PIV5</td>
</tr>
<tr>
<td>VDC</td>
<td>V delta C</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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dsRNA Signaling in Innate Immunity and Viral Inhibition

Abstract

by

LENETTE LIN LU

The innate immune system helps determine the outcome of pathogen invasion of a host because it mediates recognition of and initiates the host response against the pathogen. The balance of the system is delicate. Too little signaling is ineffective in clearing the pathogen and too much is pathological for the host. Therefore, regulation is important for both parties.

dsRNA is a molecular pattern associated with viral infections which stimulates the innate immune response. Host recognition of dsRNA include Toll like receptor 3 (TLR3) and the RNA helicases retinoic acid inducible gene (RIG-I) and melanoma differentiation associated gene (mda-5). These receptors signal to activate many transcription factors including Interferon (IFN) Regulatory Factor 3 (IRF3) which mediates the expression of IFN stimulated genes (ISGs). Of the hundreds of ISGs, ISG56 is consistently one of, if not the most, highly induced, indicating an importance in antiviral immunity. Indeed, p56, the protein product of ISG56, inhibits host translation and in doing so prevents viral replication.

To determine the physiological significance of p56, we sought to conditionally disrupt the mouse ISG56 gene. (Chapter 3) Unfortunately, problems were encountered in generating an embryonic stem cell containing a correctly integrated targeting construct.
To discover novel and to distinguish previously characterized regulatory mechanisms of IRF3, we established a cell survival assay that can identify both activators and inhibitors of IRF3 via TLR3 signaling. (Chapter 4) Using this system, we found that V proteins encoded by select RNA Paramyxoviridae viruses could inhibit IRF3 activation by acting as alternative substrates for the IRF3 kinases inhibitor of κ B kinase epsilon (IKKe) and TANK binding kinase (TBK1). (Chapter 5) In addition, we identified novel open reading frames from the DNA viruses cytomegalovirus and murine γ-herpesvirus 68 that could inhibit IRF3 activation. (Chapter 6) Mechanistic studies of these inhibitors revealed complexities in IRF3 activation, distinguished TBK1 and IKKe as points of convergence and divergence for many pathways and indicated the disparate needs of viruses to both activate and inhibit immune signaling. (Chapter 7) These investigations have implications not only in antiviral therapy development but also in understanding host virus coevolution.
CHAPTER 1

INTRODUCTION

Innate and Adaptive Immunity

The immune system consists of both innate and adaptive responses to infections.\textsuperscript{1} The innate branch presents the first line of defense against pathogens such as viruses, bacteria, fungi and parasites in the form of passive physical barriers like the skin and mucosa. If the pathogen penetrates this defense, the innate immune system is also responsible for the initial recognition of an infection and the subsequent generation of an active anti-microbial response which begins immediately and can persist hours to days. This is accomplished at the cellular level by monocytes such as macrophages and dendritic cells which produce interferons (IFNs) and cytokines, granulocytes such as neutrophils which phagocytose and kill bacteria and, finally, lymphocytes such as natural killer (NK) cells that kill infected host cells with aberrant major histocompatibility complex (MHC) Class I expression. Although effective, the innate immune response does not improve with repeat exposures to the same pathogens. This is because surveillance is limited to a set of pattern recognition receptors (PRR) that recognize only general constituents of either foreign origin or the stressed host.\textsuperscript{2}

Innate immunity also serves to initiate the adaptive branch of the immune system, which is necessary if the pathogen persists beyond the initial neutralization steps. This occurs in part through T and B lymphocytes following activation by antigen presenting cells (APC) such as monocytes. Lymphocyte activation leads to the generation of a diverse repertoire of T cell receptors and antibodies via gene rearrangement that provides more specific responses. In addition, the gene rearrangement also provides
immunological B and T cell memory and, as a result, more rapid and enhanced recall responses upon re-infection when compared to the innate response.

Innate immunity alone is sufficient for simple organisms like single cell eukaryotes such as the social amoeba which evolved 1-2 billion years ago. However as organisms have evolved, immunity too has become more complex with the development of adaptive immunity in jawed (but not jawless) fishes and higher vertebrates over the past 500 million years. During that time co-evolution of adaptive and innate immune systems under the constant selection pressure for host fitness applied by pathogens has led to the generation of a highly complex system of relationships.

Pathogen Associated Molecular Patterns and their Receptors in Innate Immunity

One of the most critical aspects of the innate immune system is recognition of pathogen associated molecular patterns (PAMPs), originally defined as molecules unique to invading microorganisms. These include but are not limited to lipopolysaccharide (LPS), lipotechoic acids (LTA), peptidoglycan (PGN), flagellin, lipoproteins/peptides, β-glucan and nucleic acids from bacteria and fungi (Table 1.1). Virus specific PAMPs include double (ds) and single stranded (ss) RNA and DNA as well as virion proteins such as glycoprotein B from human cytomegalovirus (hCMV), the envelope protein from mouse mammary tumor virus (MMTV) and Measles virus hemmaglutinin (HA) protein. In addition, endogenous proteins and nucleic acids present in a stressed host in either abnormal compartments or concentrations are also capable of initiating innate immune responses. These ligands are known as danger associated molecular patterns (DAMPs).
The PRRs are host proteins responsible for recognizing and inducing a cytokine response to PAMPs and DAMPs. They can be organized into toll like receptors (TLRs), nucleotide binding oligomerization domain (NOD) like receptors (NLR), the RNA helicases, lectins and the DNA binding protein DNA-dependent activator of IFN-regulatory factors (DAI) (Table 1.1).

Vertebrate TLRs were originally discovered as homologues of the Drosophila Toll gene which is involved in embryonic development and antifungal immunity. To date, 11 TLRs have been identified in humans and 12 in mice. As type I transmembrane proteins, they span the plasma membrane once and have extracellular N termini and cytoplasmic C termini. TLRs can further be identified by a leucine rich repeats (LRR) in their extracellular domains responsible for ligand recognition and a Toll-interleukin 1 receptor (TIR) domain in their cytoplasmic segment responsible for protein-protein interactions necessary for downstream signaling. TLR2, 4 and 5 are expressed on the plasma membrane and classically recognize lipoprotein and peptidoglycan, LPS and flagellin respectively. TLR3, 7 and 9 are localized to endolysosomes and classically recognize dsRNA, ssRNA and CPG DNA respectively (Table 1.1). For the identified 11 members of the family, two adaptor proteins serve as scaffolding to mediate interactions for gene induction: TIR domain containing adaptor inducing IFNβ (TRIF), used by TLR3 and 4, and myeloid differentiation primary response gene 88 (MyD88), used by the other TLRs.

The remaining PRRs are structurally distinct from TLRs in a number of ways. NLRs also contain LRRs for recognition of their ligands which are bacterial peptidoglycans and even viral DNA. However, they are not transmembrane proteins
Table 1.1: Pathogen/Danger Associated Molecular Patterns and their Pattern Recognition Receptors.

Adapted from “Toll-like receptor signaling” and “How dying cells alert the immune system to danger.”

<table>
<thead>
<tr>
<th>Microbial components</th>
<th>Organisms</th>
<th>Pattern Recognition Receptor</th>
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<td><strong>Bacteria</strong></td>
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<tr>
<td>LPS</td>
<td>Gram negative bacteria</td>
<td>TLR4</td>
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<td>Diacyl lipopeptides</td>
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<td>bacteria and mycobacteria</td>
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<td>RNA</td>
<td>bacteria</td>
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<td>dsRNA</td>
<td>Viruses such as Reovirus, HSV</td>
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<td>RNA viruses</td>
<td>TLR7 and TLR8</td>
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<tr>
<td>Envelope proteins</td>
<td>RSV, MMTV</td>
<td>TLR4</td>
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<td>Hemagglutinin protein</td>
<td>Measles virus</td>
<td>TLR2</td>
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<td>Glycoprotein B</td>
<td>ICMV</td>
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<td>RNA</td>
<td>Latent EBV</td>
<td>RIG-I</td>
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<tr>
<td>RNA</td>
<td>HCV</td>
<td>RIG-I and LGP2</td>
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<tr>
<td>Heat shock protein 60, 70</td>
<td>TLR2? and TLR4?</td>
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<tr>
<td>Fibrinogen</td>
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<td>Uric acid crystals</td>
<td>NALP3, TLR2? and TLR4?</td>
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<td>TLR3 and mda-5</td>
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<td>pI</td>
<td>TLR7, TLR8 and TLR3</td>
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<tr>
<td>Imidazoquinoline compounds (R837, R848)</td>
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<td>In vitro transcribed RNA</td>
<td>RIG-I</td>
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nor do they contain a TIR domain. Instead, downstream signaling through NLRs is mediated by either a pyrin, baculoviral inhibitor of apoptosis repeat (BIR), or caspase-recruitment domain (CARD). The RNA DExD/H box helicases retinoic acid-inducible gene (RIG-I), melanoma differentiation associated gene (mda-5) and LGP2 also contain CARD domains for protein-protein interactions in downstream signaling,\textsuperscript{15-17} but their helicase domain is responsible for the recognition of nucleic acids in place of LRRs. The lectin Dectin 1 binds to β-glucan in fungal walls via its C-type lectin like domain\textsuperscript{18} and is a type II transmembrane protein. Like type I transmembrane proteins, type II span the plasma membrane only once, however they utilize an immunoreceptor tyrosine based activation motif (ITAM) on their cytoplasmic N-terminus for downstream signaling. Finally, DAI (also known as tumor stroma and activated macrophages protein, DLM-I, and Z DNA binding protein 1, ZBP-I) is a cytoplasmic protein which binds and responds to dsDNA via its N terminal DNA binding domain and signals to downstream components via its C terminus.\textsuperscript{19}

PRRs mediate all their important effects via the downstream signaling events they initiate. In general, signaling initiated by NLRs activates the transcription factor nuclear factor κ B (NFkB). TLRs, RNA helicases, Dectin 1 and DAI activate the transcription factors interferon regulatory factors (IRFs) and general activator protein 1 (AP1) in addition to NFkB. The repertoire of genes and also microRNAs induced through these transcription factors includes many with diverse functions which can be both directly and indirectly anti-microbial.\textsuperscript{20} A majority of the genes induced through IRFs are classified as interferon-stimulated genes (ISGs) based on their historic characterization in the context
of type I IFN signaling and despite the fact that their induction through PAMP-signaling can be IFN-independent.

Signaling is not limited to direct gene induction either. Proinflammatory cytokines are produced to regulate extracellular signaling such as interleukins (ILs), tumor necrosis factor (TNF) and IFNs. Through paracrine and autocrine signaling this heightens the antiviral state by IFN-dependent augmentation of ISG expression, and initiates the adaptive immune response by promoting dendritic cell maturation, memory T cell proliferation and B cell differentiation. Finally, independent of transcription PRR signaling can also induce Bax and caspase mediated apoptosis. Thus, the antiviral state is established by cell death in certain circumstances as well as by direct and indirect (via cytokine amplification) gene induction from PAMPs activating their receptors.

**Receptors that respond to dsRNA**

During virus infection, dsRNA capable of acting as a PAMP/DAMP can be produced from several sources: dsRNA virus genomes, replication intermediates of ssRNA viruses, convergent DNA virus transcription products, defective viral particles and debris from necrotic cells. In addition to the TLR3 and RNA helicases, several IFN inducible proteins bind and respond to dsRNA with direct antiviral activity: dsRNA dependent protein kinase (PKR), protein activator of the IFN-induced PKR (PACT), 2-5 oligoadenylate synthetase (2-5OAS) and adenosine deaminase (ADAR). These proteins respectively function to phosphorylate eukaryotic initiation factor 2a (eIF2a) leading to inhibition of mRNA translation, bind to dsRNA as well as bind and activate PKR, synthesize oligoadenylates that activate RNaseL causing RNA degradation, and deaminate adenosines thereby destabilizing mRNA.
While PKR\textsuperscript{31} and 2-5OAS\textsuperscript{32} have been implicated in IFN induction in response to RNA, the TLRs and RNA helicases are thought to be the main effector molecules for this function. TLRs 7 and 9 have been shown to be essential for plasmacytoid dendritic cell production of IFN in response to viruses. TLR3 through its recognition of extracellular dsRNA has been shown to be important for IFN induction in conventional dendritic cells and macrophages. Lastly, for both these cell types and also fibroblasts, RIG-I and mda-5 have been shown to be essential for IFN induction in response to intracellular dsRNA.\textsuperscript{33,34}

**Extracellular dsRNA and TLR3**

Extracellular RNA is endocytosed in a clathrin dependent manner\textsuperscript{35} and binds directly and reversibly to the LRRs in the TLR3 ectodomain.\textsuperscript{36} This interaction is thought to take place in endolysosomes which is where TLR3 is translocated from the plasma membrane and endoplasmic reticulum (ER) upon dsRNA stimulation. This is accomplished with the help of the \textit{C. elegans} homologue of B1 (UNC93B1).\textsuperscript{37-39} Consistent with this location of interaction, the binding affinity between RNA and receptor increases with acidic pH. This affinity also increases with length of RNA, up to 62bp.\textsuperscript{40} Many dsRNA ligands have been shown for TLR3. These include the synthetic mimic Polyinosinic-polycytidylic acid (pIC),\textsuperscript{41} siRNA (sequence and target independent),\textsuperscript{42} ssRNA (secondary structure dependent),\textsuperscript{43} total bacterial mRNA and mitochondrial mRNA released from necrotic and not apoptotic cells.\textsuperscript{28} Interaction between receptor and ligand induces the multimerization of TLR3 with or without the myeloid specific leucine rich glycoprotein CD14 which is thought to function more in the recognition of LPS by TLR4.\textsuperscript{44} Multimerized TLR3 associates with c-Src in endosomes.
where signaling occurs through an interaction with TRIF via the TIR domains on each protein. This interaction is transient as the two proteins colocalize only initially after stimulation. During this process TRIF distribution changes from diffusely cytoplasmic to punctuate or “speckled” as it acts as a scaffolding protein for further signaling to activate NFkB and IRFs (Fig 1.1A).

For NFkB, TRIF recruits receptor interacting protein (RIP1) into these cytoplasmic speckles where they interact via the RIP homotypic interaction motif (RHIM) of RIP1. RIP1 is then phosphorylated and ubiquitinated at non-degradative K63 sites by the E3 ubiquitin ligase TNFR associated factor (TRAF6). Ubiquitinated RIP1 is then able to recruit and activate TAK1 and TAK1 binding proteins (TAB) 1, 2 and 3 via the scaffolding proteins ubiquitin conjugating enzyme (Ubc13), Ubc E2 variant (Uev1A) and evolutionarily-conserved signaling intermediate in Toll pathway (ECSIT). Activated TAK1 then phosphorylates IkB kinase (IKK) complex of the kinases IKKa and IKKb and their scaffolding protein IKKg/NEMO. Finally, phosphorylated IKKa and b phosphorylate IkB, leading to its proteosomal degradation and release of NFkB subunits p50 and p65/RelA. P65/RelA has also been implicated as a substrate for IKKa and b kinase activity. However the mechanistic significance is less clear. TAK1 also phosphorylates the mitogen activated protein kinase (MAPK) pathway of Jun kinases (JNK), p38 and extracellular signal regulated kinase (ERK) to activate the AP1 transcription factor complex of ATF2 and c-Jun.

For IRF activation, TRIF recruits the scaffolding protein TRAF3 which interacts with the non canonical IKK kinases: TRAF family member-associated NFkB activator (TANK) binding kinase (TBK1) (also known as T2K and NFkB activating kinase, NAK)
and IKKe/IKKi. TBK1 and IKKe then oligomerize\(^{52}\) and are able to bind via their ubiquitin like domain (ULD)\(^{53}\) to and directly phosphorylate IRF3 with the aid of three other adaptors that contain TANK binding domains (TBD) essential for interaction with TBK1: TANK\(^{54}\), Nck associated protein (NAP1)\(^{55}\) and similar to NAP1 TBK1 adaptor (SINTBAD).\(^{56}\) IRF3 phosphorylation is also aided by cyclophilin B\(^{57}\) which is thought to relieve autoinhibition between the N and C termini and promote homo and hetero dimerization with IRF7 via the C terminal IRF association domain (IAD).\(^{58}\) IRF3 dimers then localize to the nucleus, however for transcription activation further signals are necessary from phosphatidylinositol 3-kinase (PI3K) originating at the receptor level\(^{59}\) and histone deactylases (HDACs).\(^{60}\) Finally, via its IAD, IRF3 in its dimerized state binds to CREB binding protein (CBP) with the help of PKCa,\(^{61}\) as well as p300, and then to DNA via its N terminal DNA binding domain. ISRE mediated transcription may be further enhanced by other coactivators such as glucocorticoid receptor interacting protein (GRIP1) which also interacts with IRF3 via its IAD.\(^{62}\)

TLR3 is basally expressed in the lung, brain and kidney as well as the placenta and pancreas.\(^{12,63}\) Immune cells that express TLR3 include dendritic cells,\(^{64}\) macrophages, natural killer cells, mast cells and T cells as well as microglia, the macrophages of the central nervous system (CNS).\(^{65}\) Furthermore, TLR3 is itself an ISG. Therefore, its expression is upregulated by IRF activation through virus. Influenza A,\(^{66,67}\) Respiratory Syncytial Virus (RSV),\(^{68}\) Simian immunodeficiency virus (SIV),\(^{69}\) Measles,\(^{70}\) Rhinovirus (RV)\(^{71}\) and Hepatitis C virus (HCV)\(^{72}\) all increase TLR3 expression upon infection, and therefore at least theoretically heighten responsiveness to dsRNA.
Fig 1.1: Signaling induced by the RNA pattern recognition receptors.

A.) Toll like receptor 3 (TLR3) recognizes extracellular dsRNA endocytosed into the cell, represented by the synthetic analogue pIC, and the RNA helicases B.) retinoic acid inducible gene (RIG-I) and melanoma differentiation associated gene 5 (mda-5) recognize intracellular dsRNA in the cytoplasm. While both use different adaptors, the signaling pathways converge upon the IRF3/7 activation complex which includes IKKe and TBK1 and NFkB activation complex which includes IKKa, b, and g/NEMO. Together with activating protein 1 (AP1), these factors transcribe the interferons (IFN) and cytokines essential to an innate immune response. Adapted from “Snapshot: Pattern-recognition receptors.” 73
The role of TLR3 in immunity and pathology is complex. Reovirus dsRNA,\textsuperscript{63} Theiler’s murine encephalomyocarditis virus (EMCV),\textsuperscript{74} Herpes simplex virus (HSV) produced dsRNA\textsuperscript{75} and HCV RNA-dependent RNA polymerase NS5B\textsuperscript{76} all induce genes through TLR3. However, the effects of TLR3-mediated gene induction are varied. Some experiments show no difference in immune response or survival during viral infection in mouse models,\textsuperscript{77} others paradoxically show protective as well as detrimental effects for TLR3.

Protective roles for TLR3 have been identified in infections with RV, RSV, EMCV, mouse cytomegalovirus (mCMV), HSV and, in the CNS, influenza A. TLR3 is also required for the host immune response against RV.\textsuperscript{71} For RSV, TLR3 is not only induced but also required for protection against the virus in the lung.\textsuperscript{78} For EMCV, dsRNA released from virus infected cells primes dendritic cells and induces cross presentation and activation of cytotoxic cells for an efficient antiviral response.\textsuperscript{79} This may be how TLR3 prevents higher EMCV titres in the heart.\textsuperscript{80} In the case of mCMV, TLR3 along with TLR9 but not TLR7 are essential components for the immune response and critical to survival of infection.\textsuperscript{81} Finally, within the CNS, TLR3 expression is protective for human astrocytes,\textsuperscript{82} further TLR3-dependent pIC stimulation suppresses relapsing demyelination in a murine experimental autoimmune encephalomyelitis model.\textsuperscript{83} An autosomal dominant negative mutation in TLR3 has been linked to HSV-1 encephalitis in otherwise healthy children.\textsuperscript{84} This, aside from autosomal recessive UNC93B deficiency,\textsuperscript{85} is the most common genetic etiology of isolated herpes simplex encephalitis (HSE), the most common form of sporadic encephalitis in western countries.
Finally, a loss of function missense mutation in TLR3 is linked with influenza A induced encephalopathy in patients.\textsuperscript{86}

However, not all TLR3 induced signaling is protective and excessive signaling, or more specifically the resulting immune/inflammatory response may cause pathology. In contrast to the previous example where TLR3 in the CNS appears protective for the host in influenza A infections, upregulation of TLR3 in the lung with the same virus causes acute pneumonia and is actually detrimental for the host.\textsuperscript{66, 87} TLR3 expression also correlates with the development of autoimmune diabetes in a mouse model\textsuperscript{88} and in the infiltrating antigen presenting cells and glomerular mesangial cells involved in lupus nephritis.\textsuperscript{89} Finally, TLR3 null mice have increased resistance to infection with hepatotropic phlebovirus Punta Toro virus\textsuperscript{90} and are more resistant to lethal encephalitis caused by West Nile Virus.\textsuperscript{91} This indicates that there is an optimal level of TLR3 signaling that benefits the host and therapeutic potential in the development of both TLR3 antagonists and agonists.

Indeed in animal models, dsRNA treatment appears to protect against genital herpes infection,\textsuperscript{92} is beneficial as a vaccine adjuvant for influenza\textsuperscript{93} and, when complexed with cationic liposomes, is even able to control the growth of melanomas.\textsuperscript{94} In human studies, Ampligen, a synthetic nontoxic pIC analog, pI:C\textsubscript{12}U, is in a Phase III trial to treat chronic fatigue syndrome and Phase II trial to treat HIV.\textsuperscript{95}

**Intracellular dsRNA and RNA helicases**

Intracellular dsRNA, including that from many viruses is recognized by the cytosolic RNA DExD/H box helicases RIG-I and mda-5. For RIG-I, it is thought that in the inactive state, the C terminal repression domain (RD) and linker helicase domain
interact with and prevent function of the N terminal CARD domain. Signaling begins when interaction between ligand and the C terminal domain relieves this inhibition.\textsuperscript{96} The now exposed CARD domain is then K63 ubiquitinated by tripartite motif containing protein (TRIM25)\textsuperscript{97} thereby enabling it to bind to the adaptor, mitochondrial antiviral signaling protein (MAVS; also known as virus induced signaling adaptor, VISA; IFN\textbeta{} promoter stimulator 1, IPS1; and CARD adaptor inducing IFN\textbeta{}, Cardif) via an interaction between the CARD domains of the two proteins. Interestingly, MAVS appears to reside in the mitochondria and this localization is apparently essential for function.\textsuperscript{98} MAVS once itself is ubiquitinated by an as yet unidentified mechanism\textsuperscript{99} can recruit IKKe.\textsuperscript{100} The activation of the IRF3/7 kinases IKKe and TBK1 is, like TLR3, dependent on the scaffolding proteins TRAF3, TANK, SINTBAD and NAP1, which appears to localize in areas around the mitochondria.\textsuperscript{101} TANK also mediates interaction between TBK1/IKKe and IKKg/NEMO.\textsuperscript{102} These interactions then lead to activation of IRF3 as previously described for TLR3 mediated transcription. NFkB activation is also mediated by MAVS, but through interaction with FAS associated via death domain (FADD). This MAVS interaction with FADD leads to cleavage of Caspase 8/10 which then activates the canonical IKKa, b, g to phosphorylate and degrade IKB for p65 and p50 release and nuclear localization (Fig 1.1B).

While RIG-I is generally expressed at low levels it is highly induced by retinoic acid and IRF activators such as LPS and IFN.\textsuperscript{103} mda-5 is inducible in the same manner but is also basally expressed in the spleen, pancreas and placenta.\textsuperscript{15,104} RIG-I is essential for the antiviral response to what is most likely the short dsRNAs from Flaviviruses, Paramyxoviruses, Orthomyxoviruses, and Rhabdoviruses, \textit{in vitro} transcribed RNA
without host modifications such as capping and base modification and ssRNA with 5’ triphosphates. Conversely, mda-5 is essential for the signaling response to Picornoviruses and longer dsRNA, pIC.\textsuperscript{105-107} This specificity in response corresponds to increased vulnerability to the Flavivirus Japanese encephalitis virus (JEV) and the Picornovirus EMCV in RIG-I and mda-5 null mice respectively.\textsuperscript{108, 109} In addition RNA from the DNA virus EBV as well as dsDNA itself have been shown to be able to activate pathways that signal using the RNA helicases.\textsuperscript{110, 111}

**The IFN Regulatory Factor (IRF) family of transcription factors**

IRF3 and 7 which are involved in the initial response to dsRNA recognition are two of nine mammalian members of the Interferon Regulatory Factor (IRF) family of transcription factors: IRF1, IRF2, IRF3, IRF4 (also known as LSIRF, PIP and ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP) and IRF9 (also known as p48). These proteins share in common an amino terminus DNA binding domain of the helix-turn-helix motif which mediates the recognition of their DNA binding site, the IFN-stimulated response element (ISRE). At the carboxy terminus is an IRF association domain (IAD) which is responsible for homo- and hetero- dimeric interactions between members of the family and is homologous to the C terminal domains of the mothers against decapentaplegic homologue (SMAD) family of transcription factors.\textsuperscript{112} Nuclear localization (NLS) and export signals (NES) help guide the nuclear-cytoplasmic shuttling of the protein. In IRF3 specifically, the transactivation domain (aa 134-394) which contains the NES, a proline rich segment, and the IAD is alone sufficient to drive transcription. Finally, two inhibitory domains, one at the amino terminus (aa 134-197)
and the other at the carboxy terminus (aa 407-414), are thought to mediate intramolecular interactions to mask the IAD, DBD and NLS.\textsuperscript{113, 114}

At least six of the nine IRF members are positive regulators of type I IFN genes: IRF1, 3, 5, 7, 8 and 9. IRF3 as described previously is mainly involved in the cellular response to dsRNA and LPS via TLR 3, TLR4, RIG-I and mda-5. IRF7 is involved in the dsRNA response but in addition plays a role in TLR7 and 9 signaling. IRF5 is also involved in TLR4, 7 and 9 signaling. Via MyD88, IRF8 is a positive regulator of TLR4 and 9 responses. IRF9 is part of the ISGF3 complex with STAT1 and STAT2 and mediates signaling induced by type I IFN. Similarly, IRF1 can mediate transcription of type I IFN and also interacts with the adaptor MyD88. Finally, while IRF6 has not been extensively studied, it apparently interacts with MyD88 like IRF1. However the regulatory implications of this interaction are not yet known.\textsuperscript{112}

Negative regulation of IRFs is in part accomplished by other family members. IRF2 attenuates the type I IFN response mediated by IRF1. IRF4 competitively inhibits IRF3 binding to the adaptor Myd88. In addition, non-IRF transcription factors such as B lymphocyte induced maturation protein (Blimp-1) competes with IRF1, 2 and 3 in binding to ISREs and serves as negative regulators of IRF signaling.\textsuperscript{115} Thus, the IRF family of proteins represents an important group of transcription factors essential for the host immune response against pathogens with multiple levels of control within and between members.

**Interferon stimulated response element (ISRE) mediated signaling**

IRFs bind to ISRE sites to mediate the transcription of ISGs. While ISREs in general confer positive responsiveness to IFN with the common motif of
GAAANNGAAAA, the actual sequences of specific ISREs can vary. These variances may account for the variations in ISG expression upon different stimuli which activate different transcription factor complexes, dsRNA as compared to IFN. In addition, ISRE variation may explain differences in induction even with the same stimulus. IFN for instance requires IKKe phosphorylation of STAT1 to mediate the induction of a specific subset of genes which may be identified by subtle differences in their ISREs. The relevance of these differences is apparent in an influenza mouse model.116

The ISG56 family of proteins

The ISG56 family of genes is characterized by their promoter in that they all contain ISREs that are necessary and sufficient for induction.117-120 Thus, IRFs are the primary transcription factors that mediate expression. ISG56 family members are not basally expressed. However, as discussed in more detail in Chapter 4, ISG56 mRNA is one of the most if not the most highly upregulated messages by ISRE-mediated transcription24, 25. Although ISG56 was originally cloned from human fibroblasts as an interferon inducible gene,121, 122 it is also strongly upon activation of TLR3, RIG-I/mda-5,123-126 TLR4.127 As mentioned previously, in the context of dsRNA signaling ISG56 induction can be both direct via IRF3 and indirect via IFN feedback through ISGF3.123, 124

ISG56 has a prodigious number of aliases. They include G10P1, GARG-16, IFI-56, IFNAI1 and RNM561. The ISG56 family of genes include human ISG56, 54, 60 and 58 and mouse ISG56, 54, 49 as well as homologues in goldfish, the chinese hamster, rat and sheep.118, 120, 121, 128-130 (Fig 1.2) Intriguingly, all the genes of the family members lie next to each other on chromosome 10 in humans131 and 19 in mouse.117 This tandem gene arrangement suggests the common use of regulatory mechanisms, functional redundancy.
and gene duplication events underlying the evolution of the family. The genetic structure is similar overall as well, with a short first exon comprising just the start codon, an intervening intron and the second exon which comprises the remaining coding region. However, the actual redundancy within this family is still not known because knockdown attempts have not been completely successful.

The members of the P56 family of proteins (Fig 1.2) contain multiple 34 amino acid tetratricopeptide repeat (TPR) motifs and a proteosome-COP9-signalsome-and-initiation-factor (PCI) domain responsible for protein-protein interactions. (Fig 1.3) These TPR motifs mediate interaction with the eukaryotic translation initiation factor subunits (eIF3) to inhibit translation. The first two TPR motifs in the protein product of mouse ISG56 (mp56) and the first TPR of mp54 are sufficient to bind to eIF3c. This prevents the formation of the 48S preinitiation complex between 40S ribosomal subunit and 20S complex. In comparison, the last two motifs, TPRs 5 and 6, of human p56 (hp56) binds to the e instead of c subunit of eIF3. This prevents the formation of the ternary complex which is also essential for translation. Finally, the protein product of human ISG54 (hp54) binds to both the e and c subunits of eIF3, inhibiting both steps (Fig 1.4). In summary, the ISG56 family of genes and the proteins for which they encode represent part of a vast repertoire of genes upregulated upon activation of IRFs. To be sure, these genes mediate antiviral/antipathogen activities. However the mechanisms by which they function do not limit them to such processes and the function of ISGs in other biological processes is an important area to address.
Figure 1.2 Phylogenetic relationships between members of P56 family of proteins.

Multiple sequence alignments and phylogenetic tree for the P56 family of proteins were obtained from HOVERGEN database. (http://pbil.univ-lyon1.fr/databases/hovergen.html) Hu: human; Mu: mouse; Ra: rat; CI: chimpanzee; GF: goldfish. Adapted from “Novel functions of proteins encoded by viral-stress inducible genes.”{134}
Figure 1.3: Members of the P56 family of proteins are structurally homologous with tetracopeptide repeat (TPR) motifs.

Both human P56 (hp56) (GenBank accession P09914) and mouse P56 (mp56) (GenBank accession Q64282) contain six TPR motifs represented by grey bars. TPR2 is expanded to show conserved amino acid residues shaded in grey, similar amino acids shaded in black and nonconserved residues in white. Key consensus residues that define a TPR motif are indicated by large open traingles indicating large hydrophobic residues and dark small triangles indicating small hydrophobic residues. Adapted from “Mouse p56 blocks a distinct function of eukaryotic initiation factor 3 in translation initiation.”132
Figure 1.4: The P56 family of proteins binds to elongation initiation factor (eIF) subunits to inhibit translation.

hp56 and hp54 bind to the e subunit of eIF3 to prevent the formation of the eIF-ternary complex. hp54 and mp56 and mp54 bind to the c subunit of eIF3 to prevent the formation of the 48S preinitiation complex which comprises of the 40S ribosomal subunit, eIF3 ternary complex, mRNA and eIF4F. Adapted from “Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56.”125
Negative Regulation of Innate Immunity

Host Mechanisms

To protect against excessive innate immune responses that is in part mediated by PRRs, multiple methods of downregulation have evolved. As important as this may be to pathogen fitness, this is also necessary to prevent the immune system from becoming pathologic to the host, as seen with autoimmunity, as well as infections such as West Nile virus encephalitis,

For receptor level regulation, the third member of the RNA helicase family, LGP2 inhibits both RIG-I and mda-5 by sequestering dsRNA through its helicase domain in the absence of a CARD domain for signaling. Like many negative regulators, LGP2 is induced by signaling and serves as part of a negative feedback mechanism. In contrast, dihydroxyacetone kinase (DAK) inhibits mda-5 but not RIG-I basally by binding to its CARD domain. This inhibition is released upon mda-5 activation mediated by dsRNA binding to its helicase domain.

More examples of regulatory mechanism exist at the adaptor level. Sterile alpha and heat/armadillo motifs (SAM) containing protein (SARM), one of the five members of the TIR family of adaptors, is thought to inhibit TRIF signaling by either itself competing for the TIR domain of TRIF or recruiting another protein with its SAM domain to do so. Similarly for RNA helicase signaling, the mitochondrial-localized protein NLRX1 competes with the RNA helicases to bind to the CARD domain of MAVS. For both receptors and their adaptor, the Ring finger protein (RNF125) K48 ubiquitinates and degrades RIG-I, mda-5 and MAVS. In terms of secondary scaffolding proteins in the
signaling cascade, deubiquitinating enzyme A (DUBA) via its ubiquitin interacting motif (UIM) degrades K63 ubiquitin on TRAF3 and prevents its interaction with TBK1. Tripartite motif protein (TRIM30a) through its RING domain interacts with and mediates degradation of TAB2 and 3 to inhibit NFkB activation.

At the level of the IRF3/7 kinases, suppressor of IKKe (SIKE) via its coiled coiled domains binds to TBK1 and IKKe and prevents their basal interaction with TRIF, RIG-I and TLR3 but not TRAF6 and RIP1. Like DAK, this inhibition is released upon signaling. The tyrosine phosphatase SHP2 also negatively regulates TBK1 in a phosphatase independent manner by binding to the kinase domain. Ret Finger Protein (RFP) interacts with and is a substrate for TBK1 and IKKe as well as IKKa and b upon signaling. It also interacts with IRF3, allowing for its dimerization but not nuclear localization. The NFkB induced gene, A20 inhibits both IRF3 and NFkB signaling. To prevent the IRF3 dimerization, A20 interacts with TBK1 and IKKe. To prevent NFkB activation, it deubiquitinates TRAF6 and mediates degradative K48 ubiquitination of RIP1.

Finally, at the level of the transcription factors, peptidyl-prolyl cis/trans isomerase, NIMA-interacting (Pin1) mediates signal dependent ubiquitination and degradation of IRF3. Glucocorticoid receptor (GR) inhibits ISRE transcription by competing for the co-activator GRIP1. For NFkB, TNF-related apoptosis inducing ligand receptor (TRAILR) stabilizes the inhibitory IKBa. In addition, IKBa is itself induced by NFkB to shut down signaling.
**Viral Mechanisms**

For pathogens such as viruses, host mechanisms of PRR signaling downregulation may be easily manipulated. The human immunodeficiency virus (HIV) accessory protein Vpu\textsuperscript{151} and several strains of vaccinia viruses interfere with IKB\textalpha degradation\textsuperscript{152} to prevent NFkB activation. Herpes simplex virus 1 (HSV-1) and Hepatitis C virus (HCV) induce SOCS to negatively regulate IFN signaling\textsuperscript{153,154}.

Another mechanism of signaling inhibition commonly employed by many viruses is the development of viral homologues of host proteins or their important regulatory domains. Kaposi’s sarcoma-associated herpesvirus (KSHV)/Human herpesvirus (HHV8) encodes vIRFs that prevent (but in some instances activate) the proper function of host IRF\textsuperscript{155}. Vaccinia virus A52R acts as a dominant negative for MYD88 signaling\textsuperscript{156} while A46R inhibits both MYD88 and TRIF\textsuperscript{157} because they, like the host adaptors, contain TIR domains. Finally, vaccinia virus IFNR and Myxoma virus TNFR homologues inhibit signaling of these respective host cytokine signaling pathways\textsuperscript{158}.

Cytokine and IFN receptor and function are popular targets for viral evasion, especially when their induction is not completely prevented. Human CMV (hCMV) encodes a decoy for the chemokine RANTES, chemokine receptor analogues and also a vIL10 which serves to downregulate the immune response\textsuperscript{159,160}. KSHV, too, encodes a variety of chemokine and chemokine receptors\textsuperscript{155}. Lastly, Poxviruses secrete IFN binding proteins that neutralize the actions of IFN once it is produced\textsuperscript{161}.

For signal induced gene expression, Rift Valley fever virus NS proteins and M protein of vesicular stomatitis virus (VSV) inhibits components of the host machinery to prevent transcription. VSV M1 interacts with Rae1 and Nup98 to inhibit selective mRNA
export. Finally, even though mRNA is still exported in cells infected with Poliovirus and EMCV, protein translation is inhibited.\textsuperscript{161}

One interesting characteristic of viral evasion is that points of convergence within the host signaling pathways represent common areas of inhibition for a variety of viruses. These include members of the IKK family of kinases that activate transcription factors or the factors themselves. Vaccinia virus N1L interacts with almost all the IKKs: TBK1, IKKe, IKKa and IKKb.\textsuperscript{162} P proteins of Rabies\textsuperscript{163}, Ebola\textsuperscript{164} and Borna disease\textsuperscript{165} viruses and Severe acute respiratory syndrome coronavirus (SARS-CoV) papain-like propease PLpro\textsuperscript{166} interfere with IRF3 activation, most likely its phosphorylation. Likewise, KSHV open reading frame (ORF45) interferes with IRF7 phosphorylation.\textsuperscript{167} In addition, human papillomavirus 16 (HPV16) E6 oncoprotein binds to IRF3 and inhibits its transactivation\textsuperscript{168} and Epstein Barr virus (EBV) BZLF1 inhibits IRF7 function.\textsuperscript{169} To decrease IRF levels in general, classic swine fever virus Npro inhibits IRF3 synthesis\textsuperscript{170} and rotavirus NSP1 promotes IRF3 degradation\textsuperscript{171} while KSHV RTA does the same for IRF7.\textsuperscript{172} Finally, for NFkB, the Picornovirus Foot and mouth disease virus (FMDV) LPro degrades p65\textsuperscript{173} while the African swine fever virus uses its IkB homologue A238L protein to bind to NFkB, preventing its nuclear translocation.\textsuperscript{174}

Lastly, as implicated by some of the examples above, multiple mechanisms of immune evasions are often times attributed to one pathogen protein as, especially in the case of viruses, their genomes are small and contain only a few ORFs.\textsuperscript{175} One example is influenza NS1 which binds to and sequesters dsRNA to prevent activation of PKR, binds to and inhibits PKR itself,\textsuperscript{176} activates P58IPK a PKR inhibitor,\textsuperscript{177} interacts with the receptor RIG-I to prevent its activation and inhibits selective mRNA nuclear export.\textsuperscript{176}
Another is HCV NS34A protease, which cleaves the adaptors for both TLR3 and RNA helicases TRIF and MAVS. To finish, Ebola Virus VP35 inhibits signaling by both binding to dsRNA in addition to a yet to be elucidated mechanism downstream of TBK1 and IKKe. Thus, while the immune system has developed a complex response against invading pathogens to protect the host, pathogens themselves have been forced to evolve and adapt in equally diverse ways.
CHAPTER 2
MATERIALS AND METHODS

Construction of ISG56 knockout targeting vector

All PCRs were conducted using the Expand Hi Fidelity PCR System (Roche) per the manufacturer’s instructions. Restriction enzymes (NEB) and calf intestinal phosphatase (Roche) were used according to manufacturer’s instructions. All ligations were conducted using T4 DNA ligase (5u/ul) (Roche) with glycogen (Roche). All transformations were conducted using chemically competent DH5a prepared with rubidium chloride.

The targeting vector (mISG56LOXP) was constructed in three steps. The first step involved the cloning of two fragments to form together the 5’ homologous recombination arm and ISG56 coding region (5’ ARM-EXON2). The first fragment (5’ ARM) contained the 5’ homologous arm and Exon 1 (approximately 3kb in size). This was generated by PCR using template from 129Sv DNA obtained from University of Washington ES Core and primers that introduced a loxP site in the 5’ UTR before the ATG and an Apa I site at the 5’ end for cloning purposes. The second fragment (EXON2) contained Exon 2 and was generated from the 129Sv DNA by PCR with the 5’ primer containing the loxP and Exon 1 from the first fragment and the 3’ primer introducing a SalI site for cloning purposes. These two PCR fragments were cloned into TOPO TA vectors per the manufacturer’s suggestions (Invitrogen). The EXON2 fragment was then cloned into the TOPO TA vector containing 5’ ARM using BstEII and Sacl. The resulting fragment (5’ ARM-EXON2) was then cloned into the targeting vector, pBluescript based plasmid containing a PGK-NEO cassette, pK11, gift of V. Lefebvre, using ApaI and SaI.
The second step involved the cloning of two fragments to form together the LacZ reporter gene linked to the 3’ homologous recombination arm (LACZ-3’ ARM). The first fragment (LACZ) was generated by PCR using the plasmid pNTRlacZ, gift of V. Lefebvre, as template and using primers that introduced at the 5’ end a SacII site for cloning and a loxP site. The second fragment (3’ ARM) was generated by PCR using the 129Sv DNA as template and primers introducing a NotI site at the 3’ end for cloning purposes. These two PCR fragments were cloned into TOPO TA vectors. The 3’ ARM was cloned into the vector containing LACZ using XmaI and NotI. The resulting fragment (LACZ-3’ARM) was then cloned using SacII and NotI into the pK11 vector already containing the first fragment, 5’ARM-EXON2, to form mISG56LOXP.

While restriction digest analysis of mISG56LOXP indicated that the insertion and orientations of our fragments were correct, sequencing of important regions within the vector such as the 5’ UTR and coding regions for ISG56 and LacZ indicated that the second of the two essential ISREs in the 5’ UTR was mutated. This was not a result of the genomic DNA used as the template. Therefore, a third cloning step was required to replace this mutated sequence with a correct ISRE. A region with the 5’ UTR was amplified by PCR again, the ISRE sequence confirmed to be correct and then cloned using SexAI and BstEII into the original mISG56LOXP to replace the mutated sequence.

**Screening of ES cells for recombination of the targeting vector**

The targeting vector mISG56LOXP was linearized with NotI, gel purified with QiaexII (Qiagen) and sent for electroporation into ES cells at the University of Washington at St. Louis in collaboration with Herbert Virgin III. ES cell clones were selected in G418. DNA was extracted using a standard phenol chloroform protocol.
Briefly, cell pellets were digested overnight at 55°C with proteinase K (200μg/mL) (Sigma) in 0.5M Tris-HCl pH 8.5, 25mM EDTA, 1% SDS, 1M NaCl. DNA was extracted with phenol, washed with chloroform:isoamyl alcohol (49:1), precipitated with NaOAc and ethanol and resuspended in Tris for further analytical digests.

**Southern Blot Hybridization**

The probes for Southern hybridization were amplified by PCR from 129Sv DNA using the appropriate primers and TOPO TA cloned for use. The probe fragments BGLII, targeting the 5’ non coding region of ISG56 outside the area of recombination, and ECORI, targeting the 3’ non coding region of ISG56 outside the area of recombination, were released from their vectors by EcoRI digestion, gel purified Qiaquick (Qiagen) and resuspended in TE. The purified fragments were then labeled with 32P dCTP in RediPrime II Random Primer labeling system (Amersham) per manufacturer’s instructions, denatured at 100°C 10min before use in hybridization.

10μg of ES cell DNA samples was digested overnight with the appropriate restriction enzyme (BglII or EcoRI) with 1mM Spermidine. Digested DNA fragments were precipitated with ethanol and 300mM NaOAc and resuspended in water to be separated on a 0.8% Tris Borate EDTA agarose gel. Gels were washed 4 times for 15 minutes each in water to rinse off ethidium bromide. DNA was depurinated by soaking the gel in 0.2N HCl for 15 min. Gels were washed again and then soaked in 0.4N NaOH for 15 min to denature and dehydrolyze the DNA. Capillary transfers of the DNA were performed overnight with 0.4N NaOH onto nylon transfer membranes Hybond N+ (Amersham), rinsed briefly in 2X SSC 0.2M Tris-HCl pH 8.0 and DNA crosslinked onto membrane in the UV Stratalinker 2400 (Stratagene). Membranes were prehybridized at
65°C for more than 2 hrs in Hybridization buffer (0.3M NaPO₄ pH7.2, 6.7% SDS), then hybridized overnight with a minimum of 21 million cpm of radiolabeled probe per blot in Hybridization Buffer. Membranes were washed briefly in 2XSSC 0.1%SDS at room temperature and 30 min at 65°C, then two more times 15 min each with 0.2XSSC 0.1%SDS. Membranes were sealed and exposed overnight to visualize by Molecular Dynamics PhosphorImager. Membranes were then exposed at -80°C to visualize by film.

**Cell Survival Assay**

1x10⁵ TLR3 293 561-TK cells were seeded in 35mm wells overnight, transfected with 2ug total of plasmids expressing viral ORFs and 6 uL of Fugene for 16 hrs, media changed to complete for 24 hrs and treated with dsRNA and GCV. Cells were maintained under selection 4 days to assay survival as determined by visualization with media containing selective pressures changed every 2 days. Pools of these cells expressing V proteins were passaged without dsRNA before further experiments.

**Viruses**

W3A strain wildtype and VDC PIV5 virus, gifts of B. He, were propagated in Vero cells as described previously.²₈³, ²₈⁴ Briefly, 5x10⁶ cells were seeded in 100mm plates overnight, washed with PBS with divalent cations, inoculated with virus diluted in 1% BSA in MEM with Earle’s Salts at an moi of 0.2 to infect for 1.5 hr at 37°C, 2% FBS media added and virus allowed to grow for 4 days before collection. When growth stopped increasing as monitored by hemagluttination assay, virus was harvested, cells spun down and supernatant collected to be aliquoted and quick frozen in 1% w/v BSA.
**TLR3 antiviral activity**

1x10^6 293 or TLR3 293 cells were seeded in 35mm wells overnight, treated with dsRNA for 16hrs, then infected with WT or VDC PIV5 at an moi of 0.1. Final media volume per well was 2mLs. Viral replication was monitored via hemagglutination assays daily. Supernatants were collected 6dpi.

**PIV5 Plaque Assay**

Virus yields were determined by plaque assay on Vero as described previously. Briefly, approximately 2x10^6 Vero cells were plated to 100% confluency and washed with PBS with divalent cations before infection as described above. After the infection period of 1.5 hr, virus inoculum was removed, cells washed with PBS again and overlaid with 0.8% carboxy methyl cellulose (CMC) in 2%FBS 10mM HEPES DMEM. Plaques were allowed to develop for 4-7 days before fixation with 10% formaldehyde and visualization with 1% crystal violet in 10% methanol in PBS.

**Hemagglutination assay**

Hemagglutination assays were conducted as previously described. Briefly, virus samples were serially diluted 2 fold in cold PBS in U bottom 96 well plates. Equal volume of 0.5% suspension of guinea pig red blood cells (Colorado Serum Company) in PBS was mixed into samples and the reactions were allowed to proceed at 4C 1-2hrs.

**Cells**

HT1080 derived 2fTGH wildtype V protein expressing cells, 293 and TLR3 293 have been described. To make HT1080 derived 2fTGH cell lines permanently expressing mutant viral proteins, 2fTGH cells were co-transfected with respective pEF-V protein expression vectors and a puromycin expressing vector for selection or, for
MHV68 ORFs, 2fTGH cells were transfected with just the ORF expression vector and selected by G418 resistance carried by the plasmid. To make TLR3 293 561-TK cells, TLR3 293 was transfected with 561-TK plasmid. This contains the ISG56 promoter (-654+3) \(^{124}\) driving the herpesvirus thymidine kinase (TK) \(^{187}\) which was cloned via SstI/Sall into pGL3B (Promega) to replace luciferase.

**Plasmids**

Wildtype pEF-V protein expression vectors have been described.\(^{188, 189}\) MuV V mutants were constructed with QuikChange® II XL (Stratagene). PCR products from primers targeting residues were cloned with DpnI. PIV5 VDC mutant was constructed with Expand Hi Fidelity PCR System (Roche) using wildtype pEF-V protein as template and BamHI/BgIII and NotI for cloning. The following primers were used for cloning: for C189A, 5’ GTCCGGGTCTTTGAGTGGGCCAACCCTATATGCTCACC 3’ and 5’ GGTGAGCATATAGGGTTGGGCCCCACTCAAAGACCAGGC 3’, for C214A 5’ CCCGCAAAGGCCGATCAGTC 3’ and 5’ GCACTGATCGGCCTTTGCGGG 3’, for C217A, 5’ CCCGCAAAGGCCGATCAGTGTCG 3’ and 5’ GGTCCATAATCTCGTTCGGGCCT 3’, for M-AAA (a combination of three W to A mutations) W174A 5’ GGAGGGCATAGAGCGAGGAGCGCTCAGTGGTCC 3’ and 5’ GGACCGCAGCTGAGGCTCGCTTCCCGTCTATGCCCTCC 3’, W178A 5’ CGGGGAATGGAGCCTCATAGGAGGAGGTCCCT 3’ and 5’ GAGGTCCGGGTCTTTGAGGCGTGCAACCCTATATGCT 3’, W188A 5’ GACCTCCTCCTGGACCAGCAGCTGAGCTCGCCATTCCG 3’, for E95D 5’ TGAGCATATAGGGTTGCACGCCTCAAGACGCCGGACCTC 3’,
GGAGGGACTCAGATTCCTGACCCCCTTTTTGCACAAAC 3’ and 5’
GGAGGGACTCAGATTCCTGACCCCCTTTTTGCACAAAC 3’ and 5’
GGAGGGACTCAGATTCCTAAGGCCCCTTTTTGCACAAAC 3’ and 5’
GGAGGGACTCAGATTCCTAAGGCCCCTTTTTGCACAAAC 3’, for E95R 5’
GGAGGGACTCAGATTCCTGACCCCCTTTTTGCACAAAC 3’, for V47P 5’
GGAGGGACTCAGATTCCTGACCCCCTTTTTGCACAAAC 3’, for L97P 5’
ATCCCTGGCGTTGCACCTCCACTCATTGGCAATCCAGAG 3’ and 5’
CTCTGGATTGCCAATGAGTGGAGGTGCAACGCCAGGGAT 3’ and for L97P 5’
ACTCAGATTCTGAGCCCCCCTTTTGCCACAAACAGGACAG 3’ and 5’
CTGTCTGTGGTTGGAAAAGGGGCTCAGGAATCTGAGT 3’. pEF plasmids expressing MHV68 ORFs were a gift from J. Jung and plasmids expressing mCMV and hCMV ORFs were a gift of A. Geballe. pEF-Trif-Flag\textsuperscript{190} (gift of K. Fitzgerald), wildtype and kinase inactive (K38A) IKKe (pCDNA3.1-Myc)\textsuperscript{191} (gifts of U. Siebenlist), Flag-IRF3 and IRF3 5D \textsuperscript{192} (gifts of J. Hiscott), MyD88 dominant negative, TLR3 dominant negative without a TIR domain in plasmid and lentiviral forms,\textsuperscript{193} IRF3 siRNA,\textsuperscript{23} HCV NS34A\textsuperscript{180} (gift of M. Gale) and HA-Ubiquitin (gift of S. Fuchs)\textsuperscript{194} vectors have been described.

Reagents

All transfections were carried out with Fugene6 (Roche) with 3uL of Fugene per ug of DNA and 3uL of Fugene per 2ug pIC. Ganciclovir (GCV) (Invivogen), Leptomycin B (LMB) (Sigma), MG132 (Sigma) in DMSO (Sigma), dsRNA (Amersham Pharmacia) in the media,\textsuperscript{124} transfected dsRNA and IFNβ (Calbiochem) were used in treatments at 10ug/mL, 10ng/mL, 10uM, 100ug/mL, 8ug/mL and 1U/mL respectively unless otherwise specified.
Quantitative PCR

HT1080 V protein and MHV68 ORF expressing cells were treated with dsRNA for 6hrs. Total RNA was isolated with RNA-Bee (Tel-Test) and DNase treated with DNA-free (Ambion). cDNA was synthesized with Superscript III (Invitrogen). The Q-PCR reaction amplified bp 6-354 of ISG56, control RPL32 (ribosomal protein L32) and MHV68 ORF58 bp 338-460 with SYBR Green (Applied Biosystems). ISG56 primers: 5’ TCT CAG AGG AGC CTG GCT AAG 3’ and 5’ GTC ACC AGA CTC CTC ACA TTT GC 3’. RPL32 primers: 5’ GCC AGA TCT TAT GCC CCA AC 3’ and 5’ CGT GCA CAT GAG CTG CCT AC 3’. ORF58 primers: 5’ GGG TTC CCA TGG CAC TTA TTT CAA C 3’ and 5’ GGG AAT CCA GAA GGC AGG AGA 3’.

IRF3 Dimerization assay

IRF3 dimerization was analyzed by native PAGE and immunoblotting with IRF3 antibody. Briefly, cells were lysed in buffer containing 75mM NaCl, 50mM Tris-Cl, pH 7.5, 1mM EDTA, 1% (w/v) NP40, 10mM NaF, 0.4mM PMSF, 1mM Na3VO4, 1X protease inhibitor (complete protease inhibitor tablets, Roche), protein samples loaded in 0.125m Tris-Cl, pH 6.8 20% (w/v) glycerol and 0.1mg bromophenol blue, and electrophoresed by native PAGE with 1% deoxycholate (Sigma) in the inner chamber. Electrophoresis was conducted on ice and gels were prerun for 2hrs before loading of samples. After electrophoresis, gels were soaked in running buffer containing SDS (0.25M Tris, 1.92M Glycine, 1% SDS) for 30 minutes at room temperature before transfer onto PVDF membrane.
**IRF3 apoptosis assays**

HT1080 derived cells stably expressing MHV68 ORF58 were plated overnight, transfected with 8ug/mL dsRNA and 12uL Fugene6 for 16hrs before collection and analysis of PARP cleavage by immunoblotting. For visualization, the transfection was allowed to proceed for 20 hrs and viewed under the microscope.

**IRF3 phosphorylation and nuclear localization assays**

To assess S396 phosphorylation and IRF3 nuclear localization, HT1080 cells expressing viral proteins were treated with dsRNA for 3hrs before collection of whole cell or nuclear lysates respectively described previously. For nuclear extracts, cells were washed in PBS and resuspended first in Hypotonic Buffer (20mM Tris pH8.0, 4mM MgCl2, 6mM CaCl2, 0.5mM DTT), incubated on ice for 5 minutes and then mixed with equal volume of Lysis Buffer (0.6M Sucrose, 0.2% NP-40, 0.5mM DTT) before douncing. Nuclei were pelleted by centrifugation (1000g 5 minutes) washed with Glycerol Wash Buffer (50mM Tris pH8.3, 5mM MgCl2, 0.1mM EDTA, 40% glycerol) and PBS before further lysis. To look at localization by immunofluorescence, the same cells were plated on coverslips, treated with dsRNA for 1hr and then LMB for 2hrs before fixation with 4% formaldehyde and permeabilization with 0.2% Triton X-100 (Sigma). Cells were stained for IRF3 (1:10,000) and DAPI. To determine IRF3 transcriptional activity, the same cells were treated with dsRNA for 1hr, and then LMB for 6hrs. Whole cell extracts were prepared for immunoblotting.

**Immunoblotting and Immunoprecipitation**

Unless otherwise specified, protein extracts were made by lysing cell pellets in 50mM Tris pH 7.4, 150mM NaCl, 0.1mM EDTA, 10% glycerol, 1% Triton X-100,
50mM NaF, 10mM β-glycerophosphate, 1mM PMSF, 1mM Na3VO4 and 1X protease inhibitor (complete protease inhibitor tablets, Roche) on ice, brief sonication followed by clarification by centrifugation.

Immunoblotting was performed with antibodies HA Y-11 (1:1000) (Santa Cruz Biotechnology), actin (1:500) (Sigma), p56 (1:5000),123 c-Myc 9E10 (1:1000) (Santa Cruz Biotechnology), P396 IRF3 (1:1000) (Cell Signaling), total IRF3 (1:10,000) (gift of M. David),195 histone H1 AE4 (1:1000) (Santa Cruz Biotechnology), STAT2 C-20 (2:15,000) (Santa Cruz Biotechnology), Flag M2 (1:5000) (Sigma), PARP 9541 (1:1000) (Cell Signaling), AKT 9272 (1:1000) (Cell Signaling), phospho serine 473 AKT 4051 (1:1000) (Cell Signaling), P38 9212 (1:1000) (Cell Signaling), phospho threonine 180/tyrosine 182 P38 9211 (1:1000) (Cell Signaling), p65 4764 (1:1000) (Cell Signaling), DRBP76/p90 (1:1000) raised in the Cleveland Clinic Hybridoma core against purified protein196,197 and V5 (1:1000), a polyclonal antibody raised against a full length PIV5 V-GST fusion protein at Cocalico Biologicals Inc (gift of C. Horvath).

To assay V interaction with IKKe, TLR3 293 cells were transfected with relevant expression plasmids. Immunoprecipitations (IP) were performed as described previously189 with agarose conjugated HA F7 (Santa Cruz Biotechnology), Flag M2 (Sigma) or Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) with c-Myc 9E10 at 4C. To assay for in vivo V phosphorylation, the same procedure was used to isolate V. The resulting samples were then treated with 100U calf intestine phosphatase (CIP) (USB) 37C 3hr where indicated.
**In vitro Kinase Assay**

To purify Flag-IRF3 and V_M, the respective vectors were transfected for 24 hrs into TLR3 293 cells and lysates prepared as described. Briefly, cells were lysed on ice for 30 minutes in 50mM HEPES pH 7.4, 150mM NaCl, 2mM EDTA, 5mM NAF, 0.1mM NaVO4, 1% NP-40, 10% glycerol, 5ug/mL PMSF, 5 ug/mL Leupeptide and 5ug/mL Pepstatin A. Flag-tagged proteins were immunoprecipitated from centrifuged clarified lysates (approximately 3mg) with 50uL of Flag M2 agarose (Sigma) for 2 hrs at 4C, washed 6 times with PBS, eluted with Flag peptide (Sigma) 0.2ug/uL in 10mM TRIS pH7.5 in 10% glycerol and concentrated with Microcon Filter Devices YM-3 (Millipore). Myc-TBK1 was purified similarly with c-Myc 9E10 and Trueblot IP beads (eBioscience). Quantitation of purified substrates was performed by Flag immunoblotting with Odyssey. For *in vitro* kinase assays, purified Flag-IRF3 or V_M (100ng each) were incubated with GST-IKKe (Cell Signaling) (70ng) or Myc-TBK1 in 20uCi $\gamma$32P ATP and 20mM HEPES pH7.4, 10mM MgCl2, 100uMNa3V04, 20mM β glycerophosphate, 10mM p-Nitrophenyl Phosphate , 1mM DTT, 50mM NaCl for 30 minutes at 30C. Samples were analyzed by SDS gel electrophoresis and quantitation for autoradiograph was performed by Molecular Dynamics PhosphorImager and for immunoblotting by Odyssey (Licor).

**V_M degradation**

To follow V_M degradation by IKKe, 1ug control empty or IKKe expressing vectors were cotransfected with 1ug V expressing vectors into TLR3 293 cells for 8hrs, media changed and whole cell lysates prepared 48hrs later. To follow V_M degradation by dsRNA, the same cells were simultaneously transfected with pEF-V and dsRNA-treated with either MG132 or control DMSO for 16hrs.
**IKKe ubiquitination**

To assay for ubiquitination of IKKe, 2ug empty or Vm expressing vectors were co-transfected with 1ug of HA tagged ubiquitin and 2ug of IKKe vector into 4 x 10^6 293 cells seeded in 100mM plate for 8 hrs. Cells were collected 24 hrs after transfection, lysed and 1mg of whole cell extract pre cleared with 20uL protein A/G agarose beads (Santa Cruz Biotechnology) for 2 hr at 4C. Pre cleared lysates were then mixed with 1ug of Myc 9E10 (Santa Cruz Biotechnology) for 3 hrs 4C and then 20ul of protein A/G agarose beads were added for another 2 hr at 4C. Immunoprecipitations were washed five times before eluction and analysis by SDS gel electrophoresis.

**IL8 ELISA**

1x10^6 HT1080 derived cells stably expressing MHV68 ORF58 were plated in 35mm dishes overnight and treated as indicated. Media was collected and frozen -20C to store for further analysis. IL8 ELISA (BD Pharmingen) was performed in triplicate and confirmed by at least 2 independent experiments according to manufacturers’ instructions.
CHAPTER 3

A KNOCKOUT MOUSE MODEL TO ADDRESS THE PHYSIOLOGICAL SIGNIFICANCE OF INTERFERON STIMULATED GENE 56 (ISG56)/INTERFERON-INDUCED PROTEIN WITH TETRACOPEPTIDE REPEATS 1 (IFIT1)

Summary

Interferon stimulated genes (ISGs) are induced in diverse situations. These include infection by virus, bacteria and parasites as well as other biological processes involved in autoimmunity, cancer and even the female menstrual cycle. Of the hundreds of genes that are upregulated, ISG56 is consistently one of the, if not the, strongest induced gene. The protein which it encodes, p56, has been shown to mediate antiviral activity against human papillomavirus and hepatitis C virus by inhibiting translation. To determine the physiological functions of this antiviral activity as well as other processes in which ISG56 has been implicated we sought to disrupt the mouse ISG56 gene by making a conditional knockout. Unfortunately, problems were encountered in the generation of an embryonic stem cell containing a correctly integrated targeting construct. Possible reasons for this failure, methods to improve the chances for targeting success and the predicted phenotype of the ISG56 knockout are discussed.

Introduction

ISG56 is a member of a family of genes induced by interferons (IFNs) that include human ISG54, 60 and 58, mouse ISG56, 54, 49 as well as others in goldfish, the chinese hamster, rat and sheep. The members of the protein products
of these genes, P56 family of proteins contain multiple 34 amino acid tetratricopeptide repeat (TPR) motifs responsible for protein-protein interactions.

For the P56 family of proteins, TPR motifs mediate interaction with the eukaryotic translation initiation factor (eIF3) subunits to inhibit translation. The first two TPR motifs of mouse p56 (mp56) and the first TPR of mp54 bind to eIF3c, preventing the formation of the 48S preinitiation complex between the 40S ribosomal subunit and 20S complex. The last two motifs, TPRs 5 and 6, of human p56 (hp56) bind to the e instead of c subunit of eIF3, preventing the formation of the ternary complex which is also essential for translation. Finally, hp54 binds to both the e and c subunits of eIF3, inhibiting formation of both complexes. The translational inhibition then occurs when the p56 family of proteins is expressed.

Induction of the ISG56 family of genes is mediated by interferon stimulated regulatory elements (ISREs) in their promoters which are both necessary and sufficient. Thus, interferon regulatory factors (IRFs) which bind to ISREs are the primary transcription factors responsible for expression. Although originally identified as an IFN stimulated gene (ISG), the members of the ISG56 family can also be induced in an IFN independent manner- directly from the activation of pattern recognition receptors (PRRs). These PRRs include Toll like receptor 3 (TLR3), the RNA helicases RIG-I/mda-5 and TLR4. Induction then is both direct and mediated by IRF3 which is activated by all the above mentioned PRRs, as well as indirect through IFN amplification and mediated by the IFN stimulated gene factor 3 (ISGF3) complex of STAT1, 2 and IRF9/p48. Of the vast array of such genes that are induced by these pathways, ISG56 mRNA is one of, if not the most, highly upregulated.
The fact that ISG56 in particular is so highly expressed upon infection by viruses that activate IRF3 is one indication that the p56 protein may play an important role in immunity. In situ hybridization show that mRNA of the ISG56 gene family are upregulated during infection with the Arenavirus lymphocytic choriomeningitis virus (LCMV) particularly in microglia, a CNS specific immune cell, and other infiltrating immune cells within the brain. In addition, expression of the ISG56 family of genes overlaps with and extends beyond the area of LCMV infection. This pattern also occurs during infection of the brain by the Flavivirus West Nile virus. This indicates that ISG56 expression may help to contain virus replication in these two instances. Furthermore, in response to infection by the Rhabdovirus Vesicular stomatitis virus (VSV), almost every single other organ except the heart and kidney display increased mp56. Finally the Polyomavirus John Cunningham virus (JCV), Paramyxovirus Sendai virus, Adenovirus 12 and Coronavirus severe acute respiratory syndrome (SARS) also induce the ISG56 family of genes.

Once induced, human p56 (hp56) appears to have an antiviral role against viruses such as the Flavivirus Hepatitis C virus (HCV). Virus infection induces p56 expression in whole chimpanzee as well as human and mouse tissue culture models. hp56 is associated with the initiator ribosome HCV RNA complex in IFN treated cells and suppresses HCV internal ribosome entry site (IRES) mediated translation both in vitro and in vivo through eIF3 binding. This antiviral mechanism is important in determining viral fitness as in a cell culture model a poorly replicating HCV replicon with a mutation that induced IFN and p56 evolved to replicate better and include point mutations that decreased the association of hp56 with RNA. Indeed, the HCV NS34A protease
mediates cleavage of the TLR3 and RIG-I/mda 5 adaptors TRIF and IPS1 respectively, which are essential in IRF3 activation and hp56 induction and sequence diversity in the NS3 coding region is associated with differential IRF3 activation.180, 212 These findings indicate that the antiviral activity of P56 is a selective pressure.

Together, these lines of evidence support that the ISG56 family of genes is important in the innate immune response against virus infection. Its low basal expression and high inducibility in different tissues from a wide variety of stimuli suggest that its actions are potent, have global implications and must be tightly regulated. No mouse models of any of the ISG56 family members currently exist currently. Of the family members, ISG56 and its protein product has been the most extensively studied and has been shown to be able to directly mediate antiviral effects. Attempts at knocking down ISG56 expression in tissue culture models via siRNA have never achieved 100% efficiency.213 This indicates that there may be a potentially unknown and essential role of ISG56 for the host itself. To assess this and other as yet to be identified functions of P56 in a more physiologically relevant model, we sought to develop a conditional P56 knockout mouse.

**Results**

**Targeting Strategy**

To assess the function and expression of the mouse ISG56 gene, a conditional knockout was designed. ISG56 is encoded by only two exons separated by one intron, with the first exon comprising only of the start codon.117 Conditional expression in the targeting vector was achieved by removing the intron, joining the two exons together to create a continuous ORF and flanking this ORF with the cre recombination sites loxP.214
The widely accepted purpose of an intron is for use in alternative splicing. This process was not felt to be important for the ISG56 gene since there are no indications that splicing occurs: there is only one intron within the gene and two are necessary for actual alternative splicing and we have not observed protein products by immunoblotting that may result from an alternatively spliced version. While the removal of the intron may affect the stability of the ISG56 mRNA, we did not believe this possibility outweighed the ability of being able to completely remove the ISG56 allele in the null situation.

ISG56 and the homologous recombination arms were cloned from 129/Sv embryonic stem (ES) cell DNA derived from the genotype of ES cells in which transfection and recombination would take place.

For selection, the antibiotic Neomycin (Neo) under the control of the phosphoglycerate kinase (PGK) promoter was inserted after the poly adenylation signal of ISG56. To ensure that the PGK promoter and Neo did not affect the transcription of the ISG56 present on the targeting vector, PGK Neo was inserted in the antisense direction of the ISG56 gene. In addition, frt recombination sites were placed on either side of the PGK Neo cassette for its removal after use as a selection marker. This could be accomplished either during the embryonic stem (ES) cell stage or by crossing with transgenic mice expressing the recombinase flp.

For conditional ISG56 expression, loxP sites were placed immediately 5’ of the ISG56 ATG and 3’ of the outer flp site. The creation of a null allele could be accomplished again either in the ES cell stage or by crossing with transgenic mice expressing the recombinase cre. As many transgenic mice exist that express cre from promoters which are inducible, tissue specific or developmental stage specific, the use of
Figure 3.1: Targeting strategy of the P56-/- mouse.

The white boxes detail the genomic structure of the ISG56 gene with its two exons and an intervening intron as labeled. The targeting vector is highlighted in grey and so represented in the recombination possibilities outlined. Complete recombination of targeting vector with the resident ISG56 allele are denoted with crossing black lines and partial recombination in exon 3 denoted with dashed lines. PGK NEO denotes the phosphoglycerate kinase-neomycin resistance cassette inserted in reverse orientation. LacZ denotes the promoter-less β galactosidase gene. Target sequences for frt and cre recombinases are labeled as flp and loxP, respectively. Bgl II and Eco RI restriction sites used for genotype determination by Southern blot analysis are as labeled with indicated probes and expected DNA fragments. Representative Southern blot analysis of an ES clone picked after transfection of targeting vector into cells and antibiotic selection shows a band at 12.1 kb with the BglIII probe and 10.9 kb band with the EcoRI probe, indicating the complete integration of the targeting vector into the genome at some place other than the ISG56 allele.
the cre/loxP recombination system would allow for a great number of conditions in which the ISG56 allele could be removed.

Finally, to assess the expression pattern of ISG56, the reporter gene lacZ encoding β galactosidase was inserted immediately after the 3’ loxP site. In this way, reporter gene expression would be controlled by the ISG56 promoter once cre recombination took place.216

**Embryonic Stem (ES) cell screening**

Homologous recombination of this targeting vector with the resident gene would delete the intron, 6.6 kb, from the start of the resident gene. (Fig 3.1) Restriction enzyme digestion of the inserted intronless ISG56 construct with Bgl II would produce a 5.5 kb instead of the 12.1 kb DNA fragment when hybridizing to a specific probe targeting the 5’ non-coding region of ISG56 outside of the region of recombination.

Partial homologous recombination could also potentially occur with an incorrect 5’ homologous arm containing exon 2 of ISG56 instead of the 5’ region upstream. Digestion of such a partially recombined allele with Bgl II would produce a 12.1 kb DNA fragment identical to the wildtype ISG56 allele because of the presence of the intron. Therefore, a second screening strategy was devised to detect this partial recombination using the presence of an Eco RI restriction site in the lacZ gene of the targeting vector. Eco RI digestion of DNA containing a partial (or complete) recombination of the targeting vector would produce a 5.7 kb DNA fragment hybridizing to a specific probe targeting the 3’ non coding region of ISG56 outside of the region of recombination. The same restriction enzyme would generate a 10.9 kb DNA fragment from the resident
ISG56 allele. Using both of these strategies, we could distinguish between clones in which only partial and clones in which complete recombination took place.

Proper construction of the targeting vector was confirmed by restriction digest and sequencing. It was linearized by Not1, purified and transfected into ES cells three different times. A total of 364 clones overall were picked. None were shown to contain the targeting vector integrated appropriately by Southern hybridization of genomic DNA digested with Bgl II. Further analysis of clones from the first transfection group confirmed that while the vector integrated into the genome, it was in multiple sites outside of the ISG56 locus. Unfortunately, the sites themselves were never identified.

**Discussion**

ISRE mediated signaling is activated in diverse situations, not just viral infection. These include infection with bacteria and parasites, some autoimmune diseases and cancers, and even as part of the female menstrual cycle. Although ISREs mediate the induction of hundreds of genes, ISG56 is consistently one of the, if not the strongest, induced genes. This begs the question whether ISG56 plays important and phenotypically evident roles in the diverse situations in which it is induced. Development of a knockout mouse will be critical to answering this mystery.

**Improving chances for targeting success**

It is disconcerting that out of the 364 clones we tested, none showed correct homologous recombination of the targeting vector with the resident ISG56 allele. Equally surprising was that not even partial recombination via the exon 2 homology occurred. Instead, integration of the targeting vector occurred at unknown sites. One possible explanation may be that the lengths of the arms built into the vector for homologous
recombination were too short. The 5’ arm was 3 kb and the 3’ arm was 2.9 kb. While these lengths were certainly not the shortest successfully used for recombination by others, the fact that the intervening sequences were about 9.1 kb in length, more than three times the length of either, may have decreased the overall efficiency as the frequency of recombination is inversely proportional to the length of the nonhomologous DNA insert. A second possible problem may be that the homologous arms are similar enough to other areas of the genome for recombination. While both the 5’ and 3’ arms were specifically homologous to the respective areas around the resident ISG56 allele, BLAST analysis of did indicate approximately 80% nucleotide sequence identity in 1 kb of the 2.9 kb 3’ arm to an area on chromosome 9. In addition, the 0.5 kb PGK promoter and 0.5 kb 3’ UTR for Neo are 99% homologous to areas on chromosome X within the mouse genome. While we have not specifically assessed if recombination did indeed occur at these locations, the fact that there were more than two different banding patterns shown by Southern indicate the targeting vector integrated into many and not just these two areas.

In light of these possibilities, longer arms for homologous recombination may be necessary for larger pieces of DNA to be integrated. Thus, decreasing the length of the non-homologous insert (i.e. removing the LacZ reporter gene and/or shortening the ISG56 targeting vector allele) may help improve the chances for targeting success. Another option is to use a negative selection marker such as the herpesvirus thymidine kinase (TK) at the end of the targeting vector to screen out random integration by ganciclovir antibiotic selection. This is based on the notion that random integration will incorporate TK into the genome and its subsequent expression in the presence of GCV is
lethal to the cells. However, correct homologous recombination will not incorporate TK into the genome and its lack of expression in the presence of GCV will allow survival. Finally, changing the areas used for homologous recombination to try to minimize the homology between sequences within the targeting vector and non-specific areas in the genome, particularly in the 3’ end, may be helpful in targeting success. This last option, however, cannot be utilized unless a completely new knockout strategy is devised.

**Mouse vs human ISG56**

The development of a knockout mouse would be essential in addressing many questions as to the physiological importance of mp56. However, extrapolations to its human protein must be made cautiously. While human and mouse p56 have homology in terms of tertiary structure in the location and number of TPR motifs, actual amino acid sequence homology is 53% between mouse and human ISG56 and 63% for ISG54. The induction patterns of human and mouse p56 are also slightly different when dsRNA in the media is the treatment, with much less efficient IFN independent induction of ISG56 in mouse macrophages compared to human tissue culture cell lines. Furthermore, while mouse and human p56 have similar potencies at inhibiting translation as measured by *in vitro* assays, they are different in that mp56 binds to the c subunit of eIF3 while the hp56 binds to the e subunit. These different mechanisms mediated by the different subunits, (preventing the formation of the preinitiation complex compared to the ternary complex respectively) may be more important in a whole mouse model context.
Multiple members of the ISG56 family of genes

Another inherent issue with an ISG56 knockout mouse lies in its family members. All are homologous to each other and utilize TPR motifs to interact with eIF subunits to inhibit translation. We choose ISG56 to knock out first because it is one of the highest induced and also best characterized member of its family. In addition, only hp56 has been shown to have antiviral functions against such viruses as HPV and HCV. Therefore, a mouse lacking ISG56 is more likely to have a phenotype such as increased susceptibility to viral infections as compared to other family members who either have not been shown to be antiviral in this capacity or have not been characterized. However, no phenotype may be observed if the functional conservation between family members is sufficient to compensate for the loss of one.

It is our hope though that the mouse model will indicate physiological reasons for the subtle differences between the ISG56 family members. Even though mp49 has yet to have a function associated with it, it appears to be as strongly induced as mp56 in LCMV and WNV infections. In contrast, mp54 is consistently induced to lower extents in these experiments. Similarly, in response to IFNα and VSV treatments in the mouse, mp54 appears to be less induced as compared to mp56 and in response to IFNβ but not pIC in the media or transfected into cells, human ISG54 mRNA is induced less than ISG56. However, there are instances in which ISG56 is induced less than ISG54. This is seen most notably in B cells upon stimulation with dsRNA, VSV and IFNa at both the protein and mRNA expression levels. Thus, while the ISG56 family appears to be functionally similar, these differences in induction indicate that the roles of each protein may be unique.
Predicted phenotypes for a P56 knockout mouse

Members of the ISG56 family are induced by a variety of stimuli including viral infections. While induction alone cannot be equated to importance, expression is sufficient to indicate the possibility that ISG56 may play a role in infection. In this capacity, a knockout mouse will help to more definitively establish or debunk this characterization. Of course the most attractive pathogens to test for phenotypic changes in the knockout mouse are those viruses which have already been shown to be affected by P56 in tissue culture models: HCV and HPV. Unfortunately, the lack of a good mouse model for either of these viruses makes testing them difficult. A second group of viruses worthy of examination are those which induce p56 during infection but where an antiviral function has not been elucidated. As mentioned above, these viruses include Sendai, SARS, WNV, LCMV and herpesviruses such as CMV. Interestingly, the upregulation of ISG56 in LCMV infections is correlated with impaired spatial and temporal learning (the inability to learn how to run a maze by being shocked in the foot). This indicates a possible behavioral function or phenotype related to ISG56. Furthermore, ISG56 in the rat appears to be upregulated in developed oligodendrocytes which are responsible for producing the myelin necessary for nerve conduction. These patterns of expression indicate a possible neurological as well as antiviral role in the expression of ISGs. For non-viral pathogens, bacterial PAMPS such as LPS from Neisseria and CPG DNA have been shown to induce ISG56. Based on these findings, it is not unreasonable to hypothesize that the ISG56 knockout mouse will have defects in the host immune response to bacteria and parasites in addition to viruses.
Not surprisingly, ISG56 being an ISG is also upregulated in some autoimmune diseases where the immune system is upregulated. Plasma from patients with systemic lupus erythematosus but not rheumatoid arthritis appear to induce ISG56. Therefore, a knockout mouse in this context may exhibit altered immune pathogenesis of some autoimmune diseases.

In addition, ISG56 is linked to lymphomas. Both ISG56 and ISG60 are upregulated in myodysplastic syndromes in the cells which are precursors to lymphocytic and myelomonocytic leukemias. Furthermore, patients treated with imiquimoid, a TLR7 ligand, for superficial basal cell carcinomas and cutaneous T cell lymphomas also have elevated levels of ISG56. Thus a knockout mouse model of ISG56 may have altered susceptibilities to some lymphomas and also respond differently to treatments.

Finally, ISG56 is regulated by the menstrual cycle and pregnancy in ewes. In addition, ISG56 levels cycle in the uterus in response to menstrual hormones in both mice (GDS2208) and humans. This suggests a possible role for ISG56 in reproduction and, in a very round about manner, is one justification for using an inducible knockout model to account for the possibility of reproductive defects.

In summary, the ISG56 family of proteins is unique in its high expression, regulation and possible redundancy. The various members have been shown to be important in the context of the pathogen and host relationship. However, there are numerous indications that the ISG56 family may be important for other physiological processes. The only possible way to really ascertain their role is to systematically knockdown one by one and in combination the different members. Making an ISG56 knockout will only be the first of many to come.
CHAPTER 4
A CELL SURVIVAL ASSAY THAT IDENTIFIES VIRAL INHIBITORS OF
dsRNA MEDIATED SIGNALING

Summary

dsRNA is an important pathogen associated molecular pattern whether its source is the pathogen itself or the host as cells necrose during infection. Extracellular dsRNA is recognized through TLR3 and activates IRF3 to mediate the transcription of interferon (IFN) stimulated genes (ISGs) via IFN stimulated regulatory elements (ISREs) in their promoter. This pathway is important in establishing the antiviral environment as many viruses activate IRF3 and many of the genes that are induced have been shown to have direct antiviral activity. Viruses in turn have developed a variety of mechanisms to evade this part of the immune system. To identify inhibitors of IRF3 transcriptional activity, we therefore developed a cell death assay in which IRF3 is activated by dsRNA signaling through TLR3. When dsRNA TLR3 IRF3 signaling was induced cells died. When signaling was inhibited cells survived. In this capacity, this assay can be used to identify not only inhibitors but also activators of IRF3 mediated gene transcription from a variety of different sources including viruses.

Introduction

dsRNA is an important pathogen associated molecular pattern present during viral infections either as the viral genome, as replication intermediates or as part of cellular debris released in necrosis. This is underscored by the phenotypes of the dsRNA receptor knockout mice in virus infections. Mice lacking RIG-I and mda-5, which recognize cytoplasmic or intracellular RNA, have increased susceptibility to vesicular
stomatitis virus (VSV) and Japanese encephalitis virus (JEV) for the former and EMCV for the latter.\textsuperscript{108, 109} The mouse model for TLR3, which recognizes extracellular dsRNA, appears to be protective for DNA and RNA viruses such as rhinovirus (RV), respiratory syncytial virus (RSV), encephalomyocarditis virus (EMCV), mouse cytomegalovirus (mCMV), herpes simplex virus (HSV) and influenza in the central nervous system (CNS).\textsuperscript{45, 71, 78, 79, 81, 82} Paradoxically, TLR3 also appears to be detrimental in influenza infections in the lung, Punta Toro virus, West Nile virus (WNV) and autoimmune diseases.\textsuperscript{66, 87, 90, 91}

Signaling induced through these dsRNA receptors is predominantly through the IFN regulatory factor (IRF3). Constitutively expressed IRF3 is activated directly by dsRNA. Activated IRF3 homodimerizes,\textsuperscript{113, 227} localizes to the nucleus and induces the expression of IRF7 and other ISGs. Subsequent heterodimerization between IRF3 and IRF7 serves to amplify the antiviral response.\textsuperscript{228}

These same steps of IRF3 activation provide multiple the levels of regulation for the host and virus. Full phosphorylation and nuclear localization is essential for activity and even partial inhibition of these steps is sufficient to inhibit transcription.\textsuperscript{59, 229} Host mechanisms of negative regulation as mentioned in Chapter 1 include suppressor of IKKe (SIKE) which prevents phosphorylation,\textsuperscript{142} A20 which prevents dimerization\textsuperscript{145} and Ret finger protein (RFP) which allows for dimerization but not nuclear translocation.\textsuperscript{144} In addition, IRF3 after being activated via phosphorylation can be simply degraded via a proteosome dependent pathway.\textsuperscript{148, 192} Finally, IRF3 activation is a target for many RNA and DNA viruses to inhibit as detailed in Chapter 1. The complexity of IRF3 regulation
here indicates that the transcription factor plays key roles in immune and possibly non-immune functions.

IRF3 was first identified as an important part of the host response to viral infection as part of the dsRNA-activator factor 1 (DRAF1) complex and subsequent studies have shown that a variety of other pathogens activate the same transcription factor. IRF3 null mice produce less IFN in response to virus infection and are correspondingly more susceptible to EMCV induced lethality. WNV is also able to replicate better in an IRF3 deficient situation but only in certain cell types. RNA viruses that induce IRF3 nuclear localization include Sendai via the RNA helicase RIG-I, New Castle Disease Virus (NDV), vesicular stomatitis virus (VSV), Reovirus and Measles virus nucleocapsid. DNA viruses that induce IRF3 nuclear localization include Epstein Barr Virus (EBV) small RNAs, human cytomegalovirus (hCMV) glycoprotein B, herpes simplex virus (HSV1), Kaposi’s sarcoma virus (KSHV) vIRF3 and murine γ-herpesvirus 68 (MHV68) genome fragments. Finally non-viral pathogens that activate IRF3 include bacterial components such as LPS which signals through TLR4, Mycobacteria, Chlamydia muridarum, Listeria monocytogenes DNA and Legionella pneumophila. Many of these pathogens activate IRF3 through the receptors TLR3, RIG-I and mda-5. For this reason, these receptors play an important role in inducing the antiviral environment which IRF3 helps establish.

The IRF3 mediated antiviral activity is via transcription of interferon stimulated genes (ISGs). IRF3 binds to interferon stimulated regulatory elements (ISREs) within the promoters of these genes for expression. The array of genes overlaps with those induced
by IFN\textsuperscript{231} and include genes of the ISG56 family, genes involved in signaling such as TLR3 itself, apoptosis such as the oncogene pleiomorphic adenoma gene 1, RNA synthesis, protein synthesis and processing, cell metabolism, transport, and structure such as microfibril associated glycoprotein 2 are induced. This wide array of functions indicates that IRF3 may have functional implications beyond the host immune system.\textsuperscript{25, 244}

The importance of IRF3 in the host response to virus indicates that viruses may have developed a variety of mechanisms to cope with IRF3 activation. Inhibition of activation is one such mechanism. To identify inhibitors of IRF3 transcriptional activity, we developed a cell death assay in which IRF3 is activated by dsRNA signaling through TLR3. When signaling was induced by dsRNA or specific activating mediators of signaling, cells died. When signaling was inhibited by a known viral inhibitor, dominant negative mutant of or siRNA targeting a signaling protein cells survived. Thus, in addition to identifying inhibitors of IRF3 mediated gene transcription, this cell death assay is also capable of identifying activators.

**Results**

**A cell line that dies in response to dsRNA-TLR3-ISRE mediated signaling**

To identify inhibitors of TLR3-ISRE mediated signaling, a cell line was developed to respond specifically to dsRNA in the media. Selection with this line was conferred by the expression of the *Herpesvirus* thymidine kinase (TK) gene, which when in the presence of ganciclovir (GCV) is lethal.\textsuperscript{245} To construct the plasmid (561-TK) in which TK is induced in the presence of TLR3 signaling, the ISG56 promoter (-654 to +3) was cloned immediately upstream of the TK gene. (Fig 4.1) This promoter contains two
Figure 4.1: A cell death assay that identifies inhibitors and activators of dsRNA-TLR3-IRF3 signaling.

A. A depiction of the reporter gene construct used in our assay. This construct contains the HSV-1 thymidine kinase under the control of the ISG56 also known as 561 promoter (-654-3). This promoter contains two interferon stimulated regulatory elements (ISREs) (underlined) that are sufficient and essential for induction via the transcription factor Interferon Regulatory Factor 3 (IRF3). B. A depiction of the cell line used in our death assay: TLR3 293 561-TK. Proteins or siRNA are expressed by transient transfection or lentiviral infection and the selection pressure of ganciclovir (GCV) (grey) alone or dsRNA (dotted) and GCV is added. Thus, cells die in the presence and live in the absence of signaling.
A. \textit{ISG56 promoter} \textit{Thymidine Kinase}

\[-654 \quad +3 \quad -132 \quad \text{TTTCA} \text{TTTCC} \text{C} = \text{TTTGG} \text{TTT} \quad -110\]

B. 

- Signaling inducer: extracellular dsRNA
- TLR3 → IRF3 → TK → 561-TK → cell death
- Express proteins/siRNA in cells

- Ganciclovir only
- dsRNA and ganciclovir (GCV)

- Cells die in the presence of signaling inducer.
- Cells live in the absence of signaling inducer.
- Cells live in the presence of signaling inhibitor.
- Cells die in the absence of signaling inhibitor.
ISREs which alone are essential and sufficient for IRF mediated induction by dsRNA, virus and IFN. Induction is also independent of NFkB and AP-1. Therefore, gene expression is solely mediated by IRF3 or ISGF3.\textsuperscript{119, 120, 123, 124} The cells used for this line were 293 cells stably overexpressing exogenous TLR3 (TLR3 293).\textsuperscript{186} 293 cells do not signal in response to dsRNA or IFN in the media. However, overexpression of TLR3 in this cell lines confers dsRNA responsiveness. Therefore, IRF3 but not ISGF3 is activated in response to dsRNA in the media. In addition, 293 and TLR3 293 cells do not die in response to dsRNA in the media. As a result, TLR3 293 cells expressing 561-TK die as a result of TK and GCV lethality. Thus, the dsRNA induced cell death phenotype is TLR3 signaling specific.

561-TK was co-transfected with a puromycin antibiotic selection plasmid into TLR3-293 cells. Transfected TLR3 293 cells were selected for clones that survived without and died with dsRNA in the media. Cells were subcloned three rounds to generate the cell line used in our cell survival assays TLR3 293 561-TK. In these cells, less than 20 from an original population of $10^5$ cells survived dsRNA GCV selection.

**TLR3 293 561-TK validation in dsRNA induced cell death assays**

A variety of inhibitors as well as an activator of TLR3 signaling were used to authenticate the resulting cell line (TLR3 293 561-TK). (Fig 4.2) Exogenous expression of signaling proteins downstream of TLR3, such as the adaptor TRIF, is known to activate IRF3 and induce ISG56 transcription independent of the inducer dsRNA. As expected, transient overexpression of wildtype TRIF in TLR 293 561-TK cells induced signaling and cell death even without dsRNA. Cells survive in the absence of the inducer dsRNA or when the dsRNA signal is blocked by an inhibitor. HCV NS34A protease
Figure 4.2: Authentication of TLR3 293 561-TK cells.

Authentication was performed by challenging with activators (dsRNA and wildtype TRIF (TRIF WT)) and inhibitors (dominant negative TLR3 (TLR3DTIR) expressed by plasmid or lentivirus (LV), siRNA to IRF3, and Hepatitis C virus NS3/4A (HCV NS3/4A)). Dominant negative form of MyD88 (MyD88DN) and empty vector (-) were used as negative controls. Cells were transiently transfected or infected, media changed, and treated with or without dsRNA (100ug/mL) in the presence of GCV (5ug/mL). Percentage of surviving cells as determined by eye is shown as the mean of two independent experiments except when *, where only one experiment was conducted. Error bars, when applied, represent standard error.
inhibits TLR3 signaling by cleaving TRIF. Transient expression of NS34A inhibited dsRNA TLR3 signaling induced cell death. Similarly, an siRNA construct targeting IRF3 as well as a dominant negative mutant of TLR3 lacking the TIR domain inhibited TLR3 mediated dsRNA signaling induced cell death. This inhibition was effective when both plasmid transfection and lentiviral infection were used for expression. Finally, cell survival results from a specific inhibition of TLR3 signaling and not general signaling pathways. Overexpression of a dominant negative mutant of MyD88, which is able to inhibit TLR4, 7 and 9 but not TLR3, did not inhibit signaling induced cell death. These results indicate that cell death was an accurate reflection of signaling in TLR3 293 561-TK cells and that a variety of methods of delivery and inhibition could be measured in this cell line.

Discussion

Unique assay characteristics

Several characteristics of the 293 cells and ISG56 promoter allowed for the development of the cell death assay. These characteristics involve the specificity of the response to dsRNA for both the 293 cells and the ISG56 promoter.

For the TLR3 293 cells, constitutive TLR3 signaling is not lethal in contrast to RIG-I signaling which leads to cell death. This property allowed for cell death to specifically reflect TK mediated cell death driven by IRF3. In addition, 293 cells do not respond well to IFN. Therefore, dsRNA TLR3 signaling is not amplified and transcription at the ISG56 promoter is driven by IRF3 and not ISGF3. Finally, 293 cells are highly transfectable and infectable with plasmid or lentiviral expression vectors. Exogenous expression either transiently or in stable lines does not induce ISG56 in 293
as compared to other cell types. 293 cells, then, provide a system in which exogenous proteins can be expressed efficiently to assay for inhibition of signaling.

For the ISG56 promoter, transcription is regulated by ISREs. Several characteristics help distinguish the expression pattern of ISG56 from other ISGs. First, ISG56 is induced by dsRNA both directly by IRF3 and also indirectly through IFN and ISGF3. This is in contrast to other promoters such as the 6-16, 9-27 and Mx which are upregulated not by IRF3 but only by ISGF3. Second, ISG56 mRNA expression is tightly regulated. It is not expressed basally. However, it is one of the most highly induced ISGs in cells stimulated with dsRNA or IFN. Third, the ISG56 promoter has two ISREs which are essential and sufficient for induction. This is in contrast to the complex promoter of the type I IFNs where NFkB and AP-1 binding sites are required for induction. Put together, these characteristics embody a tightly regulated promoter that when driving a strong reporter gene creates a simple assay for signaling.

**Advantages of TK negative selection**

The use of TK in negative selection has been used to identify regulators of NFkB activation by interleukin 1 and other signals. The TK GCV selection system has several advantages over other selection genes such as the E. coli 6 thioguanine (gpt) Hypoxanthine Aminopterin Thymidine (HAT) system and also other reporter genes such as luciferase or green fluorescent protein (GFP). First, only the Herpesvirus and not cellular TK sufficiently phosphorylates GCV to induce lethality. This is in contrast to the gpt system where the cellular hypoxanthine-guanine phosphoryibosyltransferase (HPGRT) functions sufficiently in the salvage pathway so that the E. coli gpt is unnecessary. Thus for the gpt selection system to work, cells must be deficient of
endogenous HPGRT. This is unnecessary for the TK system. Second, unlike the gpt system the stringency of selection can be manipulated by GCV concentration. In this context, it appears that the selection conditions used in our assays were very stringent because the best host inhibitor, the dominant negative mutant of TLR3, could only protect 25% of cells from lethality in the presence of dsRNA as compared to cells without any treatment at all. (Fig 4.2) Using this stringency, our assay would identify only the most robust inhibitors of signaling. Third, TK selection system is much easier to use than other reporter genes such as luciferase or GFP in that the cell death phenotype is much simpler to see and does not require fluorescence measurements. Furthermore, TK or GCV alone are not toxic to the cells. In contrast, GFP for example can be toxic when expressed at high levels and or probably for an extended period of time. In fact, preliminary attempts at constructing a stable cell line using GFP as a reporter gene under the ISG56 promoter resulted in gradual loss of inducible fluorescence over time, giving credence to this possibility. (Data not shown.)

High throughput assays and screens

The use of a cell death phenotype in our assays here also builds the basis for future experiments with this cell line in more high throughput assays where, again, the phenotype is much easier to measure as compared to fluorescence or β galactosidase assays. It is possible to extend our assays into a 96 well format or microarray slide with plasmids pre-fixed onto the plate for transfection and expression. An overlay of the cells, addition of selection pressure with GCV and dsRNA and finally observation of the cell survival or death would all be performed by a robot. In this way, many proteins and molecules can be assayed for inhibition of signaling at once.
Transforming the assay into a screen would be another approach to extending our system towards a more high throughput format. Forward genetic screens have identified many proteins important in a variety of processes.\textsuperscript{247} Advancements in transposon mediated mutagenesis\textsuperscript{248} and siRNA screens\textsuperscript{250} have greatly improved the process of identification of the mutation lending to selection when compared to the complementation assays necessary for chemical mutagenesis in the past. In this instance, the addition of a positive selection marker such as the antibiotic resistance gene zeocin (R)\textsuperscript{187} under the control of the ISG56 promoter would allow for easy distinction between actual inhibitors of signaling and inhibitors of TK GCV mediated cell death which would at the moment be indistinguishable in the initial assay using TK and GCV selection presented here.

**IRF3 and ISRE as a convergence point for multiple signaling pathways**

As mentioned previously, IRF3 is activated by a variety of different signals which include cytoplasmic dsDNA through DAI and other unknown receptors,\textsuperscript{19, 110, 239, 243, 251} LPS through TLR4,\textsuperscript{240} hCMV glycoprotein B through TLR2\textsuperscript{236} and Listeria monocytogenes through an unidentified mechanism.\textsuperscript{243} Furthermore, IRF3 is involved in processes such as the induction of inflammation from ischemia\textsuperscript{252} and the responses to DNA damaging agents such as doxorubicin, UV\textsuperscript{253} and DMXAA.\textsuperscript{254} The use of this cell line in screens with these inducers would allow for further elucidation of these pathways.

In addition to IRF3, ISREs represent a point of convergence for all IRFs, both positive and negative.\textsuperscript{112} Like IRF3, other IRF family members are involved in immune regulation and also a number of processes such as regulation of cell cycle, apoptosis and
tumor suppression. The use of the ISG56 promoter and the TK reporter gene would be helpful in the further examination of these pathways as well.

Finally, for known inhibitors or activators of ISRE activation (whether they be host or pathogen associated) this cell line can be used to further screen for chemical inhibitors for potential pharmaceutical development. The influenza virus NS1, for example, is an inhibitor of IRF3 activation. Subsequent screening for inhibitors of NS1 mediated IRF3 inhibition resulted in the identification of several potentially therapeutic chemicals able to reduce influenza A replication. Ideally, viral proteins identified here in 293 TLR3 561-TK cells would be stably expressed if survival were the selection criteria as in TK and GCV. Further chemical screening of these stable lines would then provide a foray into the development of possible treatments as in the case of influenza NS1.
CHAPTER 5
SELECT PARAMYXOVIRAL V PROTEINS INHIBIT IRF3 ACTIVATION BY ACTING AS ALTERNATIVE SUBSTRATES FOR IKKe/TBK1

Summary

V accessory proteins from Paramyxoviruses are important in viral evasion of the innate immune response. Here, using a cell survival assay that identifies both inhibitors and activators of IFN Regulatory Factor 3 (IRF3) mediated gene induction, we identified select Paramyxoviral V proteins that inhibited double-stranded (ds) RNA-mediated signaling; these are encoded by Mumps virus (MuV), human Parainfluenza virus 2 (hPIV2) and Parainfluenza virus 5 (PIV5), all members of the genus Rubulavirus. We showed that interaction between V and the IRF3/7 kinases, TANK-binding kinase 1 (TBK1)/ Inhibitor of κB kinase ε (IKKe), was essential for this inhibition. Indeed, V proteins were phosphorylated directly by TBK1/IKKe, and this, intriguingly, resulted in lowering of the cellular level of V. Thus, it appears that V mimics IRF3 in both its phosphorylation by TBK1/IKKe and subsequent degradation. Finally, a PIV5 mutant encoding a V protein which could not inhibit IKKe was much more susceptible to the antiviral effects of dsRNA than the wildtype virus. Because many innate immune response signaling pathways, including those initiated by TLR3, TLR4, RIG-I, MDA5 and DAI-I, use TBK1/IKKe as the terminal kinases to activate IRFs, Rubulaviral V proteins have the potential to inhibit all of them.
Background

The family *Paramyxoviridae*

The family *Paramyxoviridae* of the order *Mononegavirales*, the nonsegmented negative single stranded RNA viruses, is defined by its F protein, which fuses virus and cell membranes at neutral pH. In relation to the *Orthomyxoviridae* family, the *Paramyxoviridae* family shares the characteristic of envelope glycoproteins and with the *Rhabdoviridae* family the characteristic of a nonsegmented genome and its gene expression. Two subfamilies represent the divisions of the *Paramyxoviridae* family and are classified based on morphology, biological activities of the proteins, genome organization and sequence relationships: *Paramyxovirinae* (with genera *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Morbillivirus* and *Henipavirus*) and *Pneumovirinae* (with genera *Pneumovirus* and *Metapneumovirus*) (Fig 5.1). The *Paramyxovirinae* subfamily is distinguished by the size and shape of the nucleocapsids (diameter 18nm, length 1um, pitch 5.5nm) which is wider than *Pneumovirinae* members and its left handed helical symmetry. All within the subfamily can cross react antigenically. However, the genera differ in neuraminidase activity of the hemagglutinin neuraminidase (HN/H/G) open reading frame (ORF) product (*Respiroviruses* and *Rubulaviruses* are positive and *Morbiliviruses* and *Henipaviruses* are negative) and the coding potential of the P ORF (Fig 5.2, discussed in more detail below).

The *Paramyxovirinae* virions possess an envelope derived from the plasma membrane of host cells (Fig 5.3). This contains two surface glycoproteins, the HN/H/G for cell attachment and F involved in fusion. In addition, some members of the genera *Rubulavirus* and *Pneumovirus* express a third integral membrane protein SH thought to
Figure 5.1: International Committee on Taxonomy of Viruses classification of negative sense ssRNA viruses.

The only order established within this group is that of the non-segmented Mononegavirales. Additionally, the segmented families Orthomyxoviridae, Bunyaviridae (not shown) and Arenaviridae (not shown) without an official order are also negative sense ssRNA viruses.
negative sense ssRNA viruses

Order unassigned

Family Orthomyxoviridae (segmented)

Subfamily

Genus Influenza virus A, B, C, Thogotavirus, Isavirus

Species PIV1, 3, Sendai, Measles, Rinderpest, PIV2, 4, Mapuera, Numps, PIV5/SV5, SV41, Hendra, Nipah, Avian paramyxovirus, New Castle Disease virus, Respiratory syncytial virus, Avian + human metapneumovirus
Figure 5.2: RNA editing during transcription of the P ORF in different genera of the *Paramyxovirinae* subfamily generates a variety of accessory proteins.

The antigenome ORFs are at the top while the correlate edited transcripts are below. The white box represents common amino termini. The RNA editing site is represented by the vertical line where the indicated number of guanines (G) are inserted to generate the different accessory proteins. The shaded boxes represent unique carboxy termini fused to the common amino termini as a result of this editing. This includes the cysteine rich domain of V proteins. Transcript abundance corresponds to number of nucleotide additions with the unedited version produced the most. For Respiro, Morbilli, Avula and Henipaviruses, the unedited transcript is the P protein. For Rubulaviruses, the unedited transcript is the V protein. Adapted from “*Paramyxoviridae: The Viruses and Their Replication.*”

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Respiroviruses, Morbilliviruses
Avulaviruses, Henipaviruses

Antigenome ORFs

-0 - P ORF
-1 - V ORF
-2 - W ORF
-2 - D ORF (PIV3)

Transcripts with Inserted Gs

-0 - P protein
+1 - Cys-Rich
+2 - V protein
+2 - W protein ORF
+2 - D protein (PIV3)

Rubulaviruses

-0 - V ORF
+1 - Wp ORF
+2 - F ORF
+1/4G - V protein
+2G - Wp protein
+2G - F protein
Figure 5.3: A *Paramyxovirinae* virion not drawn to scale.

Two glycoproteins, fusion (F) and hemagglutinin neuraminidase (HN), lie within the envelope or lipid bilayer derived from the host cell. While HN mediates attachment, F promotes fusion with the host membrane to deliver the nucleocapsid (N) bound to the (-) ssRNA nonsegmented genome and also syncytia formation. The small integral membrane protein, SH, is found only in specific Rubulaviruses such as PIV5 and MuV and is thought to be able to inhibit apoptosis of the host cell. Associated with the nucleocapsid are the large (L) and phospho (P) proteins which together have RNA dependent RNA transcriptase activity. For Rubulaviruses but not other members of the *Paramyxovirinae* subfamily, the accessory protein V is also found as an internal component of the virion. Adapted from “*Paramyxoviridae*: The Viruses and Their Replication.”²⁵⁶
block virus induced apoptosis. Inside the membrane lies the viral matrix (M) which determines the architecture. Finally, the core is a 15-19 kb RNA genome encapsidated by the nucleocapsid (N) proteins. N is also associated here with phospho- (P) and large (L) proteins. Together, P and L are responsible for RNA dependent RNA transcriptase activity. For the genus Rubulavirus, the accessory protein V is also found within the virion while for other genera it is found only in virus-infected cells.

As described above, virus adsorption and entry begins with binding of HN to sialic acid containing cell surface receptors and then fusion with the F protein at neutral pH. While the F protein remains in the host membrane and facilitates fusion with other infected cells to form syncytia, the M layer disassembles and the nucleocapsid enters the cytoplasm. It is here in the cytoplasm that all aspects of the replication cycle are thought to take place as viral mRNA synthesis is insensitive to DNA-intercalating drugs such as actinomycin D and replication can occur in enucleated cells.

The RNA wound around the helical nucleocapsid serves as the template for all RNA synthesis by the viral RNA polymerase (vRNAP), formed by the complex of L which possesses all enzymatic activities and P, an essential cofactor. Synthesis early in infection by this complex yields (+) leader RNAs for the generation of viral genomes and mRNAs for protein expression. Because of imperfect re-initiation in the “stop-start” mechanism for transcription, mRNA for the 6-10 ORFs is produced in a gradient, decreasing in abundance from the 3’ end of the genome which contains the promoter region. This results in N followed by P or V being expressed the most and L the least. In addition, RNA editing or pseudotemplated addition of guanine (G) occurs in the coding region of the P ORF, yielding a faithful unedited mRNA transcript as well as several
more edited versions that code for the accessory proteins V, W/I and D (Fig 2). These, too, are expressed in a gradient, decreasing in abundance with increased editing. Once sufficient levels of viral proteins are achieved, the (-) genome is replicated to produce a full length encapsidated complementary copy, the antigenome. This intermediate is used as a template to generate the genome itself. The nucleocapsid is then assembled in the cytoplasm and the envelope at the membrane. The M protein positioned beneath the envelope contact both the RNP core as well as the enveloped glycoproteins, facilitating the incorporation of the genome into progeny virions during budding.

**Genera within *Paramyxovirinae***

Viruses representing different genera within *Paramyxovirinae* are important pathogens. For humans these include members of the genus *Morbillivirus* Measles virus (MeV) and *Rubulavirus* Mumps virus (MuV) and human Parainfluenza virus 2 (hPIV2). Indeed, Measles virus represents not only one of the most infectious but also, along with Mumps and Parainfluenza viruses, some of the most prevalent. For animals, examples include Hendra virus (HeV), a *Henipavirus* which infects flying foxes, Sendai virus (SeV), a *Respirovirus* which infects rodents, Parainfluenza virus 5 (PIV5) (also known as Simian virus 5 or SV5), a *Rubulavirus* which infects dogs and Newcastle disease virus (NDV), a highly infectious *Avulavirus* with 100% morbidity and 90% mortality in chickens.256

Parainfluenza viruses are represented by members of three different genera within the subfamily *Paramyovirinae: Respirovirus* (hPIV1 and its mouse equivalent Sendai virus and human and bovine PIV3), *Rubulavirus* (hPIV2 and its canine and simian equivalents PIV5 and SV41 as well as hPIV4) and *Avulavirus* (NDV) (Fig 5.1).257 While
NDV as described above has important economic impacts in the poultry industry, hPIV1-3 account for at least one third of the five million lower respiratory infections each year in the US in children younger than five and has an estimated cost of over $186 million. Seen annually or biennially, long term resistance is incomplete and re-infections are frequent. Spread by droplets, the virus replicates in the epithelial cells that line the respiratory tract and causes rhinitis, pharyngitis, laryngitis, trachiobronchitis, bronchiolitis and pneumonia, with more severe disease in children or the immunocompromised. In addition to CD8 cytotoxic responses against HN, P and N, neutralizing antibodies have been identified against HN and F with a specific serotype response in the primary infection that can cross react in subsequent infections with booster responses.

Although Mumps virus is also a Rubulavirus like PIV2 and 4, the associated symptoms and disease are not as limited to the respiratory tract. Hippocrates in the 5th century BC first described the classically associated swelling near the ears (the parotid or submaxillary glands) and, variably, of one or both testes (orchitis). Also transmitted by droplets, MuV is most commonly seen in children 5-9 years old although the incidence has dropped almost 99% since the advent of the attenuated Jeryl Ly vaccine strain. Routine MuV vaccination, however, does not prevent outbreaks and two recent epidemics in the UK and US in 2005 and 2006 are such examples. The natural host of MuV is the human and initially, like PIV, infects the upper respiratory mucosa. However, MuV can then spread to the lymph nodes and disseminate via T cells to the parotid glands as described above, CNS, gonads, kidneys, pancreas, heart and joints. Although one third of infections are thought to occur without symptoms, MuV is the most common cause of
unilateral acquired sensorineural hearing loss in children and, before the vaccine, encephalitis in the US.

**Paramyxoviruses and innate immunity**

Approximately 30% of children infected with PIV produce IFN in the respiratory tract. Experiments indicated that this IFN is initially inhibitory but quickly overcome by the virus. Indeed, V from Sendai and PIV5 were isolated as the protein with this function, stimulating the degradation of STAT1 which is involved in IFNa/b signaling. Finally, V mutant or knockout virus showed that V not only inhibits IFN signaling but also IFN induction.

**V proteins in innate immunity**

V proteins were first defined as accessory proteins because the Respirovirus Sendai lacking V could replicate just as effectively as wildtype in various cell lines, indicating that V is almost completely dispensable for replication *in vitro* and *in vivo*. However, repeated failed attempts at making a complete V knockout in members of the Rubulavirus genus gave reason to believe that V and also probably the other proteins encoded by the same ORF are more essential than initially believed.

The P ORF of Paramyxoviruses encodes an assortment of accessory proteins. Via RNA editing as described previously, the P ORF encodes for V, W, D and I (depending on the species) (Fig 5.2). A second mechanism to express P ORF proteins is the use of alternative translation initiation codons, which yields C and Y proteins for Respiro-, Henipa- and Morbilliviruses. A third mechanism involves ribosomal frame shifting during translation which yields an R protein for MeV. Finally, use of a presumed internal ribosome entry site yields the X protein for Sendai.
While the functions of R and X have not been fully elucidated, V, W, D, I and even P in some instances have been shown to affect the host cell cycle and downregulate viral genome replication in addition to inhibit induction and or signaling of IFN. Through unclear mechanisms, PIV5 V and Sendai C and Y proteins inhibit cellular apoptosis. V proteins of Sendai, MeV, hPIV2 and PIV5 also inhibit viral RNA transcription. This is thought to occur by way of competition between the V or C accessory protein with the binding of P to L and N proteins for RNA polymerase activity. V and P have a common amino terminus which is able to bind to RNA, L protein and P protein. In addition, the unique carboxy terminus of V can bind to N. Finally, V can also bind to kinases such as Akt, which is involved in phosphorylating and activating P.

Finally, a variety of accessory proteins, V, W and C have been shown to be able to inhibit IFN signaling. For Respiroviruses, Sendai V and C inhibit IRF3 activation. C also binds to and prevents STAT1 phosphorylation and Y inhibits IFN signaling through an as yet to be determined mechanism. For Henipaviruses, HeV V sequesters STAT1 and 2 in the cytoplasm. Nipah V, W and C are able to do the same thing and W also inhibits TLR3 mediated IRF3 activation. For Morbilliviruses, MeV C and V can inhibit IFN signaling. V binds to IFNAR2 and also STAT1, 2, 3 and IRF9/p48 to prevent nuclear localization. For Avulaviruses, NDV V can inhibit IFN signaling. For Rubulaviruses, V proteins from PIV5, MuV and hPIV2 act as E3 ubiquitin ligases to degrade STAT1, STAT1 and 3 and STAT2 respectively. In addition, V proteins bind to mda-5 (and not RIG-I) and also to dsRNA itself to inhibit intracellular dsRNA signaling and PKR activation. These functions highlight the
importance of immune signaling in the viral life cycle and indicate that V may inhibit dsRNA activation of IRF3 via TLR3.

Introduction

Innate immunity is stimulated by viruses in part via RNA. dsRNA specifically is present in several forms: viral genomes, ssRNA virus replication intermediates, DNA virus symmetric transcription products, defective viral particles and debris from lysed cells.\textsuperscript{105} While extracellular dsRNA is sensed by Toll-like receptor 3 (TLR3), intracellular dsRNA is detected in part by the RNA helicases retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation associated gene 5 (mda-5). These receptors signal through Toll-IL1R (TIR) domain containing adaptor inducing IFNβ (TRIF) and IFNβ promoter stimulator 1 (IPS1) respectively to activate the kinases TANK-binding kinase 1 (TBK1) and Inhibitor of κB kinase ε (IKKe). They, in turn, phosphorylate IFN Regulatory Factor 3 (IRF3), promoting its nuclear translocation and subsequent IFN stimulated regulatory element (ISRE) mediated transcription of IFN stimulated genes (ISG), such as ISG56, as well as IFN and other cytokines. IFN then, through JAK/STAT activation of IRF9, modulates miRNAs in addition to upregulating itself and more ISGs to heighten the antiviral state and also to initiate the adaptive immune response by promoting dendritic cell maturation, memory T cell proliferation, and B cell differentiation.\textsuperscript{5}

To control this immune response, pathogens and hosts have developed methods of downregulation and evasion at a variety of different points.\textsuperscript{136, 161} At the level of sensing infection, NS1 from Influenza virus binds to and sequesters dsRNA and also interacts with RIG-I.\textsuperscript{161, 178} At the level of signal transduction, the NS3/4A protease from Hepatitis
C virus cleaves TRIF and IPSI and vaccinia virus A46R, which contains a TIR domain, inhibits TRIF dependent activation of IRF3. At the level of IRF mediated transcription, P proteins from Ebola and Borna Disease viruses and HPV E16 interfere with IRF3 activation. For a more global effect, M protein from vesicular stomatitis virus inhibits host machinery to prevent gene transcription. Finally, at the level of IFN and cytokines binding to their receptors, human cytomegalovirus encodes a decoy for the chemokine RANTES to prevent further signaling.

The family Paramyxoviridae encompasses two subfamilies- Paramyxovirinae and Pneumovirinae. Viruses representing different genera within the Paramyxoviral subfamily are important pathogens for humans such as Measles virus (MeV), a Morbillivirus, and Mumps virus (MuV) and human Parainfluenza virus 2 (hPIV2), both Rubulaviruses. For animals, examples include Hendra virus (HeV), a Henipavirus which infects flying foxes, Sendai virus (SeV), a Respirovirus which infects rodents, and Parainfluenza virus 5 (PIV5), a Rubulavirus which infects dogs. Paramyxoviral V accessory proteins have been shown to be important in viral evasion of innate immunity. At the level of IFN signaling, V proteins act as E3 ubiquitin ligases that target STATs for degradation or sequester them, preventing their nuclear translocation and subsequent transcriptional functions. At the level of sensing infection, V proteins bind to mda-5 and also to dsRNA itself to inhibit intracellular dsRNA signaling and PKR activation, respectively. These functions highlight the importance of immune signaling in the viral life cycle. Indeed, VDC PIV5, a mutant PIV5 virus lacking the unique C terminus of V, is no longer able to inhibit IRF3 activation and causes a greater cytopathic effect in a
variety of different cells.\textsuperscript{183, 286} Taken together, these lines of evidence indicate that V may inhibit dsRNA activation of IRF3 via TLR3.

Here we show that V proteins from hPIV2 ($V_H$), MuV ($V_M$) and PIV5 ($V_P$), but not HeV and MeV, inhibit TLR3 signaling. Analysis of the underlying mechanism revealed that the inhibitory V proteins interacted with the signaling kinases TBK1/IKKe and served as their substrates, thus preventing IRF3 phosphorylation. Our results indicated that the above interaction led to modifications of both partners and their degradation. Therefore, the V proteins from \textit{Rubulaviruses} and the IRF3 activating kinases TBK1/IKKe are connected by a negative feedback loop.

\textbf{Results}

\textbf{Some, but not all, Paramyxoviral V proteins inhibit TLR3 signaling}

We used a cell survival assay to examine the potentials of V proteins from different Paramyxoviral subfamilies to inhibit TLR3 signaling. This assay uses a TLR3-expressing 293 cell line (TLR3 293) in which a selection gene has been introduced; this gene is driven by the promoter of ISG56\textsuperscript{124} and encodes the herpesvirus thymidine kinase (TK) protein.\textsuperscript{187} Any signaling that activates transcription factors containing IRF proteins, such as TLR3 signaling or type I IFN signaling, induces TK production and causes cell death in the presence of ganciclovir (GCV). If in a cell an inhibitor of signaling blocks TK production, that cell survives and proliferates even in the presence of GCV. Using this assay, we determined that V proteins from the \textit{Rubulaviruses} hPIV2 ($V_H$), MuV ($V_M$) and PIV5 ($V_P$) inhibited TLR3 signaling (Fig 5.4A, top panel, lanes 4 - 6). In contrast, V proteins from HeV and MeV were ineffective (Fig 5.4A, top panel, lanes 2 and 3), although the different V proteins were expressed at similar levels in the transfected cells.
Figure 5.4: Select Paramyxoviral V proteins inhibit TLR3-mediated gene induction. A, Paramyxoviral V proteins were transiently expressed in TLR3 293 561-TK cells, which were then treated with dsRNA and ganciclovir (GCV) for 4 days (top panel) or GCV alone (bottom panel). Estimated cell survival is shown. Lane 1: no V protein, lane 2: Hendra V protein (HeV), lane 3: Measles V protein (MeV), lane 4: hPIV2 V protein/V_H (H), lane 5: MuV V protein/V_M (M), lane 6: PIV5 V protein/V_P (P) B, HA immunoblotting of extracts from the above cells was performed to determine the levels of HA-tagged V proteins. Lanes are as described above. Approximate molecular weights are marked. C, P56 mRNA levels were measured by Q RT-PCR in untreated (lanes 1-4) or dsRNA-treated (lanes 5-8) HT1080 cells stably expressing V proteins. D, P56 protein levels were measured by immunoblotting in untreated (lanes 1-4) or dsRNA treated (lanes 5-8) cells described in C. Immunoblotting for actin and Flag-V served as controls.
(Fig 5.4B). Expression of the V proteins themselves, without any dsRNA-treatment did not affect cell survival at all (Fig 5.4A, bottom panel). The above observations suggested that Rubulaviral V proteins could block induction of cellular dsRNA-inducible genes as well, a conclusion that was confirmed by measuring the levels of ISG56 mRNA, using a quantitative RTPCR assay in HT1080 cells permanently expressing different V proteins. The mRNA was induced strongly upon dsRNA-treatment of cells (Fig 5.4C, lane 5) and the induction was almost completely blocked by V_H, V_M and V_P (Fig 5.4C, lanes 6-8). Similar inhibitions were observed for CIG5/viperin\textsuperscript{287, 288} mRNA another ISG whose transcription is mediated by IRF3 (Data not shown).\textsuperscript{231} Finally, inhibition of the induction of p56 protein was also observed (Fig 5.4D).

**V proteins block IRF3 activation**

Once we established that the three V proteins blocked TLR3 signaling, we investigated the underlying mechanism. Exogenous expression of signaling proteins downstream of TLR3 is known to activate IRF3 and induce synthesis of p56, as shown in Fig 5.5A. Expression of TRIF, IKKe and TBK1 induced p56 (lane 1) and the induction was blocked by all three V proteins. In contrast, p56 induction from expression of a constitutively active IRF3 5D mutant was not blocked (Fig 5.5A, lanes 2-4). These results indicated that the V proteins blocked a step in signaling downstream of TLR3, TRIF and the IRF3 kinases and upstream of events following activation of IRF3. Inactive IRF3 shuttles in and out of the nucleus while activated IRF3 translocates to the nucleus and induces transcription of target genes, such as ISG56.\textsuperscript{289} IRF3 was not localized to the nucleus in cells expressing V_M (Fig 5.5B, lane 4), V_H and V_P (data not shown). We then asked whether the observed block of IRF3 activation was due to a block in its
Figure 5.5: V proteins from hPIV2, MuV and PIV5 inhibit IRF3 activation.

A, Pools of TLR3 293 561-TK cells expressing V proteins were transfected with expression vectors for different components of the TLR3 pathway; the specific signaling protein expressed is shown on the left. Induced p56 levels were determined by immunoblotting. Actin served as a loading control. Lane 1: no V; lane 2: V_H (H); lane 3: V_M (M); lane 4: V_P (P). B, Nuclear extracts from control cells (lanes 1, 2) or cells stably expressing V_M (lanes 3, 4), that were untreated (lanes 1, 3) or treated with dsRNA (lanes 2, 4), were immunoblotted for IRF3, with histone as loading control. C, IRF3 phosphorylation at serine 396 (P396) was detected by immunoblotting cell extracts with a phospho-IRF3-specific antiserum, with total IRF3 as a control. Whole cell extracts were prepared from control cells (lanes 1, 3) or cells stably expressing V_M (lanes 2, 4), that were untreated (lanes 1, 2) or treated with dsRNA (lanes 3, 4). D, Control and cells stably expressing V proteins indicated were treated with dsRNA (rows 5-8). IRF3 dimerization was assayed by native gel electrophoresis followed by immunoblotting. E, Control and V_M-expressing HT1080 cells were treated with dsRNA (rows 2, 4) and Leptomycin B (LMB) (rows 3, 4). Subcellular location of IRF3 was determined by immunofluorescence. F, Cell extracts from samples, as described in E, rows 3 and 4, were used for immunoblotting to detect p56, Flag-V and actin.
phosphorylation, which is known to activate it. Indeed, phosphorylation of Ser396, a hallmark of IRF3 activation, was inhibited in the presence of V_M. (Fig 5.5C, lane 4) Unexpectedly, IRF3 was still able to dimerize in these cells as shown by native gel electrophoresis. (Fig 5.5D) These results suggested that V prevents IRF3 mediated transcription of genes in one of three ways: 1.) V prevents shuttling of IRF3 into the nucleus independent of its phosphorylation or dimerization state, 2.) V deactivates dimerized IRF3 before it can perform its function as a transcription factor, possibly by enhancing its nuclear export and or its dephosphorylation, 3.) V allows partial activation of IRF3 for dimerization but not full activation in its phosphorylation and nuclear localization.

To address these hypotheses, we first asked if IRF3 could enter the nucleus at all in the presence of V protein. By using leptomycin B (LMB), a drug that blocks nuclear export of proteins, we examined whether V_M blocked nuclear import of IRF3 or enhanced its export. As expected, in untreated cells, IRF3 was in the cytoplasm of both control and V expressing cells (Fig 5.5E, row 1). DsRNA treatment alone caused nuclear accumulation of IRF3 only in the absence of V protein (Fig 5.5E, row 2). In both untreated and dsRNA-treated cells, LMB caused similar nuclear accumulation of IRF3 in the absence and the presence of V protein (Fig 5.5E, rows 3 and 4). These results suggested that either V_M inhibited nuclear import of only activated IRF3 or V_M enhanced export of activated IRF3 through exportin 1, which is blocked by LMB. To distinguish between these two possibilities, we examined if the IRF3 sequestered within the nucleus by LMB was transcriptionally active in the presence of dsRNA. Nuclear IRF3 in LMB-
treated cells could not induce p56 in control cells unless they were dsRNA-treated as well (Fig 5.5F, lanes 1 and 3). In dsRNA-treated V-expressing cells, although LMB-treatment caused nuclear translocation of IRF3, p56 was not induced (Fig 5.5F, lanes 2 and 4). These results indicated that V inhibits import of activated IRF3 to the nucleus and did not enhance its nuclear export. Furthermore, these results indicated that 1.) IRF3 dimerization was independent of Ser396 phosphorylation, 2.) IRF3 dimerization was not sufficient for nuclear translocation and 3.) nuclear localization, although necessary, was insufficient for IRF3 to induce genes; additional activation was required by post-translational modifications of the protein. Taken together, these results indicated that V_M did not enhance IRF3 deactivation but instead allowed only partial and not full phosphorylation and activation of IRF3 by dsRNA.

Interaction of V with IKKe is essential for inhibiting TLR3 signaling

To further analyze the mechanism of V-mediated blocking of TLR3 signaling, we examined possible interactions of the viral proteins with known components of the signaling pathways. V_H, V_M and V_P did not interact with TLR3, TRIF, IRF3, Src, PI3K and IKK α, β and , as revealed by co-immunoprecipitation assays, although the same assay demonstrated their known interactions with STAT2 (Fig 5.6A and B). In contrast, all three viral proteins interacted with both IKKe (Fig 5.6C) and TBK1 (data not shown). IKKe co-immunoprecipitated with V_H (lane 3), V_M (lane 5) and V_P (lane 7) (Fig 5.6C, top panel). Note that the mobility of co-immunoprecipitated IKKe was slower than that of the protein in cell extracts. This was also the case for co-immunoprecipitated STAT2 (Fig 5.6C, middle panel, lanes 3, 5 and 7). Since the V proteins have E3 ubiquitin ligase activity, the observed mobility differences could be due to V-mediated
Figure 5.6: Interaction of V with IKKe is essential for inhibition of TLR3 signaling.

A, TRIF and V proteins were transiently expressed in TLR3 293 cells. V proteins were immunoprecipitated (IP) and co-immunoprecipitated proteins were immunoblotted to detect TRIF (indicated by arrow), HA-tagged V, stably expressed Flag-tagged TLR3 and endogenous STAT2, PI3K p85, c-Src (indicated by arrow) and IRF3 (indicated by arrow); cell extracts (W) served as controls for expression levels. B, It is the same as (A) except that immunoblotting to detect endogenous IKKα and β was used in place of other TLR3 signaling mediators. C, IKKe and V proteins were transiently expressed in TLR3 293 cells. V proteins were immunoprecipitated (IP) and co-immunoprecipitated proteins were immunoblotted to detect Myc-tagged IKKe, STAT2, and HA-tagged V; whole cell extracts (W) served as controls for expression levels. Lanes 1, 2: no V; lanes 3, 4: V_H (H); lanes 5, 6: V_M (M); lanes 7, 8: V_P (P). D, IKKe, transiently expressed with tagged ubiquitin and VM or empty vector control, was immunoprecipitated. Samples were immunoblotted to detect HA-tagged ubiquitin (top panel: IKKe^Ub). Membranes were stripped and reprobed to detect Myc-tagged IKKe (bottom panel: IKKe) at the same molecular weight. E, Flag-tagged WT and V_M mutants M-AAA (W174A/W178A/W188A), E95D, E95R, C189A, C214A and C217A were immunoprecipitated (IP) (top two panels) and co-immunoprecipitated proteins were immunoblotted to detect Myc-tagged IKKe and Flag-tagged V. The bottom two panels show corresponding analyses of whole cell extracts (W). F, Flag-tagged WT and VM C189A mutant were immunoprecipitated (IP) (lanes 2, 4) as in (D). Samples were then electrophoresed along side whole cell extracts (W) to show shifted compared to unshifted mobilities of Myc-tagged IKKe. G, P56 protein levels were measured by immunoblotting
in untreated (lanes 1, 3, 5, 7) or dsRNA treated (lanes 2, 4, 6, 8) HT1080 cells stably expressing WT or \( V_M \) mutants C189A or M-AAA. Immunoblotting for actin and Flag-V served as controls.
polyubiquitination of the co-immunoprecipitated proteins. Indeed, exogenously introduced ubiquitin was covalently bound to IKKe purified by immunoprecipitation and this ubiquitination was V_M dependent (Fig 5.6D). The functional significance of the V-IKKKe interaction was determined by testing mutant V_M. Among six mutant proteins tested (Fig 5.6E), only the V_M mutant W174A/W178A/W188A (V_M-AAA) failed to bind to IKKe (lane 3, left top panel). This mutant protein could not block TLR3 signaling as revealed by the cell survival assay (data not shown) and in HT1080 cells permanently expressing mutant V_M-AAA (Fig 5.6G, lane 8). In contrast, WT V_M and mutant V_C189A could inhibit signaling (Fig 5.6G, lanes 4 and 6). Both bound to IKKe (Fig 5.6E, lanes 7 and 8) and, as shown when electrophoresed longer, both shifted the mobility of co-immunoprecipitated IKKe, indicating its ubiquitination (Fig 5.6F lanes 2 and 4 compared to lanes 1 and 3). These results strongly indicated that the V proteins blocked TLR3 signaling by blocking the action of the signaling kinases.

V proteins are substrates for IKKe and TBK1

Because the V proteins bound to the kinases, in the next experiments we tested whether they were phosphorylated as a consequence. Indeed, both V_M (Fig 5.7A) and V_P (Fig 5.7B) were phosphorylated by IKKe in vivo. As expected, the phosphorylated V proteins migrated more slowly (Fig 5.7A and B, lane 2) and upon phosphatase treatment, they co-migrated with the unphosphorylated proteins (Fig 5.7A and B, lane 3). Similar results were seen for TBK1 (data not shown). Expression of enzymatically inactive IKKe did not cause phosphorylation of the V proteins (Fig 5.7A and B, lane 4) suggesting that IKKe directly phosphorylated V. To test this suggestion, purified V_M and IKKe were added to an in vitro protein kinase reaction in the presence of γ\(^{32}\)PATP, and
Figure 5.7: MuV and PIV5 V proteins are phosphorylated by IKKe and TBK1.

A, MuV V protein (VM) was expressed by itself (lane 1) or co-expressed with kinase active (lanes 2-3) or inactive (lanes 4-5) IKKe, immunoprecipitated, and treated with phosphatase (lanes 3, 5). HA immunoblotting was used to detect V. B, It is the same as (A) except that PIV5 V protein (VP) was used in place of VM. C, In vitro γ32PATP kinase assays were conducted with GST IKKe and V protein or IRF3. Radiolabeled proteins were visualized by autoradiography (top two panels) and immunoblotted (bottom two panels) to determine total amounts of IRF3 or V in the loaded samples. Lane 1: VM + IKKe; lane 2: IRF3 + IKKe; lane 3: IKKe ; lane 4: VM. D, In vitro γ32PATP kinase assays were performed using purified Myc-TBK1. The top two panels are autoradiographs and bottom two panels are immunoblots. Lane 1: TBK1; lane 2: VM; lane 3: TBK1 + VM; lane 4: IRF3; lane 5: TBK1 + IRF3.
radiolabeling of $V_M$ was measured. As a positive control, purified IRF3, a known substrate of IKKe, was used. Both IRF3 and $V_M$ were phosphorylated by IKKe (Fig 5.7C, lanes 1, 2). Control reactions showed that the addition of both IKKe and $V_M$ were needed to observe the radiolabeled protein (Fig 5.7C, lanes 3, 4). Quantification of the incorporated phosphates demonstrated that, on a molar basis, $V_M$ was a slightly better substrate than IRF3 (114 vs 100). Similar in vitro reactions demonstrated the ability of purified TBK1 to phosphorylate $V_M$ (130 vs 100) (Fig 5.7D, lane 3). These results established the V proteins as authentic substrates for IKKe/TBK1.

As a consequence of phosphorylation, V appeared to be destined for faster degradation (Fig 5.8A). Co-expression of V with kinase active (lane 2), but not kinase inactive (lane 3) IKKe caused major diminution of the cellular level of V, as compared to control (lane 1). Furthermore, this was not caused just by over-expression of IKKe, because dsRNA-mediated activation of the endogenous kinase caused a dramatic lowering of the level of V as well (Fig 5.8B, lane 2) and this diminution could be inhibited by the proteasome inhibitor MG132 (Fig 5.8B, lane 3). Finally, interaction between V and IKKe was required for degradation; $V_M$-AAA, a mutant that did not co-immunoprecipitate with IKKe (Fig 5.6E, lane 3), was not degraded (Fig 5.8C).

**V protein determines the efficacy of virus replication in TLR3-activated cells**

To evaluate the biological significance of our observations, we chose to use the PIV5 mutant virus VDC, which encodes a C-terminally truncated V protein. VDC was not phosphorylated in vivo by IKKe as seen in WT PIV5 V protein (Fig 5.9A). Furthermore, we determined that the truncated V protein could not block signaling induced by exogenous expression of IKKe (data not shown). Results showed that WT
Figure 5.8: Phosphorylated MuV V protein is degraded.

A, V_M was expressed alone (lane 1), or along with wildtype kinase active (WT) (lane 2) or kinase inactive (KI) (lane 3) IKKe. Levels of HA-tagged V and Myc-tagged IKKe were determined by immunoblotting of cell extracts. B, TLR3 293 cells transiently expressing V_M were treated with DMSO as a control (lane 1), dsRNA and DMSO (lane 2) or dsRNA and MG132 (lane 3). Flag immunoblotting was used to detect Flag-tagged V, with actin as control. C, V_M WT (lanes 1 and 2) and mutant M-AAA (lanes 3 and 4) were expressed with wildtype kinase active (WT) (lanes 1 and 3) or kinase inactive (KI) (lanes 2 and 4) IKKe. Levels of Flag-tagged V and Myc-tagged IKKe were determined by immunoblotting of cell extracts.
Figure 5.9: Effects of dsRNA signaling on WT and mutant PIV5 replication. A, PIV5 WT (lane 1) or mutant C-terminally truncated (VDC) (lane 2) V proteins were co-expressed with IKKe and immunoprecipitated. V5 immunoblotting was used to detect V and Myc to detect IKKe. B, Virus yields 6 dpi were measured for WT or VDC PIV5 replication in 293 cells with and without TLR3 and pre- or mock treated with dsRNA. Bars represent standard error derived from 2 independent experiments.
virus replicated better compared to the mutant virus in both TLR3-expressing and non-expressing cells (Fig 5.9B). Since the mda-5/RIG-I pathway is still intact in these cells, this difference may be due to the lack of ability of the truncated V protein to inhibit that signaling pathway.\textsuperscript{281} In cells not expressing TLR3, treatment with dsRNA did not have any effect on the replication of either virus. In contrast, in TLR3-expressing cells, dsRNA-treatment strongly inhibited the replication of the mutant virus (5 logs), whereas the effect on WT virus was minor. These results demonstrated that inhibition of TLR3 signaling by V protein was relevant for effective virus replication.

**Discussion**

**The virus-host equilibrium**

The results presented above revealed several new features of the equilibrium established in an infected cell between the virus and the host (Fig 5.10). Induction of viral stress-inducible genes, including IFNs, is blocked by Rubulaviral V proteins by inhibiting IRF3 phosphorylation, which is required for its activation because the V proteins can themselves be phosphorylated by TBK1/IKKe. A similar observation has been made by Unterstab \textit{et al} for Borna disease virus P protein.\textsuperscript{165} It is not yet clear exactly how V proteins can inhibit IRF3 phosphorylation by TBK1/IKKe. Because they themselves are substrates of the same kinases, one possibility is that V simply competes out IRF3 as a substrate. In the \textit{in vitro} kinase assays, quantitation of phosphorylation showed that IRF3 and VM were equally phosphorylated by IKKe, when present in equimolar amounts either singly or in combination (data not shown), indicating that the two proteins are equally competent substrates of the kinases. Hence, to compete out IRF3, V has to be present at a high molar excess, a possibility not unlikely because V proteins are produced in large
Figure 5.10: A negative feed-back loop between Rubulaviral V proteins and IKKe/TBK1. While Rubulaviral V proteins block TBK1/IKKe kinase activity by acting as an alternative substrate to IRF3/7, the resulting phosphorylated V protein (VPO4) is degraded. V proteins also mediate ubiquitination of TBK1/IKKe (Ub), leading to their degradation.
quantities in *Paramyxovirus*-infected cells. Alternatively, V proteins can have stronger affinity for TBK1/IKKe, thus not allowing access to IRF3. The fact that V and IKKe were co-immunoprecipitated efficiently indicated that the two proteins could interact strongly, thus providing credence to the second scenario. Although our study was designed to determine whether different Paramyxoviral V proteins could inhibit TLR3 signaling, our observations are equally relevant for other signaling pathways that converge on TBK1/IKKe. Thus, we can expect Rubulaviral V proteins to block signaling initiated by the cytoplasmic receptors RIG-I, mda-5, and DAI or the membrane receptor TLR4, that are known to be triggered by RNA, DNA or LPS. Indeed, we observed that RIG-I/mda-5 signaling to IRF3 triggered by transfected dsRNA was inhibited by V_H, V_M and V_P (data not shown).

Wildtype and VDC mutant PIV5 replication assays showed that in addition to dsRNA inhibiting the VDC mutant to a greater extent than wildtype, the expression of TLR3 alone increased VDC viral titres two log fold. This occurred only in the absence of dsRNA. (Fig 5.9) The role of P protein as an essential polymerase subunit may be one explanation for this observation. P protein phosphorylation by host factors such as Akt is important for RNA synthesis and, consequently, viral replication. In wildtype PIV5, V protein is able to regulate RNA synthesis by acting as either an alternative or competitive substrate against P for Akt kinase activity and interaction with the other RNA polymerase subunits N, L and even the viral RNA itself. For the VDC mutant PIV5, the expression of TLR3 can increase the activity of Akt and other host kinases. Without the inhibitory function of V, P polymerase activity then increases and viral replication is enhanced. However, in the presence of dsRNA, these titres are not observed because host
expression of antiviral genes is able to inhibit the virus replication. Taken together, these results show the complex factors in host-virus interactions that affect the outcome of viral replication.

An additional consequence of the action of V proteins as alternative substrates of IKKe could be inhibition of phosphorylation of STAT1. Because STAT1 phosphorylation by IKKe is essential for its ability to induce a subset of ISGs, in addition to causing STAT1 degradation, Rubulaviral V proteins may block actions of IFN using this mechanism.

The interaction with V caused ubiquitination of IKKe as indicated by the observed slower mobility of co-immunoprecipitated IKKe and its V dependent ubiquitination when co-transfected with tagged ubiquitin. A similar change in the mobility of IKKe was observed when C189A mutant of V_M was used; this was unexpected because the mutant cannot bind DDB1 (data not shown), a component of the ubiquitin ligase complex. This indicates the existence of alternative pathways for the ubiquitin ligase activity of V_M. Here, polyubiquitination of IKKe leads to its proteasome-mediated degradation. Thus, V not only blocks IRF3 phosphorylation but destroys the activating kinase as well.

What was clear from our results was that the host could fight back by degrading V proteins. Just as phosphorylation of IRF3 leads to its degradation, phosphorylation of V protein was accompanied by its destruction (Fig 5.8). This is consistent with the degradation of V_M observed over the course of a viral infection. Because V was needed for the optimum replication of viruses, even in the absence of dsRNA-signaling (Fig 5.9), its destruction should be a potent antiviral mechanism in that context as well.
Phosphorylation and degradation of V provide an opportunity for temporal regulation of IRF3 activation; when the level of V is high, IRF3 phosphorylation is blocked by competition. As more and more V is phosphorylated and degraded, the relative concentration of IRF3 will increase, leading to its phosphorylation, activation and degradation. Thus, two negative feedback loops, one between V and IRF3 and the other between IKKe/TBK1 and V, regulate the equilibrium between the virus and the cell.

It is known that IKKe interacts with STAT1 and STAT1 can interact with V. However the interaction between IKKe and V reported here is not mediated by STAT1, because VM could block TLR3 signaling in U3B cells which lack functional STAT1 (Fig 5.6G). In addition, VM C terminal mutants in the conserved cysteine residues (C189A, C214A, C217A) within the zinc finger binding domain and N terminal mutations V47P and L97P corresponding to mutations forming a canine parainfluenza virus minus (CPI-) PIV5 strain involved in binding and degrading STAT1 were used to look at the IKKe and V interaction. Co immunoprecipitation assays indicated that all of these mutants could bind to IKKe (Fig 5.6E lanes 7-10 and data not shown). Furthermore, STAT2 is not involved in the interaction between IKKe and V. Two N terminal mutants E95D and R have reduced binding to STAT2 but still interact with IKKe (Fig 5.6E lanes 4 and 5). The tryptophan residues of V that were required for IKKe binding are located in the C-terminal region of the protein. Because this region is not shared between viral V and P proteins, whose mRNAs are produced by alternative transcription of the same open reading frame (ORF), P proteins are not expected to bind to IKKe. This prediction is consistent with previous findings that V, but not P, blocks dsRNA-mediated IRF3 activation.
While the conserved triple tryptophan residues are important for the interaction between V and IKKe, other yet to be identified motifs may also be involved. One such area is the TBK1 binding region of SINTBAD, NAP1, and TANK which is essential for all these adaptors to bind to TBK1 and IKKe. Alignment with V protein however did not show areas with significant homology. V proteins also do not have the ubiquitin like domain (ULD) which TBK1 and IKKe use to bind to IRF3. In addition, V proteins are not homologous to any of the signaling mediators such as these scaffolding proteins for the IRF3 kinases, TRAF6 or PKCα. Finally, V is not homologous to any of the host negative regulators such as SIKE, SHP and RFP. Therefore, it does not appear that V inhibits TLR3 signaling by mimicking a host protein.

The complex process of IRF3 activation

The experiments with LMB (Fig 5.5E and F) illuminated subtle, but important, aspects concerning IRF3 activation. According to the current paradigm of the activation process, IRF3 is phosphorylated at multiple serine residues and this leads to its nuclear translocation, a process that is necessary for its action as a transcription factor. We found that forcing nuclear localization of IRF3 by LMB treatment of cells was not sufficient for its ability to induce genes. Therefore, there are consequences to IRF3 phosphorylation in addition to nuclear translocation that are necessary for activation.

Furthermore, while IRF3 appeared to be less activated by Ser396 phosphorylation and nuclear localization, dimerization still occurred. This was unexpected because IRF3 dimerization has been shown to be an important step for activation and in fact correlated with its association with the CBP/p300 coactivators and subsequent transcription. For dimerization to occur, it has been thought that phosphorylation at sites such as Ser396
and Ser386 are the rate limiting steps. Indeed, a S396A mutant, incapable of being phosphorylated at that residue, is unable to drive ISRE mediated transcription and cannot associate with CBP/p300. However, native gel electrophoresis shows that this same mutant is still able to dimerize and be phosphorylated at ser386. This dissociation between phosphorylation and dimerization seen also with the V protein indicates that while ser386 may be important for dimerization it is insufficient for nuclear localization. Consistent with this hypothesis, IRF3, even when artificially retained in the nucleus by LMB, was not transcriptionally active in the presence of V and dsRNA. (Fig 5.5F) One example of such a situation may already exist with the IKKe interacting protein Ret finger protein (RFP). Like V, RFP is a substrate for IKKe and TBK1 and allows for IRF3 dimerization but not nuclear localization. Thus, for V and RFP, we would predict that residue ser386 but not ser396 is phosphorylated on IRF3 and that only when both sites are phosphorylated can full activation occur.

The role of V mediated innate immune inhibition in Rubulaviruses

It is interesting to note that among the V proteins from Paramyxoviral subfamilies tested, all inhibit IFN signaling, but only members of the genus Rubulavirus were able to inhibit dsRNA-TLR3 mediated IRF3 activation. These observations seem to correlate with what seems to be a more important role for V in the life cycle of Rubulaviruses compared to others. Rubulaviral V proteins, in contrast to P in other Paramyxoviruses, represent the default ORF transcribed at the P/V locus, resulting in higher levels of basal expression. Indeed, some Respiroviruses such as hPIV1 and 3 may not even make V protein. Instead, C seems to play a more important role. Correspondingly, virions from Rubulaviruses contain a much higher level of V compared to others and are conveniently
present during the initial stages of infection and signaling. This importance of Rubulaviral V proteins may be in part compensation for the lack of W, C and Y accessory proteins which Respiro-, Morbili- and Henipaviruses encode to counteract host defense.161, 256, 274
CHAPTER 6
HERPESVIRAL PROTEINS INHIBIT IRF3 ACTIVATION

Summary

Members of the family *Herpesviridae* encompass a variety of dsDNA viruses of which the most relevant human pathogens include human cytomegalovirus (HCMV), Epstein Barr virus (EBV) and *Human herpesviruses 8* (also known as Kaposi’s Sarcoma herpesvirus) (HHV8/KSHV). One of the defining characteristics of this virus family is that all members go through latent life cycles with life long viral persistence. In this capacity, the success of viral persistence is largely due to the many mechanisms which Herpesviruses employ to regulate the host response. dsRNA signaling through TLR3 is one of many pathways shown to activate IRF3. IRF3 in turn mediates a variety of antiviral and apoptotic effects. For this reason, many viruses encode molecules that inhibit IRF3. More specifically, Herpesvirus infections do induce the transcriptional activity of IRF3. However, this activity is greatly increased when virus is inactivated by UV or protein synthesis is blocked. Thus, Herpesviruses encode inhibitors of IRF3 activation. To identify these inhibitors, we developed a cell survival assay to systematically assay the known and predicted open reading frames from the *β* herpesviruses mouse and human cytomegalovirus and the *γ*–herpesvirus murine *γ*–herpesvirus 68. We found that none of the ORFs activated IRF3 on their own and 18 out of 135 ORFs potentially inhibited IRF3. Of these 18, four were followed to confirm the inhibition of signaling. Two of these four- MHV68 ORFs 35 and 58- were examined in more depth to determine the possible mechanism. Results from these experiments revealed the complex nature by which these viral molecules can inhibit signaling and the
disparate needs of the virus to replicate and persist in the host.

Background

The family *Herpesviridae*

The *Herpesviridae* family members are characterized by the architecture of the virion. This consists of a core containing the linear dsDNA genome, an icosohedral capsid surrounding the core, a tegument and finally the envelope. (Fig 6.1) The genome ranges from 124-241kb and contains terminal signals for packaging into the capsids. Its coding capacity lies within unique long (UL) and short (US) sequences that are separated by tandem reiterated sequences (IR) and are also flanked by the same sequences at both termini (TR). These reiterated sequences vary in copy number within the same species and promote isomerization of the genome. The capsid is mainly structural in purpose and consists of capsid and portal proteins. Around that is an amorphous and sometimes asymmetric tegument that connects the capsid with the envelope. This layer matures as the virion egresses through the endoplasmic reticulum and Golgi and is where viral proteins including some glycoproteins are added to inhibit host protein synthesis, cell defense and help stimulate viral gene expression. Finally, the envelope is derived from patches of altered cellular membranes spiked with viral glycoproteins. These glycoproteins are important in attachment and entry into the host cell and also stimulate the major antibody responses during an infection.

Four significant biological properties characterize the *Herpesviridae* family. First, they encode a variety of proteins involved in nucleic acid metabolism such as thymidine kinase, DNA synthesis and protein processing. Second, synthesis of viral DNA and capsid assembly occurs in the nucleus and the virion matures as it egresses through
Figure 6.1: Herpesvirus virion structure.

A.) A three dimensional model of the cytomegalovirus virion\textsuperscript{301} and B.) a murine $\gamma$-herpesvirus 68 virion as visualized by cryo-electron tomography\textsuperscript{300} show common structural characteristics of members of the \textit{Herpesviridae} family. Both virions contain the linear dsDNA genome in their cores. This is surrounded by an icosohedral capsid (depicted as nucleocapsid in A and colored yellow in B) which provides structure. The core is covered by an amorphous tegument layer which is developed as the virion egresses using the cellular endoplasmic reticulum and Golgi systems. The tegument layer itself can consist of layers as reflected in B by the green and blue which are acquired sequentially during virion maturation. In addition to host proteins, the tegument consists of glycoproteins such as glycoprotein B and H (gB and gH in A) which are involved in entry into a host cell and also immune evasion. Finally, the envelope (membrane in A and so labeled in B) is derived from patches of altered cellular membranes spiked with viral glycoproteins.
the cytoplasm to the plasma membrane. Third, the production of progeny eventual involves cell lysis. Fourth and finally, all Herpesviruses go through latency, replicating their genome in the form of covalently enclosed circles and expressing a subset of viral genes. This differs however from chronic infection because no infectious progeny is being produced.

ORF expression occurs in three phases: α, β and γ. The α or immediate early genes are expressed with no prior viral protein synthesis. The β or early genes is totally independent of viral DNA synthesis but somewhat dependent on protein synthesis. The γ1 or leaky late genes are augmented by the onset of viral DNA synthesis and the γ2 or true late genes are totally dependent on viral DNA synthesis.

The family Herpesviridae is divided into three subfamilies based on biological properties, DNA sequence, predicted coding regions, genomic structural arrangement and immunological recognition of viral proteins. (Fig 6.2) A herpesviruses have a variable host range, short reproductive cycle, spread rapidly in culture, efficiently destroy infected cells and establish latency in sensory ganglia. B herpesviruses have a restricted host range, long reproductive cycle, spread slowly in culture, cause cytomegaly and establish latency in secretory glands, lymphoreticular cells and kidneys. Γ− herpesviruses have an even more limited host range, a long reproductive cycle in lymphoblastoid, epithelioid and fibroblastic cells and establish latency in mainly lymphoid tissues. Of the more than 130 members within the family, the main human herpesviruses are the α herpesviruses varicella zoster virus (VZV), herpes simplex 1 (HSV) and 2, the β herpesviruses human herpesvirus HHV6, 7 and human
Figure 6.2: International Committee on Taxonomy of Viruses classification of the order Herpesvirales.

The order Herpesvirales comprises Herpesviruses that infect a variety of different species. The Families are determined based on sequence based comparisons which indicate common ancestry within each group. The Family *Herpesviridae* can be further subdivided into $\alpha$, $\beta$ and $\gamma$- Herpesviruses based on biological properties, DNA sequence, predicted coding regions, genomic structural arrangement and immunological recognition of viral proteins as discussed in the text. The most relevant human pathogens include human cytomegalovirus (hCMV), Epstein Barr virus (EBV), Kaposi’s Sarcoma herpesvirus (KSHV), Herpes simplex virus 1 (HSV1), vaicella zoster virus (VZV) and human herpesvirus 6 (HHV6).
cytomegalovirus (hCMV) and the γ−herpesviruses Epstein Barr virus (EBV) and KSHV/HHV 8.

The β−herpesvirus Cytomegalovirus: Disease and Treatment

Cytomegalovirus (CMV) is ubiquitous with seroprevalence ranging from 30-70% in varying countries and the US at 59%. In the healthy, infection and latency are established without significant pathology. However in the immunocompromised, human CMV (hCMV) can replicate in almost any tissue, causing disease. Experimental models for CMV include tissue culture adapted hCMV strains and also the mouse CMV (mCMV) which shares significant amino acid homology in 78 of its predicted 170 ORFs with hCMV. While human and mouse strains are species specific, their replication characteristics and associated diseases are similar. Transmission of is horizontal via shedding in the saliva, urine and other secretions or vertical via transplacental, intrapartum or breast milk routes. hCMV and mCMV initially infect mucosal epithelium. This is followed by dissemination and latency in myeloid lineage cells. Persistence of the primary infection can last months to years and latent infection is lifelong. Disease usually occurs with reactivation from latency in immunocompromised situations such as when the recipient is in utero or when the immune system is suppressed. For neonates who have acquired the virus from their mother, periventricular calcification and other clinical CNS symptoms are the hallmarks of infection. For transplant and HIV positive patients, hCMV infection can be life threatening, causing pathology first in the transplanted organ and then spreading to many different systems. Diagnosis for hCMV is usually made by testing for pp65 one of the most highly expressed glycoproteins in the virion which also is in part responsible for immune
evasion. The most common drugs for hCMV are nucleoside analogues such as ganciclovir and cidofovir which are phosphorylated by the herpesvirus thymidine kinase and subsequently incorporated into replicating DNA strands, inhibiting replication and causing cell death.

**Cytomegalovirus-Host interactions: Natural Killer cells**

The most well studied host responses to CMV infection involve natural killer (NK) cells, T cells and IFN. NK cells play an important early role in controlling CMV infections. The inbred BALB/c strain of mice is more susceptible to mCMV infection when compared to other strains because it expresses an inhibitory NK receptor which is activated by mCMV and thus prevents NK cell mediated cytotoxicity. Similarly, there are several reports of patients (the majority of which were offspring of consanguineous parents) with NK cell deficiencies for various reasons experiencing severe hCMV as well as other Herpesviral infections.

In BALB/c mice, the LY49I receptor on NK cells binds to the mCMV protein m157, inhibiting NK mediated lysis of the infected cell. However, m157 also binds to the activating LY49H receptor which is expressed preferentially over the inhibitory LY49I in the majority of mouse strains including wild mice. Thus, it is thought that the evolutionary advantage afforded by the inhibitory function of m157 in the majority of mice outweighs its detrimental effects in the minority. In addition, certain host cell proteins when presented by the infected cell can act as ligands for NK receptors that trigger lysis of the infected cell. To inhibit the presentation of these ligands, mCMV encodes for the proteins m152, m145 and m155 and hCMV the proteins UL16, UL141, UL142 and microRNA miR-UL112.
Cytomegalovirus-Host interactions: T cells

In contrast to the effects of NK cells early in the infection, T cells play more important roles in the termination of lytic infection and establishment of latency. Adoptive transfer experiments in mouse and humans of CMV specific CD8 T cells into immunodeficient bone marrow recipients restore viral immunity.\textsuperscript{311, 312} However, mice lacking functional CD8 responses are still able to control virus replication with CD4 T cells and macrophages.\textsuperscript{313} Furthermore, CD4 T cells seem to be essential for inhibiting horizontal transmission by limiting viral replication specifically in the salivary glands.\textsuperscript{314} Thus, both arms to the T cell response are necessary for control of viral infection and its spread.

The cytolytic responses of CD8 T cells are mediated by recognition of the CMV immediate early gene products or glycoproteins from the virions. These viral ligands include pp89, or IE1, in the mouse or its functional homologue pp72 in humans and the glycoprotein pp65, the product of UL83 in hCMV mentioned earlier to be important in viral binding and entry into the host cell.\textsuperscript{311, 315, 316} These viral ligands are presented to T cells by MHC Class I and II molecules.

To evade the cytolytic T cell response, CMV must prevent MHC Class I and II presentation. However, this inhibition must not be complete because the absence of MHC Class I on the host cell surface will also induce NK cell mediated killing. CMV downregulates host MHC Class I and II presentation by retaining the complexes in the ER-Golgi or redirecting the complexes for proteolytic degradation. The viral proteins responsible for these functions include mCMV m152 encoding gp40, mCMV m06 encoding gp48, hCMV US2, 3, 6 and 11.\textsuperscript{317} Simultaneously, CMV encodes for viral
homologues of MHC Class I, mCMV m144\textsuperscript{318} and hCMV UL18, to prevent NK cell-mediated cytotoxicity. Finally, some host MHC Class I is allowed to interact with the T cell receptor by the virus. However, this is facilitated by mCMV m04 encoding for gp34 which manipulates the interaction to block the actual antigen presentation and prevent NK cell-mediated cytotoxicity.\textsuperscript{319} Thus, all these mechanisms cooperatively hide infected host cells from cytotoxic immune surveillance.

Finally, both type I and II IFNs are important in the regulation of CMV. Antibody depletion experiments show that IFN \(\gamma\) and TNF \(\alpha\) help stimulate the effector CD8 and CD4 T cell responses described above and hence are essential for the control of viral replication.\textsuperscript{320} In addition, IFN \(\gamma\) receptor null and, to an even larger extent, IFN \(\alpha\) receptor null mice are much more susceptible to mCMV lethality compared to wildtype controls.\textsuperscript{321, 322} Thus CMV induces IFN in infection and this is essential in establishing downstream host immune responses in part mediated by T and NK cells. As a result, just as CMV encodes molecules to manipulate the functions of T and NK cells, the virus also establishes a complex relationship with the host during the initial stages of infection.

**Cytomegalovirus regulation of host innate immunity**

Previous analysis of the CMV genome has identified many ORFs which are able to inhibit host innate immunity. At the receptor level, hCMV IRS1 and TRS1 as well as mCMV m142 and m143 bind to the PAMP dsRNA. As a result they inhibit PKR and RNAse L, both of which are host IFN stimulated genes (IFN) which are activated by RNA and inhibit host translation in response to viral infection.\textsuperscript{323-326} To prevent the TLR3 and TNF\(\alpha\) activation of the transcription factor NFkB, mCMV M45 binds to RIP1 and inhibits its ubiquitination. This also inhibits TNF\(\alpha\) activation of p38 which is involved in
AP1 transcription and induction of caspase dependent cell death.\textsuperscript{327} hCMV pp65 too participates through an unknown mechanism in inhibiting NFkB.\textsuperscript{328} In addition, it is able to inhibit IRF3 activation.\textsuperscript{329} At the cytokine level, hCMV encodes homologues of host negative regulatory signals such as IL10 and also induces these negative signals such as CCL22.\textsuperscript{330} Finally, once IFN is induced and signals through its own receptor, MHV68 M2 downregulates STAT1 and STAT2\textsuperscript{331} and hCMV immediate early gene 1 (IE1/p72) coimmunoprecipitates with the components of the ISGF3 complex to block binding to DNA and further amplification of the innate immune response.\textsuperscript{332} With the exception of the latently expressed MHV68 M2 protein, all these immune evasion proteins are expressed during the initial stages of viral infection. All are found within the virion except vIL10 which is secreted along with the virions. Thus, these Herpesvirus viral immune evasion proteins are present to inhibit the early induction of signaling. In addition, the majority of these proteins are non essential (IRS1, TRS1, pp65, vIL10 and M2) which indicates that as in many immune evasion molecules, existence is not essential for viral replication. Therefore, the known Herpesvirus evasion molecules for the most part embody the characteristics of a viral inhibitor of signaling- expressed early in the infection cycle and non-essential for actual viral replication.

Ironically, in some instances, immune signaling actually benefits the virus. Both hCMV and mCMV encode homologues of proinflammatory cytokines and chemokines as well as their receptors to recruit immune cells to aid in viral dissemination. These include mCMV m131-129 and hCMV UL146 which are chemokine homologues\textsuperscript{333, 334} and mCMV M33, 78 and hCMV US28 which are chemokine receptor homologues.\textsuperscript{335, 336}
Thus, Herpesviruses must delicately balance immune activation and inhibition for successful survival.

**Murine γ–herpesvirus 68 (MHV68), a model for γ–herpesvirus infections**

Murine γ–herpesvirus 68 (MHV68) (also known as murid herpesvirus 4) is the first small animal model for γ–herpesvirus infections with similar pathogenesis to Epstein Barr virus (EBV) and structure to Kaposi Sarcoma herpesvirus/Human herpesvirus 8 (KSHV/HHV8). Since γ–herpesviruses such as EBV and KSHV are difficult to work with even in tissue culture systems, MHV68 and other murine herpesviruses such as MHV70 and Herpesvirus samurai serve as experimental models.

MHV68 is a natural pathogen of rodents such as mice, voles and shrews. Approximately 70% of bank voles in the UK harbor the virus in the respiratory tract. While the natural route of infection is unknown, it is thought that MHV68 infection, like EBV, begins with an oral exposure. Experimentally, intranasal inoculations are the most popular route of administration. This route leads to lytic replication in the lungs and epithelial cells of the oropharynx, thereby reproducing the mucosal characteristics of natural herpesvirus transmission. This also parallels some nasopharyngeal EBV infections which eventually lead to cancer.

The host response to viral infection begins with an initial wave of macrophages followed by CD8 T cells. By the time the inflammation resolves, the virus has already spread to the local lymph nodes. There it infects dendritic cells, macrophages and B cells. Latency can be established as early as three days after infection mainly in B lymphocytes, like EBV, but also in dendritic cells, macrophages as well as lung epithelial cells.

Eventually, MHV68 infection leads to disease states which have been associated
with but not necessarily proven to be caused by EBV or KSHV. The original infection can spread into the spleen leading to splenomegaly. This increases the number of circulating lymphocytes, causing a mononucleosis-like syndrome reminiscent to that seen in EBV infections. About 10% of mice with MHV68 develop lymphoproliferative disease originating from B cells, another characteristic of EBV infections. Finally, like EBV and KSHV, MHV68 infection can also aggravate autoimmune arthritis and cause lethal vasculitis in immune compromised mice.

The 118 kb genome from MHV68 strain g2.4 and the laboratory passaged derivative WUMS have been sequenced and extensively analyzed. The unique region of the genome is flanked by terminal repeat regions (TR) of approximately 1.2 kb in length. The 5’ left hand TR is significant because it is similar to the EBV small early RNA (EBER) genes most abundantly expressed during viral latency. Downstream of this region, a variety of microRNAs have been predicted to be encoded by the genome. While their targets have not yet been identified, the functional importance of microRNAs is implicated by the altered latency and reactivation observed in a mutant lacking this area. Therefore many regions in addition to the protein coding areas are important in the MHV68 genome.

In addition, the majority of the 80 genes predicted to be encoded by MHV68 is collinear and homologous to other γ−herpesviruses. Approximately 90% of MHV68 genes have homologues in KSHV and 80% have homologues in EBV. In this capacity, key similarities between MHV68 and the human γ−herpesviruses lie in the genes involved in the infection process as well as the lytic and latent cycles. MHV68 ORF52 encoding gp150 is akin to the glycoprotein B encoded by other Herpesviruses that is
important in binding and entry into the cell.\textsuperscript{344} ORF50 is the MHV68 version of the replication and transcriptional activator (RTA) which initiates the lytic cycle of gene expression both during the initial infection and in reactivation from latency.\textsuperscript{345} MHV68 ORF21 encodes the viral thymidine kinase present in all other Herpesviruses that renders infected cells more sensitive to the antibiotic ganciclovir.\textsuperscript{346} Like EBV and KSHV, MHV68 uses bcl-2 and a latent associated nuclear antigen (LANA) to enter into and maintain latency: ORF73 and M11.\textsuperscript{347-350} Finally, to reactivate from latency, ORF74 is used as a G protein coupled receptor that binds to chemokines such as KC and macrophage inflammatory protein 2 (MIP2) which are essential for this process.\textsuperscript{347}

**Innate and adaptive immune responses that control MHV68**

The host immune response to MHV68 infection is not as well studied as that for CMV. Ironically, while B cells are a major target for MHV68 latent infection, the cells themselves and also their antibody producing capability do not appear to be important for lytic viral replication because deficient mice clear replicating virus from the lungs with normal kinetics.\textsuperscript{351} In addition, NK cells while activated do not appear to play a significant role in pathogenesis or viral clearance in MHV68 infections.\textsuperscript{352} CD8 T cells, however, are important in the recovery from primary infection and regulation of latently infected cells. Depletion of CD8 T cells during the primary infection results in systemic infection and higher viral titres while activation of CD8 T cells by priming reduces them.\textsuperscript{353} CD4 T cells are also important in the host response against MHV68. Through IFN $\gamma$ CD4 T cells control the proliferation of latently infected B cells that contribute to splenomegaly. Removal of both CD4 and CD8 T cells is lethal.
Similarly, innate immune responses through IFN are important in protecting the host from MHV68 pathogenesis. The lack of IFN signaling in IFNAR and IRF1 null mice is lethal, with rapid dissemination of MHV68 into the lymph nodes, blood stream and central nervous system.\textsuperscript{354} STAT1 null mice also readily succumb to infection\textsuperscript{355} and mice lacking protein inhibitor of activated stat (PIAS1) which inhibits STAT1 have decreased viral titres compared to wildtype.\textsuperscript{356} IFN$\gamma$ is important not only in CD4 T cell function as described above but also for viral clearance from smooth muscle cells in the arterial walls to prevent vasculitis.\textsuperscript{339} However, the phenotype of IFN$\gamma$R null mice is not as severe when compared to the IFNAR null mice. IFN$\gamma$R null mice do not exhibit any differences in MHV68 replication early on in the course of infection but do show increased lymphoid tissue atrophy with more persistent virus later.\textsuperscript{355, 357} IL6, IL12p40 and C3 complement protein deficient mice however appear to experience a completely normal course of infection.\textsuperscript{358} Thus, though the field is just beginning to be investigated, it is clear that while not all cytokines are essential type I and II IFNs are important in the host immune response to MHV68 infection.

The knowledge concerning MHV68 evasion of both innate and adaptive immune responses to date consists of a handful of proteins which perform a hodgepodge of functions previously ascribed to other Herpesvirus proteins. This includes the K3 protein which is a ubiquitin ligase, and like hCMV encoded US2 and US11, can independently degrade MHC class I heavy chains and also TAP.\textsuperscript{359} ORF73, like EBV EBNA-1, inhibits T cell epitope presentation, allowing for viral amplification in latency.\textsuperscript{360} M2 downregulates both STAT1 and STAT2 during the latent replication cycle of MHV68.\textsuperscript{331} M3 is a chemokine binding protein that inhibits signaling through G protein coupled
receptors and subsequent chemotaxis of lymphocytes.\textsuperscript{361} ORF45, like its KSHV counterpart, is important for viral replication because it is phosphorylated and this presumably leads to the inhibition of IRF7 activation.\textsuperscript{167, 362} Finally, MHV68 ORF4, like its homologues in KSHV and EBV, regulates complement activation.\textsuperscript{363} These immune evasion examples indicate that the MHV68 genome encodes a variety of proteins capable of many different functions that have yet to be elucidated.

**Introduction**

Herpesviruses are prolific enveloped dsDNA viruses that cause a variety of diseases.\textsuperscript{299} Of the over 130 members within the family, the most relevant human pathogens include herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella zoster virus (VZV) and Epstein Barr virus (EBV), and *Human herpesviruses 6A, 6B, 7, and 8* (also known as Kaposi’s Sarcoma herpesvirus) (HHV6A, HHV6B, HHV7, and HHV8/KSHV). Model systems used to study these viruses include tissue culture passaged strains of hCMV, mouse CMV (mCMV) and murine \(\gamma\)-herpesvirus 68 (MHV68) which resembles EBV in pathogenesis and KSHV in structure. One of the defining characteristics of this virus family is that all members go through both lytic and latent life cycles. Thus, viral persistence is life long and only in an immunocompromised situation does disease occur. In this capacity, while a virus infection activates the innate and adaptive immune systems, the success of viral persistence is largely due to the many mechanisms which Herpesviruses employ to regulate the host response.

The innate branch of the host immune response is induced by the recognition of pathogen associated molecular patterns (PAMPs)\textsuperscript{5} such as dsRNA and DNA present
either in the genome or as replication intermediates during viral infection. These PAMPs through pattern recognition receptors (PRRs) then activate transcription factors such as Interferon (IFN) Regulatory Factor 3 (IRF3) and 7, nuclear factor κ B (NFkB) and Activate protein 1 (AP1) to mediate the expression of IFN stimulated genes (ISGs) as well as IFN and cytokines. IFN then signals in autocrine and paracrine manners to induce more ISGs and amplify the antiviral environment.

Herpesviruses present a variety of PAMPs which may stimulate this innate immune response. RNA, for what is hypothesized to be structural, transcriptional or replication purposes, is present within CMV and HSV virions in the forms of host and viral mRNA and also RNA-DNA hybrids linked to the essential lytic region, oriLyt. In the case of EBV, RNA is also present during the latent replication cycle in the form of EBV encoded small RNAs (EBERs) which can induce type I IFN. Finally, DNA is present in the form of the genome. In the case of MHV68 and KSHV, the repetitive regions of their genomes have been shown to be able to induce IFN.

In addition, Herpesviruses present other molecules which have been shown to induce signaling. These include hCMV glycoproteins B and H which are expressed in the virion and necessary for viral entry.

These PRRs that recognize these Herpesvirus PAMPs include members of the Toll like receptor family such as TLR2, 3 and 9, RNA helicase family such as RIG-I and also the recently identified cytoplasmic DNA receptor DAI. TLR2 is implicated in the recognition of and IRF3 activation in response to hCMV glycoproteins. However, additional receptors may be in play for glycoproteins in particular as a dominant negative TLR2 cannot block induction. Using null mice, TLR3 and TLR9 have been shown to
be important for the induction of cytokines in response to and subsequent control of mCMV infection. In addition, mice lacking MyD88, an adaptor for TLR9 but not TLR3 show a similar lethal phenotype upon mCMV infection. Mice lacking TRIF, the adaptor for TLR3 but not TLR9, show increased susceptibility and produce less type I IFN in response to infection. Finally, KSHV appears to not only upregulate expression of but also induce IFNβ and activate NFκB through TLR3. As shown by immunoprecipitation, RIG-I binds directly to EBV EBERs and this activates NFκB and IRF3. Paradoxically, EBV EBERs are also able to bind to the ISG protein kinase R (PKR) and actually inhibit its function. Thus these viral molecules have opposing functions dependent on the host cell context. Finally, a DNA activated pathway signaling through RIG-I is implicated in the production of IFN in response to HSV-1. It is possible that this pathway is initiated by the more recently described DNA receptor DAI which has also shown to be important for the IFN induction in response to HSV-1 infection.

The induction of IFNs and ISGs in response to Herpesviral PAMPS is antiviral in nature. Treatment of wildtype macrophages with dsRNA inhibits MHV68 replication. This inhibition is not observed in the presence of IFN blocking antibody or when IFNα receptor null cells are used. IFNα, IRF1 and STAT1 null mice experience lethal MHV68 infections with rapid system wide dissemination. IFNγ receptor knockout mice have increased pathology in the lymphoid tissues. For hCMV, infection activates IRF3 and induces not only IFN but also ISGs such as ISG56, 54 and cig5/viperin. This last ISG in particular has been shown to be able to inhibit specifically hCMV replication. Like MHV68, treatment of astrocytes and brain cells in culture with
dsRNA inhibits hCMV replication from infections at a low multiplicity of infection, presumably when not enough viral evasion proteins are available to block signaling.\textsuperscript{374} IFN\textgreek{a} and \(\gamma\) receptor knockout mice are also more susceptible to mCMV lethality compared to wildtype.\textsuperscript{321, 322} These observations indicate that the host immune system is able to mount a response against the virus. That the virus persists in the face of this response indicates that it is able to inhibit some of this response.

Indeed, there are many indications that Herpesviruses are able to inhibit the initial induction of IFN. While ISG56 and other IRF3 transcribed genes are upregulated upon hCMV infection, these levels are accentuated when inhibitory viral proteins are not expressed such as in the case of UV inactivated virus or in the presence of cycloheximide which presumably blocks the translation of viral proteins.\textsuperscript{218} Furthermore, hCMV is able to complement the vaccinia virus mutant lacking E3L which is responsible for inhibition of IRF3, protein kinase R (PKR) and RNaseL activation. This complementation occurs even in the presence of the antibiotic ganciclovir which inhibits viral DNA synthesis early in the replication cycle. This indicates that even without significant viral protein production, hCMV can inhibit innate immune signaling.\textsuperscript{376} Finally, our preliminary studies indicate that MHV68 infection does not appear to activate IRF3 at all. Thus, many virally encoded molecules present at the time of infection and also expressed later in the replication cycle are responsible for this inhibition.

While not much is known about molecular determinants of MHV68 immune evasion, previous analysis of the CMV genome has identified many ORFs which possess this function. These include hCMV IRS1 and TRS1 and mCMV m142 and m143 which inhibit PKR and another dsRNA binding ISG RNAse L\textsuperscript{323-326} In addition, mCMV M45
binds to RIP1, inhibiting its ubiquitination and thus TLR3 activation of NFκB. Finally, hCMV pp65 has also been reported to inhibit NFκB and also IRF3 although the mechanisms have yet to be elucidated.

Ironically, in some instances, immune signaling actually benefits the virus. Both hCMV and mCMV encode homologues of proinflammatory cytokines and chemokines as well as their receptors to recruit immune cells to aid in viral dissemination. For MHV68, KSHV and EBV, NFκB signaling mediated by overexpression of the p65 subunit of the transcription factor has been shown to inhibit lytic viral replication but is apparently essential for entry into the latent phase. Thus, Herpesviruses must delicately balance immune activation and inhibition for successful survival.

The viral molecules responsible for immune regulation are both protein products as well as microRNAs encoded by the large genome of Herpesviruses ranging from 124-241 kb. As mentioned previously, the capacity for MHV68 to regulate immune signaling has not been extensively studied. For CMV, while many ORFs and even microRNAs have an identified function, much of the genome has yet to be characterized. Furthermore, many viral ORFs have multiple functions that are not necessarily related. Thus, even if an ORF has an assigned function, they may still have the ability to inhibit signaling. To undertake a more systematic approach to identify viral regulators of signaling, we developed a cell death assay which specifically addressed IRF3 activation by TLR3. We assessed a limited number of uncharacterized ORFs within the US22 mCMV gene family, approximately 50% of the hCMV and 50% of the MHV68 predicted ORFs. Our results indicated that no ORFs appeared to activate IRF3 on their own. However, of the 135 total ORFs assayed, 18 appeared to be able to inhibit IRF3
activation by TLR3. We confirmed this inhibition for 4 of these 18 ORFs experimentally and continued further in addressing the mechanism of inhibition for MHV68 ORFs 35 and 58, both of which are not attributed with immune regulatory functions.

Our experiments indicated that both MHV68 ORF35 and 58 inhibit the activation of IRF3 at the level of its activating kinases, TBK1 and IKKe. In addition, ORF35 appeared to be able to augment NFkB- while still inhibiting IRF3- mediated transcription. In these capacities, MHV68 ORFs 35 and 58 reflect the complexity involved in the regulation of IRF3 activation and MHV68 ORF35 exemplifies a viral protein that is capable of manipulating the host immune system in multiple ways.

**Results**

**CMV ORFs that inhibit signaling**

To identify mCMV and hCMV inhibitors of TLR3-ISRE mediated signaling, we used the cell survival assay described in Chapter 4. Briefly, a TLR3 expressing 293 cell line (TLR3 293) in which the selection gene Herpesvirus thymidine kinase (TK) driven by the promoter of ISG56 was used to determine the presence (cell death) or absence (cell survival) of dsRNA signaling. 12 putative ORFs from the US22 gene family of mCMV and 78 putative ORFs from the hCMV genome were tested individually and also in groups according to published reports indicating interactions: 1.) m142 and m143 and 2.) m139, m140 and m141. (Table 6.1) Plasmid expression vectors for each ORF or groups of ORFs were individually transfected into cells. The cells were then either treated with dsRNA and the antibiotic ganciclovir (GCV) or GCV alone. We expected inhibitors of signaling to allow cells to survive even in the presence of dsRNA
Table 6.1: Mouse and human cytomegalovirus (mCMV and hCMV) open reading frames (ORFs) assayed for inhibition of IRF3 activation.

Grey shaded cells indicate an inhibitor of dsRNA-TLR3 activation of IRF3 as determined by the cell death assay described in the text. Bolded ORF names indicate that the mutant virus grows poorly in tissue culture compared to wildtype virus. Italicized and bolded ORF names indicate that the mutant virus grows slightly poorly in tissue culture compared to wildtype virus. Non bolded italicized ORF names indicate that the mutant virus grows better in tissue culture compared to wildtype virus. ORFs were a gift of A. Geballe. Sources of information include personal communication (A. Geballe), “The Herpesviridae Family: A Brief Introduction,”\textsuperscript{299} “Virus-encoded microRNAs: novel regulators of gene expression,”\textsuperscript{341} “Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation,”\textsuperscript{379} “Functional map of human cytomegalovirus AD169 defined by global mutational analysis,”\textsuperscript{380} “Identification of microRNAs of the herpesvirus family”\textsuperscript{342} and notes from the 29th International Herpesvirus Workshop 2004.
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and activators to cause cells to die even without dsRNA. An empty vector was used as a negative control and a plasmid expressing the V protein of Parainfluenza virus 5 was used as a positive control for inhibition of dsRNA signaling. The degree of inhibition was determined by cell survival which was estimated based on confluence after 4 days. Survival varied from 10% to 90% in cells expressing the viral ORFs and scored as a positive in terms of inhibition compared to 0% in the negative control. From the mCMV ORFs tested, 1 individual and both combinations inhibited signaling. From the hCMV ORFs, 5 inhibited signaling. Expression of the viral proteins themselves did not affect cell survival.

MHV68 ORFs that inhibit signaling

Using the assay described above, we also assayed 45 putative ORFs from the MHV68 genome individually and also a group of two (ORF27 and 58) according to published reports indicating interactions for their ability to inhibit dsRNA-TLR3 signaling. (Table 6.2) From the MHV68 ORFs tested, 6 individual and 1 combination inhibited signaling. Expression of the viral proteins themselves did not affect cell survival except the protein encoded by ORF21 which caused cell death even in the absence of dsRNA. This protein was later identified via literature search to be the MHV68 homologue of TK. 346

Confirmation of MHV68 ORF35 and 58 inhibition of dsRNA signaling

Several of the more potent inhibitors of dsRNA signaling as determined by cell confluency were further analyzed to confirm their ability to inhibit signaling: mCMV M36, MHV68 ORFs 35, 37 and 58. This was achieved by creating stably expressing lines in HT1080 derived cells (2fTGH) 381 and measuring P56 induction after treatment with
Table 6.2: Murine γ–herpesvirus 68 (MHV68) open reading frames (ORFs) assayed for inhibition of IRF3 activation.

Grey shaded cells indicate an inhibitor of dsRNA-TLR3 activation of IRF3 as determined by the cell death assay described in the text. Bolded ORF names indicate that the mutant virus grows poorly in tissue culture compared to wildtype virus. Italicized and bolded ORF names indicate that the mutant virus grows slightly poorly in tissue culture compared to wildtype virus. Non bolded italicized ORF names indicate that the mutant virus grows better in tissue culture compared to wildtype virus. Epstein Barr virus (EBV) ORF names are homologues of MHV68 ORFs. HVS: Herpesvirus Samurai, KSHV: Kaposi's Sarcoma herpesvirus. ORFs were a gift of J. Jung. Sources of information include “Complete sequence and genomic analysis of murine γ–herpesvirus 68.”
<table>
<thead>
<tr>
<th>MHV68 ORFs</th>
<th>Description</th>
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<tbody>
<tr>
<td>9</td>
<td>DNA polymerase; EBV BALF5 (interacts with BBLF2/3, BMRF1, BSLF1, BBLF4) putative homologue to BXRFL1 in EBV (possible interaction with BILF1); induces cell cycle arrest G2 inhibiting Cdc2-cyclinB</td>
</tr>
<tr>
<td>20</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>21</td>
<td>EBV BTRF1 (interacts with BZLF2 and host protein phosphatase 1)</td>
</tr>
<tr>
<td>24</td>
<td>capsid protein; EBV BDLF1 (interacts with BNLF2b and host MAP kinase 7, cathepsin c, coiled coiled domain containing 14 )</td>
</tr>
<tr>
<td>26</td>
<td>encodes gp48, a type 2 transmembrane glycoprotein; putative homologue to BDLF2 in EBV (homologous to cyclin B1) (interacts with EBNA2 and host proteosome subunits), interacts with ORF45 involved in immune evasion via inhibit IRF7 phosphorylation</td>
</tr>
<tr>
<td>30</td>
<td>Possible KSHV + HVS but not EBV homologues</td>
</tr>
<tr>
<td>31</td>
<td>EBV BDLF4</td>
</tr>
<tr>
<td>32</td>
<td>EBV BGLF1</td>
</tr>
<tr>
<td>33</td>
<td>EBV BGLF2 (interacts with BSLF1)</td>
</tr>
<tr>
<td>34</td>
<td>EBV BGLF3</td>
</tr>
<tr>
<td>35</td>
<td>putative homologue to BGLF3.5 in EBV</td>
</tr>
<tr>
<td>36</td>
<td>kinase; EBV BGLF4 (interacts with BGLF1) putative homologue to BGLF5 in EBV, an alkaline exonuclease; putative homologue to SOX of KSHV, which degrades host RNA</td>
</tr>
<tr>
<td>39</td>
<td>Glycoprotein M; EBV BBRF3</td>
</tr>
<tr>
<td>42</td>
<td>EBV BBRF2 (interacts with host protein kinase c)</td>
</tr>
<tr>
<td>43</td>
<td>EBV BBRF1 putative homologue to BBLF4 in EBV, a helicase primase; conserved essential gene in alpha, beta, and gamma herpesviruses</td>
</tr>
<tr>
<td>44</td>
<td>phosphoprotein; possibly blocking IRF7 phosphorylation like its KSHV homologue; EBV BKRF4</td>
</tr>
<tr>
<td>46</td>
<td>putative homologue to BKRF3 in EBV, a uracil DNA glycosylase</td>
</tr>
<tr>
<td>48</td>
<td>EBV BKRF2</td>
</tr>
<tr>
<td>49</td>
<td>EBV BRRF1 (interacts with BSRF1)</td>
</tr>
<tr>
<td>52</td>
<td>glycoprotein B; EBV BRLF2</td>
</tr>
<tr>
<td>54</td>
<td>EBV BLLF3; dUTPase</td>
</tr>
<tr>
<td>55</td>
<td>EBV BSRF1 (interacts with BBRF1)</td>
</tr>
<tr>
<td>56</td>
<td>EBV BSLF1 (interacts with BGLF2); DNA replication protein</td>
</tr>
<tr>
<td>58</td>
<td>form protein complex in ER. ORF58 is putative homologue to BMRF2 in EBV</td>
</tr>
<tr>
<td>59</td>
<td>EBV BMRF1 (interacts with EBNA3B); DNA replication protein</td>
</tr>
<tr>
<td>60</td>
<td>EBV BaRF1 (interacts with itself); Ribonucleotide reductase, small</td>
</tr>
<tr>
<td>67</td>
<td>EBV BFRF1 (interacts with BFLF2); tegument protein</td>
</tr>
<tr>
<td>68</td>
<td>EBV BFLF1; glycoprotein</td>
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<tr>
<td>69</td>
<td>EBV BFLF2 (interacts with BFLF1)</td>
</tr>
<tr>
<td>73</td>
<td>Like EBV EBNA-1 inhibits T cell epitope presentation</td>
</tr>
<tr>
<td>29A</td>
<td>EBV BGRF1; packaging protein</td>
</tr>
<tr>
<td>29B</td>
<td>KSHV and HVS but no EBV homologue</td>
</tr>
<tr>
<td>27</td>
<td>like EBV EBNA-1 inhibits T cell epitope presentation</td>
</tr>
<tr>
<td>27 and 58</td>
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dsRNA added to the media to activate the TLR3 signaling pathway. P56 is also induced by the RIG-I and IFNb pathways. We also assayed for the inhibition of these two pathways by transfecting dsRNA into cells and adding IFNb into the media respectively. P56 was strongly induced upon dsRNA addition to the media, dsRNA transfection and IFNb treatment (Lane 1 Fig 6.3 A and B and Lanes 1 and 2 Fig 6.3C). All viral ORFs showed varying degrees of inhibition of TLR3 and RIG-I signaling (Data not shown and Lane 2 Fig 6.3B, Lane 4 Fig 6.3C). Only MHV68 ORF37 was also able to inhibit P56 induced by IFN, indicating global repression (Fig 6.3A Lanes 2 and 3). We further confirmed the inhibition of dsRNA signaling by measuring mRNA levels of ISG56 and MHV68 ORF58 via quantitative PCR. Two independently isolated clones for ORF58 were tested. Consistent with the protein analysis, dsRNA induced ISG56 mRNA and p56 protein levels were decreased in a dose dependent manner correlating to increasing levels of ORF58 mRNA (Data not shown and Fig 6.3D). These results indicated that while MHV68 ORFs 35 and 58 can block IRF3 activation by dsRNA, they cannot block ISGF3 activation by IFNb. MHV68 ORFs 35 and 58 were chosen for further analysis because they were less well characterized in the literature.

MHV68 ORF35 and 58 activation of P38 and AKT

The AP1 transcription factor complex of ATF2 and c-Jun is activated in the TLR3 pathway via a phosphorylation cascade of mitogen activated protein kinases (MAPK) Jun kinases (JNK), p38 and extracellular signal regulated kinase (ERK). To assay the effects of ORF35 and ORF58 on the activation of this pathway we measured phosphorylation of P38 by phosphospecific antibody. In cells stably expressing ORF35 or ORF58 (Fig 6.4B and C), p38 was phosphorylated to the same extent as control cells
Figure 6.3: MHV68 ORFs 35, 37 and 58 inhibit p56 induction.

A. 2fTGH cells stably expressing ORF37 (Lane 2) or empty vector control (Lane 1) were left untreated, treated with dsRNA in the media, transfected dsRNA, or IFNb. p56 protein levels were measured by immunoblotting. B. 2fTGH cells stably expressing ORF35 (Lane 2) or empty vector (Lane 1) were left untreated, treated with dsRNA in the media, transfected dsRNA or IFNb. p56 protein levels were measured by immunoblotting. C. 2fTGH cells stably expressing ORF58 (Lanes 1 and 2) or empty vector control (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with dsRNA in the media, transfected dsRNA or IFNb (Lanes 2 and 4). p56 levels were measured by immunoblotting. D. Two independently isolated 2fTGH clones expressing ORF58 (58.2 and 58.3) or control cells (-) were treated with dsRNA in the media or transfected dsRNA. p56 protein levels were measured by immunoblotting (top). ISG56 and ORF58 mRNA levels were measured by Q RT-PCR (bottom) in independently isolated clones expressing ORF58 (58.2 and 58.3) or empty vector control (-) treated with dsRNA in the media. mRNA levels are shown relative to the highest expressing sample. Bars represent standard error.
Figure 6.4: MHV68 ORFs 35 and 58 do not inhibit activation of p38 and AKT.

Empty vector control (A) or 2fTGH cells stably expressing ORF58 (B) or ORF35 (C) were treated with dsRNA in the media for the time indicated and collected for immunoblot analysis. P38 phosphorylation at threonine 180 and tyrosine 182 (p-P38) was detected by immunoblotting cell extracts with phospho-p38 specific antiserum with total P38 as a control. AKT phosphorylation at serine 473 (p-AKT) was detected by immunoblotting cell extracts with phospho-AKT specific antiserum with total AKT as a control.
without viral protein (Fig 6.4A). These results indicated that while ORF35 and ORF58 can block IRF3 activation by dsRNA, they cannot block AP1 activation.

PI3K plays an important role in the full phosphorylation of IRF3 via AKT.\(^{59}\) To assay the effects of ORF35 and 58 on the activation of this part of the pathway we measured phosphorylation of AKT by phosphospecific antibody. The amount of phosphorylated AKT was slightly decreased compared to control cells without viral protein initially at 15 minutes (Lanes 2 Fig 6.4A-C). However, phosphorylated AKT levels were comparable to control cells after 30 minutes. (Lanes 3 Fig 6.4A-C) These results indicated that ORFs 35 and 58 do not block phosphorylation of IRF3 via the PI3K/AKT pathway.

**ORF58 inhibits NFkB activation**

NFkB is also activated in the TLR3 pathway and this can in part by assayed by the nuclear localization of the p65/RelA subunit (Lane 2 Fig 6.5A). In cells expressing ORF58, dsRNA-induced p65 nuclear localization was inhibited (Lane 4 Fig 6.5A). This was not an effect of decreased basal p65 expression because total cell extracts showed equivalent amounts of the protein in cells with and without ORF58 (Bottom Fig 6.5A). In addition, gene induction by NFkB was assayed by IL8 ELISA as the promoter of IL8 contains NFkB sites.\(^{382}\) The amount of IL8 produced from dsRNA treatment was decreased by approximately 50% in cells expressing ORF58 (Lane 4 versus Lane 2 Fig 6.5B). The IL8 promoter contains NFkB as well as AP1 and other transcription factor binding sites.\(^{383},\ 384\) These results are therefore consistent with the interpretation that ORF58 can inhibit NFkB but not AP1 mediated transcription.
Figure 6.5: ORF58 inhibits NFkB activation.

A. Nuclear fractions from empty vector control cells (Lanes 1 and 2) or 2fTGH stably expressing ORF58 (Lanes 3 and 4) that were untreated (Lanes 1 and 3) or treated with dsRNA in the media (Lanes 2 and 4) were immunoblotted for p65 with the nuclear protein DRBP76 as a loading control. For comparison, total cell extracts were immunoblotted for p65 with actin as a loading control. B. Empty vector control cells (Lanes 1 and 2) or 2fTGH stably expressing ORF58 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with dsRNA in the media (Lanes 2 and 4). Media from these samples were collected to assay for IL8 by ELISA. Bars represent standard error. Data is representative of two independent experiments.
**ORF35 enhances NFkB activation**

In contrast to ORF58, NFkB activity in cells expressing ORF35 was actually increased in unstimulated and also dsRNA treated cells activating TLR3 (Fig 6.6). Basal IL8 expression without any stimulation as assayed by ELISA was approximately 10 fold more in cells expressing ORF35 (Lane 3 Fig 6.6) compared to control cells without the viral ORF (Lane 1 Fig 6.6). When dsRNA was used as an inducer, ORF35 expressing cells produced more than twice as much IL8 compared to control cells (Lane 4 versus Lane 2 Fig 6.6). Since ORF35 appears to not affect P38 or AKT phosphorylation, these results most likely suggest that NFkB and not AP1 mediated transcription is being augmented.

**ORF58 inhibits IRF3 activation**

We hypothesized that the point of inhibition for ORF58 was at the level of the IKK family of kinases TBK1 and IKKe because ORF58 could inhibit both TLR3 and RIG-I mediated signaling which converge at this point. Furthermore, the IKK family members IKKα, β and γ are in part responsible for p65 nuclear localization in NFkB activation and this was also inhibited by ORF58. Exogenous expression of the IKK family member TBK1, activated by both TLR3 and RIG-I, can by itself phosphorylate IRF3 and induce P56. Consistent with our hypothesis, this induction was inhibited in the presence of ORF58 (Fig 6.7A).

Inactive IRF3 is predominantly cytoplasmic while activated IRF3 is predominantly nuclear. Nuclear localization of IRF3 induced by both TLR3 and RIG-I signaling was inhibited in cells expressing ORF58. (Lane 4 versus Lane 2 Fig 6.7 B and C) We then asked whether the observed block of IRF3 translocation was due to a block in
Figure 6.6: ORF35 augments NFkB activation.

Empty vector control cells (Lanes 1 and 2) or 2fTGH stably expressing ORF35 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with dsRNA in the media (Lanes 2 and 4). Media from these samples were collected to assay for IL8 by ELISA. Bars represent standard error. Data is representative of two independent experiments.
Figure 6.7: ORF58 inhibits IRF3 activation.

A. 293 cells were co-transfected with expression vectors for TBK1 and ORF58 (Lane 2) or empty vector control (Lane 1). Immunoblotting was used to detect p56 levels and Myc-tagged TBK1. B. Nuclear fractions and total cell extracts from empty vector control cells (Lanes 1 and 2) and 2fTGH stably expressing ORF58 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with dsRNA (Lanes 2 and 4) and immunoblotted to determine the levels of IRF3. The nuclear protein DRBP76 and actin served as controls. C. Nuclear fractions from empty vector control cells (Lanes 1 and 2) and 2fTGH stably expressing ORF58 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with transfected dsRNA (tdsRNA) (Lanes 2 and 4) and immunoblotted to determine the levels of IRF3. The nuclear protein DRBP76 served as a control. D. Empty vector control (left) or 2fTGH cells stably expressing ORF58 (right) were treated with dsRNA in the media for the time indicated and collected for immunoblot analysis. IRF3 phosphorylation at serine 396 was detected immunoblotting cell extracts with phospho-IRF specific antiserum with total IRF3 as a control. E. Empty vector control (Lanes 1 and 2) or 2fTGH cells stably expressing ORF58 (Lanes 3 and 4) were transfected with dsRNA and samples were collected for immunoblot analysis. IRF3 phosphorylation at serine 396 was detected immunoblotting cell extracts with phospho-IRF specific antiserum with total IRF3 as a control. F. Empty vector control cells (Lanes 1 and 2) and 2fTGH cells stably expressing ORF58 (Lanes 3 and 4) were treated with dsRNA (Lanes 2 and 4). IRF3 dimerization was assayed by native gel electrophoresis followed by immunoblotting. G. It is the same as in F except cells were transfected with dsRNA.
its phosphorylation, which is known to activate it. Phosphorylation of Ser396, a hallmark of IRF3 activation in both the TLR3 and RIG-I pathways, was inhibited in the presence of ORF58. (Fig 6.7D and E) Consistent with these results, IRF3 dimerization as measured by native gel electrophoresis was reduced in cells expressing ORF58 when TLR3 was activated. (Fig 6.7F) However, the relative inhibition of dimerization was not as great as the inhibition of IRF3 nuclear localization or P56 induction. Furthermore, there was no difference in IRF3 dimerization between ORF58 expressing and control cells when dsRNA was transfected to activate RIG-I (Fig 6.7G). These results indicated that ORF58 inhibits certain aspects of IRF3 activation such as nuclear localization and Ser396 phosphorylation but not all as reflected by dimerization.

**ORF35 inhibits IRF3 activation**

Like ORF58, ORF35 was also able to inhibit p56 induction from both the TLR3 and RIG-I/mda-5 pathways. Thus, for this protein too, the point of inhibition is likely downstream of where the two pathways converge at TBK1 and IKKe. IRF3 was not localized into the nucleus in cells expressing ORF35 when treated with dsRNA floating in the media as compared to empty vector control cells (Fig 6.8A), indicating that the transcription factor was not active. However, IRF3 dimerization as assayed by native gel electrophoresis and Ser396 phosphorylation as assayed by a phosphospecific antibody, were not decreased and in fact was the same as in control cells. (Fig 6.8B and C) These results indicated that ORF35 mediated inhibition is not at the step of TBK1/IKKe phosphorylating Ser396. However, an additional level of regulation after this step is being inhibited since nuclear translocation and transcriptional activity are absent.
Figure 6.8: ORF35 inhibits IRF3 activation.

A. Nuclear fractions from empty vector control cells (Lanes 1 and 2) and 2fTGH stably expressing ORF35 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or treated with transfected dsRNA (tdsRNA) (Lanes 2 and 4) and immunoblotted to determine the levels of IRF3. B. Empty vector control cells (Lanes 1 and 2) and 2fTGH cells stably expressing ORF35 (Lanes 3 and 4) were treated with dsRNA (Lanes 2 and 4). IRF3 dimerization was assayed by native gel electrophoresis followed by immunoblotting. C. Empty vector control (left) or 2fTGH cells stably expressing ORF35 (right) were treated with dsRNA in the media for the time indicated and collected for immunoblot analysis. IRF3 phosphorylation at serine 396 was detected immunoblotting cell extracts with phospho-IRF3 specific antiserum with total IRF3 as a control.
A. nuclear fraction

- + - + dsRNA
- ORF35 cells

B. IRF3 (2)
IRF3
- + - + dsRNA
- ORF35 cells

C. 396 IRF3
IRF3
0 15 30 60 120 240 min dsRNA
0 15 30 60 120 240 ORF35 cells
ORF58 inhibits RIG-I and IRF3 mediated apoptosis

IRF3 mediates apoptosis induced by transfected dsRNA activating RIG-I. TBK1 kinase activity in this process is essential. Since ORF58 could inhibit TBK1 mediated transcriptional activation of IRF3, we asked if ORF58 could also inhibit the apoptotic pathway going through the same kinase. Controls cells when transfected with dsRNA cleaved PARP (Lane 2 Fig 6.9A) and died after 18 hrs. Both cell death estimated by visualization (data not shown) and PARP cleavage were inhibited in cells expressing ORF58. (Lane 4 Fig 6.9A) These results indicated the ORF58 could inhibit the apoptotic as well as transcriptional activity of IRF3.
Figure 6.9: ORF58 inhibits IRF3 mediated apoptosis.

Empty vector control (Lanes 1 and 2) or 2fTGH cells stably expressing ORF58 (Lanes 3 and 4) were untreated (Lanes 1 and 3) or transfected with dsRNA (Lanes 2 and 4). Total cell extracts were collected for cleaved PARP immunoblot analysis.
1 2 3 4

cleaved PARP

actin

- + - + tdsRNA
– 0RF58 cells
Discussion

A cell death assay that identifies inhibitors and activators of IRF3

We employed a cell death assay to identify inhibitors of dsRNA-TLR3 mediated signaling. We assayed a total of 135 ORFs encoded by members of the *Herpesviridae* family that are either putative or have been previously characterized. None of the ORFs were previously described in the literature to affect IRF3 activation specifically. Our results indicated that 18 ORFs are able to inhibit IRF3 activation by TLR3 (6 of 12 mCMV, 5 of 78 hCMV and 7 of 45 MHV68) (Table). Of these 18, 4 were chosen to follow up by making cell lines stably expressing the ORFs to confirm the inhibition of signaling. P56 protein induction from dsRNA activation of TLR3 was significantly decreased in cell lines expressing all four ORFs: mCMV M36, MHV6 ORF35, 37 and 58. Finally, two of these four ORFs, MHV68 ORF 35 and 58, were examined in depth to elucidate the mechanism of signaling. In the following discussion, I will discuss first the advantages and disadvantages of our assay in the context of identifying viral inhibitors and activators of signaling. Next, the focus will turn towards the results of the assay discussing in general and then taking on more specific examples from each virus, mCMV, hCMV and MHV68. Finally, I will discuss the results from experiments conducted to address the mechanisms of inhibition specifically for MHV68 ORF35 and 58.

Viral assays that identify inhibitors of signaling

Several types of systems have been used to identify viral inhibitors of innate immune signaling documented in the literature. As an example, Martinez-Sobrido *et al* identified the NP protein of lymphocytic choriomeningitis virus (LCMV) from
systematically assaying all the ORFs for inhibitors of IFN induction using reporter gene assays with plasmid constructs containing CAT, GFP or luciferase under the control of the IFN promoter.\textsuperscript{385} Similarly, Park \textit{et al} systematically assayed ORFs from NDV and Nipah viruses and identified V accessory proteins as inhibitors of signaling.\textsuperscript{273} However, instead of the reporter gene under the control of the IFN promoter, they genetically engineered NDV to express GFP as a marker for signaling- if virus grew and GFP was expressed then IFN signaling was inhibited; if virus did not grow and no GFP was expressed then IFN was effectively signaling. These two examples were feasible because of the genomes for these RNA viruses were small.

For hCMV which is approximately ten times larger in its genome compared to these RNA viruses, Child \textit{et al} used a system based on the vaccinia virus E3L (VV delta E3L) mutant which is unable to inhibit IRF3,\textsuperscript{386, 387} PKR\textsuperscript{388} and 2, 5A synthetase\textsuperscript{389} activation.\textsuperscript{323} While the situation is more complex in whole mice experiments,\textsuperscript{386} in tissue culture, PKR appears to be main host protein that inhibits VV delta E3L.\textsuperscript{390} Thus, knockdown of expression or function of PKR rescues the virus and allows for replication. The authors assembled a library of CMV genomic fragments into a vaccinia virus vector, homologously recombined this library into vaccinia virus delta E3L and selected for virus growth in tissue culture cells. This screen was performed with two different libraries created with different restriction enzymes and from this, only one positive hit was obtained. This contained a 10kb hCMV region which was narrowed down to TRS1 and IRS1, both shown to be able to inhibit PKR activation by binding to dsRNA.\textsuperscript{323} In our system, we assayed for inhibition of signaling by expressing cloned putative ORFs in transfected plasmids. For hCMV specifically, our screen of 78 ORFs resulted in 5
positives, approximately 3% of the genome. This rate of recovery is within the range to that performed by Child et al who scored 2 out of approximately 200 ORFS theoretically represented by the library, 1%. Incidentally, while TRS1 was not tested in our assays, IRS1 was. Results indicated that IRS1 did not block dsRNA TLR3 induced signaling, signifying that the ability of IRS1 to rescue VV delta E3L replication was in its inhibition of PKR or 2', 5' A synthetase as described in the paper and not IRF3 activation.

One issue taken into consideration when scoring positives was that our selection system used a herpesvirus protein, the HSV-1 thymidine kinase. Since thymidine kinase is conserved throughout the three subfamilies, it would be reasonable to expect that the majority of Herpesvirus genomes would also encode for proteins that regulate this function. If the ORFs in our assay affected the function of TK or the metabolism of GCV, false positives could arise. Indeed, the inclusion of the MHV68 TK homologue was an example of a false positive whose identity was not uncovered until after the assays were completed. Unexpectedly, our assay did not show a similar phenotype for the hCMV TK homologue, UL97. This could be due to issues with expression of the protein itself as this was not confirmed in our assay, a possible mutation in UL97 affording resistance to the antibiotic or the relative activity of the hCMV TK as compared to the TK from MHV68 from our ORF collection and HSV-1 used in our cell line TK. It may be that hCMV TK is much less efficient at phosphorylating GCV as compared to the TK of the other two subfamilies and therefore its activity would not be reflected at the concentration of antibiotic used.

The second important issue was that since we assayed each ORF expressed from a plasmid, the inhibition would not necessarily be relevant in the context of a viral
infection. This increased the importance in confirming the inhibition and addressing its role in virus replication.

A third important issue was that we did not confirm the protein expression of the ORF plasmids in our assay. Therefore, our assay was limited to the correct cloning and expression level afforded by a transient transfection from plasmids.

Finally, our screen was limited to the extent of our plasmids particularly in the prediction of putative ORFs. If any ORFs did not fall within these predictions, they would be misrepresented. In addition, these ORFs were not constructed specifically to express microRNAs even though they are predicted to be encoded by the genome. While microRNAs could still be expressed from a cloned ORF, this cannot be guaranteed. Despite these issues, the method we used did afford us several advantages.

First, because we were assaying ORFs individually and not conducting a large screen, we could identify both inhibitors and activators of signaling. Just as cells survive in the presence of an inhibitor independent of dsRNA, cells die in the presence of an activator independent of inducer. There are numerous examples of viral homologues encoded by *Herpesviruses* which stimulate the immune system for dissemination purposes exist (for instance vIL10 and viral homologues to the TNFR and chemokines). Consequently, ORFs inducing signaling would not be an unexpected finding in our assays. None of the ORFs we assayed were previously described in the literature to affect IRF3 activation specifically. In our assay, with the exception of the thymidine kinase homologues, no ORF induced cell death on its own. One reason that may explain this result is the possibility that viral proteins activating IRF3 as an effect of viral binding or entry may not be properly folded and secreted out into the medium when they are
expressed alone from a plasmid as opposed to when they are part of a virion in an actual virus infection. In addition, viruses may not utilize IRF3 to purposefully stimulate the immune system for dissemination perhaps because IRF3 mediates too potent an antiviral response. Other less potent signaling molecules then would then allow for easier viral manipulation.

Second, using plasmids to express ORFs allowed us to control the representation of the genome. Alternatives to systematic ORF expression include random library construction into vectors such as that performed by Child et al or mutagenesis of the virus itself and then assaying for inhibition of signaling during viral infection instead of using plasmids to express genes. Both of these methods are subject to biases within the construction of the library dependent on the restriction enzymes used or mutagenic technique, whether it be mediated by transposons or chemicals. Here, we are able to cover genomic ends and repetitive regions which are more difficult to assess with alternatives mentioned above. Our assay covered a limited number of ORFs for mCMV, approximately 50% of putative ORFs for hCMV and 50% for MHV68 but can be potentially extended to cover all the ORFs (and microRNA encoding areas) from these viruses.

Third, control of individual ORFs allowed us to test specific combinations easily. In instances where viral proteins work together as indicated by protein protein interactions in the literature, we were able to express all proteins in a complex together. We did find that several combinations for mCMV (the complex of m139, m140 and m141 and the complex of m142 and m143) and MHV68 (ORF27 and 58) were able to inhibit signaling. Using the cell death assay, we assessed whether each could
independently inhibit signaling or if co-expression was necessary. In all cases, each individual ORF was as equally efficient in inhibiting signaling as the combination. We did not, however, observe a cooperative effect. This may be in part due to varying expression levels– when combinations of plasmids were transfected into cells for the assay, the total DNA was kept equal so the individual amounts of plasmids per ORF were decreased by one half to one third when tested in combination as compared to alone. While controlling for the levels of expression would be important before drawing conclusions about cooperatively, we could conclude from the data we collected that the individual ORFs within the combinations were sufficient to inhibit signaling.

Fourth, ORF expression was independent of viral life cycle. The plasmid based expression gave us the potential to easily assay for late lytic and even latent genes. If these were expressed from a virus itself, the experimental procedure and results would have been complicated by the presence of all the immediate early and early genes (or other viral genes) as well as conducting the assay in cells capable of supporting latency. While early expressing genes are thought to be important in inhibiting the initial induction, the inhibition of amplification of signaling is just as important later on in the life cycle. M2 which is expressed in latency and inhibits IFN signaling is such one example.\textsuperscript{331} No known latent ORFs were assayed here, but we were able to address several late expressing ORFs in the lytic cycle. The results indicated that 3 out of 6 mCMV, 4 out of 5 hCMV and 3 out of 7 MHV68 ORFs thought to inhibit signaling were in fact expressed late in the viral life cycles.

Fifth and finally, ORFs essential or playing an important role in the virus replication cycle could be assayed. These ORFs constitute a significant part of the
45 of 162 (28%) of hCMV and 41 of 80 (51%) MHV68 ORFs are thought to be essential and 35 of 162 (22%) hCMV and 6 of 80 (8%) MHV68 ORFs when mutated have attenuated viral replication abilities. In our assay, 29% of hCMV ORFs and 69% of MHV68 ORFs fell into these two categories. (Table 6.1 and 6.2) It is interesting that the CMV ORFs which appeared by cell survival to be the strongest inhibitors of signaling were also essential: m142 and m143 for mCMV and UL122 for hCMV. From this, we may hypothesize that inhibition of signaling is an important enough selection criteria to engender these ORFs essential. Indeed, in the case of the Paramyxovirus Sendai, IRF3 alone appears to control the fate of the cells by promoting apoptosis to prevent viral persistence. This indicates that IRF3 is able to determine the outcome of infections and viral action against IRF3 may in fact be essential for survival. However, only two of the six inhibitory γ-herpesvirus MHV68 ORFs (ORFs 35 and 44) were essential to viral replication and they did not appear to inhibit significantly more than the others. Ultimately, a more complete analysis of all the ORFs from the genome would be necessary to conclude if IRF3 activation and its inhibition is actually essential in replication of β and not γ-herpesviruses.

**Members of the US22 mCMV gene family inhibit IRF3 activation**

We assayed only 12 ORFs for mCMV. Because analyses of smaller RNA viruses showed that only some but not the majority of viral proteins are involved in immune evasion, we expected the same for the larger DNA viruses such as here. Therefore, it was surprising that half of the ORFs we tested for mCMV inhibited cell death, indicating that they could prevent IRF3 activation. This however is not completely unreasonable because the US22 gene family as a whole have two properties thought to be important for
viral proteins to inhibit the initial induction of signaling from viral infection: they are either found or hypothesized to be part of the tegument\textsuperscript{378, 394} and are expressed as immediate early genes.\textsuperscript{395} Thus they are all present at the time of infection and accumulate immediate after viral penetration of the cell to potentially block initial signaling. Furthermore, the conservation of the US22 gene family across βherpesviruses suggests that their function(s), though many are yet to be elucidated, is essential to maintain through evolution and divergence. It is interesting that of the members of the US22 gene family identified to inhibit IRF3 activation, the growth of the corresponding viral ORF mutants is either severely attenuated or non existent. In comparison, viruses mutated in the six ORFs that did not inhibit signaling in this assay have much less severe viral growth defects.\textsuperscript{396} This strongly suggests the importance of inhibition of IRF3 on the fitness of viral replication.

**mCMV M36 inhibits IRF3 activation**

For M36 specifically, the mCMV ORF is a homologue of the hCMV UL36.\textsuperscript{397} While the mCMV ORF has not been very well characterized, the hCMV UL36 is also known as the viral inhibitor of caspase 8 induced apoptosis (vICA).\textsuperscript{398} Caspase 8 is involved in TK/GCV mediated cell death and chemical inhibitors of caspase 8 such as zIETD-fmk and zVAD-fmk can partially inhibit apoptosis.\textsuperscript{399} Thus, M36 could potentially be inhibiting caspase 8 in the apoptosis mediated by TK/GCV instead of the signaling that induces TK.

We tested this possibility by constructing cell lines stably expressing M36. These cells did not induce p56 after treatment with transfected dsRNA or dsRNA in the media compared to controls, confirming that M36 does indeed inhibit IRF3 activation. This
inhibition was seen in a dose dependent manner with decreased ISG56 mRNA corresponding to increasing M36 expression. (Data not shown.) Furthermore, M36 could not block IFN induction of p56, indicating that the inhibition is not global. (Data not shown.) Since M36 is one of the few ORFs in mCMV that is predicted to be spliced, this inhibition of signaling could be due to the default protein, the alternatively spliced version or both.400

**hCMV ORFs inhibit IRF3 activation**

The five hCMV ORFs identified to inhibit IRF3 activation share many characteristics with the inhibitory mCMV ORFs found in our assay. With the exception of TRL3 which has yet to be directly examined, all are present in the virion so again the proteins are present at the time of infection to prevent signaling.65, 401 In fact, UL25 and UL122 are contained at high enough levels to elicit a significant antibody response in humans.402 Of the five, two are conserved across β herpesviruses, UL24 and UL122, indicating that like the mCMV US22 gene family, the functions of UL24 and UL122 are essential enough to be maintained through evolution and divergence. The actual function of UL24 is unknown. For UL122 (also known as IE2 and IE86), it has immediate early transactivation activity which is necessary for viral gene expression. In addition, many other functions have already been described for this protein: negative autoregulation of the major immediate early promoter, induction of cell cycle from G0 to G1 and arrest at the G1/S transition, inhibiting cellular DNA synthesis.403

**hCMV UL122 inhibits signaling potentially via the IRF3 autoinhibitory domain**

In innate immunity, IE2 has also been shown to block the induction of not only IFN but also RANTES, MIG, MCP-2, MIP1a and IL8 by UV inactivated hCMV or
Sendai virus.\textsuperscript{404, 405} Since IRF3 is one transcription factor important in the induction of IFN, its inhibition by UL122 detected here may help explain how the inhibition of IFN is occurring. Like the mCMV scenario, UL122 was the highest scoring inhibitor of TLR3 signaling and also the only one essential for viral replication.\textsuperscript{380} Interestingly, searches using protein protein BLAST and GCG Wisconsin looking for hCMV areas similar to IRF3 showed that UL122 was homologous to the N terminus of IRF3. (Data not shown.) This was specifically in the N terminal inhibitory domain which is thought to bind to its C terminal equivalent to keep IRF3 in an inactive state by masking the transactivation domain consisting of the IRF association domain (IAD), nuclear export signal (NES) and a subset of residues that are phosphorylated in response to an inducer.\textsuperscript{113, 114} From this, we can hypothesize that UL122 inhibits IRF3 activation by encoding an IRF3-like inhibitory domain that binds to the actual IRF3 C terminal inhibitory domain. This masks IRF3 domains necessary for activity and thus prevents function.

**MHV68 ORFs inhibit IRF3 activation**

The MHV68 set of ORFs identified to inhibit IRF3 activation are the most diverse set of proteins in comparison to the mCMV and hCMV results. Three of seven have been reported in the literature to be found in the virion.\textsuperscript{406} For those not reported to be found in the virion, three of the four are expressed with early kinetics. Thus, with the exception of ORF35, the MHV68 ORFs identified in our screen appear to be present either by being delivered with the virion or expressed at early times to prevent the early steps of signal induction. Despite this, ORF35 does appear to be important somehow because when mutated, it along with ORF44, have shown to be essential to virus replication.\textsuperscript{392, 407}
**Putative exonuclease activity of MHV68 ORF37 may repress global gene expression**

Though not necessarily characterized, all the MHV68 inhibitory ORFs have orthologues in other γ−herpesviruses such as EBV and KSHV. MHV68 ORF37 for example is homologous to EBV BGLF5 and the SOX protein of KSHV. The published literature indicates that EBV BGLF5 and KSHV SOX are DNases involved in processing and packaging of the viral genome and, more importantly, exonucleases which globally inhibit gene expression from the host at early times in the lytic replication cycle. Consistent with this description, cell lines stably expressing ORF37 showed a reduction in p56 expression in response to all inducers used, including intracellular dsRNA activating RIG-I/MDA-5, extracellular dsRNA activating TLR3 and IFN. (Data not shown.) Thus, MHV68 ORF37 may behave like its EBV and KSHV orthologues and globally inhibit mRNA expression such as that of ISG56.

A more in depth analysis of the mechanism of inhibition for MHV68 ORFs 35 and 58 revealed new features of MHV68 evasion of the dsRNA mediated signaling. Similar to mCMV M36, induction of viral stress-inducible genes by dsRNA activating TLR3 and RIG-I but not IFN is blocked by ORFs 35 and 58. (Fig) Thus, ISG56 gene induction by IRF3 and not ISGF3 is inhibited. The point of inhibition is most likely at the step of IRF3 activation because that is where the RIG-I/MDA-5 and TLR3 pathways converge. Similar observations have been made here in Chapter 5 and by others, for example Unterstab et al for Borna disease virus P protein.

**MHV68 ORF58 inhibits IRF3 and NFkB activation**

MHV68 ORF58 inhibits IRF3 and NFkB mediated transcription induced by TLR3 and RIG-I/MDA-5 as well as IRF3 mediated apoptosis induced by RIG-I/MDA-5. Several
scenarios are possible that explain the mechanism of inhibition. One possibility is that like BDV P protein and probably Rubulavirus V proteins, ORF58 competes out IRF3 as substrates for IKKe/TBK1. Similarly, they may compete out IkB as substrate for IKKa/b.

A second possibility is that ORF58 may have stronger affinity for the IKK family members involved, thus preventing physical interaction between kinase and substrate. A third possibility is that ORF58 is acting indirectly through a host negative regulators such as the Ret Finger Protein which interacts with not only the IKK family of kinases including IKKe, TBK1, IKKa and IKKb but also IRF3 itself, allowing for dimerization but not nuclear localization.\(^{144}\) This scenario would explain the relatively normal amount of IRF3 dimerization seen in ORF58 expression cells but inhibition of IRF3 as well as p65 nuclear localization. (Figs 6.5 and 6.7) In addition, pathway leading to AP1 transcription as assayed by P38 or AKT phosphorylation is likely unaffected because IKKs are not involved in its activation as the divergence of the pathways is earlier at TAK1. IKKe kinase activity has been reported to affect a certain subset of genes stimulated by IFNb.\(^ {116}\) However, there is no experimental evidence indicating that ISG56 is a member of this subset. Thus, it is possible that ORF58 may inhibit a subset of IFN induced ISGs by inhibiting IKKe but that this inhibition may not be reflected by ISG56 specifically. Finally, we cannot rule out the possibility that the inhibitory activity of ORF58 may be the result of a non-protein product such as a microRNA. The protein product of ORF58 is membrane bound and expressed in low abundance so is difficult to detect.\(^ {410}\) In our hands, we can detect RNA by Q PCR but not protein expression via an epitope tag. Our assay is capable of detecting siRNA mediated effects directed against IRF3 (Chapter 4). While the coding region of ORF58 does not have any areas predicted
to encode a microRNA, if ORF58 did encode a microRNA that inhibited signaling, its target would most likely not be IRF3 as protein levels are comparable to control cells but rather other molecules such as TBK1 or IKKe.

Whether it be by a RNA silencing mechanism or protein-protein interactions ORF58 inhibits not only the transcriptional activity of IRF3 but also its proapoptotic activity. Sendai virus infection induces cellular apoptosis through IRF3\textsuperscript{23} and this cell death may be a host mechanism for preventing persistent infection. In addition, inhibition of apoptosis prevents the possible dissemination of danger associated molecular patterns (DAMPs) that could be released to potentially prime other cells. Therefore, it would behoove the virus to inhibit the IRF3 mediated apoptotic pathway to prolong the life of the host cell to generate more virus, prevent cross priming of uninfected cells and block IRF3 mediated antiviral gene expression in the infected cell.

ORF58 is non-essential and encodes for a protein predicted to have multiple transmembrane spanning domains. Immunofluorescence detecting protein expressed from a plasmid indicated that it localizes to the ER, Golgi and plasma membrane.\textsuperscript{392, 410} Intriguingly, localization predictions using PSORT indicated that TBK1 (but not IKK\alpha, \beta, e) may also be found in the ER.\textsuperscript{411} More importantly, TBK1 coimmunoprecipitates with Sec proteins of the exocyst, which is involved in vesicle trafficking to the plasma membrane via the Golgi and ER.\textsuperscript{412} Thus, an interaction between ORF58 and TBK1 may occur transiently at the ER leading to inhibition of the kinase whose activity is essential for both transcription and apoptosis. Co-localization and immunoprecipitation studies assaying for interactions between TBK1 and ORF58 would help to determine if this is indeed occurring.
ORF58 is expressed in low abundance.\textsuperscript{410, 413} However, its expression is present at the time of infection because the protein is thought to be incorporated into the virion\textsuperscript{414} and it is transcribed with early kinetics.\textsuperscript{407} ORF58 is essential for the localization of ORF27 to the Golgi and eventually the plasma membrane which is thought to be important for cell to cell spread of the virus.\textsuperscript{415} However, the effect of ORF27 on the localization of ORF58 is not yet clear.\textsuperscript{410} Our data showed that ORF58 inhibits IRF3 activation independent of ORF27. However, expression of ORF27 may change the functional amount of ORF58 available for inhibition. Thus, it is important to address the effect of ORF27 on the ability of ORF58 to inhibit IRF3 activation either in transient assays or in the more relevant viral context with ORF58 deficient virus that still expresses ORF27.

ORF58 deficient virus is only slightly attenuated in the tissue culture system when compared to wildtype- the difference in titres is approximately one log.\textsuperscript{410} More significant effects on viral replication may be observed however in the presence of IRF3 activation such as in an animal model when dsRNA and other components that activate the immune system are present.

**MHV68 ORF35 inhibits IRF3 but augments NFkB activity**

Like ORF58, MHV68 ORF35 inhibits IRF3 activation by TLR3 and RIG-I/mda-5. However NFkB basal and induced activation is augmented by its presence. This inhibition can be either through the inhibition of IRF3 as there is a precedent for IRF3 negatively regulating a subset of NFkB genes or completely independent of IRF3 inhibition for which of course there are also precedents.
Situations in which IRF3 negatively regulate NFkB and vice versa have been previously described. The NFkB induced A20 for instance interacts with TBK1 and IKKe to inhibit IRF3 activation.\textsuperscript{145} It also deubiquitinates Traf6 and degrades RIP1 to inhibit NFkB activity.\textsuperscript{146, 147} Elco et al described how increasing levels of IRF3 inhibited a subset of NFkB induced genes.\textsuperscript{217} This phenomenon is independent of the transcriptional activity of IRF3 since induction is not necessary and deficient IRF3 mutants are also capable of inhibition. In addition, IκB release and p65 phosphorylation appears similar to wildtype. A similar but not identical situation could be occurring here. Production of IL8 as assayed by ELISA was the endpoint to measure NFkB activation for ORF35. (Fig) However, it is unclear in microarray analysis if IL8 is suppressed by IRF3 overexpression.\textsuperscript{217} Thus, analysis of other NFkB induced genes especially those which are more clearly repressed by IRF3, such as A20, is needed. One possible mechanism of IRF3 inhibition and NFkB augmentation is via the glucocorticoid receptor interacting protein GRIP1. GRIP1 augments ISRE mediated gene induction by interacting with IRF3 and through its receptor simultaneously represses NFkB mediated transcription.\textsuperscript{62, 416} If ORF35 somehow decreases the amount of functional GRIP1, then its IRF3 inducing and NFkB repressing effects would be alleviated.

Many examples exist in the literature involving an IRF3 independent scenario for ORF35 mediated augmentation of NFkB activity. EBV LMP1 in latency for instance activates NFkB by being a TNFR homologue.\textsuperscript{417} KSHV K15-P through an unknown mechanism activates NFkB and AP1.\textsuperscript{418} HTLV Tax oncoprotein associates with the IκB kinase,\textsuperscript{419} Cocksackie virus cleaves IκBa,\textsuperscript{420} HSV1 ICP27 induces loss of IκBa and IκBb\textsuperscript{421} and Hepatitis C NS5A phosphorylates IκB.\textsuperscript{422}
One possible rationale for viral activation of NFkB and specifically IL8 may be that in certain circumstances, IL8 inhibits the interferon induced antiviral response. This is what has been suggested for the last example, HCV NS5A. Therefore, ORF35 mediated induction of IL8 may be another way to inhibit the host antiviral activity.

A second possible rationale for viral activation of NFkB and IL8 may be that it is necessary for the purposes of dissemination as discussed earlier. IL8 is traditionally a proinflammatory cytokine that induces the chemotaxis of neutrophils, lymphocytes and basophils. Viruses such as herpesviruses have been documented to activate the immune system using viral homologues of proinflammatory cytokines and chemokines as well as their receptors such as mCMV m131-129 and hCMV UL146 which are chemokine homologues and mCMV M33, 78 and hCMV US28 which are chemokine receptor homologues. ORF35 mediated induction of IL8 may just be a part of this effort to recruit host cells to the site of infection and facilitate viral spread.

Finally, a third possible rationale for ORF35 mediated activation of NFkB may be that it is necessary for the establishment of the latent replication cycle and or the promotion of oncogenesis during this latency. Inhibition of IRF3 and augmentation of NFkB are activities that have attributed to the HPV16 E6 oncoprotein. The two behaviors are thought to be independent- inhibition of IRF3 to suppress the immune response and activation of NFkB to promote the tumorogenesis involved in HPV induced cervical cancers. Like HPV, MHV68 is also associated with oncogenesis such as that in the lymphomas that arise from latently infected B cells. NFkB activity inhibits lytic replication but is absolutely required for viral latency. In this particular instance, the timing of ORF35 expression may be important. Early in the infection, ORF35 is not
present in the virion and not expressed. Thus, NFkB and IRF3 are not active and lytic replication can take place. Late in the cycle, however, ORF35 is expressed. While ORF35 continues repression of IRF3, it augments NFkB activity to facilitate the transition into latency and perhaps promote oncogenesis. ORF35 is essential for viral replication. It would be interesting to determine which if not both of these functions is related to viral fitness.

Clearly any or all of these rationales may account for the different effects seen with expression of MHV68 ORF35. As with all viral ORFs, function and purpose is dependent on host and virus in terms of the time or stage of infection and the stage of replication for the virus. Thus, viral protein functions just like the viruses themselves are ever changing.
CHAPTER 7
DISCUSSION AND PERSPECTIVE

Summary

The previous chapters have presented in detail work regarding the use of a cell survival assay to identify viral inhibitors of dsRNA TLR3 mediated IRF3 activation and then the mechanisms by which these viral inhibitors function. The discussion here begins with some of the unique strengths of the cell survival assay introduced in chapter 4 and used in chapters 5 and 6. The discussion continues with interesting aspects concerning the virus host relationship reflected by the viral inhibitors of signaling and their mechanisms of evasion presented in chapters 5 and 6: the complexity in IRF3 activation and regulation, viral inhibition of TBK1 and IKKe which represent an important point in convergence and divergence of pathways, viral manipulation of the host that is both inhibitory and activating, the bidirectional relationship of the host virus interaction that allows for virus inhibition of the host and vice versa and, lastly, the characteristics of a viral inhibitor of signaling. The discussion ends with practical applications and implications for virus host evolution derived from the work here.

A cell survival assay

Here, we have presented work involving the use and development of cell assays, to study the nature of viral inhibitors of the immune response as well as the nature of the host response to viruses and viral evasion. Our cell line featuring cell death as an inducible phenotype represents one of the easiest methods to systematically assay for inhibition of ISRE mediated signaling. Its strengths lie in 1.) the phenotype which is easily detected in an assay or a screen, 2.) the 293 parental cell line is highly
transfectable, infectable and do not respond apoptotically to many stimuli, thus allowing
the cell death phenotype to be specifically reflect signaling; and finally 3.) the promoter
driving the reporter gene is not basally active but is induced highly by many different
signals that converge onto the IRF family of transcription factors. Using this assay
system, we were able to assess the inhibitory (and activating) potential of a variety of
ORFs on dsRNA TLR3 signaling. While none of the ORFs we tested were previously
described in the literature to inhibit IRF3 activation specifically, there were indications
the some could inhibit signaling. We went on to further characterize the nature of
inhibition for several of these ORFs and discovered surprising aspects regarding the host
virus relationship.

**IRF3: complex regulation by phosphorylation**

First, IRF3 activation is complex. Unlike other transcription factors such as the
p65 subunit of NFkB or the Fos or Jun subunits of AP1, simply overexpressing
IRF3 is insufficient for activation. Thus, many post translational mechanisms of control
exist which have yet to be fully elucidated. The lack of congruence between Ser396
phosphorylation and IRF3 dimerization seen in the cases of the Paramyxoviral V proteins
or MHV68 ORFs 35 and 58 expression indicate that the currently accepted model of
IRF3 activation: phosphorylation, followed by dimerization and then nuclear localization-
is inaccurate. Our data indicate that 396 phosphorylation is not required for
dimerization. This is supported in part by other observations within the literature. It
is likely that phosphorylation or other modifications such as acetylation or methylation at
this or other residues is instead required.
**TBK1/KKe represents an important point of convergence and divergence**

Second, TBK1 and IKKe appear to be popular targets for viral inhibition of signaling. Both the interaction shown by coimmunoprecipitation between V proteins and IKKe or TBK1 as well as the preliminary studies examining signaling pathway inhibition by MHV68 ORFs 35 and 58 indicate that they inhibit at this level of the signaling cascade. This point of regulation is also popular with other viruses. As noted previously, the P proteins from Borna disease virus, Rabies, Ebola, and the PLpro of Severe acute respiratory syndrome coronavirus all appear to inhibit at this step within the pathway.163-166 TBK1/IKKe also represents a key point in the host regulation of signaling via multiple proteins including SIKE, SHP2, RFP and A20.142-145 The manipulation of this checkpoint by both host and virus may be due to either the fact that many pathways both converge as well as diverge from TBK1 and IKKe. TBK1 and IKKe are known to act in the TLR3, RIG-I, mda-5, TLR4, DAI and IFN signaling pathways. Thus, they have functional implications in a variety of immune regulatory responses. In addition, both proteins have been shown to be involved in the promotion of solid cancers and lymphomas.412, 426 Finally, mice lacking IKKe are hypersusceptible to influenza infection and mice lacking TBK1 die in utero from massive liver degeneration.116, 427, 428 Therefore, TBK1 and IKKe, the non canonical members of the IKK family, are responsible for a wide variety of functions which are reflected phenotypically.

Although TBK1 and IKKe are considered almost synonymous in the literature, it is clear that they are not functionally identical. TBK1 is basally expressed while IKKe expression is more induced. Furthermore, basal expressions of TBK1 and IKKe seem to complement each other. IKKe is expressed more in immune cells and organs such as the
spleen, thymus and peripheral blood leukocytes while TBK1 is expressed in other
tissues. \(^{429, 430}\) Finally, deleting TBK1 in mice is lethal while the same for IKKe gives a
much more subtle phenotype with influenza infection. \(^{427, 428}\) It is clear from these
phenotypes that the two kinases are not completely functionally redundant. It is curious
then how these two kinases are so different when structurally they both contain similar
domains (ubiquitin like domains, kinase domains, coiled coiled domains) and
functionally in terms of their kinase abilities, they appear to be able to act on common
substrates. Identifying interacting partners and other possible functions would help in
discriminating between these two non-canonical IKKs.

Intriguingly, the IKK family is well conserved throughout all eukaryotes from
vertebrates such as Homo sapiens to plants such as Arabadopsis thaliana to amphibians
such as Xenopus laevis to pathogenic protozoa such as Trichomonas vaginalis. \(^{431, 432}\)
Drosophila encodes two members of the IKK family: Dmikkb/ird5 and ik2. Dmikkb/ird5
phosphorylates a Drosophila homologue to NFkB, relish, the host response to bacterial
infection. \(^ {433}\) ik2 is most closely related to TBK1 and IKKe and its kinase domain is
essential for viability. It is characterized with in NFkB independent role in mRNA
localization for dorsal ventral patterning during oogenesis. This is based on regulation of
microtubules in the oocyte cortex involved in actin assembly and cell structure. \(^ {434}\) Thus,
in addition to their more documented functions in immunity and cancer, human TBK1
and IKKe activation may potentially have additional undocumented impacts. We do not
know if viral proteins that can inhibit the kinase activity of TBK1 and IKKe directed
towards IRF3 can inhibit other functions of TBK1 and IKKe. We do not even know if
TBK1 or IKKe may have non-kinase related activities, although a function within the
ubiquitin utilization pathways is implicated by their ubiquitin like domains and their role in K63 Ub of TANK. Again, identifying more interacting partners with these kinases as well as the modifications which they undergo would then be an important step in elucidating regulation of TBK1/IKe and potential novel non-kinase functions. How viruses would interact in these capacities would then shed more light on the importance of TBK1/IKe as a convergence point.

There are numerous examples of viral proteins inhibiting at levels of signaling that are not TBK1 and IKe such as HCV NS34A cleaving TRIF. Interestingly, however, NS34A appeared to inhibit signaling only weakly in our cell death assay compared to V proteins and MHV68 ORFs. Therefore, by selecting for the strongest inhibitors to further characterize, we may have biased our group of proteins towards inhibitors of TBK1 and IKe. Nevertheless, these inhibitors still represent an important aspect of pathogen control and immune evasion.

**Viral activation and inhibition of host signaling pathways**

Third, viral manipulation of the host is both inhibitory and activating. Numerous examples from the literature describe viral homologues of host proteins that act to stimulate the immune system for the proposed purposes of dissemination or viral replication that requires host genes. Here, ORF35 represents an MHV68 gene that both inhibits IRF3 and augments NFκB activation. There are several rationales for this which are not mutually exclusive. One conjecture that they can have different functions-inhibition of IRF3 evade the host immune system and stimulation of NFκB to aid in the entry of MHV68 into latency and/or to promote tumorogenesis. A similar situation is documented with the HPV16 E6 oncoprotein which inhibits IRF3 signaling but activates
Another explanation is that activation of NFkB and inhibition of IRF3 induces some but not all proinflammatory cytokines and this is necessary to recruit host cells to aid the dissemination of the virus. Finally, activation of IL8, an NFkB driven gene, may inhibit IFN mediated antiviral environment. Thus, ORF35 within MHV68 may in part reflect the disparate needs of the virus to survive in the host and how it may go about meeting them with a single protein.

The need to be able to activate specific pathways for viral persistence may be one reason why viral or host inhibition of the other is never really complete. In the evolution between host and virus, room for adaptation is necessary. In some circumstances, the benefits from activation of signaling by viruses may outweigh the negative effects resulting from stimulation of the host immune response. Similarly the disadvantages of mounting too strong an immune response that may eventually kill the host may outweigh the advantages of complete viral clearance. Therefore viral and host regulation of each other reflects the homeostasis of a relationship which is ever evolving.

**A single point of viral host interaction can be both inhibitory and activating**

An alternative explanation for incomplete inhibition may be that the host and virus uses the same point of interaction to oppose the other. Therefore, virus host interactions are not necessarily completely in favor of either the virus or the host. We showed that V protein interaction with TBK1 and IKKe results in inhibition of IRF3 activation by ubiquitination of the kinases or acting as alternative substrates for them. We also showed that TBK1 and IKKe mediated phosphorylation of V, just as in IRF3, results in its degradation. Therefore, V protein inhibition of signaling may be an active viral mechanism of host evasion and TBK1/IKKe phosphorylation of V may be an active host...
mechanism to suppress the virus. The literature describes a similar relationship between the host antiviral protein PKR and Hepatitis C virus (HCV). PKR can inhibit HCV IRES mediated translation by phosphorylating the host eukaryotic initiation factor (eIF2). HCV via its own internal ribosomal entry site (IRES) can inhibit dsRNA activation of PKR by competing for the activation site itself and or mediating interaction between viral non structural protein NS5A and PKR that also inhibits the ability of PKR to be activated by dsRNA. Thus, while it is difficult to prove, the relationship between PKR and the HCV genome, just like that between TBK1/IKKe and V, may be one which is exploited by both virus and host to fight the other. Since the protein product of human ISG56, P56, also interacts with the HCV ribosome RNA complex to inhibit IRES mediated translation like PKR, a similar relationship between P56 and HCV is possible. Finally, it would be interesting to see if the yet to be elucidated molecular mechanisms by which MHV68 ORFs 35 and 58 inhibit signaling are also exploited by the host to inhibit the virus.

**Viral evasion at multiple points within the viral replication cycle**

Fifth and finally, the results presented here in part address the nature of a viral inhibitor of signaling. Paramyxoviral V proteins described here have characteristics which are thought to be important to all viral inhibitors of signaling. They are expressed in large amounts at the beginning of the viral infection cycle. Rubulavirus V proteins are expressed highly as part of the virion. In addition, most but not all V proteins are considered accessory proteins whose functions are not required in viral replication per se but are necessary to interact with a potentially negative environment. However, as exemplified by the MHV68 ORFs, these characteristics do not necessarily apply to all
viral inhibitors of signaling. Unlike V proteins, MHV68 ORF58 is expressed at extremely low levels. However, it is present in the virion. Perhaps this small amount is sufficient for the inhibition of signaling at the initial stage of infection. In contrast, MHV68 ORF35 is neither within the virion nor expressed with early kinetics. Thus, it is not present when signaling is initiated. However, it may still play an important role later in the infection when possibly the initial viral inhibitors of signaling such as ORF58 are degraded, occupied with other functions such as manipulation of the host cytoskeleton to aid in cell to cell viral spread or when the amount of signaling simply becomes overwhelming.

Finally, viral evasion molecules are not just accessory proteins that are dispensable when the virus is grown in a permissive environment. Like mCMV US22 genes m139-m143 and M36, hCMV UL122 and MHV68 ORF44 identified in our assay, MHV68 ORF35 is essential for virus replication. Indeed, V proteins specifically for Rubulaviruses also appear to be essential as a complete knockout has not been successfully achieved. The reason for this has been speculated to be that Rubulavirus V proteins may also function in viral replication. However, the nature of this function is unknown. Alternatively, Rubulavirus V proteins may be essential because inhibition of innate immune signaling is much more important than one may first imagine. Indeed, it is clearer now that like many host signaling proteins, a single viral evasion molecule encodes for many different functions. The Paramyxoviral V proteins inhibits IFN, mda-5 and TLR3 signaling with at least four different targets, STAT1, mda-5, TBK1 and IKKe. MHV68 ORF35 may be functioning in two different mechanisms to inhibit IRF3 and augment NFkB activation. Finally, MHV68 ORF58 blocks not only IRF3 activation but also apoptosis. Thus, the nature of a viral inhibitor of signaling is as diverse as the
mechanisms by which they function and in this way viruses may encode for magnitudes more of host inhibition then previously imagined.

**Practical applications**

The identification of viral inhibitors of signaling and their mechanisms of action have many practical as well as theoretical implications. Work on the influenza NS1 protein is an excellent example of how understanding the molecular basis for viral pathogenesis may have therapeutic impacts. NS1 has been shown to be able to inhibit both innate and adaptive immunity via a variety of different mechanisms.\textsuperscript{439} This has been shown to be in part responsible for the extensive pathology seen in the 1918 variant of influenza.\textsuperscript{440} An understanding of the mechanisms of inhibition and relative importance to viral replication has thus lead to the identification of inhibitory drugs which may be used for treatment\textsuperscript{255} and also the establishment of attenuated viral strains for vaccine purposes.\textsuperscript{441} Such strategies may be applied to other viruses and pathogens as well for which we do not yet have effective therapy or means of prevention.

**Evolution of the virus host relationship**

Furthermore, the study of virus host interactions serves to help us better understand the evolution of both organisms. It is interesting that the immune system has not evolved to completely extinguish pathogens and that the successful pathogens have not evolved to completely exterminate their hosts. Indeed, it is thought that viruses such as human immunodeficiency virus (HIV) with more persistent infection courses, able to evade and/or downregulate the immune response, are more adapted for survival as compared to those viruses such as Ebola which replicate prolifically resulting in a strong enough immune response to kill the host. In fact, these highly pathogenic viruses only
survive because of a reservoir of host species which do not react as strongly and thus can support persistent infection.

This viral persistence can be in certain circumstances beneficial to the host. Infection with MHV68 and CMV, two DNA viruses which have co-evolved with their hosts for over 40 million years, appears to protect the host against other bacterial infections by constitutively stimulating a low level of IFN expression.\textsuperscript{442} Extending one step beyond this, polydnaviruses exhibit an obligate symbiotic relationship with their Hymenoptera hosts, the wasps. These viruses are transmitted vertically in a Mendelian fashion from mother to progeny instead of horizontally and their genomes exist in both integrated and episomal forms. The wasp host is itself parasitic during part of its life cycle in Lepidoptera, caterpillars. For the wasp, polydnaviruses play an essential role in development because they produce factors necessary to inhibit the caterpillar immune response to the invading parasite. Thus, in this example, not only is the host absolutely essential for viral replication and persistence but the virus is essential for the propagation of the host.\textsuperscript{443} Finally, the mitochondria represents the most classic example of the co-evolution of pathogen and host. From the invasion of a proteobacterium-like ancestor with oxidative capabilities followed by the genome reduction of the bacterium and its subsequent dependence on the host arise the mitochondria and chloroplast organelles that define eukaryotes today.\textsuperscript{444} Perhaps 40 million years from now we will see that CMV and MHV68 are no longer considered pathogens but rather virus like genetic elements that aid in the regulation of the immune system.

Eukaryotic life is thought to have originated about 2.5 billion years ago (2.5 bya). Components of the innate immune system are thought to have arisen approximately 1.5
bya with protists and single celled organisms and that of the adaptive 500 million years ago (mya) with the diversification of jawless fishes. One of the events that is hypothesized to be essential for the rise of the adaptive immune system is the invasion of an immunoglobulin like gene by a transposable element. This then facilitates recombination which generates the diversity that today characterizes the antibody and T cell receptor genes. The recombination activating genes (RAG) which are responsible for recombination have functional and genetic similarities to transposons, retroviruses and recombinases from bacteria and phages. It would be ironic if viral or bacterial elements 500 mya somehow evolved within the host to become the essential recombination component of the adaptive immune system that fights off infections today.
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