ABSTRACT

Stewart, Michael J. PKR, Myocarditis and the Cardiac Response to Reovirus Infection. (Under the direction of Dr. Barbara Sherry).

Viral myocarditis is an important human disease associated with many viruses. Mechanistically, cardiac damage associated with viral myocarditis can be immune mediated and/or the result of direct cytopathic effect. Reovirus induced myocarditis is not immune mediated, and thus, provides an excellent model for the study of direct cytopathic effect in the heart. Previous work has demonstrated that reovirus myocarditic potential reflects induction and/or sensitivity to interferon (IFN). Specifically, nonmyocarditic reoviruses induce greater IFN-β and/or are more sensitive to the antiviral effects IFN than are myocarditic reoviruses. Importantly, IFN mediates its antiviral effects through the induction of interferon-stimulated genes (ISGs); ISGs function as the antiviral effector proteins that block replication in the host cell.

The work presented in this dissertation further defines the cardiac response to reovirus infection. In chapter 2, we examined the double-stranded RNA activated protein kinase PKR: the role of PKR in cardiac IFN induction and protection against reovirus-induced myocarditis. We demonstrate that PKR is critical to the robust induction of IFN-β in primary cardiac myocyte cultures (PCMCs). Additionally, we show that nonmyocarditic reoviruses become myocarditic in PKR-null mice, even though reovirus growth in PKR-null hearts is similar to that in wild type mouse hearts. Finally, we demonstrate that relative to wild type mice, reoviruses induce significantly greater morbidity in PKR-null mice.
In the following chapter, we compare the IFN response in PCMCs: a non-replenished, critical cell type, to that in primary cardiac fibroblast cultures (PCFCs): a readily replenished cardiac cell type. By quantitative real-time PCR, we find that PCMCs express IFN-β and the ISGs IRF-7 and 561 constitutively at higher levels than PCFCs. We also identify constitutive IFN as a primary means of constitutive ISG expression. Additionally, we demonstrate that PCFCs, more so than PCMCs are more dependent on IFN-mediated protection against reovirus infection.

The regulation of IFN-β expression is achieved primarily through the actions of Interferon Regulatory Factors (IRFs). In Appendix 1, we show contributions to a publication demonstrating that IRF-1 is dispensable for IFN-β induction in PCMCs, and yet critical for defense against reovirus-induced myocarditis in the mouse. Finally, a summary of this work is provided.
PKR, MYOCARDITIS AND THE CARDIAC RESPONSE TO REOVIRUS INFECTION

by

MICHAEL JUDE STEWART

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

MICROBIOLOGY

Raleigh

2003

APPROVED BY:

__________________________       ____________________________
Chair of Advisory Committee
DEDICATION:

I would like to dedicate this dissertation to the people I love the most. My parents, whose love and support has been a constant, and without whom, this work would not have been possible. My brother Tom, for always being there when I needed him. My brother Robert, for teaching me what is truly important in life. To all my friends, for helping me forget my troubles and keep my perspective.
PERSONAL BIOGRAPHY

Michael Jude Stewart was born in Amityville, New York on March 1st, 1976. He lived in various towns on Long Island until he graduated from Eastport High School in 1994. After graduation, he moved to Rocky Mount, North Carolina to begin his undergraduate studies at North Carolina Wesleyan College. In 1998, he graduated from NCWC with a Bachelors of Science of Mathematics. Upon graduation, Michael moved back to Long Island to begin graduate work in the Department of Pharmacology at Stony Brook University. After two semesters at Stony Brook, Michael realized that Long Island was no longer for him, and returned to North Carolina. In 1999, Michael began graduate work at North Carolina State University in the department of Microbiology. Here, he joined the laboratory of Dr. Barbara Sherry and studied the cardiac response to viral infection. Upon completion of this dissertation, Michael will continue his training as a postdoctoral scientist.
ACKNOWLEDGEMENTS

I’d like to acknowledge the following people for their support of this work:

Dr. Barbara Sherry, for her support and expertise throughout this project, and most importantly, for her acceptance of my gambling “itch”. MA Blum, for all her assistance in and out of the laboratory as well as for introducing me to the wonders of caffeine. To my “Big Sister” Kathleen Azzam-Smoak, for always being there when I needed her, whether inside the lab or out. Thanks to all members of the Sherry lab, past and present, without all of you, this work could not have been done.

I’d also like to thank the members of my committee: Dr. Scott Laster, Dr. Fred Fuller, and Dr. Tim Petty. Your insight and guidance has been very much appreciated. Special thanks to Dr. Scott Laster, for being my mentor in the Preparing the Professoriate program. I’d also like to thank the Microbiology Department and the members of the Pylon Research facilities for their support.

Finally, I’d like to thank “Amore Pizza”. You have the best pizza in town, and easily have provided me with 50% of my graduate school lunches.
TABLE OF CONTENTS:

<table>
<thead>
<tr>
<th>List of Tables</th>
<th>vii</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
</tbody>
</table>

CHAPTER 1. LITERATURE REVIEW:

Reovirus 1
Myocarditis 3
Myocarditis and Interferon 6
IFN-β Gene Expression Regulation 8
IRF-1 Regulation of IFN-β 9
IRF-2 Regulation of IFN-β 10
IRF-3 Regulation of IFN-β 11
IRF-7 Regulation of IFN-β 13
IRF-9 Regulation of IFN-β 15

Interferon Stimulated Genes:

PKR 15
561 19
IRF regulation of ISGs 21
Negative Regulation of ISGs: SOCS 23
Summary 24
References Cited 27

CHAPTER 2. PKR’S PROTECTIVE ROLE IN VIRAL MYOCARDITIS 49

Abstract 50
Introduction 51
Results 53
Discussion 56
Materials and Methods 60
References Cited 65
CHAPTER 3. CONSTITUTIVE AND REOVIRUS-INDUCED INTERFERON-β AND IFN-β STIMULATED GENE EXPRESSION ARE CELL TYPE SPECIFIC IN THE CARDIAC PROTECTIVE RESPONSE

Abstract 79
Introduction 81
Materials and Methods 84
Results 89
Discussion 94
References Cited 99

APPENDICES

APPENDIX 1. INTERFERON REGULATORY FACTOR-1, INTERFERON-β AND REOVIRUS-INDUCED MYOCARDITIS 117

Abstract 118
Materials and Methods 119
References Cited 123

SUMMARY 126
LIST OF TABLES

CHAPTER 2. PKR’S PROTECTIVE ROLE IN VIRAL MYOCARDITIS

TABLE 1. Survival of reovirus infected mice 77
LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

FIGURE 1. Signaling through the JAK/STAT pathway 43
FIGURE 2. Formation of the IFN-β enhanceosome 44
FIGURE 3. IRF-3 induction and IRF-7 amplification of IFN-β 45
FIGURE 4. PKR’s antiviral activity 47
FIGURE 5. Regulation of IFN-β and ISGs 48

CHAPTER 2: PKR’S PROTECTIVE ROLE IN VIRAL MYOCARDITIS

FIGURE 1. PKR is critical for induction of IFN-β in PCMCs 73
FIGURE 2. Viral induction of cardiac lesions 74
FIGURE 3. Reovirus induction of myocarditis 75
FIGURE 4. Increased myocarditis does not reflect greater replication in the hearts of PKR-null mice 76

CHAPTER 3: CONSTITUTIVE AND REOVIRUS-INDUCED INTERFERON-β AND IFN-β STIMULATED GENE EXPRESSION ARE CELL-TYPE SPECIFIC IN THE CARDIAC PROTECTIVE RESPONSE

FIGURE 1. Constitutive and induced IFN-β is greater in PCMCs than in PCFCs 106
FIGURE 2. IRF-7 expression in PCMCs and PCFCs 108
FIGURE 3. 561 expression in PCMCs and PCFCs 110
FIGURE 4. IFN-α/β treatment of PCMCs and PCFCs induces IRF-7 expression similar to reovirus T3D 111
FIGURE 5. IFN-α/β treatment of PCMCs and PCFCs induces 561 expression similar to reovirus T3D 112
FIGURE 6. PCFCs are more dependent on the IFN response than PCMCs 114
FIGURE 7. Reovirus directly induces 561 to similar extents in PCMCs and PCFCs 115
FIGURE 8. Constitutive IFN is a determinant of constitutive ISG expression 116

APPENDIX 1: INTERFERON REGULATORY FACTOR-1, INTERFERON-β, AND REOVIRUS-INDUCED MYOCARDITIS

FIGURE 1. Reovirus T3D induces pβlux in 129Sv/Ev and C57BL/6J derived primary cardiac myocyte cultures 124
FIGURE 2. IRF-1 can protect against viral myocarditis 125
SUMMARY:

FIGURE 1. Proposed mechanisms of cardiac protection 129
CHAPTER 1
LITERATURE REVIEW:

Reovirus:

The early observation that reovirus genomic RNA was resistant to the single-stranded specific nuclease S1 suggested that reovirus genomic RNA was not only double stranded, but also collinear and complimentary (104, 152), i.e. the reovirus genome is fully double stranded. These observations were later confirmed by X-ray diffraction and genome base experiments that revealed isolated genomic RNA adopted a right-handed double helix (2) and had a G/C and A/U ratio of one (12).

The reovirus genome consists of ten dsRNA segments classified as small (S) medium (M) and large (L) based upon their migration on a polyacrylamide gel (152). The small gene segments (S1-S4) encode three structural proteins (σ1, σ2, and σ3) and two nonstructural proteins (σ1s and μNS). The medium gene segments (M1-M3) encode two structural proteins (μ1 and μ2) and one nonstructural protein (μNS). The large gene segments (L1-L3) encode three structural proteins that include the RNA-dependent RNA polymerase (L1) and a guanylyltransferase (L2). Interestingly, all reovirus genes are monocistronic with the exception of S1, which is dicistronic; S1 encodes the cell attachment protein σ1 and as a result of a frameshift, a small nonstructural protein with unknown function.

The reovirus genome is enclosed by two concentric icosahedral capsids. The outermost capsid primarily consists of 200 heterohexameric complexes of the penetration protein μ1 and its protector protein σ3 (19, 85). Additionally, the outermost capsid contains the cell attachment protein σ1 as well as the λ2 guanylyltransferase. The
innermost capsid contains the RNA dependent RNA polymerase $\lambda_3$, as well as $\lambda_1$ and $\mu_2$, which also have roles in viral transcription. Additionally, the innermost capsid contains the dsRNA binding protein $\sigma_2$. Together, the innermost capsid proteins and the dsRNA genome comprise the transcriptionally active viral core.

Reovirus entry into the host cell is a multistep process involving successive proteolytic degradation of viral proteins (reviewed by in (19)). After attaching to a target cell via $\sigma_1$ interactions with a receptor such as JAM and/or cell surface sialic acid (8, 9, 27), reovirus enters the target cell by endocytosis. In the endocytic vesicle, $\sigma_3$ is degraded by host acid dependent proteases such as Cathepsin B and L (42). This proteolytic degradation converts the reovirus virion into an intermediate subvirion particle (ISVP). Within the endocytic vesicle, the reovirus penetration protein $\mu_1$ undergoes a rearrangement exposing a protease-sensitive, myristlyated hydrophobic molecule required for endocytic membrane penetration (19). Also during penetration, $\mu_1$ conformational changes/cleavages are accompanied by the loss of $\sigma_1$. The process of penetration results in the formation of transcriptionally active cores in the cytoplasm of the host cell that lack $\sigma_1$, $\sigma_3$ and $\mu_1$.

Mammalian reoviruses can infect a broad range of hosts including humans, but reovirus induced disease is restricted to the very young (45, 152). The most common route of reovirus infection is fecal/oral and the type of pathogenesis induced by the virus is dependent upon serotype. For instance, after oral inoculation of newborn mice, both reovirus T1 Lang (T1L) and T3 Dearing (T3D) are taken up by intestinal M cells (161) and undergo primary replication in lymphoid tissue of the Peyers Patches; but while reovirus T1L spreads to the central nervous system and infects ependymal cells, reovirus
T3D spreads to the central nervous system, infects neurons, and causes a lethal encephalitis. Analysis of T1L X T3D reassortant viruses revealed that neuronal tropism segregated with the viral attachment protein σ1 (81, 158). Interestingly, the S1 gene also segregates with differential pathogenesis in the pancreas; while T1L damages the islet cells resulting in the production of autoantibodies to insulin, T3D directly infects the insulin producing beta cells (108, 109). In addition to encephalitis and pancreatic dysfunction, reoviruses can induce pathology in a variety of organs including the heart, liver, intestine, and lungs.

**Myocarditis:**

Viral myocarditis is an important human disease affecting 5-20% of the human population (162). Viral myocarditis can be fatal in infants as well as children (41), and although usually resolved in older individuals, can progress to chronic myocarditis and/or dilated cardiomyopathy and cardiac failure (17, 69, 101). Extreme cases of viral myocarditis require mechanical circulatory support and/or transplantation (41).

Enteroviruses and adenoviruses account for the majority of human viral myocarditis cases (94). Enterovirus-induced myocarditis is predominantly immune mediated (28, 124); the intensity of the inflammatory response correlates well with disease. However, enteroviruses are directly cytopathic in murine cardiac myocytes and can induce myocarditis in mice with severe combined immunodeficiency (SCID mice) (25). Furthermore, clinical trials showed that immunosuppressive agents were largely ineffective as a treatment for enterovirus associated myocarditis (95).

In addition to enteroviruses, many other viruses have been implicated in the disease. Adenovirus induced myocarditis is poorly understood, but is not believed to be
immune mediated (94). Likewise, the degree of cardiac damage in human immunodeficiency virus (HIV) associated myocarditis correlates poorly with the inflammatory response (32). Thus, given that many viruses have access to the heart, and that cardiac myocytes are not replenished, understanding the mechanisms by which viruses directly damage the heart will only further our understanding of viral myocarditis and its potential treatments.

To date, all evidence suggests that reovirus-induced murine myocarditis is not immune mediated. Widespread destruction of cardiac tissue occurs in the absence of a robust inflammatory response (52, 138, 143). In fact, reovirus can induce myocarditis in both nude (138) and SCID mice (137). Furthermore, passive transfer of virus specific antibodies is protective; specifically, prior to challenge, passive transfer of monoclonal antibodies against capsid proteins on the highly myocarditic reovirus 8B (136) prevents myocarditis in neonatal mice (137).

Similar to the humoral response, the cellular arm of the immune system was also demonstrated to be protective against reovirus-induced myocarditis. While naïve spleen cells do not, passive transfer of reovirus-immune spleen cells (prior to challenge) protected neonatal mice from 8B-induced myocarditis (137). Additionally, passive transfer of reovirus immune spleen cells depleted of CD4$^+$ or CD8$^+$ T cells provided protection against subsequent viral challenge (137). Thus, both T cell subtypes are able to provide independent protection against reovirus-induced cardiac damage.

Consistent with a model of direct cytopathic effect (CPE), reovirus-induced CPE in cardiac myocytes, but not cardiac fibroblasts correlates with viral myocarditic potential (10). Moreover, 8B antigen co-localizes with areas of cardiac damage in vivo (35, 138).
Recently, reovirus induced myocarditis was demonstrated to reflect virus-induced apoptosis of cardiac cells (35). Indeed, if a protease inhibitor was used to block the cysteine protease calpain, a known inducer of apoptosis in the heart (23), 8B-induced myocarditis was dramatically attenuated in neonatal mice (35). Moreover, treated neonates had reduced levels of serum creatine phosphokinase: a marker for cardiac injury (35). Finally, treated animals experienced better weight gain than their non-treated counterparts; thus suggesting the use of calpain inhibitors as a potential therapy for myocarditis.

Currently, there exists no reverse genetics system for reovirus. However, since the reovirus genome consists of ten independent dsRNA gene segments, reassortant viruses can be generated via co-infection with multiple reovirus strains. These reassortant viruses can then be used to map a particular phenotype to a reovirus gene segment. Utilizing this approach, reovirus myocarditic potential was mapped to the M1 and L2 genes (135, 136). M1 encodes μ2: a core protein whose function is not completely understood. However, μ2 is an RNA binding protein (18), as well as a microtubule-associated protein (MAP) (111). Moreover, recent evidence suggests μ2 to have a key role in both the formation, (111) and rate of formation (98) of viral inclusion bodies. L2 encodes the viral guanylyltransferase protein λ2. This core protein forms a hollow channel from the viral core to the host cytoplasm; as newly synthesized RNAs are extruded through this channel they are given a 5’ cap (120). In addition to M1 and L2, if one controls for the contribution of M1, the L1 gene is also implicated as having a role in reovirus induced myocarditis (135). L1 encodes the viral RNA-dependent RNA polymerase and thus, has a critical role in viral RNA synthesis. Interestingly, M1, L1,
and L2 are all reovirus core proteins and each has been demonstrated to have a role in RNA synthesis.

Given that core proteins were demonstrated to be determinants of reovirus-induced myocarditis, one could surmise that quantity of RNA produced during a cardiac infection would also be predictor of myocarditic potential. Indeed this is the case. Northern blotting analysis revealed that those viruses with the greatest myocarditic potential also had the greatest positive strand, and positive strand to negative strand ratio (indicator of RNA synthesis rate) for RNA synthesis in primary cardiac myocyte cultures (134). Interestingly, while viral RNA synthesis correlated with myocarditic potential, generation of infectious virus did not; that is, in single round replication studies both myocarditic and nonmyocarditic reoviruses grew to similar titers in primary cardiac myocyte cultures (134). Moreover, these results indicated that viral RNA synthesis does not correlate with the quantity of infectious virus produced (134); thus implicating some other aspect of RNA synthesis in determining myocarditic potential.

Myocarditis and Interferon:

Viral infection induces the production of proteins known as interferons. These cytokines were named for their ability to interfere with viral replication in previously uninfected cell cultures (66). There are two categories of interferons (IFNs): Type I IFN and Type II IFN (66). Type I IFNs include IFN-α and IFN-β; IFN-α is produced mainly by dendritic cells while IFN-β is produced by numerous virally infected cell types (80). They bind a common cellular receptor: the IFNα/β receptor. In addition to viral infection, Type I IFN can also be produced in response to dsRNA and lipopolysaccharide
(LPS (22, 150)). In contrast, Type II IFN or IFN-γ, binds the IFN-γ receptor and is only produced by activated natural killer (NK) and T cells (15, 84, 116, 130).

Interestingly, IFNs also function as mediators of apoptosis. Treatment of cells with IFN-α inhibits cell cycle kinases (78). This results in the accumulation of non-phosphorylated retinoblastoma protein (pRB) (78). In its non-phosphorylated state, RB protein sequesters cell cycle transcription factors such as E2F, thereby preventing cell cycle progression (22, 78). In many cell types, lack of progression through the cell cycle is a trigger for apoptosis. In addition to roles in apoptosis and innate immunity, IFNs have also been demonstrated to have cell differentiative and anti-angiogenic effects (144).

Type I IFN binding to the IFNα/β receptor ultimately results in the upregulation of genes termed interferon stimulated genes (ISGs; Figure 1). Using microarray technology, more than 300 genes have been identified as ISGs (22, 34, 37). Examples of these ISGs and their mechanism of induction will be discussed in detail later in this chapter. Classical ISGs include the dsRNA protein kinase PKR, 2’-5’ oligo “A” synthetatse, and Mx family GTPases. ISGs such as these are the antiviral effector proteins that function to stop viral replication in the infected cell. Furthermore, the induction of ISGs in neighboring, uninfected cells limits viral spread by rendering these cells inhospitable to viral replication.

In primary cardiac myocyte cultures (PCMCs), reovirus induction of, and sensitivity to, IFN-β correlates with viral myocarditic potential. Specifically, nonmyocarditic reoviruses induce more IFN-β and/or are more sensitive to the antiviral effects (mediated through ISGs) of IFN-β than are myocarditic reoviruses (139).
Furthermore, reovirus spread through PCMCs was found to be IFN-β dependent and to correlate with their myocarditic potential (139). The importance of IFN as a determinant of myocarditic potential was also demonstrated invivo: a nonmyocarditic reovirus became myocarditic when neonatal mice were pretreated with anti-IFN antibody, but did not in the presence of control antibody (139). Similar to myocarditic potential, induction of IFN correlated with the M1 and L2 reovirus gene segments. However, unlike myocarditic potential, IFN induction also correlated with the S2 gene; S2 encodes an abundant inner core protein with demonstrated dsRNA binding ability (152).

Recently, IFN-β has been demonstrated to be a powerful therapy against persistent viral infection in the heart (76). In a phase two clinical trial, 22 patients with left ventricular dysfunction that tested positive for adenovirus or enterovirus genomes were treated with IFN-β for 24 weeks; remarkably, viral clearance from the heart was observed in 22 of 22 patients (76). Furthermore, improved left ventricular function was observed in 15 of 22 patients. These results are in accordance with earlier reports demonstrating interferon-α (IFN-α) and thymic agents that increase endogenous IFN levels and stimulate natural killer and T-cell activities can decrease viral load, improve cardiac function, and increase survival rates in patients with viral myocarditis (29, 59, 99, 100, 145)

**Interferon-β Gene Expression Regulation:**

The regulatory region of the IFN-β gene promoter contains multiple positive regulatory domains (PRDs) that bind a variety of transcription factors (40, 49, 63). Specifically, following viral infection, a large nucleoprotein complex termed the enhanceosome forms (151). The enhanceosome includes the transcription factors NF-κB,
ATF-2/c-Jun, and interferon regulatory factors (IRFs) bound to PRDII, PRDIV, and PRDI-III respectively (165). Similarly, the IFN-β gene promoter contains multiple AT rich regions that allow binding of high mobility group proteins (HMG).

The formation of a functional enhanceosome is a coordinated, multistep process (Figure 2). Following viral infection, HMG I binds PRDII and PRDIV and initiates a chromatin restructuring that allows the binding of NFκB and ATF-2 (40). Moreover, both ATF-2 and NFκB physically interact with HMG-I to facilitate the formation of the enhanceosome complex (165). Similarly, IRF-1 and IRF-3 are recruited to the enhanceosome and interact with HMG-I. The creation of this large, multimeric protein complex finally creates a docking site for CREB binding protein (CBP), CREB associated proteins such as PCAF (157, 166), and the polII holoenzyme (165). Histone acetylase activity of CBP then stimulates the initiation of transcription from the IFN-β promoter.

In addition to initiating IFN-β transcription, CBP is also critical to the disassembly of the enhanceosome complex. Once transcription is complete, CBP, but not PCAF acetylation of HMG I results in the disassembly of the enhanceosome (102, 103). This disassembly ultimately results in the abrogation of IFN-β transcription.

IRF-1 Regulation of IFN-β:

Interferon regulatory factors (IRFs) were so named because they could regulate transcription from the IFN-β gene. Currently, nine human IRFs and three Kaposi’s sarcoma-associated herpesvirus encoded IRFs (vIRFs) have been identified (7). All human IRFs share similar structure in that the amino terminus contains a DNA binding domain while the carboxy terminus contains a dimerization domain that allows IRFs to
form both hetero and homodimers. Similarly, all IRFs bind to a consensus sequence known as an IRF-E via a unique tryptophan cluster located within an N terminal helix turn helix motif (43).

IRF-1, the first characterized IRF, is expressed in a variety of cell types, has a relatively short half-life (~30 min), and binds to PRDI of the IFN-β regulatory region. IRF-1 has been demonstrated to be primarily a modulator of IFN induction. That is, IRF-1 is not strictly required for the induction of IFN-β. For example, depletion of IRF-1 simply reduces Newcastle disease virus (NDV) induced levels of IFN-β transcript (121); specifically, NDV can induce expression of the IFN-β gene in IRF-1-null mice as well as in both IRF-1-null stem cells and mouse embryonic fibroblasts (97, 121). However, dsRNA induction of IFN-β is compromised in IRF-1-null mouse embryonic fibroblasts (MEFs), thus, suggesting-stimulus specific pathways for IFN-β induction exist.

Similar to the case for NDV, IRF-1 was found to be dispensable for reovirus induction of IFN-β. Specifically, reovirus induced an IFN-β reporter plasmid to similar extents in wild type and IRF-1-null derived PCMCs (5). Interestingly, with respect to myocarditis, even though similar IFN-β induction was demonstrated in PCMCs, more severe cardiac lesions were found in the hearts of IRF-1-null mice than in wild type mice following reovirus challenge, suggesting that IRF-1 plays some other protective role against reovirus-induced myocarditis (5).

IRF-2 Regulation of IFN-β:

Interestingly, IRF-2 competes with IRF-1 for binding to the same IRF-E on the IFN-β promoter. However, in contrast to IRF-1, IRF-2 has a relatively long half-life (~8 hrs) and dependent upon cell type, can either induce or repress induction of IFN-β
expression. That is, overexpression of IRF-2 was found to induce IFN-β expression in undifferentiated C2C12 cells (67, 133), but to repress viral induction of IFN-β in MEFs (57). Furthermore, IRF-2 was found to be a negative regulator of ISGs; that is, IRF-2-null mice displayed increased levels of ISGs such as 2’5’ oligoadenylate synthetase and IRF-7 (62). Interestingly, these elevated levels of ISGs were abolished following introduction of a null mutation for ISGF3 component IRF-9; thus indicating IRF-2 as an antagonist of ISGF3 mediated transcription (62). Additionally, it is important to note that IRF-2-null mice also have elevated levels of IFN-inducible chemokines such as IP-10 and MIG, and spontaneously develop an autoimmune skin disease (62).

IRF-2’s role in viral myocarditis has yet to be elucidated. However, overexpression of IRF-2 in PCMCs was found to repress IFN-β expression (133) while, in accordance with previous results, induce IFN-β in undifferentiated C2C12 skeletal muscle cells and in undifferentiated primary skeletal myocyte cultures (unpublished results). Thus, IRF-2’s role in myocarditis is likely interesting given its repressor function in cardiac muscle cells, but inducer function in skeletal muscle cells.

**IRF-3 Regulation of IFN-β:**

IRF-3 was originally discovered in a database search for IRF-1 and IRF-2 homologs (149). IRF-3 is both ubiquitously, as well as constitutively, expressed, and unlike IRF-1 and IRF-2 is present in uninfected cells in an autoinhibitory conformation (119). Recently, the crystal structure of IRF-3 was solved and this elucidated a basis for the autoinhibition (89, 146). In its autoinhibitory state, the IRF-3 dimerization and DNA binding domains are in a condensed, non-functional conformation. However, several phosphorylations of an “autoinhibitory loop” induce conformational changes that expose
active dimerization and DNA binding domains (119). Although IRF-3 is phosphorylated on seven independent sites, phosphorylation of Ser385 and Ser386 are strictly required for activation (166). Recently, after years of searching (140), the kinase responsible for activating IRF-3 was identified to be cell type specific: either IκKε or Tank kinase (132).

IRF-3 induction of IFN-β is a multistep process (Figure 3a). Following viral infection, dsRNA is recognized by Toll-like receptor 3 (TLR3) (44, 159). Activation of TLR3 leads to recruitment of the adaptor protein TRIF (110) and through an unknown mechanism, the activation of IκKε. Activated IκKε (or Tank kinase) phosphorylates IRF-3. Once phosphorylated, IRF-3 is released from its autoinhibition, dimerizes, (87, 89) and translocates into the nucleus where it binds PRDIII of the IFN-β regulatory region. In the nucleus, IRF-3 associates with CBP/p300; this interaction facilitates enhanceosome formation, histone acetylation, and ultimately expression of IFN (71, 157).

Indeed, many studies have demonstrated IRF-3 to be critical for robust interferon induction of IFN expression. IRF-3-null MEFs have dramatically reduced IFN levels following NDV treatment (126). Moreover, through a TLR4 pathway, IRF-3 was found to be required for induction of IFN-β following LPS treatment of macrophages (125). With respect to myocarditis, transfection with a plasmid expressing an IRF-3 dominant negative mutant results in abrogation of IFN-β induction following infection with reovirus in PCMCs, but only reduced induction in murine L929 cells (107). In summation, these results suggest that IRF-3 is indeed critical to IFN induction, but that alternate, IRF-3 independent pathways also exist.
**IRF-7 Regulation of IFN-β:**

The induction of IFN-β is a biphasic process. The initial phase of IFN induction is predominantly IRF-3 dependent (Figure 3a). Since latent IRF-3 is present in the cytoplasm of the host cell, only viral infection and not *de novo* synthesis is needed for a rapid initial IFN response. However, the initial quantity of IFN produced is relatively minor and needs to be further amplified for the robust and sustained stimulation of ISGs. In essence, the cell requires a “late” phase of interferon amplification. This late phase of interferon induction is accomplished by IRF-7 (92).

In contrast to IRF-3, IRF-7 protein is constitutively expressed only in cells of lymphoid origin (3). In other cell types, IRF-7 expression can be strongly induced following addition of Type 1 IFN; this again differs from IRF-3, whose mRNA level remains constant following IFN or virus treatment (4, 90). Thus, while IRF-3 is regulated post-translationally, IRF-7 is predominantly regulated at the level of gene expression. Importantly, IRF-7 mRNA half-life is relatively short and it must be continuously produced to stimulate IFN genes (126). However, similar to IRF-3, full IRF-7 activation ultimately requires carboxy terminal serine phosphorylations by IκKε or Tank kinase (132). It is these phosphorylations that ultimately allow IRF-7 to dimerize and translocate into the nucleus of the infected cell.

Since IRF-7 is transcriptionally regulated through the interferon-stimulated JAK/STAT pathway, it is considered an ISG (Figure 1 and Figure 3b). Following secretion from cells, Type 1 IFN binds to the IFNα/β receptor. Interferon binding results in receptor dimerization and activation of receptor associated kinases of the Janus kinase family (JAKs). Specifically, Jak1 and Tyk2 become activated and subsequently
phosphorylate tyrosine residues on Stat1 and Stat2 (60, 115, 129, 144). These newly phosphorylated Stat proteins form a heterodimer and associate with interferon regulatory factor 9 (IRF-9). This multimeric protein complex is known as ISGF3 and can translocate into the nucleus to bind gene promoter regions containing an interferon stimulated response element (ISRE) (144). IRF-7’s promoter region contains two tandem ISREs, and ISGF3 binding to an IRF-7 ISRE ultimately results in transcriptional stimulation (91, 149). Importantly, IRF-7 transcription is solely regulated by ISGF3: fibroblasts lacking IRF-9 (discussed below) and thus unable to form ISGF3 were incapable of transcribing IRF-7 in response to IFN stimulation (127).

IRF-7 amplification of the IFN response is a coordinated interplay of IRF-3, IFN, and ISGF3 (Figure 3b); in essence, there exists a positive feedback loop. Following an initial IRF-3 mediated induction, IFN is released from a virus infected cell and binds to the IFNα/β receptor in an autocrine or paracrine fashion. Binding to the IFNα/β receptor activates the JAK/STAT pathway and formation of ISGF3, which subsequently stimulates IRF-7 transcription. IRF-7 mRNA is translated; IRF-7 protein is then phosphorylated, dimerizes, and translocates into the nucleus. Once nuclear, dimerized IRF-7 binds both PRDI and PRDIII of the IFN enhancer, thereby stimulating an amplification of interferon response (88).

Interestingly, there remains controversy whether IRF-7 binds to the IFN regulatory region as a homodimer or as a heterodimer with IRF-3. Following Sendai virus infection, the presence of functional IRF-7/IRF-3 heterodimers was observed in 293 human embryonic kidney (HEK) cells (88), but in murine fibroblasts, IRF-7 homodimers and not IRF-7 heterodimers were found to stimulate IFN-α gene transcription (93). Thus,
IRF-7 homo/heterodimerization may be cell type specific. Regardless of its associations, IRF-7 has been demonstrated to be critical for efficient IFN induction (50). For example, even in the absence of IRF-3, splenocytes but not MEFs, are able to efficiently induce IFN-α/β following NDV infection (58); these results raise the intriguing possibility that there exist cell type specificities for IRF roles in IFN induction.

**IRF-9 Regulation of IFN-β:**

Compared to previously discussed IRFs, IRF-9 is less characterized, but still shown to indirectly regulate IFN-β transcription. IRF-9 is expressed in a variety of cell types, (31) and as previously mentioned, is an integral component of the transcription factor complex ISGF3. In fact, IRF-9 functions as the ISGF3 DNA binding subunit (154). As such, IRF-9 is required for ISGF3 function (31) and is therefore critical to the induction of IRF-7 and the subsequent amplification of the IFN response (127).

**Interferon Stimulated Genes:**

**PKR:**

The double-stranded RNA activated protein kinase (PKR) is one of the most extensively studied ISGs. The PKR amino terminus contains two dsRNA binding motifs (dsRBM I & II) (53, 113). dsRBM I is more flexible and displays greater affinity for dsRNA than dsRBM II. In contrast, dsRBM II directly interacts with the PKR kinase domain and may function as an autoinhibitor of PKR (105, 113). Interestingly, it has recently been proposed that activation of PKR is a multi-step process involving successive binding of dsRNA. In short, it is thought that dsRBM I initially contacts dsRNA. This initial interaction subsequently allows cooperative binding of dsRBM II to dsRNA (105). Concurrent binding of dsRNA to these two motifs results in a
conformational change that may expose the kinase domain (105), allow PKR to bind ATP, (16), autophosphorylate, dimerize, and become activated (122, 123, 153). Importantly, in response to cellular stress, recent evidence demonstrates PKR can also be activated in a dsRNA independent manner by dimerizing with PACT (protein activator of PKR) (112, 114, 117).

Once activated, PKR can phosphorylate a variety of downstream targets including the alpha subunit of eukaryotic initiation factor 2 (Figure 4; (46, 61)). Normally, eIF2 functions in protein synthesis by binding initiator Met-tRNA to the small ribosomal subunit. As a prerequisite, eIF2-GDP must first form a ternary complex with the initiator met-tRNA and GTP (142, 153). Phosphorylation of the alpha subunit on serine 51 effectively converts eIF from a substrate to an inhibitor of the guanine exchange factor eIF2B. Specifically, phosphorylated eIF2-GDP forms a stable interaction with eIF2B, thereby preventing the exchange of GTP for GDP on eIF2 (153). This ultimately results in the abrogation of cellular translation (61) and termination of cell growth (33).

In addition to phosphorylating eIF2α, PKR has a number of other downstream substrates including IκK (Figure 4). Phosphorylation of IκK leads to a subsequent phosphorylation of the NFκB inhibitor IκB (51, 83). Phosphorylation of IκB results the release of its inhibitory hold on NFκB. Once released from sequestration, NFκB can translocate into the nucleus and upregulate a multitude of genes including IFN-β (Figure 4). Thus, in addition to functioning downstream of IFN induction as an ISG, PKR can also function upstream of IFN as an activator of NFκB. Interestingly, PKR has also been shown to directly phosphorylate IκB (77); similarly, this phosphorylation results in the release and nuclear translocation of NFκB.
Importantly, PKR has also been implicated as a mediator of apoptosis (36). HeLa cells overexpressing PKR readily undergo apoptosis (82) and PKR overexpression in yeast suppresses growth (160). Similarly, PKR-null MEFs were significantly less apoptotic than wild type following treatment with dsRNA, TNFα, or LPS (36). Moreover, when fibroblasts were engineered to express PKR under an inducible promoter, these cells readily underwent apoptotic cell death (36).

The exact mechanism underlying PKR mediated apoptosis has not been elucidated. However, PKR’s ability to induce apoptosis may be linked to interferon regulatory factor 1 (IRF-1; discussed later in this chapter). In PKR-null cells, IRF-1 is incapable of binding DNA (36); this is significant given that IRF-1 is a demonstrated tumor suppressor, and that IRF-1-null cells also do not undergo apoptosis (148). Additionally, PKR may also mediate apoptosis through its activation of NFκB; many genes with prominent roles in apoptosis (e.g. FAS/FASL) contain NFκB consensus sequences (22). Finally, PKR phosphorylation of eIF2α could also be a signal of cellular stress that results in apoptosis (22).

Given PKR’s prominent roles in apoptosis and innate immunity, it is not surprising that many viruses have evolved strategies to circumvent PKR’s antiviral activities (reviewed in (48)). For instance, adenovirus encodes a 260 nucleotide RNA transcript (VAI) that directly interacts with the RNA binding domains of PKR (96); in essence, high concentrations of adenovirus VAI are decoy RNAs that out-compete double-stranded RNA for PKR binding. Interestingly, there still remains controversy on whether low concentrations of VAI can actually induce PKR activation (47, 48). Similar to VAI, Epstein-Barr virus encodes an inhibitor RNA known as EBER-1 RNA (26). This
competitor RNA is hypothesized to function by binding PKR RBD I but not RBD II, thereby preventing the activation of PKR (105). Interestingly, overexpression of EBER-1 can compensate for mutant adenoviruses lacking VAI (13, 14).

In addition to competitively inhibiting dsRNA binding to PKR, viruses have evolved a myriad of other strategies to inhibit the protein kinase. Vaccinia virus encodes a pseudosubstrate for PKR known as K3L (11), and also E3L, a dsRNA scavenging protein (20). Similar to vaccinia virus E3L, the reovirus encoded σ3 protein also acts as a dsRNA binding protein that can prevent the effective activation of PKR (30, 65, 128). Additionally, viruses have evolved strategies to prevent PKR activation/function by preventing PKR dimerization, interaction with substrate, and by providing counter-regulation of eIF2-α. For example, Herpes simplex virus (HSV) encoded protein γ1.34.5 targets a phosphatase to reverse PKR phosphorylation of eIF2-α; this creates an environment where functionally active PKR is rendered useless in terminating host cell translation (48).

PKR is constitutively expressed in all cell types; the promoter of its gene is TATA-less and contains a novel element known as the kinase conserved sequence (KCS). The 15-bp KCS is located four basepairs upstream of a fully functional ISRE (155). Interestingly, it has been reported that the KCS sequence is required for optimal basal transcription of the PKR gene (73, 75); specifically, it has been demonstrated that the 5’ region of the KCS contains a low affinity Sp1 site that is required for maximum basal expression of PKR.

In contrast, robust induction of PKR following the addition of IFN requires both the KCS and the ISRE. To these promoter regions, an inducible protein complex forms
and drives transcriptional activation of PKR. This inducible protein complex includes ISGF3 binding to the PKR ISRE (155) as well as Sp3 binding to the KCS. Indeed, insertion of additional nucleotides between the KCS and the ISRE results in diminished PKR transcription, indicating both elements are required for optimal transcriptional activity. Moreover, it is important to note that Sp3, but not Sp1 is a component of the inducible protein complex, possibly interacting with STAT1 and STAT2 to facilitate the recruitment of ISGF3 (155). Thus, it appears Sp1 is more critical for basal PKR induction while Sp3 is required for optimal IFN-mediated induction.

In addition to ISGF3 and Sp family binding sites, the PKR promoter contains consensus sequences for several other transcription factors. The regulatory region can bind STAT1/STAT2 heterodimers via a single gamma IFN-activated sequence (GAS sequence). Similarly, the PKR promoter contains both an NFκB and an NF-IL6 binding site. Interestingly, even though the PKR promoter contains a GAS site as well as an NF-IL6 site, treatment of L929 cells with either IFN-γ or IL-6 results in PKR induction (147). In contrast to the KCS and ISRE sites, the PKR promoter also contains a negative regulatory domain (NRD). The NRD is a 40 bp sequence located approximately 400 bp upstream of the KCS; although the mechanism is largely unknown, the NRD functions in a KCS dependent manner to negatively regulate PKR transcription (74).

561:

In contrast to PKR, ISG 561 is only somewhat characterized. This ISG encodes a 56-kDa protein (P56) and its expression is strongly induced following virus, interferon, or dsRNA treatment of cells (56, 64). In fact, microarray analysis demonstrates that following Type I IFN treatment, 561 is the (human) gene whose expression is most
greatly induced in human cells (37). Interestingly, there appears to be multiple pathways governing 561 induction. For instance, expression of 561 was strongly induced following addition of dsRNA to a mutant cell line devoid of Tyk2, JAK1, and ISGF3 component IRF-9 (6). However, IFN treatment of this mutant cell line did not induce 561 transcript, indicating dsRNA and IFN can utilize distinct pathways for the induction of 561 (6). Interestingly, addition of IRF-1 anti-sense RNA abolished dsRNA induction of 561 in these cells (see below; (6)). Thus, it appears IFN mediated 561 induction is ISGF3 dependent, while induction by dsRNA is IRF-1 dependent.

It is highly likely that the full spectrum of 561-encoded P56 function is not known, but similar to PKR, 561 has recently been proposed to have a role in cessation of protein synthesis. Specifically, P56 is thought to interact with eukaryotic initiation factor 3 (eIF3): a cellular protein that interacts with eIF2 and the 40S ribosomal subunit to stabilize the ternary complex required for translation initiation (21). Furthermore, eIF3 functions in associating eIF4-mRNA with the 43S ribosomal complex, therein creating a translation competent 48 S complex (79). In vitro studies utilizing purified P56 suggest that it specifically exerts its antiviral function by inhibiting eIF3’s stabilization of the ternary complex, but not its association with eIF4-mRNA (64). Additionally, P56-mediated translation inhibition was found to be effective only in a cap-dependent manner; invitro, translation mediated by the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) was unaffected by addition of purified P56 (64).

Indeed, overexpression of P56 in HT1080 cells resulted in approximately a 25% reduction in protein synthesis. However, overexpression of P56 in these cells was not sufficient to inhibit vesicular stomatitis virus (VSV) EMCV replication (55). In contrast,
overexpression of the ISG encoding 2'5' (A) synthetase was able to inhibit EMCV but not VSV replication (55). Thus, the role of an individual ISG in host defense is likely complex. Interestingly, in contrast to IRF-7, the 561 promoter is capable of being induced directly by viruses (see “IRF Regulation of ISGs); in GRE cells, which are devoid of Type I IFN genes, induction of 561 was demonstrated following infection by VSV, EMCV, and Sendai virus. Furthermore, Sendai virus was able to induce 561 expression in a mutant cell line that is non-responsive to both Type I IFN as well as dsRNA (55). In accordance with these results, reovirus was capable of inducing 561 in PCMCs and primary cardiac fibroblast cultures (PCFCs) derived from IFN-α/β receptor-null mice (see chapter 3).

**IRF Regulation of ISGs:**

The 561 regulatory region contains a TATA box and two tandem ISRE sequences that confer binding to ISGF3 (156). However, as previously mentioned, multiple IRFs are also implicated in regulating transcription from the 561 promoter. This is because there exists significant sequence homology between the IRF-E and the ISRE; that is, both the IRF-E and the ISRE have repeating GAAA sequence motifs (86).

Importantly, multiple lines of evidence demonstrate IRF regulation of ISGs is gene specific (6, 118, 163, 168). Partially explaining this phenomenon is the observation that different IRFs recognize slightly different DNA consensus sequences. Utilizing random oligonucleotides, it was revealed that IRF-3 had a strong affinity for DNA containing the sequence GAAANNGAAANN. In contrast, the IRF-7 consensus sequence is seemingly less restrictive; IRF-7 binds to the same consensus sequence as IRF-3, but unlike IRF-3, can also bind to this sequence if one of the GAAA repeats is not
perfect (86). Thus, a one-nucleotide substitution in the GAAA repeat confers preferential binding of IRF-7.

Given these results, the mechanism for preferential IRF-3 binding to an ISG’s ISRE (e.g. 561 and RANTES) remains unclear. Similarly, these results are interesting given that IRF-3 has been implicated much more so than IRF-7 in the regulation of ISGs; in fact, IRF-7 has only been implicated in the regulation of ISG 15 (168). Thus, IRF regulation of ISGs is complex and may specifically relate to an IRF’s ability to interact with other transcription factors; indeed, IRF-3 but not IRF-7 interacts with CBP to initiate gene transcription (86).

However, increased affinity for an ISG promoter does appear to have biological consequences. For instance, in electrophoretic mobility shift assays (EMSA), IRF-3 binds the RANTES ISRE with higher affinity than IRF-7. In accordance with these results, when transfected into 293 cells, a constitutively active IRF-3 induced a RANTES reporter plasmid 150-fold while constitutively active IRF-7 induced RANTES only 38-fold (86). However, it is important to note that overexpression of IRF-3, more so than IRF-7, induces IFN; thus, increased RANTES reporter activity may simply reflect increased ISGF3 production.

Not surprisingly, IRFs can also negatively regulate the transcription of ISGs. Furthermore, this negative regulation does not necessarily have to equate with direct IRF binding to an ISRE. For example, IRF-2 has been implicated in the negative regulation of MHC class I genes; mechanistically, IRF-2 achieves MHC downregulation by forming a transcription incompetent complex with the transcription factor NFκB (39). In contrast, IRF-1 associates with NFκB to stimulate transcription from the MHC I promoter (38).
Interestingly, it has recently been shown that IRF-2 heterodimerizes with IRF-1 to downregulate ISRE transcription (24, 131); moreover, it has been proposed that IRF-2 dimerization with other factors negatively affects ISG transcription because IRF-2 has a low association rate for ISREs (24, 131). Similarly, a truncated spliced isoform of IRF-3 can form a nonfunctional heterodimer with full length IRF-3 to negatively regulate IFN-β transcription (70). Given these results, it is interesting to surmise that nonfunctional IRF complexes with other transcription factors may represent a primary means by which ISG expression is negatively regulated.

Finally, it is important to mention that not all ISGs are regulated by IRFs. For example, the ISG IRF-7 is solely regulated through the production of ISGF3. In accordance with this, reovirus does not directly induce IRF-7 in cultures derived from IFN-α/β receptor null mice (IFN-R-null mice; Chapter 3 and unpublished data).

**Negative Regulation of ISGs: SOCS**

In addition to negative regulation by IRFs, ISG transcription is also repressed by suppressors of cytokine signaling (SOCS; (68, 72, 141)). SOCS family members interfere directly with the JAK/STAT signaling pathway to eliminate the production of ISGF3 (Figure 5). Importantly, like ISGs, SOCS family members are regulated through the JAK/STAT pathway. Thus, this pathway is subject to an autoinhibitory loop (106); IFN activation of the JAK/STAT pathway not only induces the expression of ISGs whose products have antiviral activities, but also the SOCS proteins that will ultimately squelch the IFN response.

Currently, there are eight identified members of the SOCS family of proteins (reviewed in (1)). Structurally, each member has a central SH2 domain; this domain is
viral myocarditis is an important human disease affecting an estimated 5-20% of the human population. In infants, the disease can be fatal. In adults, although usually resolved, it can progress to dilated cardiomyopathy, chronic myocarditis, and even cardiac failure. Extreme cases of viral myocarditis, especially in children, can require the use of mechanical circulatory support.

Adenoviruses and enteroviruses are responsible for the majority of human viral myocarditis cases. Interestingly, the cardiac damage that occurs during viral myocarditis can either reflect immune-mediated damage or direct cytopathic effect in the heart. That
is, enterovirus-induced myocarditis is primarily immune mediated, but cardiac damage is also observed in the hearts of SCID mice. Similarly, the exact mechanism of adenovirus-induced myocarditis is not known, but is probably not immune mediated. Importantly, direct cytopathic effect in the heart remains largely unexplored.

Reovirus-induced viral myocarditis is not immune mediated, but rather reflects virus-induced apoptosis of cardiac cells. That is, reovirus induces myocarditis in both nude and SCID mice and both arms of the immune system were demonstrated to be protective against reovirus-induced myocarditis. Furthermore, immune cells do not determine the myocarditic potential of a reovirus. Rather, reovirus induction of, and sensitivity to, Type I IFN determines myocarditic potential; nonmyocarditic reoviruses induce more Type I IFN and are more sensitive to the antiviral effects of Type I IFN (mediated by ISGs) than are myocarditic reoviruses.

Type I IFN binding to the IFNα/β receptor results in the induction of antiviral genes known as ISGs. The product of one such ISG is PKR; PKR is activated upon binding dsRNA, and subsequently phosphorylates numerous downstream targets, including eIF2α. Phosphorylation of eIF2α results in the cessation of host cell translation. Similarly PKR phosphorylation can lead to the nuclear translocation of NFκB, which then upregulates numerous other antiviral genes, including IFN-β. Therefore, PKR functions both upstream as an IFN inducer, as well as downstream as an IFN effector protein (ISG).

Similarly, ISG 561 is strongly induced following virus or Type I IFN treatment. And again, similar to PKR, 561 protein (P56) also functions as an antiviral by limiting host cell translation. However, P56 limits cellular translation by interacting with eIF3,
thereby destabilizing the ternary complex required for translation initiation. Interestingly, while overexpression of P56 results in a 20-30 percent inhibition of host cell translation, P56 overexpression has little effect on the replication of many viruses. Thus, the role individual ISGs have in host defense is likely complex.

The induction of IFN and subsequent induction of ISGs is a highly regulated process. IRFs, due to consensus sequence similarities, are involved in the transcriptional regulation of both pathways. That is, IRFs can both positively and negatively regulate IFN and ISG induction. In addition to IRFs, other pathway/promoter specific factors can regulate induction. For example, SOCS proteins directly inhibit the JAK/STAT signaling pathway to down-regulate ISG expression. In sum, IRFs, other transcription factors, and proteins such as SOCS work in concert to ensure both the proper induction and subsequent repression of IFN signaling.
REFERENCES:


27


TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol. 4:491-6.


123. **Romano, P. R., S. R. Green, G. N. Barber, M. B. Mathews, and A. G. Hinnebusch.** 1995. Structural requirements for double-stranded RNA binding,


163. **Xi, H., and G. Blanck.** 2003. The IRF-2 DNA binding domain facilitates the activation of the class II transactivator (CIITA) type IV promoter by IRF-1. Mol Immunol. **39:**677-84.


165. **Yie, J., M. Merika, N. Munshi, G. Chen, and D. Thanos.** 1999. The role of HMGI(Y) in the assembly and function of the IFN-β enhancesome. The EMBO J. **18:**3074-3089.


Figure 1. Signalling through the JAK/STAT pathway. Type I IFN binds the IFNα/β receptor and results in the activation of receptor associated kinases of the Janus kinase family. Jak 1 phosphorylates STAT1, while Tyk 2 phosphorylates STAT 2. Phosphorylated STAT1 and STAT2 associate and subsequently form a multimeric complex with IRF-9. This protein complex is known as ISGF3 and can translocate into the nucleus and stimulate transcription via binding an ISG’s ISRE(s). Ultimately, upregulated ISGs will function to block viral replication in the host cell.
**Figure 2. Formation of the IFN-β enhanceosome.** (A) Multiple AT rich regions within the IFN-β promoter allow for the binding of HMG I proteins. (B) HMG I binding initiates a chromatin restructuring within the IFN-β promoter. (C) This restructuring subsequently allows the binding of ATF-2/c-Jun, IRFs, and NF-κB to PRDIV, PRDII and PRDII-III respectively. Addition of IRF-3 to the IFN-β enhanceosome is critical in that it associates with CBP300; CBP300 has the histone acetylase activity required for *pol II* transcription.
Figure 3A. IRF-3 mediated IFN-β induction. Virus infects a host cell and during its replication cycle dsRNA intermediates are created. dsRNA binds to TLR3 and through an unknown mechanism results in the activation of IkKε. Activated IkKε (or Tank Kinase) phosphorylates several serine residues on latent IRF-3 protein found in the cytoplasm of the host cell. These phosphorylations release IRF-3 from an autoinhibitory conformation and allow it to homodimerize (not shown for simplicity). IRF-3 homodimers translocate into the nucleus, join the enhanceosome complex, and stimulate an initial round of IFN-β induction.
Figure 3B. IRF-7 mediated amplification of IFN-β. Following an initial IRF-3 mediated IFN-β induction, IFN-β binds the IFNα/β receptor, activates the JAK/STAT pathway—ultimately resulting in ISGF3 production. ISGF3 binds to the IRF-7 ISRE and stimulates its transcription. Once transcribed and translated, IRF-7 protein is activated via IκKε phosphorylation. IRF-7 then either homodimerizes or heterodimerizes with IRF-3 (not shown), translocates into the nucleus, and amplifies the IFN-β response.
Figure 4. PKR’s antiviral activity. Virus infects a host cell and during its replication cycle dsRNA intermediates are created. dsRNA binds to latent PKR found in the cytoplasm of the host cell and triggers an activating autophosphorylation. Activated PKR subsequently phosphorylates numerous downstream targets including serine 51 on eIF2α. Phosphorylation of eIF2α results in the cessation of host cell translation. Additionally, activated PKR can also phosphorylate IκK or IκB resulting in the nuclear translocation of the transcription factor NFκB. NFκB then upregulates numerous antiviral genes including IFN-β.
Figure 5. Regulation of IFN-β and ISGs. Viral replication produces dsRNA intermediates that are recognized by TLR3. Signaling events through TLR3 lead to the activation of IkKε and the phosphorylation of IRF-3. Activated IRF-3 stimulates an initial round of IFN-β induction and directly induces certain ISGs (dashed arrows). Following IFN induction, IFN binds the host cell in an autocrine fashion activating the JAK/STAT pathway, thereby producing ISGF-3. This multimeric transcription factor complex stimulates the transcription of ISGs, including IRF-7, which both further amplifies the IFN response and induces some ISGs directly. In addition to upregulating antiviral ISGs, ISGF3 also upregulates the SOCs proteins that will ultimately dim the IFN response.
CHAPTER 2
PKR’s Protective Role in Viral Myocarditis

Michael J. Stewart¹, Mary Ann Blum², and Barbara Sherry ¹,²*

Department of Microbiology, College of Agriculture and Life Sciences¹ and the Department of Molecular Biomedical Sciences, College of Veterinary Medicine², North Carolina State University, Raleigh, NC, 27606

Published in Virology, 314(1):92-100; 2003.

*Corresponding footnote: Barbara Sherry, (919) 515-4480, FAX (919) 515-3044, email barbara_sherry@ncsu.edu

Running title: PKR and Myocarditis
ABSTRACT:

Reovirus-induced murine myocarditis provides an excellent model for the human disease. Previously, we showed that reovirus induction of and sensitivity to interferon-β (IFN-β) are important determinants of protection against cardiac damage. IFN-β induces a number of genes with antiviral activities, including the dsRNA-activated protein kinase, PKR. Once bound to viral dsRNA, PKR becomes activated and phosphorylates eukaryotic initiation factor-2α (eIF2α) leading to the cessation of host cell translation. Additionally, activated PKR can exert its anti-viral effects by inducing phosphorylation of IκB, leading to the activation of the transcription factor NFκB and subsequent induction of IFN-β. Thus, activated PKR can both induce and be induced by IFN-β. Recently, numerous reports have shown PKR to be dispensable for both induction of IFN as well as protection against disease. However, both PKR’s role in the heart in response to viral infection and its ability to prevent cardiac damage have gone largely unexplored. Here, we demonstrate PKR to be critical for viral induction of IFN-β in primary cardiac myocyte cultures. Additionally, we show that loss of PKR leads to an increase in virulence for both myocarditic and nonmyocarditic reoviruses. Finally, we demonstrate PKR to be critical for protection against reovirus induced viral myocarditis.

Key Words: myocarditis; reovirus; interferon; PKR.
INTRODUCTION:

Viral myocarditis is an important human disease affecting 5-20% of the human population (67). Viral myocarditis can be fatal in infants as well as children (17), and although usually resolved in older individuals, can progress to chronic myocarditis and/or dilated cardiomyopathy and cardiac failure (5, 30, 46). Extreme cases of viral myocarditis require mechanical circulatory support and/or transplantation (17). In recent years, clinical trials of antiviral agents have been tested in hopes of alleviating the disease. Specifically, interferon-α (IFN-α) and thymic agents that increase endogenous IFN levels and stimulate natural killer and T-cell activities have demonstrated an ability to decrease viral load, improve cardiac function, and increase the survival rate in patients with the disease (13, 25, 44, 45, 60). However, none of these treatments are able to completely restore cardiac function.

Many viruses have been implicated in human myocarditis (67) with enteroviruses and adenoviruses most likely accounting for the majority of cases (41, 50, 64). Enterovirus-induced myocarditis is both immune mediated (12, 50) and due to direct cytopathic effect (8, 27) while adenovirus-induced myocarditis is most likely not immune mediated (41). Given that many viruses have access to the heart, and that cardiac myocytes are not replenished, the innate response of cardiac cells to viral insult may be a significant determinant of the extent of cardiac damage. However, the mechanisms by which viruses directly damage the heart and cardiac cells respond to viral infection have gone largely unexplored.

Reovirus-induced myocarditis is not mediated by the immune system (56, 57), but instead reflects virally induced apoptosis of cardiac cells (14). Thus, reovirus-induced
myocarditis provides an excellent model for the exploration of cardiac damage as a result of direct viral cytopathic effect. Previously, we showed reovirus induction of and sensitivity to IFN-β are important determinants of protection against cardiac damage (58). Specifically, in primary cardiac myocyte cultures, we have found that nonmyocarditic reoviruses induce more IFN-β and are more sensitive to the effects of IFN-α/β than myocarditic reoviruses. In addition, nonmyocarditic reoviruses induce myocarditis in mice pretreated with anti-IFN-α/β antibody (58). Interestingly, addition of anti-IFN-α/β antibody inhibits reovirus spread in primary cardiac myocyte cultures, but not in differentiated C2C12 (skeletal muscle) cell cultures (58), suggesting a possible unique role of IFN in the heart.

IFN binding to the IFN-α/β receptor results in the transcription of numerous interferon stimulated genes including 2’, 5’oligoadenylate synthetase, RNase L, Mx protein GTPases, and the 65 kd serine/threonine kinase PKR, which is thought to have a unique role in the innate response to viral infection. PKR is the only known protein kinase that is activated upon binding double-stranded RNA (18), or stem loop structures of some single-stranded RNAs. Once activated, PKR autophosphorylates and subsequently phosphorylates a variety of downstream targets including the alpha subunit of eukaryotic initiation factor 2 (18, 26), thereby abrogating translation initiation (26). In addition, PKR may also exert its antiviral effects by inducing the phosphorylation of IκB (20, 34, 35, 39) ultimately resulting in the activation and nuclear translocation of the transcription factor NFκB, which can then upregulate a multitude of antiviral genes including IFN-β. Moreover, PKR has been implicated in the induction of apoptosis (16, 22, 37, 59), differentiation (29), and cell growth (7, 33, 43).
Although PKR has been implicated in a variety of critical cellular defense mechanisms, and despite the fact that many viruses have evolved strategies to inhibit PKR (19), PKR is not always critical for protection against viral infection (see discussion). Neither the role of PKR in the heart in response to viral infection nor its ability to protect against specific tissue damage have been explored. Since cardiac myocytes are not replenished, the heart likely provides a unique environment where the innate response to viral infection is critical in determining disease outcome. Thus, we investigated the role of PKR in the cardiac response to viral infection using a panel of reoviruses differing in their myocarditic potentials (i.e. degrees of cardiac damage). We show that PKR is critical to both the induction of IFN-β in primary cardiac myocyte cultures and protection against reovirus-induced myocarditis. This provides the first evidence of PKR as an upstream inducer of IFN-β in differentiated primary cell cultures and the first evidence that PKR is a critical antiviral effector in the heart.

RESULTS:

Induction of Interferon is severely compromised in PKR⁻/⁻ Primary Cardiac Myocyte Cultures.

Several studies have demonstrated PKR to be dispensable for the induction of IFN (see discussion). However, none of these studies used non-dividing cells where the innate response to viral infection is likely vital. Given our previous evidence that reovirus induction of IFN-β is an important determinant of viral myocarditis, and that IFN-β’s role
is cell type specific, we asked here whether PKR is critical for viral induction of IFN-β in primary cardiac myocyte cultures.

Primary cardiac myocyte cultures were derived from PKR−/− and parental C57BL/6J mice. Co-transfection with a control plasmid constitutively expressing firefly luciferase (pGL3-C) and a normalization plasmid constitutively expressing renilla luciferase (pRLSV40) revealed no significant difference in control reporter expression between PKR−/− and C57BL/6J derived cultures (FIG 1a, p = 0.35).

Primary cardiac myocyte cultures were then co-transfected with a plasmid containing the IFN-β regulatory region upstream of firefly luciferase (pβlux), and the normalization plasmid pRLSV40. One day later, myocyte cultures were either mock- or T3D- infected. Reovirus T3D was specifically chosen for these experiments because it is known to induce high levels of IFN-β in a variety of cell types including primary cardiac myocyte cultures (48). Eighteen hours post-infection, cells were harvested and analyzed for luciferase activity. A control plasmid lacking a promoter (pGL3-basic) was not induced by T3D in primary cardiac myocyte cultures (data not shown), nor was the constitutively expressing luciferase plasmid pGL3-control ((2, 48) and data not shown). T3D induced pβlux in C57BL/6J cultures as expected, and induction of pβlux was statistically significantly higher in these cultures than in PKR−/− cultures (Figure 1b), ranging from 3.0 to 6.1 fold in C57BL/6J cultures, but only 1.1 to 1.6 fold in PKR−/− cultures. Therefore, PKR is critical to reovirus induction of IFN-β in cardiac cells.
Wild type C57BL/6J mice are more resistant to reovirus infection than are PKR−/− mice.

Parental C57BL/6J and PKR−/− neonates were injected intramuscularly with 1x10^6 PFU of a panel of reoviruses varying in myocarditic potentials. Mice were to be euthanized on day seven for subsequent analysis, however all PKR−/− mice infected with myocarditic viruses (with the exception of EW93) died and/or displayed overt signs of intense discomfort as early as day 3 (Table 1), and were therefore euthanized prematurely. Strikingly, increased morbidity and mortality were also observed in PKR−/− litters injected with two non-myocarditic reoviruses (Table 1). Specifically, PKR−/− mice injected with EW50 exhibited 50 percent mortality five days postinjection whereas 100 percent of wild type mice remained robust through seven days post-injection. Similarly, PKR−/− mice injected with DB93 exhibited 50 percent mortality by day 6 whereas again, 100 percent of wild type mice remained robust. Thus, reovirus was dramatically more virulent in PKR−/− than in C57BL/6J mice.

Nonmyocarditic reoviruses become myocarditic in PKR−/− animals.

Parental C57BL/6J and PKR−/− animals were euthanized following injection (1x10^6) with a panel of reoviruses differing in myocarditic potentials. While all hearts from euthanized mice were subjected to morphometric analysis, only those taken from mice seven days postinjection could accurately be compared. At earlier times, lesions are more variable and do not provide accurate indications of myocarditic potential. For example, the intensely myocarditic reovirus 8B (55) generated few lesions in the PKR−/− animal following euthanization on day three (data not shown). Similarly, EW112
generated significant cardiac lesions (30.5%) at seven days postinjection in wild type mice, while there was little cardiac damage (1.9%) in PKR\(^{-/-}\) mice following euthanization at four days post-injection (data not shown).

Importantly, at seven days post-injection, nonmyocarditic viruses induced significantly more cardiac lesions in PKR\(^{-/-}\) mice than in C57BL/6J mice (Figures 2 and 3). Specifically, the nonmyocarditic reoviruses DB95, EW 27, DB188, and EW29 induced a statistically significant 4.4-16.8 fold greater cardiac damage in PKR\(^{-/-}\) mice than in wild type mice (Figure 3). While T3D induced four-fold greater cardiac damage in PKR\(^{-/-}\) mice than in wild type mice, there was overall less cardiac damage in comparison to the other viruses tested (data not shown). This four-fold increase was not statistically significant, most likely due to the difficulties in accurately measuring very small lesions. Interestingly, EW93, the only myocarditic reovirus that allowed survival of PKR\(^{-/-}\) mice to seven days post-injection yielded similar percentages of lesions in null and wild type mice (data not shown).

To determine whether changes in cardiac damage correlated with changes in viral cardiac titer, C57BL/6J and PKR\(^{-/-}\) mice were injected with 1x10\(^2\) PFU of the nonmyocarditic reoviruses T3D, EW29, EW27, DB188, and DB95, and viral titers in the heart determined at seven days post-injection. While an increase in titer was observed in hearts derived from PKR\(^{-/-}\) mice (Figure 4), only the increased titer of DB188 was statistically significant (p= .002).

**DISCUSSION:**

Our results indicate PKR to be critical for reovirus induction of IFN-\(\beta\) in primary cardiac myocyte cultures. In three independent experiments, T3D induced an IFN-\(\beta\)
reporter (pβlux) 3.0 – 6.1 fold in wild type cardiac myocyte cultures, but only 1.1 – 1.6 fold in PKR\(^{-/-}\) cardiac myocyte cultures (data summarized in Figure 1b). These data are consistent with a report of impaired EMCV induction of both IFN-\(\alpha\) and IFN-\(\beta\) mRNA in promonocytic U937 cells following either transient transfection with a dominant negative PKR construct or the stable introduction of a PKR antisense gene (15). Similarly, our data are consistent with that of impaired induction of IFN-\(\alpha\) and IFN-\(\beta\) message by dsRNA in PKR\(^{+/+}\) MEFs (68). PKR can induce the phosphorylation of I\(\kappa\)B (20, 34) for activation of NF\(\kappa\)B, (21, 34, 70) and subsequent induction of IFN-\(\beta\) (62, 63, 69).

Interestingly, reovirus activates NF\(\kappa\)B in HeLa cells and MEFs, and activation of NF\(\kappa\)B is required for reovirus-induced apoptosis in those cell types (10, 11). Given the precedent for PKR activation of NF\(\kappa\)B in induction of IFN-\(\beta\) and the precedent for reovirus activation of NF\(\kappa\)B, it is likely that the role of PKR in reovirus induction of IFN-\(\beta\) is through activation of NF\(\kappa\)B.

Although we and others have demonstrated PKR to be critical, some have shown PKR to be dispensable for induction of IFN\(\alpha/\beta\). For example, spleen, lung, and liver IFN-\(\alpha\) and IFN-\(\beta\) mRNA levels were similar in wild type and PKR\(^{-/-}\) mice following challenge with NDV (68). Similarly, EMCV challenge resulted in comparable IFN serum levels in wild type and PKR\(^{-/-}\) mice (68). Additionally, following NDV challenge, analogous levels of IFN-\(\alpha\) and IFN-\(\beta\) message were found in wild type and PKR\(^{-/-}\) MEFs (68). Moreover, PKR was found to be nonessential for the IFN response to influenza virus (1). These discrepancies could reflect differences between *in vitro* and *in vivo* environments, the specific cell types involved, stimulus, or a combination thereof. In addition, it is not clear in each of these cases whether measurements reflect direct
induction of IFN-β, or further amplification through “priming”. That is, induction of low levels of IFN-α/β can result in autocrine or paracrine stimulation for further induction of much higher levels of IFN (40, 52). The mechanism and level of this amplification is cell-type specific (24). For example, spleen cells are more dependent on IRF-7-mediated IFN-β priming than are fibroblasts (24). Therefore, PKR may be critical for either the initial viral induction of IFN-β in primary cardiac myocyte cultures, or for subsequent amplification through priming, or both.

PKR was critical for protection against viral myocarditis in vivo, as evidenced by an increase in severity of reovirus-induced cardiac damage in PKR<sup>−/−</sup> mice relative to C57BL/6J mice (Figures 2 and 3). This PKR-mediated protection could reflect PKR’s critical role in induction of IFN-β (Figure 1b), or PKR’s function as an IFN-induced antiviral effector, given that both reovirus induction of and sensitivity to IFN-β are determinants of reovirus myocarditic potential (58). The increase in frequency of reovirus-induced cardiac lesions in PKR<sup>−/−</sup> mice was not accompanied by a statistically significant increase in viral titer for four of the five viruses tested (Figure 4), thus viral replication alone does not account for the observed cardiac damage. These results contrast with a report demonstrating that Foot and Mouth Disease Virus replicates to higher titers in PKR<sup>−/−</sup> MEFs as well as 2-aminopurine treated porcine and bovine cells (6). Our results, however, are consistent with previous studies of reovirus-induced myocarditis, where introduction of inhibitors of apoptosis protected against reovirus myocarditis in vivo without dramatically decreasing viral cardiac titer (14). A number of studies in vitro support the possibility that reovirus-induced apoptosis occurs through signals independent of viral replication. For example, UV-inactivated, replication-
incompetent reovirus virions can induce apoptosis in vitro (66), suggesting that virus-cell surface interactions alone, without accompanying viral replication, can induce apoptosis. Moreover, reovirus binding to cell surface sialic acid is critical for induction of apoptosis, and strain-specific differences in reovirus induction of apoptosis are not correlated with viral yield (10). Additionally, Bcl-2 over-expression can inhibit reovirus-induced apoptosis in vitro without affecting viral yield (49). While reovirus can activate both the death receptor and mitochondrion-associated apoptotic pathways (9, 31, 32), the role of viral replication in this process has not yet been examined.

All reoviruses tested increased in virulence in PKR\textsuperscript{−/−} mice. While nonmyocarditic reoviruses induced more cardiac lesions in PKR\textsuperscript{−/−} mice (Figures 2 and 3), myocarditic viruses became increasingly lethal (Table 1). These results are in agreement with earlier findings demonstrating PKR to be critical in the defense against vesicular stomatitis virus (3, 61), influenza virus (3), and herpes simplex virus type 1 (38) in mice. Similarly, pretreatment with IFN-γ or double-stranded RNA increased survival of wild type but not PKR\textsuperscript{−/−} mice following challenge with EMCV (68). Moreover, our results are in accord with a report demonstrating inhibition of VSV protein synthesis following overexpression of PKR (36). However, our findings differ from those demonstrating PKR\textsuperscript{−/−} mice, similar to wild type, develop only subclinical Sindbis virus infection (51). Our data also contrast with a report demonstrating PKR overexpression in cell culture has no effect on polio virus protein synthesis (36). Since reoviruses can induce a variety of fatal diseases in mice (65), the cause of increased mortality in these mice is unclear. Hearts from moribund mice did not have exceptional cardiac lesions (data not shown), suggesting that mortality did not reflect myocarditis and that PKR protects against
reovirus damage in other tissues as well. This could reflect reduced serum IFN-β in PKR< sup>-/-</sup> mice, or a more direct role in specific tissues. Future experiments will further define the role of PKR in reovirus myocarditis and reovirus virulence in the mouse.

**MATERIALS AND METHODS:**

**Mice and Inoculations:** PKR<sup>-/-</sup> and C57BL/6J parental wild type mice were generously provided by Dr. Bryan Williams (68), The Cleveland Clinic Foundation, Cleveland Ohio. For inoculations with reovirus, indicated mice were mated, housed individually in isolator cages, and checked daily for births. Neonates (2 days old) were inoculated in the left hind limb with 20 µl of virus (1.0 x 10<sup>6</sup> PFU for lesion analysis; 1 x 10<sup>2</sup> PFU for plaque assay analysis) diluted with gel saline. At 7 days postinjection or earlier if mice were moribund, mice were euthanized. Hearts were removed to 10% buffered formalin for morphometric analysis, or 2 ml gel saline and frozen at -70°C for plaque assay. Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

**Cell Cultures:** To generate primary cardiac myocyte cultures from Cr:NIH(S), C57BL/6J, or PKR<sup>-/-</sup> mice, term fetuses or one to two day-old neonates were euthanized and the apical two-thirds of the hearts were removed, minced, and trypsinized (4). Cells were plated at a density of 1.25 x 10<sup>6</sup> cells per well in six-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg,
supplemented with 7% fetal calf serum (HyClone, Logan, Utah), 0.06% thymidine (Sigma Co., St. Louis, Mo.), and 50 µg of Mezlin (Miles Labs) per ml, (“supplemented media”) and plated as indicated for each procedure. Myocyte cultures contained 5-20% fibroblasts (4), consistent with levels reported by others (23, 28, 42), and consistent with cell heterogeneity in the heart. Mouse L929 cells were maintained in suspension culture in minimal essential medium (MEM) (Gibco BRL) supplemented with 5% fetal calf serum (HyClone) and 2mM L-glutamine (Gibco BRL) (“supplemented media”).

Viruses: All reovirus stocks (triply plaqued and passaged twice in mouse L929 cells) were characterized previously for their myocarditic phenotypes (53, 55, 57). Virus 8B is a reassortant virus derived from a mouse infected with serotype 1 Lang (T1L) and serotype 3 Dearing (T3D) (57). All other reassortant viruses were derived from mouse L929 cells infected with 8B-, T1L-, or T3D derived viruses (55). Viral myocarditic potentials were determined by injecting 2 x 10^5 to 5 x 10^7 PFU into two 2-day-old Cr:NIH(S) mice and examining their hearts for macroscopic lesions (gross myocarditis) at death or 14 days postinjection (53, 55).

Plasmids: The pβlux reporter plasmid was constructed as previously described (48), and contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter), and pGL3-Control (expressing firefly luciferase constitutively from an SV40 promoter) were purchased (Promega). DNA was purified for transfection using Qiagen’s Maxiprep system (Qiagen Inc., Valencia, Calif.).
**Transfections:** Primary cardiac myocyte cultures were plated at a density of $1.0 \times 10^5$ cells per well in 0.5 ml in 48-well tissue culture plates (Costar). Cultures were allowed to adhere for one day prior to transfection. Transfection was performed as previously described (2, 47, 48) using FuGENE6 according to the manufacturer’s protocol (Boehringer Mannheim/Roche Molecular Biomedical, Indianapolis, Ind.). Transfection conditions were optimized previously using a β-galactosidase reporter plasmid that demonstrated that myocytes in primary cardiac myocyte cultures were transfected, although overall transfection efficiency was low (47). Each well received 0.01µg pRLSV40 mixed with 0.5µg pβlux. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 0.5 µg of plasmid DNA per well required 1 µl of FuGENE6 per well).

**Infections of transfected wells:** Infections were performed one day post-transfection. Primary cardiac myocyte cultures were washed twice with supplemented DMEM immediately prior to infection. For transfections, two wells were trypsinized and viable cells were determined by trypan blue exclusion. Wells were infected with reovirus T3D at 25 plaque-forming units per cell in 150µl supplemented media or were mock infected. Cultures were incubated for 1 h at 37°C in 5% CO₂, and 350µl supplemented DMEM was then added.

**Dual-luciferase assay:** The dual-luciferase assay was performed according to the manufacturer’s protocol (Promega) with the following exceptions: cells were washed
twice with phosphate-buffered saline prior to the addition of lysis buffer, cells were allowed to remain in lysis buffer at 4 °C for at least 15 min, and the surfaces of the wells were then scraped with a rubber policeman from Fisher Scientific. Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity.

**Morphometric analysis:** Hearts were sectioned and stained with hematoxylin and eosin (H&E). Blinded slides (≥ 5 hearts per virus dose, and ≥ 5 sections per heart) were subjected to morphometric analysis using an Olympus Vannox workstation running Image Pro Plus morphometric analysis software.

**Plaque assay:** Hearts (≥ 4 hearts/virus) in gel saline were subjected to two additional freeze-thaw cycles and sonicated utilizing a Heat Systems, Ultraonic Inc. sonicator. Virus titers were determined by plating serial dilutions on mouse L-cell monolayers, overlaying with agar, and staining with neutral red as previously described (54).

**Statistical analysis:** A Student’s one-tailed $t$ test and pooled variance was used for statistical analysis. Results were considered significant at $P \leq 0.05$. Data were subjected to outlier analysis (Systat 9.0).
ACKNOWLEDGEMENTS:

The author is indebted to Kathleen Azzam-Smoak for her stimulating discussions, and Mac Law for his digital imaging of cardiac sections. We would also like to thank Dr. Bryan Williams for generously providing the C57BL/6J and PKR−/− mice. This work was supported by NHLBI grant HL57161.
REFERENCES:


Figure 1a. A control plasmid is similarly expressed in C57BL/6J and PKR-null cultures Primary cardiac myocyte cultures were generated from PKR−/− mice or parental C57BL/6J mice. After one day, cultures were co-transfected with a control plasmid (pGL3-C) and a normalization plasmid (pRLSV40). Two days later, cells were harvested for luminometry, and normalized luciferase determined.
Figure 1b. PKR is critical for IFN-β induction in cardiac cells. Primary cardiac myocyte cultures were generated from PKR⁻/⁻ mice or parental C57Bl/6J mice. After one day, cultures were transfected with the IFN-β reporter plasmid (pβlux) and a normalization plasmid (pRLSV40). One day later, cultures were mock- or reovirus T3D-infected (MOI 25 PFU/cell). After 18 hours cells were harvested for luminometry. Fold induction of pβlux was determined by dividing normalized luciferase activity in T3D infected wells by that in mock infected wells. Each bar is the average of three independent experiments, each including triplicate wells, performed in the indicated cell type. Numbers above indicate p value as determined by student T test.
Figure 2. Viral induction of cardiac lesions. Control C57BL/6J (A) or PKR\(^{−/−}\) (B) 2-day old mice were injected intramuscularly with the nonmyocarditic reovirus (1X10\(^{6}\) PFU) EW29. Arrow (A) and bracket (B) indicate examples of cardiac lesions present at 10X magnification.
Figure 3. Reovirus induction of myocarditis. Control C57Bl/6J (hatched) or PKR⁻/⁻ (solid bars) 2-day old mice were injected intramuscularly with the indicated nonmyocarditic reoviruses (1X10⁶ PFU). At 7 days postinjection, mice were euthanized, hearts fixed and sectioned (≥5 hearts per virus ≥5 sections per heart), and H&E-stained sections were examined for lesions by morphometric analysis. Percent lesions denotes the lesioned area divided by the total area. Bars represent the average plus the standard error of the mean. * indicates significant difference between PKR⁻/⁻ mice and wild type mice (Students T test, DB95: p= 0.018; Ew27: p= 0.031 DB188: p= 0.001; EW 29 p= 0.036; T3D: p> 0.05). Numbers above bars indicate fold increase by viral injection.
Figure 4. Increased myocarditis does not reflect greater replication in the hearts of PKR-null mice. C57BL/6J (hatched) and PKR<sup>-/-</sup> (solid bars) mice were euthanized at seven days post-injection with 1X10^2 PFU of the nonmyocarditic reoviruses T3D, EW29, EW27, DB188, and DB95. Hearts were removed, sonicated, and viral titers were determined by plaque assay. * indicates significant difference between PKR<sup>-/-</sup> mice and wild type mice.
TABLE 1.
Survival of Reovirus Infected Mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Phenotype</th>
<th>Viable Mice, Day Seven Post-Injection</th>
<th>Early Euthanasia of PKR&lt;sup&gt;-/-&lt;/sup&gt; Mice&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild Type</td>
<td>PKR&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3D</td>
<td>Nonmyocarditic</td>
<td>100%</td>
<td>86%</td>
</tr>
<tr>
<td>DB95</td>
<td>Nonmyocarditic</td>
<td>75%</td>
<td>100%</td>
</tr>
<tr>
<td>EW29</td>
<td>Nonmyocarditic</td>
<td>89%</td>
<td>71%</td>
</tr>
<tr>
<td>DB93A</td>
<td>Nonmyocarditic</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>DB188</td>
<td>Nonmyocarditic</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>EW27</td>
<td>Nonmyocarditic</td>
<td>100%</td>
<td>83%</td>
</tr>
<tr>
<td>EW50</td>
<td>Nonmyocarditic</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>8B</td>
<td>Myocarditic</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>EW112</td>
<td>Myocarditic</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>DB181</td>
<td>Myocarditic</td>
<td>89%</td>
<td>0%</td>
</tr>
<tr>
<td>DB62</td>
<td>Myocarditic</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>EW93</td>
<td>Myocarditic</td>
<td>100%</td>
<td>86%</td>
</tr>
<tr>
<td>DB69</td>
<td>Myocarditic</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup> PKR<sup>-/-</sup> mice displaying decreased growth and generalized lack of responsiveness were euthanized before day seven. Dashes indicate no mice euthanized.

<sup>b</sup> Phenotypes are described as myocarditic or nonmyocarditic on the basis of infection resulting in macroscopic external cardiac lesions on or before day 14 postinjection, as previously determined in wild type mice.

<sup>c</sup> PKR<sup>-/-</sup> mice are described as being robust based on size and responsiveness.
Chapter 3

Constitutive and Reovirus-Induced Interferon-β (IFN-β) and IFN-β-Stimulated Gene Expression are Cell-Type Specific in the Cardiac Protective Response.

Michael J. Stewart¹, Kathleen Smoak¹, Mary Ann Blum², and Barbara Sherry¹,²*

Department of Microbiology, College of Agriculture and Life Sciences¹ and the Department of Molecular Biological Sciences, College of Veterinary Medicine², North Carolina State University, Raleigh, NC, 27606

*Corresponding footnote: Barbara Sherry, (919) 515-4480, FAX (919) 515-3044, email barbara_sherry@ncsu.edu
ABSTRACT:

Viral myocarditis is an important human disease, with a wide variety of viruses implicated. Cardiac myocytes are not replenished and yet are critical for host survival, and thus may have a unique response to infection. Previously, we determined that the extent of reovirus induction of IFN-β and IFN-β-mediated protection in primary cardiac myocyte cultures inversely correlated with the extent of reovirus-induced cardiac damage in a mouse model. Surprisingly, and in contrast, the IFN-β response did not determine reovirus replication in skeletal muscle cells. Here, we compared the IFN-β response in cardiac myocytes to that in primary cardiac fibroblast cultures, a replenishable and non-essential cardiac cell type. We compared constitutive and reovirus-induced expression of IFN-β, IRF-7 (an interferon-stimulated gene [ISG] that further induces IFN-β), and another ISG (561) between the two cell types, using real time RT-PCR. Constitutive IFN-β, IRF-7, and 561 expression was higher in cardiac myocytes than in cardiac fibroblasts. Reovirus T3D induced greater expression of IFN-β in cardiac myocytes than in cardiac fibroblasts, but equivalent expression of IRF-7 and 561 in the two cell types (though fold induction for IRF-7 and 561 was deceptively higher in fibroblasts than in myocytes because of the differences in constitutive expression). Interestingly, while reovirus replicated to equivalent titers in cardiac myocytes and cardiac fibroblasts, removal of IFN-β had a 10-fold greater impact on reovirus replication in the fibroblasts than in the myocytes. Together the data suggest that the IFN-β response controls reovirus replication equivalently in the two cell types. In the absence of reovirus-induced IFN-β, however, reovirus replicates to higher titers in cardiac fibroblasts than in cardiac
myocytes, suggesting that the higher constitutive IFN-β and ISG expression in myocytes may play an important protective role.
INTRODUCTION:

Viral myocarditis has occurred in an estimated 5 to 20% of the human population. It is often fatal in infants, and although usually resolved in adults can progress to chronic myocarditis, dilated cardiomyopathy, and/or cardiac failure (58). In recent years, clinical trials of antiviral agents have shown promise in treatment of the disease. Specifically, interferon-α (IFN-α) (8, 16, 32, 33, 52) and IFN-β (23) have demonstrated an ability to decrease viral load, improve cardiac function, and increase the survival rate in patients with the disease. However, none of these therapies can completely restore cardiac function.

Many viruses have been implicated in human myocarditis, (58) with enteroviruses and adenoviruses accounting for the majority of cases (29, 39, 40, 55). Enterovirus-induced myocarditis is both immune mediated (7, 39) and due to direct cytopathic effect (5, 19). In adenovirus (29) and HIV associated human myocarditis, the severity of disease correlates poorly with the presence of inflammatory infiltrate, suggesting a direct viral effect. However, non-immune mediated mechanisms of viral myocarditis remain largely unexplored.

Reovirus-induced myocarditis is not immune mediated (46, 47); indeed, the immune system is protective against cardiac damage (47). Instead, reovirus-induced myocarditis reflects virally induced apoptosis of cardiac cells (11). Thus, reovirus infection provides an excellent model for studying direct cytopathic effect in the heart. Previously, we demonstrated that in primary cardiac myocyte cultures (PCMCs), reovirus induction of, and sensitivity to, interferon-β (IFN-β) is a determinant of viral myocarditic
potential. Specifically, nonmyocarditic reoviruses induce more IFN-β and/or are more sensitive to the antiviral effects of IFN-β than are myocarditic reoviruses (49).

Importantly, IFN-β mediates its antiviral effects though the induction of interferon stimulated genes (ISGs). Following secretion from infected cells, IFN-β binds to the IFNα/β receptor resulting in receptor dimerization and activation of receptor associated kinases of the Janus kinase family (JAKs). Specifically, Jak1 and Tyk2 are activated and phosphorylate tyrosine residues on STAT1 and STAT2 (17, 35, 43, 50). These newly phosphorylated STAT proteins associate with interferon regulatory factor 9 (IRF-9 or p48), to form the multimeric protein complex ISGF3, which migrates into the nucleus to bind gene promoter regions containing an interferon stimulated response element (ISRE) (50).

To date, more than 300 genes have been identified as ISGs (4, 10, 12). Classical ISGs include those encoding PKR, 2’-5’ oligo “A” synthetase, and Mx family GTPases. Notably, while PKR is critical for protection against reovirus-induced myocarditis (51), the role of other ISGs in protection against myocarditis is unclear. The ISG 561 encoded protein, p56, is the most strongly induced human gene following addition of Type I IFN (12) to human cells, and similar to PKR, it inhibits translation in virally affected cells (14, 21). Specifically, p56 interacts with eukaryotic initiation factor 3 (eIF3), thereby limiting its interactions with eIF2 and the 40S ribosomal subunit and compromising the translation initiation complex (3, 21, 24).

In addition to upregulating ISGs that are directly antiviral, ISGF3 also binds to the interferon regulatory factor seven (IRF-7) promoter, which contains two tandem ISRE sequences that confer ISGF3 binding (27, 53). Importantly, IRF-7 is a transcription
factor responsible for an amplification of the IFN response. While initial IFN-β induction is predominantly IRF-3 dependent (22, 25, 26, 57), robust and sustained IFN-β production is IRF-7 dependent (28). Unlike many other ISGs, IRF-7 is solely regulated via ISGF3: fibroblasts which lack IRF-9, and thus are unable to form ISGF3, do not generate IRF-7 following IFN treatment (42).

Cardiac myocytes are exposed to a variety of viruses, are not replenished, and yet are critical to survival, suggesting that this highly specialized cell type may have evolved a unique response to viral infection. We have previously shown that reovirus induction of IFN-β and IFN-β mediated protection in PCMCs inversely correlate with viral spread in PCMCs and the degree of cardiac damage in a mouse model (49). Surprisingly, and in contrast, the IFN response in skeletal muscle cells is not a determinant of viral replication (49), suggesting a cell type specific role for IFN-β in antiviral protection. Here, we compare the IFN-β response in PCMCs to that in primary cardiac fibroblast cultures (PCFCs), a non-essential, replenished cardiac cell type. Results demonstrate that IFN-β, and the ISGs IRF-7 and 561 are each expressed at constitutively higher levels in PCMCs than in PCFCs. Moreover, while reovirus T3D induced expression of IFN-β to significantly higher levels in PCMCs than PCFCs, it induced similar expression of IRF-7 and 561 between the cell types (though fold induction for IRF-7 and 561 was deceptively higher in PCFCs than in PCMCs because of differences in constitutive expression). Interestingly, while reovirus replicated to equivalent titers in PCMCs and PCFCs, removal of IFN-β resulted in significantly greater reovirus replication in PCFCs than in PCMCs. Together these data suggest that the IFN-β response controls reovirus replication equivalently in the two cell types. However, in the absence of IFN-β reovirus
replicates to higher titers in PCFCs than in PCMCs, suggesting higher constitutive IFN-β and ISG expression in PCMCs may play a protective role.

MATERIALS AND METHODS:

Mice and Inoculations: Timed pregnant Cr:NIH (S) mice were obtained from the National Cancer Institute. IFN-α/β receptor−/− (34) and wild type parental 129Sv/Ev mice were maintained in breeding colonies for timed matings to generate fetuses for primary cultures. Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

Cell Cultures: To generate primary cardiac myocyte and fibroblast cultures from Cr:NIH(S), IFN-α/β receptor−/− (34), or 129Sv/Ev mice, term fetuses or one to two day-old neonates were euthanized and the apical two-thirds of the hearts were removed, minced, and trypsinized (2). Cells were plated at a density of 1.25 x 10^6 cells per well in six-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to isolate rapidly adhering cardiac fibroblasts. Fibroblasts were trypsinized from six well clusters and resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 7% fetal calf serum (HyClone, Logan, Utah), and 50 µg of Mezlin (Miles Labs) per ml, (“supplemented media”). Fibroblasts were then plated as indicated for each procedure. The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 7% fetal calf serum (HyClone, Logan, Utah), 0.06% thymidine (Sigma Co., St. Louis, Mo.), and 50 µg of Mezlin (Miles Labs) per ml, (“supplemented
media") and plated as indicated for each procedure. Myocyte cultures contained 5-20% fibroblasts (2), consistent with levels reported by others (15, 20, 30), and consistent with cell heterogeneity in the heart. Mouse L929 cells were maintained in suspension culture in minimal essential medium (MEM) (Gibco BRL) supplemented with 5% fetal calf serum (HyClone) and 2mM L-glutamine (Gibco BRL) ("supplemented media").

**Viruses:** All reovirus stocks (triply plaqued and passaged twice in mouse L929 cells) were characterized previously for their myocarditic phenotypes (44, 46, 48). Virus 8B is a reassortant virus derived from a mouse infected with serotype 1 Lang (T1L) and serotype 3 Dearing (T3D) (48). All other reassortant viruses were derived from mouse L929 cells infected with 8B-, T1L-, or T3D derived viruses (46). All EW-series reassortant viruses were derived from 8B and EW121. Viral myocarditic potentials were determined by injecting $2 \times 10^5$ to $5 \times 10^7$ PFU into two 2-day-old Cr:NIH(S) mice and examining their hearts for macroscopic lesions (gross myocarditis) at death or 14 days postinjection (44, 46).

**Infections for RNA harvests:** Reovirus infected a similar percentage of cells in PCMCs and PCFCs by immunocytochemistry (data not shown). PCMCs were plated at confluency ($1 \times 10^6$) and PCFCs were plated at half confluency ($5 \times 10^5$) in a 24 well cluster; PCFCs were confluent by day of infection ($1 \times 10^6$). Two days post-plating, PCMCs or PCFCs were washed twice with supplemented DMEM immediately, and then infected with reovirus T3D (Dearing) at 10 PFU per cell in 300 µl supplemented media. After incubation for 1 h at 37°C in 5% CO$_2$, 700 µl DMEM was added and these cultures
were harvested at the indicated times. Mock infected refers to cultures washed with supplemented DMEM and harvested immediately, or five to six hours post overlay with 1 ml supplemented DMEM. Harvests performed at 0 hrs were not incubated for 1 h at 37°C in 5% CO₂; virus was added, immediately removed, and cells harvested.

**Infections for viral replication studies:** PCMCs and PCFCs were plated at a density of 4.0 x 10⁵ cells and 2.0 x 10⁵ cells per well in 300 µl in 96-well tissue culture plates (Costar). At two days post-plating, cultures were infected with a panel of parental and reassortant reoviruses at 0.1 PFU per cell. Cultures were treated with either: 3 ul of antibody containing 165 National Institute of Health neutralizing units of rabbit-anti-mouse IFN-α/β (catalog no. 21032; Lee Biomolecular Research, Inc., San Diego, CA.) or control antibody. 7 days post-infection, cell monolayers were frozen at –70°C, and subjected to additional freeze thaw cycles. Cultures were then lysed in 0.5% Nonidet P-40 (NP-40) and titers determined by plaque assay on mouse L cell monolayers as previously described (45).

**RNA harvest and Reverse Transcription:** At indicated times post-infection, supernatants were aspirated. Cells were lysed directly from the culture plates, homogenized using Qiashredders (Qiagen), and total RNA was isolated using an RNeasy kit (Qiagen). Contaminating genomic DNA was removed by DNase treatment using an RNase-free DNase Set (Qiagen). Total RNA was extracted and quantified in initial experiments using the RiboGreen total RNA detection kit (Molecular Probes; Eugene, OR). RNA from each well was then subjected to reverse transcription using the
following components at final concentrations for a total reaction volume of 50 µl: 5 µM oligo-dT (Promega), 1X Taq buffer, 7.5 mM MgCl₂, 1 mM DTT, 1 mM each dNTP, 0.5 U/µl RNA guard (Pharmacia), 0.22 U/µl avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega).

**Real-time PCR:** Approximately 10% of the reverse transcription reaction was then amplified by real-time PCR. Experiments were performed in duplicate 25 µl reactions in 96 well plates using Quantitech master mix (Qiagen) spiked with 10 nM fluorescein to optimize fluorescent data quality and analysis. Duplicates of PCR were carried out in 96-well plates with optical sealing tape (Bio-Rad). Amplification, quantification, and melt curve analysis (detection of specific products) were performed on an iCycler iQ Fluorescence Thermocycler (Bio-Rad) with the following cycle profile: 95°C (PCR initial activation step 13.5 min), followed by 50 cycles of 95°C for 10 sec (denaturation) and 59°C for 60 sec (annealing, synthesis, and fluorescent data collection step), followed by a melt curve protocol designed for decrement temperatures of 0.5°C with a starting temperature of 95°C and ending at 50°C.

The sequences for the primers were selected using online software designed by Steve Rosen and Helen J. Skaletsky (1998): [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers were checked for base complementarity using [http://oligos.qiagen.com/oligos/toolkit.php](http://oligos.qiagen.com/oligos/toolkit.php) (software provided by Qiagen, 1996-2003). Primers were then designed as follows:

IRF-7 forward primer: 5’CCCATCTTCGACTTCAGCAC 3’

IRF-7 reverse primer: 5’TGTAGTGTTGGTGACCCTTGC 3’
561 forward primer: 5’ TGGCCGTTTCCTACAGTTT 3’
561 reverse primer: 5’ TCCTCCAAGCAAAAGGACTTC 3’

IFN-β forward primer: 5’ GGAGATGACGGAGAAGATGC 3’
IFN-β reverse primer: 5’ CCCAGTGCTGGAGAAATTGT 3’

GAPDH forward primer: 5’ CAACTACATGGTCTACATGTTC 3’
GAPDH reverse primer: 5’ CTCGCTCCTGGAAGATG 3’

Note that the sequence of the IRF-7 amplicon is present in all three IRF-7 spliceoforms (α, β, γ); therefore all spliced variants are detected. To determine copy number of experimental samples, standard curves of known concentrations of DNA were generated for each gene of interest on each plate, using four 10-fold dilutions of PCR generated DNA fragments which included the amplicon sequence. To generate these DNA fragments, the following primers were used to amplify sequences from mouse DNA.

IRF-7 forward primer: 5’ TGGGTTCCTGGATGTGAC 3’
IRF-7 reverse primer: 5’ TTCACCAGGATCAGGGTC 3’

561 forward primer: 5’ CTGAGGCCCACATTTGAGAT 3’
561 reverse primer: 5’ GGAGCATTGGAACACTTGGT

IFN-β forward primer: 5’ GCGTTCCTGCTGTGCTTC 3’
IFN-β reverse primer 5’ CCATCCAGGCGTAGCTG 3’

GAPDH forward primer: 5’ GTGAAGGTCGGTGTGAACGG 3’
GAPDH reverse primer 5’ GTGGCAGTGATGGCATGGAC 3’

These DNA fragments were then purified by agarose gel electrophoresis and quantified by fluorometry. ICycler software was used to analyze data (confirming single point melt curve) and generate standard curves on each plate, and then copy numbers for the gene of
interest were expressed relative to GAPDH expression. Finally data were further normalized to compensate for 3.0 fold higher constitutive expression of GAPDH in PMCs than PCFCs (data not shown). Controls using water or reverse transcribed water were included for each primer set and were always negative.

Statistical analysis: A Student’s one-tailed t test and pooled variance was used for statistical analysis. Data were subjected to outlier analysis (Systat 9.0). For analysis of viral replication, the nonparametric Kruskal-Wallis (Systat 9.0) was used. In all cases, results were considered significant at $p \leq 0.05$.

RESULTS:

Reovirus T3D induces greater IFN-$\beta$ in PMCs than in PCFCs.

The possibility that the innate cardiac response to viral infection is cell type-specific had not previously been explored in any viral system. Here, we compared viral induction of IFN-$\beta$ gen expression in PMCs to that in PCFCs. PMCs and PCFCs were derived from Cr:NIH(S) (wild type) mice. Two or three days post plating, cultures were mock treated or virally infected, and total RNA was extracted and reverse transcribed, and cDNAs were then analyzed by real-time PCR to determine IFN-$\beta$ absolute expression (mRNA copy number). Constitutive IFN-$\beta$ expression was 3.0 fold higher in PMCs than in PCFCs (Fig 1A; $p < 0.001$). In addition, at multiple time-points post-infection, T3D induced IFN-$\beta$ expression (copy number) to significantly higher levels in PMCs relative to PCFCs (Fig 1B: 4 hr =3.8 fold, $p = 0.03$; 8 hr =12.8 fold, $p = 0.01$). When results from Fig. 1A and Fig. 1B were combined to express data as fold
induction, again T3D induced the expression of IFN-β more in PCMCs than in PCFCs (Fig 1C, fold induction).

**Constitutive IRF-7 is higher in PCMCs than in PCFCs, but reovirus induction of IRF-7 is similar.**

Constitutive and reovirus-induced expression of IFN-β was greater in PCMCs than PCFCs; thus, we inquired whether constitutive and/or induced ISG expression was similarly higher in PCMCs. IRF-7 was specifically chosen for this experiment because it is an ISG solely regulated by ISGF3 (42), and furthermore, because it is critical to the robust amplification of the IFN-β response. Reverse transcription and real-time PCR were performed on RNAs from mock and virus-infected PCMCs and PCFCs as above. Constitutive expression of IRF-7 was dramatically higher in PCMCs than in PCFCs (12.7 fold; p < 0.001; Fig. 2A). In contrast, reovirus T3D induced similar IRF-7 expression (copy number) in PCMCs and PCFCs except at 8 hours post-infection where the difference was only 1.9 fold (with no increase in difference at later times, data not shown). When data in Fig. 2A and Fig. 2B were combined, fold induction of IRF-7 was dramatically higher in PCFCs (3.5-17.5 fold) compared to PCMCs (0.63- 2.3 fold, Fig. 2C). Thus, dramatically different constitutive levels of IRF-7 expression between the two cell types resulted in a deceptively large difference in fold induction, despite a minimal difference in final copy number.

**Constitutive expression of ISG 561 is higher in PCMCs than in PCFCs, but reovirus induction of 561 is similar.**

The ISG 561 is the most strongly induced ISG following treatment of cells with Type 1 IFN (12), but unlike IRF-7, virus has been shown to directly induce expression
from the 561 promoter in the absence of IFN (14, 56). This direct induction of 561 is most likely mediated by IRF-3 or IRF-1 binding to the 561 ISRE (1, 6, 9, 13, 14, 36). Importantly, direct induction of 561 is cell type and/or stimulus specific (1, 18). Similar to IRF-7, constitutive expression of 561 was higher in PCMCs than in PCFCs (3.6 fold; p < 0.001; Fig. 3A). However, in contrast to IRF-7, reovirus T3D induced 561 with cell-type specific kinetics (Fig 3B) resulting in a significant difference in 561 expression at 8 hours post-infection (4 fold; p < 0.001). 561 expression remained elevated in PCMCs at 10 and 12 hours post-infection (data not shown). As for IRF-7, when data in Fig. 3A and Fig. 3B were combined, fold induction provided a different curve because of the differences in constitutive expression. At 4 hours post-infection, despite little difference in absolute copy number, fold induction differed dramatically: 12.1 fold in PCMCs compared to 52.7 fold in PCFCs (Fig 3C). Moreover, while at 8 hours post-infection PCMCs expressed 4-fold more 561 than did PCFCs (Fig 3b), there was a negligible difference in relative induction of 561 between the cell types (Fig 3c). Thus, similar to IRF-7, differences in constitutive 561 expression between PCMCs and PCFCs resulted in a curve for fold induction that did not reflect actual final copy numbers.

**IFN-α/β treatment is similar to viral infection.**

To determine whether viral induction of IRF-7 and 561 in PCMCs and PCFCs reflected additional virus-specific events other than induction of IFN-β, PCMCs and PCFCs were overlaid with media containing IFN-α/β and then harvested for analysis by real-time PCR. While IFN-β induced the expression of IRF-7 (Fig 4A) more rapidly than T3D did (Fig 2B), the overall pattern of induction was similar, in that IRF-7 copy number was comparable in PCMCs and PCFCs but fold induction (Fig 4B) was dramatically
different due to differences in constitutive expression. In contrast, while IFN-α/β induction of the ISG 561 in PCFCs (Fig 5A) followed similar kinetics to reovirus induction of 561 in that cell type (Fig 3B), IFN-α/β (Fig 5A) and reovirus (Fig 3B) induction of 561 followed different kinetics in PCMCs. Specifically, while 561 expression remained elevated following reovirus infection, 561 expression rapidly decreased in PCMCs following IFN-α/β stimulation, similar to 561 kinetics in PCFCs. Again differences in the fold induction of 561 expression (Fig 5B) between PCMCs and PCFCs were largely determined by differences in constitutive expression.

**PCFCs are more dependent on the IFN response than PCMCs.**

Data presented in Figures 1-5 suggest that relative (fold) induction of ISGs is determined, at least in part, by constitutive ISG expression. Specifically, PCMCs may rely on high constitutive ISG levels thereby eliminating a need for extensive induction of ISGs; while in contrast, PCFCs express little constitutive ISGs, but achieve protective ISG levels through rapid and substantial IFN-mediated induction. These data suggest that PCFCs may rely more than PCMCs on the IFN mediated response for antiviral protection.

To address this possibility, PCMCs and PCFCs were infected with a large panel of reoviruses in the presence of control or anti-IFN antibodies. After incubation to allow viral replication and spread, cultures were harvested and viral titers determined. In the presence of control antibody, reovirus replicated to similar titers in PCMCs and PCFCs (Fig 6A) and, as previously observed, myocarditic viruses replicated to higher titers than nonmyocarditic viruses did in cardiac cells (49). However, in the presence of anti-IFN antibody, reovirus replicated to significantly higher titers in PCFCs than in PCMCs.
Specifically, anti-IFN antibody increased viral replication an average of 50 fold in PCMCs, but 550 fold in PCFCs (Fig 6B p < 0.001). These results demonstrate that induced IFN is more important for restriction of reovirus growth (protective) in PCFCs than in PCMCs. Given that viruses replicated equivalently in PCMCs and PCFCs in control cultures, but that viruses replicated to titers 10-fold higher in the absence of IFN in PCFCs than in PCMCs, the data suggest that constitutively higher ISG levels in PCMCs may provide a degree of protection even in the absence of IFN (see discussion).

Viruses can directly induce ISGs such as 561 (14, 56), and the mechanisms can be cell-type specific. That is, it was possible that the residual protection in the absence of IFN in PCMCs relative to PCFCs reflected greater direct induction of ISGs in PCMCs than PCFCs. Accordingly, we tested whether reovirus can directly induce expression of 561 in PCMCs and PCFCs derived from IFN-α/β receptor null (IFN-R-null) mice. PCMCs and PCFCs were derived from IFN-R-null mice and infected with reovirus T3D. Cultures were harvested at 0 hrs and 8 hrs post-infection. By real-time PCR analysis, reovirus directly induced 561 expression in both PCMCs and PCFCs, with no significant difference between cell types (Fig 7, p = 0.11). Additionally, reovirus T3D did not induce IRF-7 expression in IFN-R-null derived PCMCs (data not shown). Therefore, differences in viral replication in PCFCs compared to PCMCs in the absence of IFN-α/β is not associated with direct viral induction of 561 or IRF-7.

What determines greater constitutive ISG expression in PCMCs relative to PCFCs? PCMCs express higher constitutive IFN-β than do PCFCs (Fig 1A), providing a possible explanation for differences between PCMCs and PCFCs for IRF-7 (Fig 2A) expression and 561 expression (Fig 3A). Indeed IFN-R-null PCMCs and PCFCs express
dramatically lower levels of constitutive 561 (Fig 8A) and IRF-7 (Fig 8B) than wild type Cr:NIH(s) cultures (similar results were obtained for mock-treated parental 129 Sv/Ev PCMCs and PCFCs, data not shown). Together, these data strongly suggest constitutive expression of IFN is a primary determinant of constitutive ISG expression, and constitutive ISG expression is an important determinant of antiviral protection in PCMCs, but not PCFCs.

DISCUSSION:

Cardiac myocytes are not replenished, and yet are critical for survival. Given that many viruses gain access to the heart, these highly specialized cells may have a unique response to viral infection. Specifically, cardiac myocytes may be more dependent on the innate immune response than cell types that are non-essential, and/or replenished. Indeed, we have previously shown the innate IFN response is critical in determining viral spread in PCMCs, but not in differentiated skeletal muscle cells (49).

In this report, we demonstrate that PCMCs express greater levels of constitutive IFN-β than do PCFCs (Fig 1A). In addition, reovirus induces greater IFN-β expression in PCMCs than in PCFCs, as indicated by both copy number and relative fold induction (Fig 1A and Fig 1B). Cells “primed” with small amounts of IFN-α/β and then virally infected, express more IFN-α/β than non-primed cells (41). This priming is IRF-7-dependent; that is, following viral infection, spleen cells lacking IRF-3, but IFN-primed to express IRF-7, induce IFN-α/β mRNA to levels similar to those of wild type spleen cells (54). Given that constitutive IRF-7 expression was significantly higher in PCMCs than PCFCs (12 fold), our data support a mechanism whereby relatively high constitutive
IFN-β expression in PCMCs results in similarly high constitutive IRF-7 expression, which “primes” PCMCs to induce greater IFN-β in response to viral infection. Thus, PCMCs, non-replenished and critical, are “pre-armed” to respond to viral infection.

Surprisingly, even though reovirus induced more IFN-β in PCMCs than PCFCs (Fig 2B), reovirus induction of IRF-7 expression (copy number) was similar at all timepoints. Additionally, kinetics of IRF-7 induction was identical between these cell cultures. These data suggest that there may be an upper limit to IRF-7 expression in cardiac cells, and that those with higher constitutive expression (PCMCs) are less responsive to viral infection and/or IFN-α/β stimulation.

Similar to IRF-7, peak expression of the ISG 561 was comparable between PCMCs and PCFCs (Fig 3B), but unlike IRF-7, the kinetics of 561 induction were cell type specific. In virally infected PCFCs, peak 561 expression was achieved at 4 hrs, and then sharply declined by 8 hrs post-infection; while in PCMCs, 561 expression steadily increased to 8 hrs (Fig 3B), and slowly declined at later timepoints (data not shown). Interestingly, following IFN treatment, kinetics and levels of 561 expression were identical between the cell types (Fig 5A), resembling that in PCFCs following reovirus infection. It is possible that differences in kinetics of induction of 561 reflect differences in virally activated IRF-3. That is, IRF-3 may remain activated longer, or may be more efficient at induction of 561 expression in PCMCs than PCFCs. Indeed, infection with herpes simplex virus type 1 results in IRF-3 activation that is more robust and sustained in human foreskin fibroblasts than in human fetal lung fibroblasts (38). Also consistent with this possibility, IRF-7 was not induced in PCMCs derived from IFN-R-null mice (data not shown), and no difference in kinetics of induction of IRF-7 were observed.
Constitutive levels of IRF-7 and 561 expression were higher in PCMCs than PCFCs (Fig 2A and Fig 3A). Importantly, our data indicate that constitutive expression of IFN is a primary determinant of constitutive ISG expression (Fig 8). The greater decrease in IRF-7 expression (426-fold and 20-fold in PCMCs and PCFCs, Fig 8B) than 561 expression (58-fold and 16-fold in PCMCs and PCFCs, Fig 8A) in IFN-R-null cultures likely reflects IRF-7’s greater dependence on ISGF3 mediated transcription (data not shown, (42)); in contrast, 561 can be also be induced by IRFs (1, 6, 9, 13, 14, 36) and thus, is likely less affected by loss of ISGF3. The greater decrease in ISG expression in PCMCs than PCFCs likely reflects the higher constitutive expression levels of ISGs (IRF-7 and 561) in PCMCs than PCFCs.

Interestingly, our results in IFN-R-null derived cultures demonstrate that like vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), and Sendai virus, (14), reovirus T3D can directly induce expression of 561 (Fig 8A). Moreover, these cultures display no statistical difference in 561 expression levels at 8 hours post-infection; indicating that in the absence of JAK/STAT signaling, reovirus induces 561 expression to similar extents in PCMCs and PCFCs. Interestingly, these results contrast with a report indicating that reovirus does not directly induce the expression of p56 (encoded by 561) in GRE cells (14). Thus, reovirus direct induction of ISGs may be cell type specific, occurring in cardiac cells but not in human glioblastoma cells.

Viruses replicated to higher titers in PCFCs than PCMCs in the absence of induced IFN-β (Fig 6). Together with data demonstrating higher constitutive ISG expression in PCMCs (Figs 2-5), this suggests that PCMCs benefit from a “pre-arming” while PCFCs are more dependent on induced expression of IFN-β and ISGs. Others have
shown that mutant cell lines expressing high constitutive levels of IFN also express high constitutive levels of the ISGs 6-16, 9-27 and 2’5’ oligoadenylate synthetase (31). Our results are also in agreement with observations that in the absence of IFN mediated protection, cells stably transfected with an IRF-1 transgene are significantly more resistant to VSV, EMCV, and Newcastle disease virus (NDV) than wild type cells (37). Importantly, because “pre-arming” requires higher levels of constitutive IFN, and IFN is anti-proliferative, it is likely that this mechanism of protection is restricted to non-dividing cell types such as cardiac myocytes.

Finally, our results demonstrate that relative induction of gene expression can be misleading, in that final absolute expression (copy number) may differ minimally, while relative induction differs drastically due to differences in constitutive expression. That is, reovirus or IFN-β always induced equivalent or more copies of 561 and IRF-7 in PCMCs than in PCFCs (Figs 2B, 3B, 4A, 5A), but relative induction was always higher in PCFCs than PCMCs (Figs 2C, 3C, 4B, 5B,). These differences were often dramatic: for example, while IFN induced equivalent IRF-7 copies in PCMCs and PCFCs at 5 hours post-treatment (Fig 4A), there was a 10-fold greater relative induction in PCFCs than PCMCs (Fig 4B; 47-fold induction for PCFCs vs. 4 fold induction in PCMCs). This discrepancy was also apparent when comparing 561 expression between wild type and IFN-R-null derived cultures. At 8 hrs post-infection in PCMCs derived from IFN-R-null mice, there was greater relative induction of 561 (Fig 7, 26 fold) than at 8 hrs in wild type cultures (Fig 3C, 22 fold), yet wild type cultures had dramatically higher absolute expression of 561 (47-fold: wild type = 0.47 GAPDH normalized copies; IFN-R-null = 0.01 GAPDH normalized copies). Even at 8 hours post-infection, IFN-R-null derived
PCMCs still expressed less than wild type cultures expressed constitutively (constitutive wild type = 0.02 GAPDH normalized copies; 8 hr IFN-R-null = 0.01 GAPDH normalized copies). This indicates that relative induction data such as that obtained from reporter gene and microarray analyses should be interpreted carefully, especially when comparing multiple cell types (including null to wild type). Moreover, these data further demonstrate that constitutive expression can be a principle determinant of induced expression.

Future experiments will continue to define the molecular differences between PCMCs and PCFCs following viral infection and/or IFN-β treatment. Of particular interest will be the STAT-signaling cascade. Given that relative induction of ISGs is higher in PCFCs than PCMCs, one could imagine a more robust and/or sustained STAT activation in PCFCs.

**ACKNOWLEDGEMENTS:**

The authors are indebted to Tim Petty for a very helpful discussion, as well as Fred Fuller and Matthew Breen for their technical assistance. This work was supported by NHLBI grant HL57161.
REFERENCES:


A.

![Bar graph of IFN-ß Copies normalized by GAPDH](image)

- **PCMCs**
- **PCFCs**

```
Hours Post-Infection:
- 2 hr
- 4 hr
- 8 hr

IFN-ß Copies: GAPDH Normalized X 10^5
3.0x*
```

B.

![Line graph of IFN-ß Copies normalized by GAPDH](image)

- **PCMCs**
- **PCFCs**

```
Hours Post-Infection:
- 2 hr
- 4 hr
- 8 hr

IFN-ß Copies: GAPDH Normalized X 10^2
```

* Denotes significant difference
C.

Figure 1. **Constitutive and induced levels of IFN-β mRNA are greater in PCMCs than in PCFCs.** PCMCs and PCFCs were derived from Cr:NIH(S) mice. Cultures were mock treated or infected with reovirus T3D at an MOI of 10 PFU/cell. Total RNA was extracted and quantified by fluorometry. Equivalent RNA was reverse transcribed, and cDNAs were then analyzed by real-time PCR. * indicates a significant difference in IFN-β expression between PCMCs and PCFCs. (A) Constitutive expression of IFN-β in PCMCs and PCFCs; bars are the averages of six replicates from three independently-derived myocyte and fibroblast cultures + SEM. (B) Reovirus T3D induction of IFN-β in PCMCs and PCFCs. Each timepoint is the average of six replicates from three independently derived myocyte and fibroblast cultures (except for 2hr and 4hr in PCFCs; average of four replicates from two cultures), + SEM. (C) Data from (A) and (B) are combined to express results as fold induction over mock expression. Note, since these data are ratios of data from the two previous panels, there are no SEM values.
A.

1. **Bar Graph:**
   - **Y-Axis:** IRF-7 Copies; GAPDH Normalized x 10^2
   - **X-Axis:** PCMCs, PCFCs
   - **Title:** 12.7x*

2. **Line Graph:**
   - **Y-Axis:** IRF-7 Copies; GAPDH Normalized x 10
   - **X-Axis:** Hours Post-Infection
   - **Data Points:** 0 hr, 2 hr, 4 hr, 8 hr
   - **Legend:**
     - PCMC
     - PCFCs
   - **Trend:**
     - PCMCs showing a slight increase over time.
     - PCFCs showing a more pronounced increase, especially after 4 hr.
     - Significant difference indicated by * at 8 hr.

---

107
Figure 2. IRF-7 expression in PCMCs and PCFCs. Reverse transcription and real-time PCR were performed on RNA from mock and T3D-infected PCMCs and PCFCs as for Figure 1. * indicates a significant difference in IRF-7 expression between PCMCs and PCFCs. (A) Constitutive expression (copy number) of IRF-7 in PCMCs and PCFCs; bars are the average of 17 or 18 replicates from at least eight independently-derived primary cell cultures + SEM. (B) Reovirus T3D induction of IRF-7 (copy number) in PCMCs and PCFCs. Each timepoint is the average of 6 to 8 replicates from 3 to 4 myocyte and fibroblast cultures (except 2 hr and 4 hr in PCFCs; average of four replicates from two cultures) + SEM. (C) Data from (A) and (B) are combined to express results as fold induction over mock expression. Again, because these data are expressed as ratios of data from the two previous panels, there are no SEM values.
A.  

![Graph showing gene expression](image1)

B.  

![Graph showing gene expression over time](image2)
Figure 3. 561 expression in PCMCs and PCFCs. Reverse transcription and real-time PCR were performed on RNA from mock and T3D-infected PCMCs and PCFCs as for Figure 1. * indicates a significant difference in 561 expression between PCMCs and PCFCs. (A) Constitutive expression (copy number) of 561 in PCMCs and PCFCs; bars are the average of 20 or 16 replicates (respectively) from at least eight-independently derived primary cell cultures + SEM. (B) Reovirus T3D induction of 561 (copy number) in PCMCs and PCFCs. Each timepoint is the average of 6 to 10 replicates from 3 to 5 myocyte and fibroblast cultures + SEM. (C) Data from (A) and (B) are combined to express results as fold induction over mock expression. Again, because these data are expressed as ratios of data from the two previous panels, there are no SEM values.
Figure 4. IFN-α/β treatment of PCMCs and PCFCs induces IRF-7 expression similarly to reovirus T3D. Reverse transcription and real-time PCR were performed on RNA from mock and IFN-treated PCMCs and PCFCs as for Figure 1. * indicates a significant difference in IRF-7 expression between PCMCs and PCFCs. (A) IFN-α/β induction of IRF-7 (copy number) in PCMCs and PCFCs. Each timepoint is the average of 4 to 8 replicates from two or four myocyte and fibroblast cultures + SEM. (B) Data from Fig 2A and (A) are combined to express results as fold induction over mock expression. Again, because these data are expressed as ratios of data from the two previous panels, there are no SEM values.
Figure 5. IFN-α/β treatment of PCMCs and PCFCs induces 561 expression similarly to reovirus T3D. Reverse transcription and real-time PCR were performed on RNA from mock and IFN-treated PCMCs and PCFCs as for Figure 1. * indicates a significant difference in 561 expression between PCMCs and PCFCs. (A) IFN-α/β induction of 561 (copy number) in PCMCs and PCFCs. Each timepoint is the average of 4 to 6 replicates from 2 to 3 myocyte and fibroblast cultures (expect at 4 hrs; 2 replicates from a single experiment) + SEM. (B) Data from Fig 3A and (A) are combined to express results as fold induction over mock expression. Again, because these data are expressed as ratios of data from the two previous panels, there are no SEM values.
Figure 6. **PCFCs are more dependent on the IFN response than PCMCs.** Replicate wells of PCMCs and PCFCs were infected at an MOI of 0.1 and overlaid with anti-IFN-α/β antibody or control antibody. At 7 days post-infection cultures were lysed and viral titers determined by plaque assay. (A) Results expressed as mean viral yield of duplicate wells plus standard deviation. (B) Results from PCMCs and PCFCs expressed as ratio of viral yield in anti-IFN-α/β treated relative to that in control-treated wells.
Figure 7. Reovirus directly induces 561 to similar extents in PCMCs and PCFCs. PCMCs and PCFCs were derived from IFN-R-null mice. Cultures were infected with reovirus T3D at an MOI of 10, and harvested at 0 hrs and 8 hrs post-infection. Total RNA was extracted and reverse transcribed. Resultant cDNAs were analyzed by real-time PCR. Each bar is the average of 10 replicates from 5 experiments in PCMCs, or 6 replicates from 3 experiments in PCFCs, + SEM.
Figure 8. Constitutive expression of IFN is a determinant of constitutive ISG expression. (A) Constitutive levels of 561 expression from Fig 3A compared to 0 hr timepoint 561 expression from Figure 7. (B) Constitutive IRF-7 expression from Fig 2A compared to 0 hr IRF-7.
Appendix 1

INTERFERON REGULATORY FACTOR-1, INTERFERON-β, AND REOVIRUS-INDUCED MYOCARDITIS

Kathleen Azzam-Smoak¹, Diana L. Noah², Michael J. Stewart¹, Mary Ann Blum², and Barbara Sherry ¹,²*

Department of Microbiology¹ and the Department of Microbiology, Pathology, & Parasitology, College of Veterinary Medicine², North Carolina State University, Raleigh, NC, 27606

Published in Virology, 298(1):20-29)2002)

*Corresponding footnote: Barbara Sherry, (919) 515-4480, FAX (919) 515-3044, email barbara_sherry@ncsu.edu
ABSTRACT

Viral myocarditis is an important human disease, and reovirus-induced myocarditis in mice provides an excellent model to study direct viral damage to the heart. Previously, we showed that reovirus induction of, and sensitivity to, interferon-β (IFN-β) is an important determinant of viral pathogenicity in the heart, and that the transcription factor interferon regulatory factor-3 (IRF-3) is required for reovirus induction of IFN-β in primary cardiac myocyte cultures. Given several lines of evidence suggesting a possible distinctive environment for IRFs in the heart, we have now focussed on IRF-1. Previous studies demonstrated that viruses, double stranded-RNA (dsRNA), and IFN-α/β can each induce IRF-1, and that IRF-1 plays a role in dsRNA, but not viral, induction of IFN-α/β. Importantly, none of these studies used a virus with a dsRNA genome (such as reovirus), none of them used a highly differentiated non-lymphoid cell type, and none of them addressed whether viral induction of IRF-1 is direct or is mediated through viral induction of IFN-β. Indeed, as recently as this year it has been assumed that viral induction of IRF-1 is direct. Here, we found that reovirus induced IRF-1 in primary cardiac myocyte cultures, but that IRF-1 was not required for reovirus induction of IFN-β. Surprisingly, we found that reovirus failed to induce IRF-1 in the absence of the IFN-α/β response. This provides the first evidence that viruses may not induce IRF-1 directly. Finally, nonmyocarditic reovirus strains induced more cardiac lesions in mice deficient for IRF-1 than they did in wild type mice, directly demonstrating a protective role for IRF-1. Together, the results indicate that while IRF-1 is downstream of the IFN-β response, it plays an important protective role against viral myocarditis.
MATERIALS AND METHODS:

Mice and Inoculations: C57BL/6J mice were generously provided by Dr. Bryan Williams (11), The Cleveland Clinic Foundation, Cleveland Ohio. For inoculations with reovirus, indicated mice were mated, housed individually in isolator cages, and checked daily for births. Neonates (2 days old) were inoculated in the left hind limb with 20 µl of virus (1.0 x 10^6 PFU for lesion analysis; 1 x 10^2 PFU for plaque assay analysis) diluted with gel saline. At 7 days postinjection or earlier if mice were moribund, mice were euthanized. Hearts were removed to 10% buffered formalin for morphometric analysis, or 2 ml gel saline and frozen at -70°C for plaque assay. Mice were housed according to AAALAC recommendations and all procedures were approved by NCSU IACUC.

Cell Cultures: To generate primary cardiac myocyte cultures from C57BL/6J, 129 Sv/Ev, or IRF-1-null mice, term fetuses or one to two day-old neonates were euthanized and the apical two-thirds of the hearts were removed, minced, and trypsinized (2). Cells were plated at a density of 1.25 x 10^6 cells per well in six-well clusters (Costar, Cambridge, Mass.) and incubated for 1.5 to 2 h to remove rapidly adherent cells (predominantly fibroblasts). The remaining cells (predominantly myocytes) were resuspended in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 7% fetal calf serum (HyClone, Logan, Utah), 0.06% thymidine (Sigma Co., St. Louis, Mo.), and 50 µg of Mezlin (Miles Labs) per ml, (“supplemented media”) and plated as indicated for each procedure. Myocyte cultures contained 5-20% fibroblasts (2), consistent with levels reported by others (3-5), and consistent with cell heterogeneity in the heart. Mouse L929 cells were maintained in suspension culture in
minimal essential medium (MEM) (Gibco BRL) supplemented with 5% fetal calf serum (HyClone) and 2mM L-glutamine (Gibco BRL) (“supplemented media”).

**Viruses:** All reovirus stocks (triply plaqued and passaged twice in mouse L929 cells) were characterized previously for their myocarditic phenotypes (8-10). Virus 8B is a reassortant virus derived from a mouse infected with serotype 1 Lang (T1L) and serotype 3 Dearing (T3D) (10). All other reassortant viruses were derived from mouse L929 cells infected with 8B-, T1L-, or T3D derived viruses (9). Viral myocarditic potentials were determined by injecting $2 \times 10^5$ to $5 \times 10^7$ PFU into two 2-day-old Cr:NIH(S) mice and examining their hearts for macroscopic lesions (gross myocarditis) at death or 14 days postinjection (8, 9).

**Plasmids:** The pβlux reporter plasmid was constructed as previously described (7), and contains the murine IFN-β regulatory region inserted into pGL3-Basic (Promega). pRL-SV40 (expressing renilla luciferase constitutively from an SV40 promoter), and pGL3-Control (expressing firefly luciferase constitutively from an SV40 promoter) were purchased (Promega). DNA was purified for transfection using Qiagen’s Maxiprep system (Qiagen Inc., Valencia, Calif.).

**Transfections:** Primary cardiac myocyte cultures were plated at a density of $1.0 \times 10^5$ cells per well in 0.5 ml in 48-well tissue culture plates (Costar). Cultures were allowed to adhere for one day prior to transfection. Transfection was performed as previously described (1, 6, 7) using FuGENE6 according to the manufacturer’s protocol (Boehringer
Mannheim/Roche Molecular Biomedicals, Indianapolis, Ind.). Transfection conditions were optimized previously using a β-galactosidase reporter plasmid that demonstrated that myocytes in primary cardiac myocyte cultures were transfected, although overall transfection efficiency was low (6). Each well received 0.01µg pRLSV40 mixed with 0.5µg pβlux. FuGENE6 was used in a volume equal to twice the total micrograms of plasmid DNA to be transfected per well (e.g., 0.5 µg of plasmid DNA per well required 1 µl of FuGENE6 per well).

**Infections of transfected wells:** Infections were performed one day post-transfection. Primary cardiac myocyte cultures were washed twice with supplemented DMEM immediately prior to infection. For transfections, two wells were trypsinized and viable cells were determined by trypan blue exclusion. Wells were infected with reovirus T3D at 25 plaque-forming units per cell in 150µl supplemented media or were mock infected. Cultures were incubated for 1 h at 37°C in 5% CO_{2}, and 350µl supplemented DMEM was then added.

**Dual-luciferase assay:** The dual-luciferase assay was performed according to the manufacturer’s protocol (Promega) with the following exceptions: cells were washed twice with phosphate-buffered saline prior to the addition of lysis buffer, cells were allowed to remain in lysis buffer at 4 °C for at least 15 min, and the surfaces of the wells were then scraped with a rubber policeman from Fisher Scientific. Measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Oakridge, Tenn.) and
autoinjection. Normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity.
REFERENCES:


Figure 1. Reovirus T3D induces pβlux in 129Sv/Ev and C57BL/6J derived primary cardiac myocyte cultures. PCMCs were generated from C57BL/6J mice (left), or 129Sv/Ev mice (right), were transfected with pβlux and the normalization plasmid pRL-SV40. One day post-transfection, the cultures were mock infected (hatched) or infected with reovirus T3D (solid). Luciferase activity was measured 14 h postinfection. For each well, normalized luciferase activity was determined by dividing firefly luciferase activity by renilla luciferase activity. Each bar shows the mean of three wells (± the standard error of the mean). Asterisks denote a significant increase between mock- and virus-infected cultures (C57BL/6J, \( p = 0.003 \); 129Sv/Ev, \( p = 0.048 \)).

** Contributions to this figure include all data obtained from C57BL/6J cultures.
Figure 2. IRF-1 can protect against viral myocarditis. C57BL/6J ( ), 129 Sv/Ev ( ), or IRF-1−/− ( ) neonatal mice were inoculated intramuscularly with either 1 x 10^3 PFU reovirus strain 8B (myocarditic; C57BL/6J not injected) or 5 x 10^5 - 1 x 10^6 PFU reovirus strain DB95 (nonmyocarditic). At 7 days postinjection, blinded H&E-stained cardiac sections were examined for microscopic lesions utilizing morphometric analysis. Percent lesion refers to the average area of lesion divided by the average total area, x 100, ± standard error of the mean. The asterisk denotes a significant increase between DB95 infected C57BL/6J, 129 Sv/Ev, or IRF-1−/− mice; p=0.05 for C57BL/6J vs. IRF-1−/−; p=0.005 for 129 Sv/Ev vs. IRF-1−/−.

** Contributions to this figure include the development of the morphometric analysis technique used to quantify all lesions, and the data obtained from C57BL/6J mice.
SUMMARY

The data presented in this dissertation further define our understanding of the cardiac response to viral infection. PKR was demonstrated to be critical to the induction of IFN-β in primary cardiac myocyte cultures (PCMCs, Chapter 2). However, while experiments in PCMCs addressed PKR’s role upstream of IFN-β induction, its antiviral role downstream of IFN-β induction (as an ISG) remains to be characterized. Therefore, it would be interesting to generate PCMCs from PKR-null mice, IFN prime these cultures, and infect with reovirus at a low MOI. These experiments would assay for viral spread in the absence of PKR antiviral activity (i.e. translation inhibition, apoptosis), but independent of its role in IFN-β induction.

In PKR-null mice, nonmyocarditic reoviruses became myocarditic (Chapter 2). Reovirus-induced myocarditis reflects virally induced apoptosis of cardiac cells, and following reovirus infection, NFκB is anti-apoptotic in the heart. Thus, increased myocarditis could reflect a decrease in functional NFκB in PKR-null mice. Gel-shifts using lysates from PKR-null hearts would be helpful in testing for NFκB activity. However, even if gel-shifts were negative, these results would only further suggest a role for NFκB, and would not rule out other PKR-mediated activities (i.e. translation inhibition) contributing to increased myocarditis in PKR-null mice. Furthermore, because the mechanism of reovirus-induced apoptosis is largely undefined, gel-shifts would lend little insight into NFκB’s anti-apoptotic activity. For instance, NFκB may be anti-apoptotic in the heart simply because it can induce IFN-β, which then stops reovirus
replication before it triggers apoptosis in cardiac cells. Thus, accessing PKR’s antiviral role in the heart is complex.

Interestingly, increased myocarditis in the hearts of PKR-null mice was not accompanied by a dramatic increase in reovirus replication (Chapter 2). Importantly, these results are in accord with previous work showing inhibition of reovirus-induced apoptosis prevents myocarditis without an appreciable decrease in viral replication. Together, these results suggest that pathways governing reovirus replication and apoptosis are distinct.

In this dissertation we also show that reovirus induction of IFN-β, and IFN-β mediated protection is cardiac cell type specific (Chapter 3). Results demonstrate that IFN-β, and the ISGs IRF-7 and 561 are each expressed at constitutively higher levels in PCMCs than in PCFCs. Moreover, while reovirus T3D induced expression of IFN-β to significantly higher levels in PCMCs than PCFCs, reovirus induced similar expression of IRF-7 and 561 between the cell types. Interestingly, while reovirus replicated to equivalent titers in PCMCs and PCFCs, removal of IFN-β had a much greater impact on reovirus replication in PCFCs than in PCMCs. Together these data suggest that the IFN-β response controls reovirus replication equivalently in the two cell types, but in the absence of induced IFN-β, higher constitutive ISG expression in PCMCs may be protective.

However, while our data suggests that increased constitutive ISG expression is protective in PCMCs relative to PCFCs (Figure 1), it cannot yet be proven. Most notably, our work has only examined the expression of two ISGs. Moreover, due to limitations in reagents, we did not examine whether increased constitutive expression of
mRNA correlated with greater constitutive expression of protein. Thus, future experiments comparing constitutive expression of message and protein for additional ISGs would strengthen this hypothesis. Moreover, it would be interesting to generate PCMCs and PCFCs from IFN-R-null mice and infected with a panel of reoviruses. Since IFN-R-null derived PCMCs and PCFCs have minimal constitutive ISG expression, reovirus replication should be equivalent in these cultures.

Is increased constitutive IFN-β and ISG expression unique to cardiac myocytes? Given that IFN-β is anti-proliferative, it is possible that this mechanism of protection is restricted to non-dividing cell types such as cardiac myocytes. Therefore, it would be interesting to compare constitutive IFN-β and ISG expression in PCMCs to that in additional primary cell cultures (both dividing and non-dividing). Results from these experiments would clarify whether elevated constitutive IFN-β and ISG expression is restricted to non-dividing cells. Additionally, these experiments would determine whether the cardiac response to viral infection is unique.
Figure 1. Proposed mechanisms of cardiac protection. (A) PCMCs have relatively large constitutive ISG protection. Therefore, these cells need relatively little IFN-mediated induction of ISGs (designated as “fold”) to achieve antiviral protection. (B) Conversely, PCFCs have relatively little constitutive ISG protection. Therefore, PCFCs are more dependent on IFN-mediated induction of ISGs for antiviral protection.